ORIGIN OF DNA METHYLATION PATTERNS IN THE MALE GERM LINE: ROLES OF THE NOVEL DNA METHYLTRANSFERASES IN MALE GERM CELLS

by

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Avec tout l'amour qu'il se doit, cette thèse est dédiée à :

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ABSTRACT

Formation of gametes capable of supporting development is dependent on a number of genetic and epigenetic events. DNA methylation is an epigenetic modification catalyzed by enzymes named DNA methyltransferases (DNMTs). In the mouse, methylation of DNA is associated with the control of gene expression and proper embryo development. Methylation patterns are established in a sex- and sequence-specific manner during male and female germ cell development and further modified during early embryonic development. Even though new DNMTs have recently been identified, little information is known on the origin of the methylation marks during male germ cell development (spermatogenesis). The main goal of the work presented in this thesis was to gain a better understanding of the enzymes involved in creating the epigenetic program of the male genome. The first step in doing so involved comparing the temporal expression profiles of DNA methyltransferases in the developing testis and ovary. The expression profiles obtained indicated that in the male, DNMT3a and DNMT3L could be involved in *de novo* methylation while DNMT3b and DNMT1 could be responsible for maintaining methylation patterns following DNA replication. Next, characterization of *Dnmt3a* and *Dnmt3b* expression in isolated postnatal male germ cells revealed how tightly regulated the expression of these genes is during spermatogenesis: specific transcript variants and protein isoforms of each DNMT are differentially expressed during male germ cell development. Finally, assessing the effect of *Dnmt3L* inactivation on the male germ line exposed the presence of a mitotic defect in germ cells lacking this protein. DNA methylation analyses revealed that many loci throughout the genome are marked for methylation by DNMT3L, indicating a more global role for this enzyme than that previously reported in genomic methylation patterning in the male germ line. As methylation patterns instituted during spermatogenesis have to be properly established for accurate transmission of epigenetic information to the next generation, the studies presented here contribute to our knowledge of the events leading to the creation of the epigenetic program necessary for the formation of healthy gametes.

RÉSUMÉ

La formation de gamètes capable de favoriser le développement de la vie dépend de nombreux évènements génétiques et épigénétiques. La méthylation de l'ADN est une modification épigénétique catalysée par des enzymes nommés ADN méthyltransférases (DNMTs). Chez la souris, la méthylation de l'ADN est associée avec le contrôle de l'expression des gènes et est requise pour un développement embryonnaire approprié. Les patrons de méthylation sont élaborés en fonction du genre de l'organisme et de la nature des séquences à méthyler; ces patrons sont initialement établis durant le développement des gamètes mâles et femelles et sont subséquemment consolidés au début du développement embryonnaire. Bien que de nouvelles DNMTs aient récemment été identifiées, bien peu est connu sur l'origine des patrons de méthylation durant le développement des cellules germinales mâles (spermatogenèse). Le but principal des travaux présentés dans cette thèse était d'approfondir notre compréhension de la nature des enzymes responsables de l'élaboration du programme épigénétique du génome mâle. Pour ce faire, il s'agissait premièrement de comparer l'expression temporelle des ADN méthyltransférases durant le développement du testicule et de l'ovaire. Les patrons d'expression obtenus indiquaient que, chez le mâle, DNMT3a et DNMT3L pourraient être responsable de l'établissement de nouveaux patrons de méthylation, tandis que DNMT3b et DNMT1 pourraient être impliqués dans la propagation de ces patrons suivant la réplication de l'ADN. Puis, caractériser l'expression de *Dnmt3a* et *Dnmt3b* dans des cellules germinales mâles isolées durant le développement post-natal a révélé à quel point l'expression de ces gènes est strictement régulée durant la spermatogenèse : des transcrits et des isoformes spécifiques de ces deux DNMTs sont exprimés de façon distincte durant le développement du gamète mâle. Finalement, déterminer l'effet de l'inactivation de *Dnmt3L* sur la lignée germinale mâle a permis d'exposer la présence d'aberrations mitotiques chez les cellules dépourvues de cette protéine. Analyser le niveau de méthylation de l'ADN a révélé que plusieurs locus répandus à travers le génome sont marqués par DNMT3L pour être méthylés ultérieurement, indiquant un rôle

plus global de cet enzyme dans l'établissement des patrons de méthylation génomiques que précédemment rapporté. Les patrons de méthylation institués durant la spermatogenèse doivent être établis de façon appropriée afin de permettre une transmission exacte de l'information épigénétique à la prochaine génération. Les résultats présentés contribuent à notre connaissance des évènements menant à l'élaboration du programme épigénétique nécessaire à la formation de gamètes compétents.

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FORMAT OF THE THESIS

This thesis is composed of five chapters, three chapters of which are scientific data chapters in the form in which they were submitted for publication. Chapter I is a review of the literature that pertains to the subject of this thesis and provides general background information; also included is a description of the rationale and objectives of the research presented. Portions of Chapter I are reproduced from La Salle and Trasler (2006), in *The Sperm Cell: Production, Maturation, Fertilization, Regeneration*, eds. De Jonge, C. & Barratt, C., pp. 279-322. Chapters II to IV contain experimental data presented in manuscript format. Chapter II and Chapter III have been published in *Developmental Biology* in 2004 (268: 403-415) and 2006 (296: 71-83), respectively. Chapter IV is a manuscript that has been submitted for revision to *Human Molecular Genetics*. Connecting texts between the data chapters are provided in accordance to McGill University's "Guidelines for Submitting a Doctoral Thesis" (Section 1C). Discussed in Chapter V are a general overview of the findings presented in this thesis and the possible future experimental directions this work could take. The thesis is completed by a reference list and relevant appendices.

CONTRIBUTION OF AUTHORS

For all the work described in this thesis, the candidate participated actively in experimental planning and was responsible for all data analyses. She wrote all three manuscripts with assistance from the co-authors involved. All of the work was conducted under the supervision of Dr. Jacquetta M. Trasler.

In **Chapter II**, the candidate performed the tissue collections and RNA extractions relevant to the Real-Time RT-PCR (qRT-PCR) experiments; she also designed the primers and setup the conditions used for qRT-PCR. Dr. Carmen Mertineit performed all immunocytochemistry experiments. Dr. Teruko Taketo provided guidance for early gonad isolation and histological examination, as well as genetic sexing of embryos. Dr. Peter B. Moens provided antibodies and protocols for the preparation and immunostaining of meiotic chromosome spreads. Dr. Timothy H. Bestor provided the PATH52 antibody and the Dnmt1 probes for the Northern. Guylaine Benoit conducted the Northern Blot analyses.

In **Chapter III**, the candidate carried out all of the germ cell isolations, tissue collections and experimental procedures.

In **Chapter IV**, the candidate was responsible for establishing and maintaining all the mouse colonies used for the various experiments, in addition to genotyping all mice used. She performed all qRT-PCR experiments, in addition to the germ cell isolations and tissue collections. Histological sectioning was performed by Dr. Xinying He. Oana R. Neaga did the serial sectioning and the immunostaining experiments pertaining to the germ cell count analysis. The candidate conducted the immunofluorescence study. Establishing the quantitative Analysis of DNA Methylation using real-time PCR assay, or qAMP, was a collaborative effort between the candidate and Christopher C. Oakes. While the candidate participated in primer design, collected the cells and extracted the DNA, Christopher C. Oakes performed the assay. Dr. Deborah Bourc'his was responsible for creating the *Dnmt3L* knockout mouse model under the supervision of Dr. Timothy H. Bestor at Columbia University (New York, NY).

ABBREVIATIONS AND NOMENCLATURE

The most common abbreviations found in this thesis include:

A	type A spermatogonia
ART	assisted reproductive technologies
B	type B spermatogonia
BSA	bovine serum albumin
C_t	threshold cycle value
ΔC_t	delta threshold cycle value
DMR	differentially methylated region
DNMT	DNA methyltransferase
dpc	day post-coitum
dpp	day post-partum
E	embryonic day
ES cell	embryonic stem cell
FACS	fluorescence activated cell sorting
FSH	follicle stimulating hormone
GCNA1	germ cell nuclear antigen-1
GFP	green fluorescent protein
IAP	intracisternal A particle
ICF	immunodeficiency, centromeric region instability and facial anomalies syndrome
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilization
L/Z	leptotene/ zygotene spermatocytes
LH	luteinizing hormone
LINE	long interspersed nuclear element
LTR	long terminal repeat
MI, MII	meiosis I, meiosis II
P	pachytene spermatocytes

PA	primitive type A spermatogonia
PBS	phosphate buffered saline
PGC	primordial germ cell
PL	preleptotene spermatocytes
PP	prepubertal pachytene spermatocytes
qAMP	quantitative analysis of DNA methylation using real-time PCR
qPCR, qRT-PCR	quantitative or Real-Time PCR, RT-PCR
RB	residual bodies/ elongating spermatids
RLGS	restriction landmark genomic scanning
RNAi	RNA interference
RS	round spermatids
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	standard deviation
SEM	standard error of the mean
T-DMR	tissue specific- differentially methylated region

The **nomenclature** used throughout this thesis is based on the conventions described by Maltais *et al.*, (2002), Roberts *et al.*, (2003) and Wain *et al.*, (2002). Mouse genes are written in lowercase italics (e.g. *Dnmt3a*), transcripts are in lowercase letters (e.g. Dnmt3a), while proteins are in uppercase letters (e.g. DNMT3a); human genes, transcripts and proteins are distinguished by the use of uppercase letters. Only genes are italicized. A lower case suffix is used when referring to the murine protein (e.g. DNMT3a). The only exception to these conventions is for *Dnmt3L*, as the "L" will always be written using an uppercase letter because the use of a lowercase "l" can be confused with the number "one".

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CHAPTER I

Introduction

1 Preface

The birth of a healthy child is intimately related to the genetic contribution of both parents. Although years of research have contributed to better our understanding of the underlying genetic mechanisms governing mammalian development, multiple issues appearing to go beyond the information encoded in our DNA remain unresolved. Over the last decade, mounting interest in the field of epigenetics, described as the next "frontier" in genetics, has emerged. The term *epigenetics* encompasses all of the processes that affect gene expression without changing the DNA sequence. Studies investigating these processes have established that epigenetic programming is a requirement of normal development, in addition to showing that there are crucial windows of epigenetic programming taking place during gametogenesis and embryogenesis (Li, 2002). The fundamental theme of this thesis is the creation of the epigenetic program relevant to the production of healthy gametes capable of supporting development.

Statement of Investigation

Epigenetic modifications lead to heritable changes in gene expression without altering the underlying DNA sequence. To date, three main mechanisms, namely RNA-associated silencing, histone modifications and DNA methylation, have been associated with epigenetic silencing of gene expression. Recent advances made in our understanding of the relationships between these systems have illustrated how they interact and stabilize each other, emphasizing how disrupting one or more of these interacting systems could lead to inappropriate expression or silencing of genes, resulting in "epigenetic diseases" such as cancer.

The best-characterized modification of DNA associated with modulation of gene expression is the methylation of cytosine residues within CpG dinucleotides by DNA methyltransferases (DNMTs). DNA methylation, a post-replicative, reversible and heritable mark, takes place at approximately 30 million CpG sites throughout the genome (Bestor and Tycko, 1996). Methylation in the promoter

region of genes is commonly associated with transcriptional repression, indicating that DNA methylation provides a means to control gene expression. More so, a number of highly specialized biochemical processes, such as allele-specific gene expression (genomic imprinting), transcriptional silencing of parasitic sequence elements and X-chromosome inactivation, depend on or involve DNA methylation (Goll and Bestor, 2005). Germ cell and early embryo development are critical times when patterns of DNA methylation are initiated and maintained. During gametogenesis, genomic DNA methylation patterns are acquired in a sex- and sequence-specific manner; the most striking sex-specific methylation differences are observed at imprinted gene loci and repetitive sequence elements (Bestor, 2000; Reik *et al.*, 2001). Imprinted genes are expressed exclusively from one of the parental alleles. So far, DNA methylation remains the best-studied epigenetic mark known to distinguish the alleles from one another.

Characterizing the expression of the first identified mammalian DNA methyltransferase (DNMT1) during gametogenesis revealed some interesting sex-specific features, but raised questions regarding its actual role in methylation pattern acquisition in the germ line (Jue *et al.*, 1995; Mertineit *et al.*, 1998; Howell *et al.*, 2001). As I undertook the work presented in this thesis, the members of the DNMT3 family of DNA methyltransferases had just been discovered. Although little was known about their expression in the germ line, they were postulated to be the long-sought *de novo* DNA methyltransferases responsible for methylation pattern establishment in germ cells. Thus, my initial studies consisted in characterizing the expression of the genes coding for the DNMT3 enzymes in both the male and female germ lines (Chapter II), as well as in isolated populations of male germ cells (Chapter III). The objective of the work described in Chapter IV was to study the consequences of altering the expression of one of the *Dnmt3* genes in the male germ line.

This introductory chapter will provide an overview of the aspects of DNA methylation relevant to the work presented in this thesis, as well as a description of the enzymes capable of methylating DNA. The main events leading to the production of mature male and female germ cells in the mouse will also be

outlined. Much of the literature on DNA methylation is based on work done in the mouse, so this dissertation will largely focus on studies using this model organism. As well, since the main focus of this thesis is the male germ line, more emphasis will be placed on this system rather than on its female complement.

1.1 Gametogenesis in the mouse

Gametogenesis is a unique system by which spermatozoa (in the male) and oocytes (in the female) are produced. These highly differentiated cells retain the genetic information necessary to direct the development of all cell lineages in the new organism following their union at fertilization. Gametogenesis forms the fragile link between generations, and is central to the survival and evolution of a species. It is in this respect that gametogenesis, and more so germ cells, carry the potential for both totipotency and immortality.

1.1.1 Fetal Germ Cell Development

Primordial germ cells (PGCs) are the founder cells of the germ line and their descendants form the functional gametes of the adult animal. In the mouse, at about 6.5 days post-coitum (dpc), a small cluster of PGCs migrates from the endodermal yolk sac epithelium towards the developing genital ridge, which they begin to colonize at about 10.5 dpc. Throughout this period of migration, PGCs proliferate mitotically at a rather uniform rate; they continue to divide within each genital ridge, reaching a maximum number of about 25,000 by 13.5 dpc in the sexually-differentiated gonad (Tam and Snow, 1981; Donovan *et al.*, 1987). The appearance and the behaviour of PGCs are undistinguishable in male and female embryos throughout the migratory period, but their fate diverges as they colonize the genital ridges (McLaren, 1995). Gonads are morphologically indifferent prior to 12.5 dpc, but the sex of an embryo can be determined by the presence of testicular cords and a coelomic vessel in the male after this time (Hogan *et al.*, 1986).

Germ Cells of the Fetal Testis

Once the PGCs have colonized the testicular cords (the precursors of seminiferous tubules), they are referred to as gonocytes or prospermatogonia. During this period of pre-spermatogenesis, they are situated centrally within the seminiferous cords in the space where the lumen will later develop. Gonocytes continue to proliferate until about 14.5 dpc following which they enter a period of mitotic arrest until birth (Vergouwen *et al.,* 1991; Nagano *et al.*, 2000). They resume their mitotic activity only shortly after birth (about 1.5 to 2 days post-partum (dpp)), at which point they descend towards the basement membrane to give rise to spermatogonial stem cells and, eventually, spermatogonia that will proceed through spermatogenesis (Bellvé *et al.*, 1977b; Nagano *et al.*, 2000). A detailed description of spermatogenesis will be presented in the next section.

Germ Cells of the Fetal Ovary

In contrast, the female pathway is characterised by a single wave of proliferation followed by entry into meiosis prior to birth (Wassarman and Albertini, 1994). Oogonia (fetal germ cells of the ovary) enter meiosis at about the same time as gonocytes enter mitotic arrest. Once they have gone through their final mitotic division, oogonia undergo one further round of DNA replication, known as the preleptotene stage, before they enter prophase I of meiosis to become oocytes. The first leptotene oocytes, characterised by condensing chromosomes, are detected around 14 dpc. By 17 dpc, all oogonia have entered meiosis and are found at various stages of early prophase: leptotene, zygotene (synapsis or pairing of homologous chromosomes), pachytene (crossing-over and recombination), or diplotene (segregation of homologous chromosomes) (Wassarman and Albertini, 1994). Oocytes enter and progress through meiosis with some degree of synchrony, a fetal ovary of a given age containing a majority of oocytes in one or two stages of meiosis (Bakken and McClanahan, 1978). After birth, the majority of oocytes are found arrested in late diplotene, at which point they are termed dictyate oocytes. Unlike the male, where meiosis is a continuous process, oocytes progress through meiotic prophase collectively only

once, and they remain arrested at the dictyate stage until they are recruited into the growth phase; meiosis resumes shortly before ovulation upon hormonal stimulation.

1.1.2 Male Germ Cell Development

A number of investigators have described the intricacies of the male reproductive system, including the regulation, kinetics and dynamics of spermatogenesis (Clermont, 1972; Ewing *et al.*, 1980; Hecht, 1998; de Rooij and Russell, 2000; Grootegoed *et al.,* 2000; Cooke and Saunders, 2002; Dadoune *et al.*, 2004). Therefore, only an overview of the aspects relevant to the work presented in this thesis will be addressed.

1.1.2.1 The Male Reproductive Tract

The testes, epididymides, excurrent ducts and seminal vesicles make up the male reproductive tract. The functions of the testis are controlled by two gonadotropins produced by the anterior pituitary: 1- luteinising hormone (LH) and 2- follicle-stimulating hormone (FSH). In turn, by producing several steroids, the testis functions as an endocrine gland. Considered to be an extremely proliferative tissue, the testis yields male germ cells in response to this hormonal stimulation.

Testis Structure

A trilayered capsule named the tunica encloses the testicular parenchyma, which is comprised of seminiferous tubules and interstitial tissue (Figure 1.1). The seminiferous tubules are a series of convoluted tubules in which *spermatogenesis*, the process of formation of male gametes, takes place. The tubules are encircled by the basal lamina, leading into the seminiferous epithelium. The epithelium is a stratified structure; in the adult, it is made up of non-proliferating Sertoli cells and proliferating germ cells. A cross-section of a tubule shows concentric circles of successive germ cell generations (Figure 1.1).

The most immature spermatogonia are found closest to the basal lamina, while the more mature germ cells are found in the following order as they are positioned inward from the basal lamina to the inside of the tubule: spermatogonia, spermatocytes, spermatids and immature spermatozoa.

The only type of somatic cell found within the seminiferous tubule is the Sertoli cell. Distributed randomly along the basal epithelium, Sertoli cells form a non-dividing stable population of cells in the mature testis. These cells play a central role in nourishing germ cells, in addition to compartmentalizing the seminiferous epithelium by dividing the tubule into the basal and adluminal regions to form the blood-testis barrier (Figure 1.1) (reviewed by Hutson, 1997). The intimate contact between Sertoli cells and germ cells is critical to germ cell maturation; between 30-50 germ cells, all at various stages of development, are in contact with a given Sertoli cell (reviewed by Siu and Cheng, 2004). Other functions of Sertoli cells include the regulation of the spermatogenic cycle, the secretion of proteins, the mediation of hormonal effects, the secretion of fluid to form the tubular lumen, and the phagocytosis of residual bodies and damaged germ cells (reviewed in Sharpe, 1994; Griswold, 1995; Mruk and Cheng, 2004).

As for the connective interstitial tissue, it is mostly composed of testosterone-producing Leydig cells (~12%), as well as macrophages, lymphatic channels, nerves and blood vessels. Leydig cells not only contribute to the high levels of testosterone present in the testis, they are also thought to secrete oxytocin, which is involved in the peristaltic movement of seminiferous tubules (reviewed by Hutson, 1997). Most importantly, spermatogenesis absolutely requires the proper interaction of germ cells with the somatic cells of the testis (reviewed by Syed and Hecht, 2002; Saunders, 2003).

1.1.2.2 Spermatogenesis

Spermatogenesis is an elaborate process of cell proliferation and differentiation whereby fully differentiated spermatozoa are produced starting from mitotically-dividing spermatogonial stem cells (Figure 1.2). In mammals, this process continues throughout life.

Spermatogenesis can be broken down into three distinct phases - proliferation, meiosis and spermiogenesis – each involving a unique class of germ cells that are coordinately arranged in the seminiferous epithelium (Figure 1.2 A) (reviewed by Bellvé *et al.*, 1977b). Germ cells mature and progress from the periphery of the tubule towards the lumen, passing through all of these phases. Incomplete cytokinesis following mitosis and meiosis causes all the spermatids derived from a single spermatogonium to be linked via intercellular bridges; they remain so associated until the end of differentiation.

The initial phase of spermatogonial proliferation and stem cell renewal takes place in the basal compartment. Although the mechanisms of stem cell renewal remain controversial, there are currently two hypotheses that predominate: the *reserve stem cell theory* by Clermont and Bustos-Obregon (1968), and the *single stem cell theory* by Huckins (1971) and Oakberg (1971) (reviewed by de Rooij and Russell, 2000). Common to both theories is the existence of a subset of stem cells which is less vulnerable to insult due to their capacity to remain quiescent for prolonged periods of time.

Spermatogonia are mainly categorised according to the amount of chromatin lying along the nuclear envelope. Rapidly mitotically dividing type A spermatogonia sit at the base of the epithelium with Sertoli cells. The type A spermatogonia further divide and mature into intermediate and type B spermatogonia. Commitment to differentiation is associated with division of type B spermatogonia into preleptotene spermatocytes, which undergo one last round of DNA replication before entering meiosis. Meiosis is characterized by two reductive divisions of spermatocytes to produce haploid spermatids. In brief, primary spermatocytes undergo the lengthy process of meiosis I during which they go through the same stages as seen for the female. In the male, however, pachytene is by far the longest stage of all, lasting about one week. The first reductive division ends with the formation of secondary spermatocytes, short-lived cells that quickly enter the second reductive division to form haploid spermatids.

Spermiogenesis is the culminating point of spermatogenesis, whereupon spermatids undergo a series of cytological changes during which they acquire the unique morphological characteristics of spermatozoa. In the mouse, the spermiogenic process includes sixteen precisely timed steps that allow for nuclear condensation and elongation, acrosome formation, excess cytoplasm removal (shedding of the residual body) and the development of the flagellum (Figure 1.2 B). Extensive chromatin remodelling occurs during spermiogenesis to allow for the tightly packed chromatin configuration characteristic of the spermatozoon. DNA packaging is facilitated by the replacement of histones with transition proteins and protamines (reviewed by Sassone-Corsi, 2002; Dadoune *et al.*, 2004). Due to these changes in chromatin structure, the global transcription machinery shuts down during mid-spermiogenesis. Thus, post-meiotic cells either synthesize and store important proteins prior to termination of transcription, or store the transcripts encoding these proteins until a later time when they can be translated (reviewed in Sassone-Corsi, 1997; Hecht, 1998). In the final steps of spermiogenesis, the intercellular bridges maintaining communication between germ cells are finally broken off, as excess cytoplasm is released in the form of residual bodies. Sperm are then liberated into the fluid-filled lumen and transported to the epididymis, where they acquire the capacity to fertilize an ovum. In all, the spermatogenic process lasts approximately 35 days in the mouse (Oakberg, 1956).

The Stages of Spermatogenesis

Within the seminiferous epithelium, different generations of germ cells are found together in specific cellular associations named *stages*. Because of the precise and regular timing of spermatogenesis, spermatids at a specific step in spermiogenesis are always associated with the same types of spermatogonia and spermatocytes (reviewed by Russell *et al.*, 1990). Even in the prepubertal testis, the coordinate entry of spermatogonia into meiosis is time-dependent. In the mouse, twelve distinct stages, or cellular associations, are observed and any given germ cell passes through all 12 stages about 4.5 times (Figure 1.2 B). By

definition, at any one point in a seminiferous tubule, progression to the next stage involves the synchronous maturation of each gem cell type to the succeeding step of development; this is rendered possible via the intercellular bridges that link germ cells.

Hormonal Requirements of Spermatogenesis

As previously mentioned, the testis produces male germ cells in response to stimulation by hormones such as testosterone and FSH. In mice, FSH appears dispensable to sperm production, as FSH-deficient mice are fertile albeit with reduced testis size (Kumar *et al.*, 1997). Administration of FSH to neonatal mice stimulates spermatogenic production due to increased Sertoli cell proliferation, but as rodents age, spermatogenesis becomes increasingly dependent on testosterone (Singh and Handelsman, 1996; reviewed in Zirkin, 1998). Testosterone, produced by Leydig cells in response to LH, is found in high local concentrations in the testis. This androgen promotes the adhesion of spermatids to Sertoli cells, favouring their maturation; consequently, testosterone is required for spermatogenesis and, more so, fertility (Cameron and Muffly, 1991; Sharpe, 1994; Perryman *et al.*, 1996).

1.1.3 Oocyte Growth and Maturation

The highly specialized and regulated process by which an oogonium differentiates into a mature egg capable of being fertilized by sperm and competent to support embryo development is referred to as *oogenesis* (Figure 1.3). Although the outcome of oogenesis and spermatogenesis is the same – the creation of haploid gametes – these two processes differ in many respects. For example, oogenesis is characterized by a period of meiotic arrest, while spermatogenesis happens in an uninterrupted sequence. Also, the male produces four haploid spermatids from one primary spermatocyte, whereas the female produces only a single mature egg (and two smaller polar bodies) from one oocyte due to unequal cytokinesis. Exploring the uniqueness of oogenesis

goes far beyond the scope of this thesis, therefore only an overview of some of the key events will be provided.

Oocyte Growth

By 5 dpp, oocytes are found arrested at the dictyate stage, at which point in time they measure between 10 to 20 μm in diameter. Although oocytes undergo prolonged meiotic interruption until they are stimulated to resume meiosis, they are not quiescent. Oocytes experience a period of dramatic growth lasting two to three weeks in the mouse, during which the oocyte grows from 20 to 80 μm in diameter (excluding the zona pellucida). Entry into the growth phase appears to be triggered by the size of the pool of non-growing oocytes (Wassarman and Albertini, 1994). A number of structural changes characterize the growth phase, such as an enlargement in nucleus and nucleolus diameter, an increase in the number of mitochondria, ultra-structural changes in both mitochondria and Golgi complex, the accumulation of ribosomes, in addition to the formation of cortical granules and the zona pellucida (Mehlmann *et al.*, 1995). These structural changes are also accompanied by an increase in overall RNA and protein synthesis.

Folliculogenesis

Oocyte growth is associated with folliculogenesis, the proliferation and differentiation of the follicular cells that surround the oocyte. Oocyte development is dependent on these support cells (Eppig, 1992). A non-growing oocyte enclosed in a single layer of flattened cells is referred to as a primordial follicle. As the oocyte grows, it becomes surrounded by several layers of granulosa cells, distinguished by their cuboidal shape, to form the secondary follicle. A preantral follicle is characterized by both inner and outer layers of differentiated follicle cells that surround the oocyte-granulosa cell complex. Until the preantral stage, both oocyte and follicular growth are independent of hormonal regulation. Stimulation by FSH and LH is required for further development of the follicle and formation of the antrum, the fluid-filled cavity characteristic of antral or Graafian follicles

(Eppig, 1985). LH stimulation also prompts the resumption of meiosis and the completion of oocyte development.

Meiotic Maturation

The transition from a fully grown oocyte arrested at the dictyate stage of prophase I to an ovulated unfertilized MII egg is referred to as meiotic maturation. The capacity to resume meiosis is acquired during oocyte growth, as it is related to oocyte diameter; thus, mouse oocytes measuring more than 60 μm are competent to do so.

Germinal vesicle breakdown (GVBD) commences the process of meiotic resumption, whereby the membrane surrounding the nucleus is dissolved. Chromatin condensation, separation of homologous chromosomes and extrusion of the first polar body by asymmetric cleavage of the ooplasm follow. The ovulated oocyte then undergoes its second meiotic arrest and blocks at meiosis II (MII); completion of meiosis and extrusion of the second polar body occurs upon fertilization (Wassarman *et al.*, 1979).

In the next section, the nature of DNA methylation pattern establishment and maintenance in the germ line will be discussed. The first reports examining genome-wide DNA methylation differences in germ cells were published in the 1980s and identified the sperm genome as being more highly methylated than that of the oocyte (Monk *et al.*, 1987; Sanford *et al.*, 1987).

1.2 DNA Methylation

1.2.1 Roles of DNA Methylation in the Mammalian Genome

DNA methylation consists in the covalent addition of a methyl group to the 5' carbon of the cytosine ring within the context of a CpG dinucleotide to form 5-methylcytosine (Figure 1.4). While it is commonly accepted that DNA encompasses only four bases (adenine, cytosine, guanine and thymine), 5-methylcytosine is often regarded as a fifth base because of the additional degree

of information it confers. Methylation of DNA occurs throughout the genome following DNA replication and is both reversible and heritable (Bestor and Tycko, 1996). Unmethylated CpGs are mostly found in CpG islands, short sequences relatively rich in C + G (>55%) and associated with the promoter region of genes (Goll and Bestor, 2005). DNA methylation is generally associated with transcriptional repression as well as genome organization and stabilization. As mentioned previously, it is also associated with a number of specialized biochemical processes, such as allele-specific gene expression (genomic imprinting) and X-chromosome inactivation (Goll and Bestor, 2005).

Regulation of Gene Expression

DNA methylation was first postulated to be involved in regulating gene expression in 1975 by Holliday and Pugh. It was later established that methylation of cytosines in the promoter region of a gene is generally associated with transcriptional repression. A number of mechanisms are thought to function to regulate gene expression through DNA methylation. Accessibility of target sequences can be directly blocked by cytosine methylation, preventing binding of transcription factors. Gene expression can also be repressed through several methyl-CpG-binding proteins that recognize and "read" methylation patterns. For instance, transcription can be repressed in a methylation-dependent manner via formation of a complex between the methyl-binding protein MeCP2, histone deacetylases (HDACs) and a co-repressor protein, Sin3a (Nan *et al.*, 1998; Jones *et al.*, 1998). MBD2, another methyl-CpG-binding protein, forms a complex with NuRD, a multisubunit complex containing an ATP-dependent chromatin-remodeling protein, Mi-2, and HDACs (Zhang *et al.*, 1999; Wade *et al.*, 1999); the MBD2-NuRD complex, formerly known as MeCP1, represses transcription and remodels methylated chromatin with high efficiency (Ng *et al.*, 1999; Feng and Zhang, 2001). Finally, DNA methyltransferases have also been shown to directly interact with histone deacetylases and are able to repress transcription through this association (reviewed by Burgers *et al.*, 2002).

Genome Stability

Repetitive parasitic DNA elements, such as endogenous transposable elements, ALU elements and LI elements, make up a large part of the mammalian genome (up to 40%). Promoter activation of some of these elements can result in their random transposition and integration into the surrounding area, leading to the induction of new mutations and the deregulation of neighbouring genes. Interestingly, the majority of methylated CpG dinucleotides are found within these regions of "junk DNA" (Yoder *et al.*, 1997b). It has been postulated that DNA methylation evolved to protect the genome from such sequences and ensure their inactivity (Smit, 1996; Yoder *et al.*, 1997b; Walsh *et al.*, 1998). DNA methylation within the promoter region of these elements could function in interfering with the expression of the genes necessary for their transposition. Methylation of DNA also participates in maintaining genomic integrity by ensuring chromosome stability. Proper methylation of satellite sequences localized to the centromere is thought to play an important role in centromere function and kinetochore assembly (Viegas-Pequignot and Dutrillaux, 1976; O'Neill *et al.*, 1998; Xu *et al.*, 1999).

1.2.2 The DNA Methyltransferases

DNA methyltransferases (DNMTs) are the enzymes involved in the transfer of a methyl group donated by the cofactor S-adenosylmethionine (SAM) to the 5'-carbon of cytosines within CpG dinucleotides. In mammals, three families of DNMTs have been described and are classified according to similarities found in their C-terminal catalytic domains (Figure 1.5) (Goll and Bestor, 2005). The predominant mammalian DNA methyltransferase is DNMT1, although four other enzymes, including DNMT2 (Yoder and Bestor, 1998), DNMT3a, DNMT3b (Okano *et al.*, 1998) and more recently DNMT3L (Aapola *et al.*, 2001; Bourc'his *et al.*, 2001), have been characterized. Of these, only DNMT1, DNMT3a and DNMT3b are thought to be capable of methylating DNA *in*

vivo; nonetheless, recent reports suggest that DNMT2 might have residual methylation activity (Hermann *et al.*, 2003; Goll *et al.*, 2006).

Three types of enzymatic activities are required to erase, establish and perpetuate DNA methylation patterns: demethylation, *de novo* methylation and maintenance methylation. Demethylation can occur passively if methylation is not maintained at the time of replication, or it can come about actively by some yet unidentified mechanism *in vivo*. *De novo* methylation refers to the addition of methyl groups by DNMTs on an unmethylated substrate; this occurs mainly within CpG dinucleotides (i.e. the 3' carbon atom of the cytosine is linked by a phosphodiester bond to the 5' carbon atom of the guanine). DNMTs with maintenance activity are required to ensure the accurate propagation of DNA methylation patterns at the time of cell division by faithfully copying the methylation status of the mother strand onto the daughter strand after replication.

All of the known mammalian DNA methyltransferases share similarities in their C-terminal catalytic domain, characterized by the 10 conserved amino acid motifs implicated in their catalytic function (reviewed by Bestor, 2000; Hermann *et al.*, 2004; Goll and Bestor, 2005). In addition, DNMT1, DNMT3a and DNMT3b contain large N-terminal regulatory domains (Figure 1.5). The mammalian DNMTs show little evidence of specificity for selected DNA sequences and thus how and why certain sequences become methylated has been a longstanding question in the field (for review, see Goll and Bestor, 2005). An equally important question is what prevents certain sequences from becoming methylated at different times. Recent results suggest that methylation of DNA at specific sites is the result of multiple inputs including the presence of repeat sequence elements in the DNA, interactions between RNA and DNA and histone modifications; precisely how these different mechanisms interact in germ cells to target specific sequences for methylation is unknown and an important area for future studies. Mouse gene-targeting studies have been particularly useful in shedding light on the biological functions of the different DNMTs (summarized in table 1.1). In the following section, current knowledge on the DNMTs, including

their expression in germ cells and early embryos, as well as the reproductive consequences of their disruption, are discussed.

DNMT1

The first identified and perhaps best characterized mammalian DNA methyltransferase, DNMT1, was purified and cloned from mouse cells in the late 1980's (Bestor *et al.*, 1988); to date, it remains the sole DNA methylating enzyme to have been identified by means of biochemical purification. Earlier studies showed DNMT1 to prefer hemimethylated DNA (Bestor, 1992; Yoder *et al.*, 1997a), which caused it to be assigned a function in maintenance methylation. Although its activity on unmethylated DNA substrates is greater than that of DNMT3a and DNMT3b, the postulated *de novo* methyltransferases (Okano *et al.*, 1998), it remains unresolved whether DNMT1 has *de novo* methylation activity *in vivo*.

Expression of the mouse *Dnmt1* gene is controlled by the use of sex-specific exons and the use of alternative splicing to produce two major protein products, DNMT1 and DNMT1o, as well as an untranslated transcript, Dnmt1p (Mertineit *et al.*, 1998). The full-length somatic form originally characterized by Bestor and colleagues, DNMT1, is produced from exon 1s (Bestor *et al.*, 1988). Initiation of transcription through exon 1o results in a degradation-resistant form of DNMT1 only expressed in mouse oocytes (Carlson *et al.*, 1992; Mertineit *et al.*, 1998). This oocyte-specific form, DNMT1o, lacks the first 118 amino acids of the N-terminus of the somatic form and accumulates at very high levels in non-cycling oocytes and preimplantation embryos. In fact, DNMT1o is the only form of DNMT1 detected in oocytes following birth, coincident with their entry into the growth phase (Mertineit *et al.*, 1998; Howell *et al.*, 2001; Ratnam *et al.*, 2002). A tissue-dependent differentially methylated region (T-DMR) has recently been found in the 5' region of *Dnmt1o,* but not in that of Dnmt1s/1p; the *Dnmt1o* T-DMR is completely methylated in all tissues examined except for oocytes and early developing embryos (Ko *et al.*, 2005). Production of Dnmt1p, a non-translated mRNA transcript only expressed in pachytene spermatocytes, is

initiated from exon 1p (Trasler *et al.*, 1992; Mertineit *et al.*, 1998). Monitoring of the translational status of this transcript as well as sequence analyses show that production of active DNMT1 is highly improbable and expression of this transcript correlates with the fall in DNMT1 content in maturing pachytene spermatocytes (Trasler *et al.*, 1992; Mertineit *et al.*, 1998).

DNMT1 is the major methyltransferase in all somatic tissues, but levels of *Dnmt1* mRNA are higher in the testis and the ovary than in any other adult tissue (Trasler *et al.*, 1992). More so, DNMT1 expression is tightly regulated throughout both spermatogenesis and oogenesis (Trasler *et al.*, 1992; Benoit and Trasler, 1994; Jue *et al.*, 1995; Mertineit *et al.*, 1998; Sakai *et al.*, 2001; Howell *et al.*, 2001; Ratnam *et al.*, 2002). In the male, a detailed developmental study has shown that DNMT1 can be found throughout the nucleoplasm in proliferating spermatogonia (Jue *et al.*, 1995). Entry into meiosis coincides with the formation of DNMT1 nuclear foci only detected in leptotene/zygotene spermatocytes, after which point the presence of DNMT1 gradually decreases to become completely absent at pachynema; following meiosis, DNMT1 remains undetectable (Jue *et al.*, 1995; Mertineit *et al.*, 1998). As mentioned previously, down-regulation of DNMT1 protein is linked to the expression of Dnmt1p transcript in pachytene spermatocytes (Trasler *et al.*, 1992; Jue *et al.*, 1995; Mertineit *et al.*, 1998). DNMT1 is predicted to play a role in maintaining DNA methylation patterns in the germ line; whether it plays other functional roles not directly linked to its DNA methyltransferase activity, such as in early meiotic cells, is currently unknown.

The importance of DNA methylation in embryonic development was first demonstrated by gene targeting of *Dnmt1* (Li *et al.*, 1992). Homozygous targeted partial ($Dnmt^{n/n}$ and $Dnmt^{s/s}$) and complete ($Dnmt^{c/c}$) loss-of-function mutations in *Dnmt1* cause growth retardation and mid-gestational lethality; the $Dnmt^{c/c}$ mice display only ~5% of wild-type DNA methylation levels (Li *et al.*, 1992; Lei *et al.*, 1996). In addition, biallelic expression of imprinted genes (Li *et al.*, 1993), ectopic X-chromosome inactivation (Panning *et al.*, 1996), reactivation of normally silent IAP sequences (Walsh *et al.*, 1998) and increased levels of apoptosis (Li *et al.*, 1992) are all induced by *Dnmt1* deficiency in embryos.

Based on the high levels of DNMT1 in the testis and evidence of tight regulation during male germ cell development, one could predict that *Dnmt1* inactivation would also affect spermatogenesis; because targeted disruption of *Dnmt1* is embryonic lethal, germ cell-specific inactivation of *Dnmt1* or knock-down approaches will be required to determine the precise role(s) of DNMT1 in male germ cells.

DNMT3a and DNMT3b

The mammalian genome encodes two functional cytosine methyltransferases of the DNMT3 family, DNMT3a and DNMT3b, which are more closely related to multispecies DNMTs than to DNMT1 or DNMT2 (Bestor, 2000; Goll and Bestor, 2005). They are postulated to function primarily as *de novo* methyltransferases, without any sequence specificity beyond CpG dinucleotides (Okano *et al.*, 1998). DNMT3a and DNMT3b are encoded by two different essential genes and are highly expressed in undifferentiated embryonic stem (ES) cells but their expression is down-regulated upon differentiation (Okano *et al.*, 1998 and 1999); genetic evidence indicates that the functions of DNMT3a and DNMT3b are distinct (Okano *et al.*, 1999).

Organization of the *Dnmt3a* and *Dnmt3b* genes is complex, as is the regulation of their expression (Okano *et al.*, 1998; Chen *et al.*, 2002; Weisenberger *et al.*, 2002; Ishida *et al.*, 2003). Originally, several tissue-specific transcripts of *Dnmt3a* were identified by Northern Blot analysis (Okano *et al.*, 1998), pointing to the possible existence of more than one protein product. It was later determined by Chen and colleagues (2002) that two distinct proteins originate from the *Dnmt3a* gene, DNMT3a and DNMT3a2. DNMT3a is the full-length protein initially characterized and shown to possess *de novo* methylation activity (Okano *et al.*, 1998), while DNMT3a2 is encoded by a transcript initiating from a downstream intronic promoter and is a shorter protein product lacking the first 219 N-terminal amino acids residues of DNMT3a (Chen *et al.*, 2002). Although their cytosine methyltransferase activity is very similar *in vitro*, they display different subcellular localization patterns suggesting that while DNMT3a

associates with heterochromatin, DNMT3a2 associates with euchromatin (Chen *et al.*, 2002).

Several different transcripts of the *Dnmt3b* gene, resulting from alternative splicing of exons 11, 22 and/or 23, have also been reported (Okano *et al.*, 1998; Aoki *et al.*, 2001; Chen *et al.*, 2002; Weisenberger *et al.*, 2004). In all, there are potentially eight different splicing variants, each producing a slightly different protein product. Only DNMT3b1 and DNMT3b2 are capable of methylating DNA and are presumably both *de novo* methyltransferases, whereas DNMT3b3 appears unable of transferring methyl groups, despite its ability to bind DNA (Okano *et al.*, 1998; Aoki *et al.*, 2001; Weisenberger *et al.*, 2004). The other isoforms are most likely inactive, as they lack some of the conserved motifs conferring catalytic activity, but their specific roles remain elusive (Chen *et al.*, 2002; Weisenberger *et al.*, 2004); some have been postulated to act as negative regulators of DNA methylation (Saito *et al.*, 2002).

Dnmt3a and *Dnmt3b* are highly expressed in undifferentiated ES cells and, to a lesser extent, in a range of adult tissues (Okano *et al.*, 1998). They are also expressed in a stage- and cell-specific manner during embryogenesis, possibly reflecting distinct functions during embryonic development (Okano *et al.*, 1998 and 1999; Watanabe *et al.*, 2002). Immunostaining studies indicate that DNMT3a2 is present in the nuclei of prenatal gonocytes at the time when methylation patterns are initially laid down in the male, whereas DNMT3a expression is restricted to the nuclei of Sertoli cells and surrounding tissues at all stages examined (Sakai *et al.*, 2004); postnatal expression of DNMT3a has also been detected but appears to be restricted to type B spermatogonia (Watanabe *et al.*, 2004). DNMT3b is expressed at low levels in prenatal gonocytes (Sakai *et al.*, 2004) and expressed at higher levels specifically in type A spermatogonia after birth (Watanabe *et al.*, 2004). Certain transcript variants of Dnmt3b have been reported to be expressed in the testis (Chen *et al.*, 2002; Watanabe *et al.*, 2004), but their specific nature or role is still unclear. As for *Dnmt1*, detailed developmental studies are needed to fully understand these enzymes in the context of male germ cell development.

Some of the key functions of DNMT3a and DNMT3b have been identified by gene-targeting experiments. DNMT3a-deficient mice survive to term but are underdeveloped, die 3-4 weeks after birth and have impaired spermatogenesis; their global methylation levels appear normal (Okano *et al.*, 1999). Inactivation of *Dnmt3b* has more deleterious consequences, resulting in a more severe, mid-gestation embryonic lethal phenotype with demethylation of minor satellite repeats (Okano *et al.*, 1999). Combination of DNMT3a and DNMT3b deficiencies act synergistically, where embryos fail to develop past gastrulation and show global demethylation of their genomes (Okano *et al.*, 1999). Conditional inactivation of *Dnmt3a* in germ cells has revealed the crucial role it plays in establishing *de novo* methylation patterns in male germ cells, more specifically at paternally imprinted loci (Kaneda *et al.*, 2004). Deletion of the *Dnmt3a* gene in male germ cells causes demethylation of the differentially methylated regions (DMRs) of *H19* and *Gtl2-Dlk1* but does not affect the methylation of *Rasgrf1* (Kaneda *et al.*, 2004). Whether the demethylation defect is restricted to the DMRs of imprinted genes or affects other regions of the genome is still unknown. Germ cell-specific inactivation of *Dnmt3b* does not produce any apparent phenotype; viable offspring are produced from these *Dnmt3b* conditional mutants and the methylation status of all analyzed sequences is not perturbed (Kaneda *et al.*, 2004).

DNMT3B in ICF syndrome

DNMT3B is the only DNA methyltransferase gene associated with a human disease. Various mutations in *DNMT3B* cause a human autosomal recessive genetic disorder characterized by immunodeficiency, centromeric instability and facial anomalies known as ICF syndrome (Xu *et al.*, 1999). Cytogenetic abnormalities affecting predominantly the pericentric regions of chromosomes 1, 9 and 16 are observed in ICF patients; these regions contain a type of satellite DNA that is normally methylated but is almost completely demethylated in the DNA of ICF patients (Jeanpierre *et al.*, 1993). Demethylation of CpG islands on the inactive X has also been reported in ICF patients (Kondo *et*

al., 2000). None of the patients reported are homozygous for null alleles of *DNMT3B*, suggesting that complete loss of function of DNMT3B is lethal, as is seen in the mouse model (Okano *et al.*, 1999). A specialized role in methylation of certain repeated sequences and CpG islands on the inactive X chromosome can thus be ascribed to DNMT3B. Effects on fertility have not been reported in patients with ICF syndrome.

DNMT3L

DNMT3L (DNA methyltransferase 3-Like) is related to DNMT3a and DNMT3b in both N- and C-terminal domains but lacks some of the key amino acid residues conferring catalytic activity (Figure 1.5) (Aapola *et al.*, 2000 and 2001; Bourc'his *et al.*, 2001; Hata *et al.*, 2002). DNMT3L does not possess methyltransferase activity, but genetic studies have demonstrated its importance for the establishment of a subset of methylation patterns in both male and female germ cells (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Webster *et al..*, 2005). *In vitro* and *in vivo* studies suggest that DNMT3L interacts with DNMT3a and/or DNMT3b to stimulate their methyltransferase activity, implying that DNMT3L could by a cofactor to both proteins and a stimulator of *de novo* methylation (Chédin *et al.*, 2002; Hata *et al.*, 2002; Margot *et al.*, 2003; Suetake *et al.*, 2004).

The mouse *Dnmt3L* gene was identified *in silico* based on its homology with *Dnmt3a* and *Dnmt3b* (Aapola *et al.*, 2001). Recent studies have described the promoter region of *Dnmt3L*, characterized by a TATA-less and CpG-rich minimal promoter (Aapola *et al.*, 2004). This genomic organization is reminiscent of the human *DNMT3A* and *DNMT3B* genes, which were shown to contain several promoters that are either CpG-rich or CpG-poor and typically lacking TATA consensus sequences (Yanagisawa *et al.*, 2002). Methylation analysis of the *Dnmt3L* promoter region showed that in a highly expressing tissue such as the testis, all CpG sites studied are fully unmethylated (Aapola *et al.*, 2004).

Like other DNMT family members, *Dnmt3L* is highly expressed in ES cells, but its expression becomes restricted to a few tissues upon differentiation, namely the chorion of embryonic day (E) 7.5 and E8.5 embryos and the gonads

(Aapola *et al.*, 2001; Bourc'his *et al.*, 2001; Hata *et al.*, 2002). Activity of a reporter gene controlled by the endogenous *Dnmt3L* promoter suggests DNMT3L is only present in pre- and peri-natal prospermatogonia and that expression of DNMT3L in spermatogonia is for the most part extinguished by 6 days post-partum (Bourc'his *et al.*, 2001; Bourc'his and Bestor, 2004; Webster *et al.*., 2005). However, using a similar approach, Hata and colleagues (2002) also detect DNMT3L in differentiating spermatocytes of newborn and adult mice. In the female, *Dnmt3L* expression appears to be restricted to growing oocytes (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Lucifero *et al.*, 2004b). Immunostaining studies will prove decisive in determining the exact endogenous expression and localization of DNMT3L in male germ cells.

The central role played by *Dnmt3L* in reproduction was clearly demonstrated by gene-targeting studies. While homozygous *Dnmt3L* null mice are viable, both males and females are sterile (Bourc'his *et al.*, 2001; Hata *et al.*, 2002). Homozygous oocytes can be fertilized but the resulting heterozygous progeny die at mid-gestation, showing demethylation of DMRs of maternally methylated imprinted genes and corresponding biallelic expression of normally paternally expressed imprinted genes (Bourc'his *et al.*, 2001; Hata *et al.*, 2002). The reproductive impact of DNMT3L deficiency is different in males, as they suffer from hypogonadism and are azoospermic (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Webster *et al.*., 2005). Seminiferous cords appear normal at 1 week of age postnatally but there are few differentiated spermatocytes in the testes of *Dnmt3L*-deficient mice by 4 weeks (Hata *et al.*, 2002; Webster *et al.*., 2005). Targeting of the *Dnmt3L* gene causes partial demethylation of the *H19* and *Rasgrf*1 DMRs (Bourc'his and Bestor, 2004; Webster *et al.*, 2005), while it clearly prevents methylation of both LINE-1 and IAP elements, leading to their massive transcription in spermatogonia and spermatocytes (Bourc'his and Bestor, 2004). While DNMT3L is dispensable for female meiosis, abnormal synapsis accompanies DNMT3L deficiency in the male, triggering an apoptotic checkpoint that prevents spermatocytes from progressing to pachynema (Bourc'his and Bestor, 2004).

1.2.3 DNA Methylation in the Germ Line

Experimental evidence in mice suggests that genomic methylation patterns inherited from the parental gametes are erased upon entry into the reproductive cycle, re-established in immature germ cells according to their fate as either male or female gametes and further consolidated and maintained during early embryonic development, giving rise to methylation profiles that will be preserved throughout adulthood (Figure 1.6).

1.2.3.1 Dynamics and Timing of DNA Methylation Programming during Gametogenesis and Early Embryogenesis

Erasure of Methylation Patterns

The founding cells of the germ line, the primordial germ cells (PGCs), are thought to carry full complements of parental methylation profiles when they begin migrating towards the genital ridge. Upon their entry into the genital ridge, around 10.5 days of gestation, they undergo extensive genome-wide demethylation. Early studies employing methylation-sensitive restriction enzymes and Southern Blot and PCR approaches indicated that PGCs have completely demethylated genomes by 13.5 days of gestation (Monk *et al.*, 1987; Chaillet *et al.*, 1991; Kafri *et al.*, 1992; Brandeis *et al.*, 1993). Recent work done using bisulfite sequencing to examine the methylation status of imprinted genes and repetitive sequences has helped better define the timing and sequence-specificity of these early epigenetic events in PGCs. A number of imprinted genes, including *Peg3*, *Kcnq1ot1* (also known as *Lit1)*, *Snrpn*, *H19*, *Rasgrf1* and *Gtl2*, as well as non-imprinted genes such as *α-actin*, were shown to become demethylated between 10.5 and 13.5 days of gestation (Hajkova *et al.*, 2002; Li *et al.*, 2004). However certain sequences (at least some repetitive elements) appear to be treated differently: IAP, LINE-1 and minor satellite sequences are only subject to partial demethylation, whereas most imprinted and single-copy

genes become demethylated (Hajkova *et al.*, 2002; Szabo *et al.*, 2002; Lane *et al.*, 2003; Lees-Murdock *et al.*, 2003).

Assessing the expression of imprinted genes has also been used by investigators to ascertain the state of epigenetic reprogramming of germ cells. Monoallelic expression would be expected prior to erasure of epigenetic marks on imprinted genes in PGCs, while biallelic expression would be an indication of ongoing or complete erasure of these marks. Using this approach, Szabo and colleagues (2002) found monoallelic expression of the four imprinted genes examined, including *H19* and *Snrpn*, in PGCs at 9.5 days of gestation. In contrast, *Snrpn* was biallelically expressed at day 10.5 as were the other genes examined by day 11.5. Consistent with the DNA methylation data this expression study points to the time of entry into the genital ridge as the period when demethylation of imprinted genes takes place in PGCs. Another approach used to examine epigenetic reprogramming in the early germ line has been analysis of embryos derived from somatic cell nuclear transfer (SCNT) using PGC nuclei (Lee *et al.*, 2002; Yamazaki *et al.*, 2003). Although no viable offspring were obtained when PGC nuclei were used for cloning experiments, careful examination of these embryos indicated that methylation imprints were being erased between 10.5 and 12.5 days of gestation (Lee *et al.*, 2002; Yamazaki *et al.*, 2003).

Overall, it appears that rapid and possibly active genome-wide erasure of methylation patterns takes place between 10.5-12.5 days of gestation, leaving PGCs of both sexes in an equivalent epigenetic state by embryonic day 13.5 (Szabo and Mann, 1995; Kato *et al.*, 1999; Hajkova *et al.*, 2002; Lee *et al.*, 2002; Szabo *et al.*, 2002; Li *et al.*, 2004).

Programming of the Male and Female Genomes

Following demethylation in PGCs, male and female gametes acquire sex- and sequence-specific genomic methylation patterns (Reik *et al.*, 2001). For non-imprinted genes and repeat sequences, DNA methylation can be assessed directly. For imprinted genes, determination of DNA methylation status and

assessment of mono- or biallelic expression of the genes of interest in the resulting embryos are used. Nuclear transplantation and cloning experiments using germ cell nuclei at different stages have also been used to study the timing of acquisition of methylation on imprinted genes by assessing postimplantation embryos for monoallelic expression of the genes of interest (Obata *et al.*, 1998; Bao *et al.*, 2000; Obata and Kono; 2002).

The timing of acquisition of methylation patterns differs greatly between the two germ lines. In the male, techniques like Southern Blotting and bisulfite genomic sequencing have shown that acquisition of DNA methylation patterns begins before birth, in prospermatogonia, and is completed for most of the sequences after birth, before the end of the pachytene phase of meiosis (Kafri *et al.*, 1992; Walsh *et al.*, 1998; Davis *et al.*, 1999 and 2000; Ueda *et al.*, 2000; Lees-Murdock *et al.*, 2003; Li *et al.*, 2004). Initial acquisition of methylation occurs between 15.5 and 18.5 days of gestation; at this time germ cells begin to stain positively with an antibody directed against methylated cytosine, indicating increases in overall genomic methylation (Coffigny *et al.*, 1999). *H19*, a maternally expressed gene that becomes methylated in the male germ line (and remains unmethylated in oocytes) has been extensively characterized (Bartolomei *et al.*, 1991 and 1993; Ferguson-Smith *et al.*, 1993; Tremblay *et al.*, 1995; Davis *et al.*, 1999 and 2000; Ueda *et al.*, 2000). A DMR spanning a 2 kb region roughly 2-4 kb from the transcription start site of the gene begins to acquire its methylation between days 15.5 and 18.5 of gestation in prenatal male germ cells; methylation is completed postnatally by pachynema, persisting in sperm (Davis *et al.*, 1999 and 2000; Ueda *et al.*, 2000). Additional experiments demonstrated that the paternal alleles of *H19* become methylated prior to the maternally inherited alleles in male germ cells, suggesting that paternal alleles may "remember" their origin as methylated alleles (Davis *et al.*, 1999 and 2000).

Two additional imprinted genes in the mouse, *Gtl2* (Takada *et al.*, 2002) and *Rasgrf1* (Yoon *et al.*, 2002), have also been reported to be methylated on the paternally inherited allele. Their respective DMRs become progressively methylated between 12.5 and 17.5 days of gestation, but not to the extent seen in

mature sperm (i.e. fully methylated), suggesting ongoing postnatal *de novo* methylation as seen for *H19* (Li *et al.*, 2004). Other types of sequences are also known to become methylated in prospermatogonia: repetitive DNA elements such as LINE-1, IAP and minor satellite sequences are fully methylated by 17.5 days of gestation (Walsh *et al.*, 1998; Lees-Murdock *et al.*, 2003). Further proof that male germ cells have completely acquired their methylation prior to the haploid phase of spermatogenesis comes from studies using intracytoplasmic round spermatid and sperm injections (Shamanski *et al.*, 1999); expression of imprinted genes is similar in embryos derived from round spermatids as compared to embryos derived from epididymal spermatozoa. Unique to the male germ line is the fact that acquired methylation patterns also have to be maintained when DNA replication takes place. Male germ cells therefore possess both *de novo* and maintenance methylation activities (Figure 1.6B).

In contrast to the male, female germ cells only begin to acquire their gametic DNA methylation postnatally, following the pachytene phase of meiosis (Chaillet *et al.*, 1991; Ueda *et al.*, 1992; Brandeis *et al.*, 1993; Stoger *et al.*, 1993; Kono *et al.*, 1996; Walsh *et al.*, 1998; Lucifero *et al.*, 2002 and 2004b; Obata and Kono, 2002). DNA methylation analysis of imprinted genes and nuclear transplantation studies have both pointed to the oocyte growth phase as the time when functional imprints are acquired in the female (Bao *et al.*, 2000; Obata and Kono, 2002; Lucifero *et al.*, 2002 and 2004b).

Reprogramming in Early Embryos: Maintenance of DNA Methylation Patterns

A second genome-wide demethylation event occurs in the early embryo. Marks established on imprinted genes and some repeat sequences must be faithfully maintained during preimplantation development at a time when the methylation of non-imprinted sequences is lost (Figure 1.6). The male pronucleus faces a rapid, presumably active demethylation process within about four hours of fertilization (Mayer *et al.*, 2000; Oswald *et al.*, 2000; Santos *et al.*, 2002), while the maternal genome becomes demethylated more slowly, presumably through a passive process whereby maintenance methylation does

not take place following cell division (Howlett and Reik, 1991; Rougier *et al.*, 1998). Preservation of methylation at imprinted loci during the wave of preimplantation demethylation is postulated to be important for subsequent postimplantation embryo development, allowing for appropriate allele-specific, monoallelic expression of genes required for embryonic development (Olek and Walter, 1997; Tremblay *et al.*, 1997; Reik and Walter, 2001; Hanel and Wevrick, 2001). The overall methylation level of non-imprinted genes reaches a minimum at the blastocyst stage of preimplantation development, following which initiation of genome-wide *de novo* methylation takes place, coincident with the time of the first differentiation event after the fifth cell cycle (Santos and Dean, 2004).

1.2.3.2 DNA Methylation of Testis-Specific Genes

That DNA methylation could be an important mechanism for the regulation of tissue-specific gene expression was proposed a number of years ago (Riggs, 1975; Holliday and Pugh, 1975). The role of DNA methylation in the regulation of testis-specific gene expression has been the subject of a number of studies. In examples of early mouse studies, genes including transition protein 1, phosphoglycerate kinase-2, apolipoprotein A1, Oct-3/4, and lactate dehydrogenase C, were methylated in non-expressing tissues and became demethylated at some CpG sites prior to their expression (Trasler *et al.*, 1990; Ariel *et al.*, 1994; Bonny and Golberg, 1995; Kroft *et al.*, 2001). For a few testis-specific genes, increases in methylation precede expression. For example, some CpG sites in the coding region of mouse protamine 1 and the 5' upsteam region of mouse protamine 2, increases in methylation in meiotic prophase prior to expression in spermatids (Trasler *et al..*, 1990; Choi *et al.*, 1997). More recently, careful developmental time-course studies using bisulfite genomic sequencing on isolated germ cells have shown that a decrease in the methylation of the promoter region of phosphoglycerate kinase-2 is one of the earliest events, taking place before changes in chromatin structure, DNase I hypersensitivity, tissue-specific binding of factors to the enhancer region and transcriptional activation in meiotic male germ cells (Geyer *et al..*, 2004). Interestingly, phosphoglycerate

kinase-2 is remethylated during transit through the epididymis; it is currently the only gene whose methylation status has been examined for change between testicular and cauda epididymal sperm (Geyer *et al.*., 2004). The purpose of a remethylation event occurring in the epididymis is unclear at present since most DNA methylation patterns (except those at imprinted genes and retrotransposons) are removed from the paternal genome shortly after fertilization. Although more epididymal DNA methylation studies need to be done on other genes, it has been postulated that non-specific genome-wide alterations in DNA methylation might occur in the epididymis to prepare the sperm genome for post-fertilization and early developmental epigenetic events (Geyer *et al.*, 2004). Although there are a number of examples of testis-specific genes that show a correlation between hypomethylation and germ cell expression, it is presently unresolved whether alterations in DNA methylation associated with germ cell-specific expression of genes are a cause or consequence of expression (reviewed by MacLean and Wilkinson, 2005).

1.2.4 Germ Line Epigenetic Inheritance

1.2.4.1 Genomic Imprinting

The functional non-equivalence of the maternal and paternal genomes is underscored by the failure of uniparental embryos to develop normally, confirming the requirement of both parental genomes for normal development (reviewed by Reik and Walter, 2001). As previously mentioned, genomic imprinting regulates the expression of a subset of mammalian genes by restricting their expression to one of the parental alleles. Differential epigenetic marking of the parental alleles takes place during gametogenesis or in the first few divisions after fertilization; differential DNA methylation and chromatin structure, e.g. histone modifications and hypersensitivity sites, and asynchronous replication timing are common features of the maternally and paternally inherited alleles of imprinted genes (reviewed by Bartolomei and Tilghman, 1997; Reik *et al.*, 2001; Reik and Walter, 2001). Extensive studies

have unveiled some of the characteristic features shared by a subset of imprinted genes, namely genomic clustering, regulation by antisense transcripts and the presence of repeat elements near or within their DMR. The clustering of imprinted genes into large imprinted domains raises the possibility that a common imprinting mechanism may regulate an entire imprinted area, allowing coordinated regulation of genes in a given chromosomal region. Supporting this concept is the discovery that *cis*-acting imprinting control elements or imprinting centers (IC) have been found in some clusters and shown to have an impact on genes many kilobases away (Spahn and Barlow, 2003).

The H19 Subdomain: a Model for Paternally Inherited Imprinting Marks

A well characterized imprinted cluster is found on human chromosome 11p15.5, spanning a region of 1Mbase separated into two independent imprinting subdomains, the *H19* subdomain and the *KCNQ1OT1* subdomain (reviewed by Verona *et al.*, 2003); mouse distal chromosome 7 shows conserved genomic organization and imprinting of this cluster. The *H19* subdomain includes the *H19*, *IGF2* and *INS* genes; *H19* encodes a nontranslated RNA with exclusive maternal expression while *IGF2* is reciprocally imprinted and paternally expressed. Both the mouse and human *H19* genes exhibit paternal-specific methylation of a region located 2kb upstream of the *H19* promoter known as the DMR or the imprinting control region (ICR), and this DMR is thought to harbour the imprinting mark that distinguishes the parental alleles of *H19* (Bartolomei *et al.*, 1991; Tremblay *et al.*, 1995 and 1997; Frevel *et al.*, 1999). In the mouse, studies suggest the *H19* DMR acts as a methylation-sensitive insulator element in regulating the imprinting of *Igf2* by blocking promoter-enhancer interactions through binding of the methylation-sensitive insulator factor CTCF (Bell and Felsenfeld, 2000; Hark *et al.*, 2000; Kanduri *et al.*, 2000). Together, these data have led to the elaboration of the following model to account for reciprocal imprinting of H19 and Igf2 (Figure 1.7): on the maternal allele, binding of CTCF to the unmethylated DMR insulates the *Igf2* promoter from enhancer elements situated 3' of *H19*, allowing *H19* exclusive access to these elements and ensuring

maternal expression of *H19*. On the paternal allele, methylation of the DMR prevents binding of CTCF and formation of an insulator, resulting in paternal expression of *Igf2* by allowing access to downstream enhancers. Another imprinted cluster on human chromosome 14 and mouse chromosome 12 is reminiscent of this arrangement; the paternally expressed gene *Dlk* is flanked by a maternally expressed gene *Gtl2*, which is a non-coding RNA, and these genes contain DMRs that are reciprocally imprinted (Schmidt *et al.*, 2000; Takada *et al.*, 2000; Wylie *et al.*, 2000). The factors that regulate the establishment of parental imprints at these loci remain to be identified.

1.2.4.2 Epigenetic Modulators with Potential Reproductive Implications

Chromatin Modification and Remodelling Factors

Numerous types of chromatin modifying factors including histone modification enzymes have been described and are postulated to be involved in modulating transcription, X-chromosome inactivation, genome stability and chromosome events during meiosis (Hendrich and Bickmore, 2001; Goll and Bestor, 2002: Li, 2002). Chromatin modification and chromatin remodelling are the principal mechanisms by which global gene silencing and higher order chromatin structure are established and maintained. Discussed here are additional factors with chromatin remodelling or modifying activities that greatly influence genome stability and, perhaps, DNA methylation patterning during gametogenesis.

a) SWI2/SNF2 Remodelling Proteins

LSH (lymphoid-specific helicase) is a member of the SNF2 family of chromatin remodelers and is involved in mammalian development and cellular proliferation (Geiman *et al.*, 2001; Raabe *et al.*, 2001). More specifically, LSH is required for genome-wide methylation, normal histone methylation and formation of heterochromatin (Dennis *et al.*, 2001; Yan *et al.*, 2003). Targeted deletion of *Lsh* results in perinatal lethality with substantial loss of methylation throughout the

genome. Various sequences are affected by LSH deficiency, including repetitive elements such as satellite sequences, LINE-1s and IAPs, as well as single-copy genes, suggestive of a role for this enzyme in maintenance methylation during development (Dennis *et al.*, 2001). Recent studies indicate that LSH is specifically required to maintain the methylation status of the imprinted gene *Cdkn1c* but not of other imprinted genes such as *H19* or *Igf2r* during development, implying that LSH is only crucial to the maintenance of imprinting marks at specific loci (Fan *et al.*, 2005). So far, the only nonlymphoid tissue found to express *Lsh* at very high levels is the adult testis, both in mouse and human (Geiman *et al.*, 1998).

ATRX (*α*-thalassaemia/ mental retardation syndrome, X-linked) localizes to pericentromeric heterochromatin and to the short arms of human acrocentric chromosomes. It contains a PHD-like zinc-finger domain shared by the DNMT3 family members and a SNF-like helicase domain, suggesting a role in transcriptional regulation (McDowell *et al.*, 1999). Mental retardation, facial dysmorphism and *α*-thalassaemia result from mutations in the human *ATRX* gene. Patients suffering from the ATR-X syndrome present with specific changes in the pattern of methylation of several highly repeated sequences including hypomethylated rDNAs and hypermethylated Y-chromosome repeats (Gibbons *et al.*, 2000). More recently, ATRX was shown to be required for proper chromosome alignment and meiotic spindle organization in mouse metaphase II oocytes (De La Fuente *et al.*, 2004); it is currently unknown if this protein plays a similar role during spermatogenesis.

b) Histone Modification Enzymes

Modifications of histone tails by acetylation, phosphorylation and methylation have a fundamental role in gene regulation. Several classes of histone methyltransferases have been identified, one of which includes H3-K9 methyltransferases. Three members of this class are of particular interest: G9a, Suv39h1 (human homologue: *SUV39H1*) and Suv39h2. These histone methyltransferases appear to direct H3-K9 methylation to distinct chromatin

domains: G9a targets euchromatin while Suv39h1 and Suv39h2 target heterochromatic regions of the genome (Rice *et al.*, 2003). Gene-targeting studies in mice have shown *G9a* to be essential to normal development, *G9a*-deficient embryos showing a drastic decrease in H3-K9 methylation and severe growth retardation leading to embryonic lethality (Tachibana *et al.*, 2002). More recently, studies using mouse *G9a*-deficient ES cells have shown that the maintenance of CpG methylation at the Prader-Willi syndrome imprinting center requires the function of G9a (Xin *et al.*, 2003), while X-inactivation is properly maintained in *G9a*-deficient embryos (Ohhata *et al.*, 2004). The *Suv39h* genes display overlapping expression profiles during mouse embryonic development, but *Suv39h2* expression becomes restricted to the testis of adult mice (O'Carroll *et al.*, 2000). Peters and colleagues (2001) demonstrated that the Suv39h histone methyltransferases regulate H3-K9 methylation of pericentromeric heterochromatin. Combined disruption of the *Suv39h* genes severely impairs viability, induces chromosomal instabilities and causes sterility in males; spermatogenic failure largely results from nonhomologous chromosome pairing (Peters *et al.*, 2001). Interestingly, a recent report has directly linked Suv39h-associated H3-K9 methylation to DNMT3b-dependent DNA methylation at pericentric repeats (Lehnertz *et al.*, 2003). Together, these data demonstrate an evolutionarily conserved pathway between H3-K9 methylation and DNA methylation in mammals. Furthermore, they provide an attractive avenue to pursue to explain the role these proteins play during spermatogenesis.

1.2.4.3 Errors in Erasure, Acquisition or Maintenance of DNA Methylation

Imprinting Disorders

Loss of function of a number of imprinted genes has been linked to human genetic diseases, progression of certain cancers and has also been implicated in a number of neurological disorders. Some of the most extensively studied disorders involving imprinted genes are Prader-Willi syndrome (PWS), Angelman syndrome (AS), Beckwith-Wiedemann syndrome (BWS) and Russell-Silver

syndrome; the clinical features as well as the specific molecular determinants associated with these imprinting disorders will not be considered here (reviewed by Lucifero *et al.*, 2004a).

Diverse molecular events can lead to phenotypic abnormalities involving imprinted genes (reviewed by Jiang *et al.*, 2004). Generally, uniparental disomy can result in overexpression or underexpression of imprinted genes implicated in the control of fetal growth and postnatal development. Another general mechanism involves the deletion of one or more genes or point mutations affecting a specific locus. Epigenetic defects can also give rise to imprinting disorders, in which case a chromosome of one parental origin has an abnormal epigenetic status (DNA methylation, chromatin structure and gene expression), often that of the opposite parental origin. Mutations or deletions in the imprinting center can be associated with imprinting diseases, and this is particularly well characterized in the case of PWS and AS; these genetic events can have secondary epigenetic effects resulting in the *de novo* acquisition of epigenetic marks. Finally, imprinting defects can also be epigenetic in origin (gain or loss of imprinting at a specific locus) when no identifiable genetic defect is observed. Imprinting defects have the potential to arise at any step of epigenetic reprogramming and could result from problems with the enzymes responsible for erasing, establishing and maintaining imprints. Alternatively, the methylation status or chromatin structure may be affected by epigenetic insults leading to abnormal imprinted gene expression.

Of note, loss of imprinting is also involved in the progression of a number of cancers (Tycko and Morison, 2002). Tumours that show imprinting defects include Wilm's tumour, where loss of function of the maternal allele leading to the suppression of *H19* and biallelic expression of *IGF2* appears to be involved. Some imprinted genes act as tumour suppressor genes, the best characterized being *IGF2R* and *WT1*; in the case of imprinted tumour suppressor genes, inactivation of only one allele is required to cause loss of function, compared to non-imprinted tumour suppressor genes where loss of function requires mutation of both alleles.

Male Germ Cell Culture and Assisted Reproductive Technologies

Epigenetic marks, in particular DNA methylation patterns, tend to be unstable and amenable to modification by culture conditions and cellular manipulations. A number of animal studies have examined how different types of media or the addition of serum to culture media affect the methylation status and expression of imprinted genes of cultured preimplantation embryos (Doherty *et al.*, 2000; Khosla *et al.*, 2001); significant DNA methylation perturbations were found in both cases. Introduction of intracytoplasmic sperm injection (ICSI) has greatly improved chances to conceive for couples with previous fertilization failures using in vitro fertilization (IVF). The success of ICSI with freshly ejaculated spermatozoa has been extended to epididymal spermatozoa, and even to elongating and round spermatids (reviewed by Tsai *et al.*, 2000). It is unlikely that assisted reproductive technologies themselves, involving the use of haploid gametes, interfere with either erasure or acquisition of genomic imprints, as both processes appear to be complete by the spermatid phase of spermatogenesis.

Nonetheless, there is cause for concern when abnormal or more immature gametes are used in assisted reproductive technologies to overcome male infertility. In mice, multiple approaches have been developed to overcome spermatogenic arrest, including injection of secondary and primary spermatocyte nuclei into oocytes (Kimura and Yanagimachi, 1995; Kimura *et al.*, 1998; Sasagawa *et al.*, 1998). Upon electrical activation of the oocyte, oocyte and spermatocyte chromosomes complete meiosis (producing two pronuclei and two polar bodies) and participate in embryogenesis; but it appears that mouse primary spermatocytes nuclei complete meiosis with less efficiency than secondary spermatocytes. A number of reasons could explain poor embryo outcome: suboptimal culture conditions, unsuccessful DNA repair or incomplete paternal genomic imprint acquisition. Nevertheless, *in vitro* culture of spermatocytes has been proposed to overcome spermatogenic arrest in humans (Tesarik *et al.*, 1998 and 1999) and the first live births using both primary and

secondary spermatocytes have been reported respectively in 1999 (Tesarik *et al.*) and 1998 (Sofikitis *et al.*).

To date, there is only one report linking an imprinting defect to disruptive spermatogenesis. Marques *et al.*(2004) found that 17-30% of moderate to severe oligozoospermic (very low sperm counts) patients with an infertility diagnosis presented defective *H19* methylation, with most patients having normally methylated and hypomethylated alleles in the same semen sample; all patients presented complete erasure of the maternally methylated gene *MEST*. Since maternal imprints appear to be properly erased, abnormal genomic imprinting could indicate changes in DNA methyltransferase activity or DNA methylation modulating factors. Recent studies suggest a possible link between the increased incidence of imprinting disorders and human assisted reproductive technologies (reviewed by Lucifero *et al.*, 2004a). However, it is unclear whether the imprinting disorders are due to underlying infertility (i.e. that there exist epigenetic causes for infertility) or the techniques being used. Interestingly, a recent study reported an increased prevalence of imprinting defects in patients with Angelman syndrome born to subfertile couples; the findings suggest that imprinting defects and subfertility may have a common cause (Ludwig *et al.*, 2005). In light of the apparent instability of epigenetic marks, studies are required to look for epigenetic causes of infertility and examine the effects of culture conditions on spermatogenic cell maturation. These studies should provide an understanding of male germ cell epigenetic plasticity, as well as the safety of procedures used in assisted reproduction.

Epimutations

As mentioned previously, epigenetic changes can also play a major role in the development of human cancer. For example, a high proportion of patients with sporadic colorectal cancers with microsatellite instability display abnormal methylation and silencing of the gene *MLH1* (Kane *et al.*, 1997). Germ line "epimutations" in the *MLH1* gene have been proposed to predispose individuals carrying aberrant methylation patterns to multiple cancers (Gazzoli *et al.*, 2002;

Suter *et al.*, 2004). In some patients, the epimutation is detected in normal somatic tissues derived from the three founding cell lineages (buccal mucosa - endoderm; blood - mesoderm; hair follicles - ectoderm), implying that the event occurred in the germ line (Suter *et al.*, 2004); more so, spermatozoa display the same promoter-associated methylation found in tumours, evidence that the epimutation can be transmitted through the germ line (Suter *et al.*, 2004). More examples of epimutations are likely to be reported in the future, some of which may be associated with infertility or subfertility.

1.3 Rationale for Thesis Studies

Approximately 15% of couples suffer from infertility problems, of which male factor infertility accounts for roughly 30 to 50% of the infertility (Cooke and Saunders, 2002). Although unaccounted for in most situations, male factor infertility may result from genetic defects impairing the maturation or the motility of the sperm, or the integrity of chromatin, leading to chromosomal abnormalities (reviewed in Agarwal and Said, 2003). Just as genetic causes may underlie some male infertility so may defects in epigenetic modifications. To date, DNA methylation remains the best-studied epigenetic phenomenon and gametogenesis is the only period during which sex-specific patterns of DNA methylation can be established. As elaborated previously, the dynamics of DNA methylation are tightly regulated during both spermatogenesis and oogenesis. The functional importance of DNA methylation to gametogenesis is undeniably demonstrated by the deleterious effects observed consequently to DNA methyltransferase inactivation (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Bourc'his and Bestor, 2004; Kaneda *et al.*, 2004). However, these models do not permit a systematic developmental analysis of the contribution of these enzymes due to the severity of the phenotypes.

Recent evidence in humans suggests that poor sperm outcome and inability of sperm to fertilize could be related to abnormal DNA methylation pattern acquisition (Marques *et al.*, 2004). As well, infertility may not be impaired

by slight perturbations in DNA methylation, but the embryo or the progeny may encounter more detrimental consequences. Disruption of DNA methylation patterns in male germ cells using cytosine analogs has severe consequences for both germ cells and progeny outcome (Doerksen and Trasler, 1996; Doerksen *et al.*, 2000; Kelly *et al.*, 2003). Decreased sperm counts, decreased fertility, increased preimplantation loss and decreased sperm DNA methylation are all consequences of chronic exposure to 5-azacytidine and 5-aza-2'deoxycytidine.

Germ cell development is therefore a crucial developmental window during which epigenetic programming takes place. The purpose of this dissertation is to advance our current understanding of the key factors involved in the elaboration of the male germ cell epigenetic program. To do so, the following hypotheses were tested: **a) the expression of the newly discovered DNA methyltransferase genes, *Dnmt3a*, *Dnmt3b* and *Dnmt3L*, is tightly regulated in the germ line and b) the DNMT3s play important but distinct roles in establishing and/ or maintaining DNA methylation patterns in male germ cells**. The following questions were asked to test these hypotheses:

1. Are the DNMTs differentially expressed in the developing testis and ovary?
2. How are the postulated *de novo* DNMTs expressed during spermatogenesis?
    a) Which types of germ cells express *Dnmt3a* and *Dnmt3b*?
    b) Are there cell-specific transcript variants and protein isoforms?

3. How is the "regulator of methylation" DNMT3L regulated in the male germ line and what are the consequences of perturbing its expression?

Multiple techniques were used to address these questions. To answer the first question, ovaries and testes at different times during embryonic and postnatal development were collected. The time points were chosen to span the important DNA methylation events taking place in both germ lines.

Immunocytochemistry and Northern Blotting were mainly used to assess expression during the prenatal period, whereas real-time RT-PCR (qRT-PCR) was used to allow for precise examination of the relative expression levels of *Dnmt3a*, *Dnmt3b* and *Dnmt3L* in the developing testis and ovary.

For the second question, we focussed our attention on the male germ line since both events of DNA methylation acquisition and maintenance can be studied in this system. Different populations of postnatal male germ cells were isolated using the sedimentation velocity cell separation technique. qRT-PCR and Western Blots were used to determine the expression levels of *Dnmt3a* and *Dnmt3b,* their transcript variants and their protein isoforms in these cells.

Finally, questions regarding the expression of *Dnmt3L* in the male germ line were addressed using a similar approach as for the second question. Expression experiments were also done on prenatal male germ cells isolated by fluorescence activated cell sorting (FACS) from transgenic mice expressing green fluorescent protein (GFP) in germ cells. In addition, a mouse model deficient in DNMT3L was used to study the consequences of perturbing the expression of this enzyme on the establishment of methylation patterns in male germ cells. Detailed histological and immunocytochemical analyses were conducted to determine the precise timing and nature of the testicular defect. The DNA methylation status of a number of sequences, including whole chromosomal regions, was analysed using qAMP, as this assay can be used to analyse the methylation status of almost any sequence in the genome.

Together, these studies highlight the tightly regulated expression of DNA methyltransferases during spermatogenesis. More importantly, they provide the ground work necessary for the design of tailored gene inactivation experiments to study the subtleties of DNA methyltransferase function and action during male germ cell development.

Table 1.1 Dissecting the mammalian DNA methyltransferases

Gene	Function	Mouse mutant phenotype	Mouse/ human homology *DNA % (protein %)*	Associated human disease	References
Dnmt1	Maintenance methylation	Genome-wide demethylation; developmental arrest at E8.5.	86.5 (86.4)	-	*Li et al., 1992* *Lei et al., 1996*
Dnmt2	Unknown	Normal	87.4 (85.5)	-	*Yoder et al., 1998*
*Dnmt3a**	*De novo* methylation	Spermatogenesis defects; die at ~ 4 weeks of age.	91.6 (96.8)	-	*Okano et al., 1999*
		Germ cell-specific inactivation affects imprint establishment, perturbing both spermatogenesis and oogenesis.			*Kaneda et al., 2004*
*Dnmt3b**	*De novo* methylation	Demethylation of minor satellite DNA; embryonic lethality at ~E14.5– E18.5.	88.4 (91.2)	ICF syndrome	*Okano et al., 1999*
		Germ cell-specific inactivation does not affect spermatogenesis or oogenesis.			*Kaneda et al., 2004*
		* Double mutant: failure to initiate de novo methylation after implantation; developmental arrest at E8.5.	-		*Okano et al., 1999*
Dnmt3L	No catalytic activity; Regulation of methylation	Failure to establish maternal imprints; no viable progeny obtained from *Dnmt3L*$^{-/-}$ dams. Complete spermatogenic arrest (infertility); partial methylation of some paternally imprinted genes and complete absence of methylation of certain repeat sequences.	83.0 (69.8)	-	*Bourc'his et al., 2001* *Hata et al., 2002* *Bourc'his and Bestor, 2004* *Webster et al., 2005*

E, embryonic day

Figure 1.1 ***Schematic representation of seminiferous tubules within the testis.*** Germ cell populations are shown in the context of their localization in the tubule, with immature spermatogonia lying along the basement membrane and spermatids near the lumen. The basal and adluminal compartments of the testis are also shown. Adapted from Gilberts (2000) and reprinted with permission of Sinauer Associates Inc.

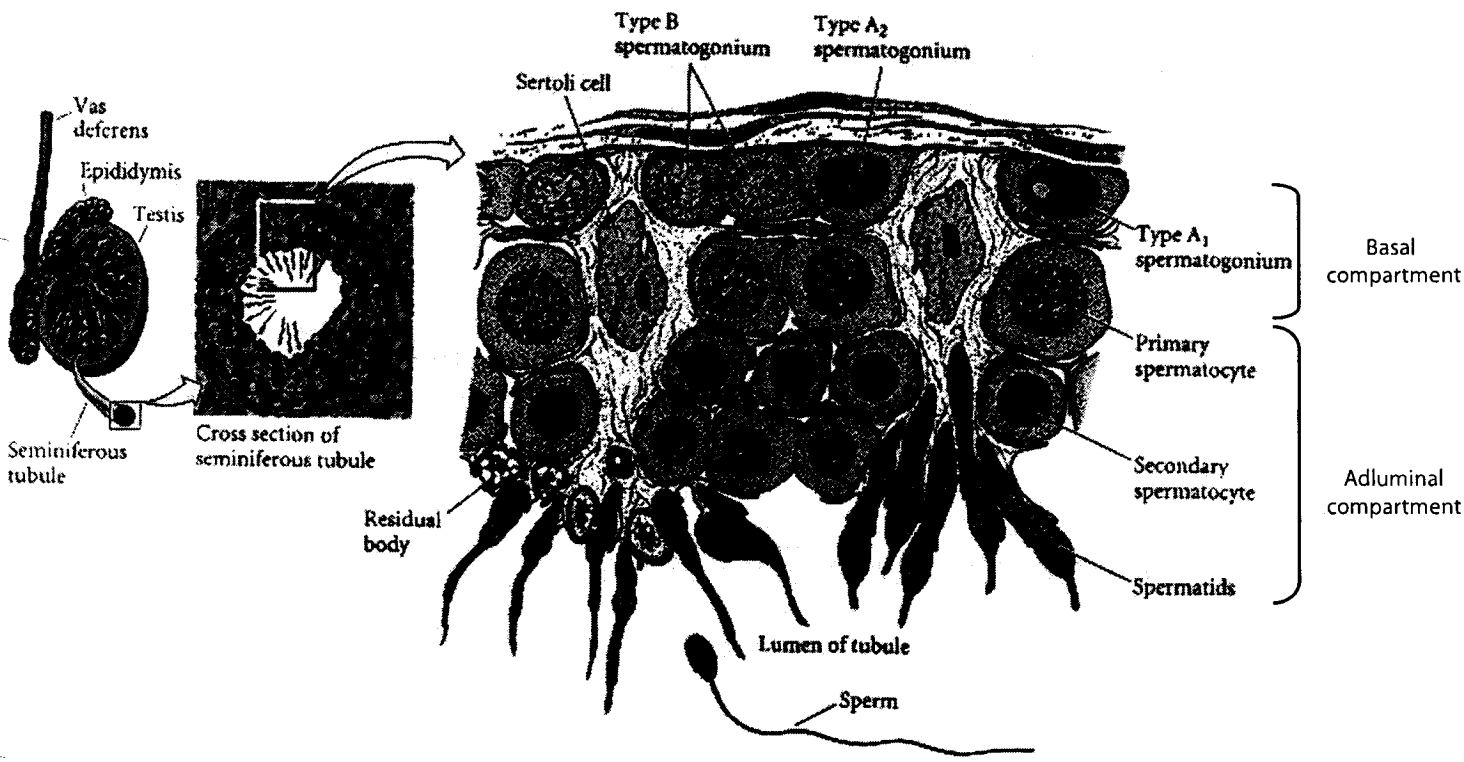
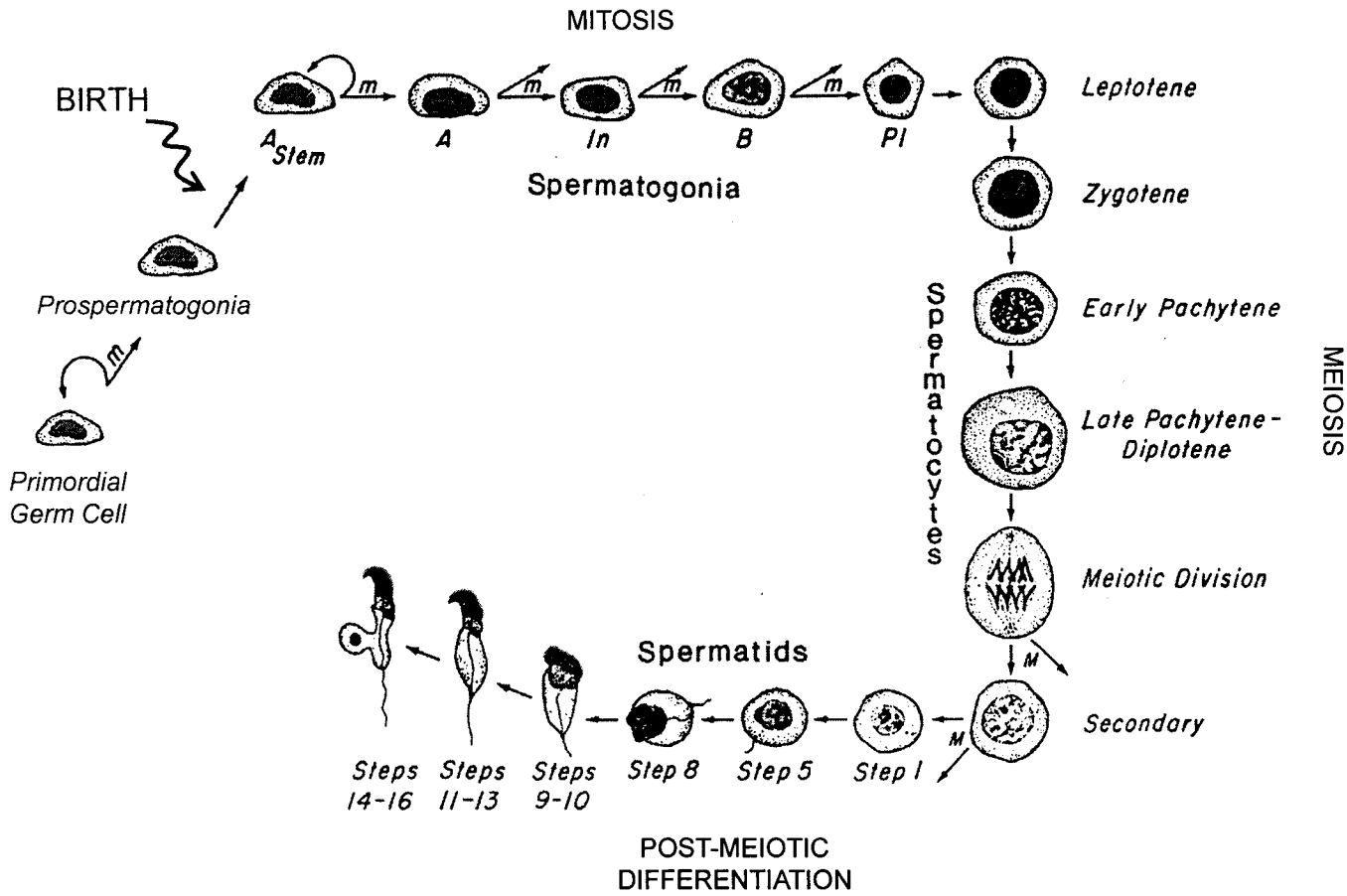

Vas deferens
Epididymis
Testis
Seminiferous tubule
Cross section of seminiferous tubule
Type B spermatogonium
Type A_2 spermatogonium
Sertoli cell
Type A_1 spermatogonium
Primary spermatocyte
Secondary spermatocyte
Spermatids
Residual body
Lumen of tubule
Sperm
Basal compartment
Adluminal compartment

Figure 1.2 ***Spermatogenesis and the stages of the seminiferous epithelium.*** **A)** Formation of haploid sperm, or spermatogenesis, results from germ cells passing through three phase: proliferation, meiosis and spermiogenesis. *m*, mitotic division; *M*, meiotic division; *A*, type A spermatogonia; *In*, intermediate spermatogonia; *B*, type B spermatogonia; *Pl*, preleptotene spermatocytes. Adapted from Meistrich (1977) and reproduced with permission from Elsevier. **B)** The twelve stages of the cycle of the mouse seminiferous epithelium are characterized by distinct cellular associations. Roman numerals indicate the stages of spermatogenesis, while numbers indicate the steps of spermiogenesis. Stages are classified according to the type of spermatid present. *m*, mitotic and meiotic divisions; *A*, type A spermatogonia; *In*, intermediate spermatogonia; *B*, type B spermatogonia; *Pl*, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes. Adapted from Russell *et al.* (1990) and reprinted with permission from Cache River Science, an imprint of Quick Publishing.

A)


MITOSIS
BIRTH
m
A Stem
A
In
B
Pl
Leptotene
Spermatogonia
Zygotene
Prospermatogonia
Early Pachytene
Spermatocytes
MEIOSIS
Late Pachytene - Diplotene
Primordial Germ Cell
Meiotic Division
M
Spermatids
Secondary
Steps 14-16
Steps 11-13
Steps 9-10
Step 8
Step 5
Step 1
POST-MEIOTIC DIFFERENTIATION

B)

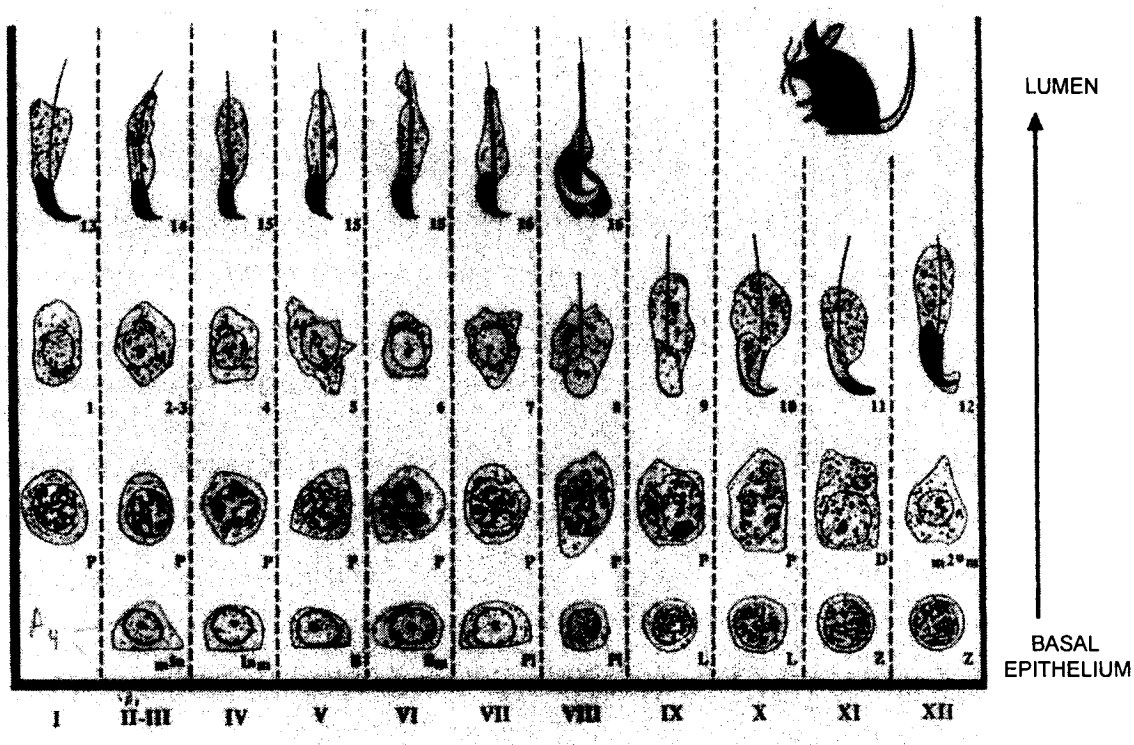

LUMEN
BASAL EPITHELIUM
I
II-III
IV
V
VI
VII
VIII
IX
X
XI
XII

Figure 1.3 ***Diagram of mammalian oogenesis.*** Depiction of the events leading to the formation of a mature egg. The period of oocyte growth and the formation of the zona pellucida are also represented. Reproduced from Alberts *et al.* (2002) and reprinted with permission from Garland Science/ Taylor & Francis Group.

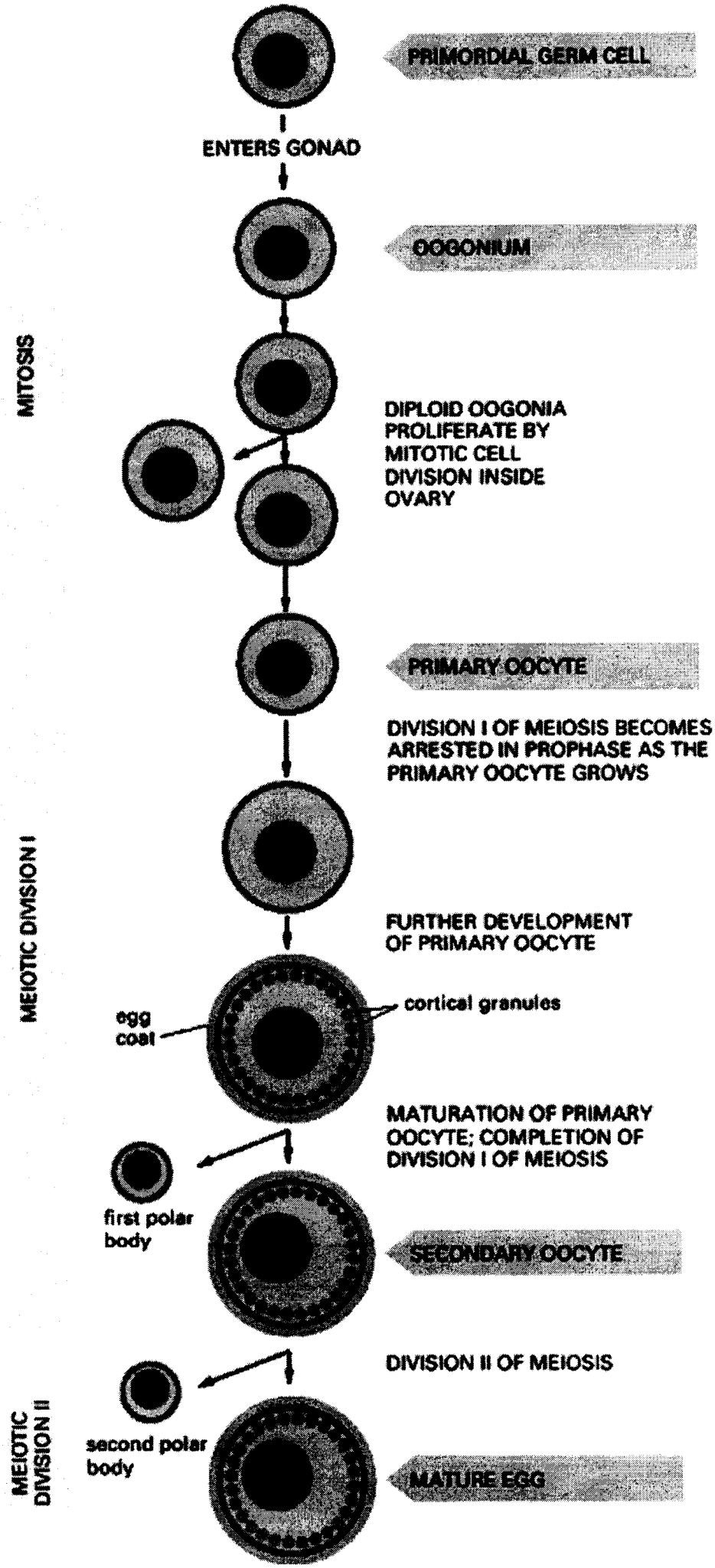
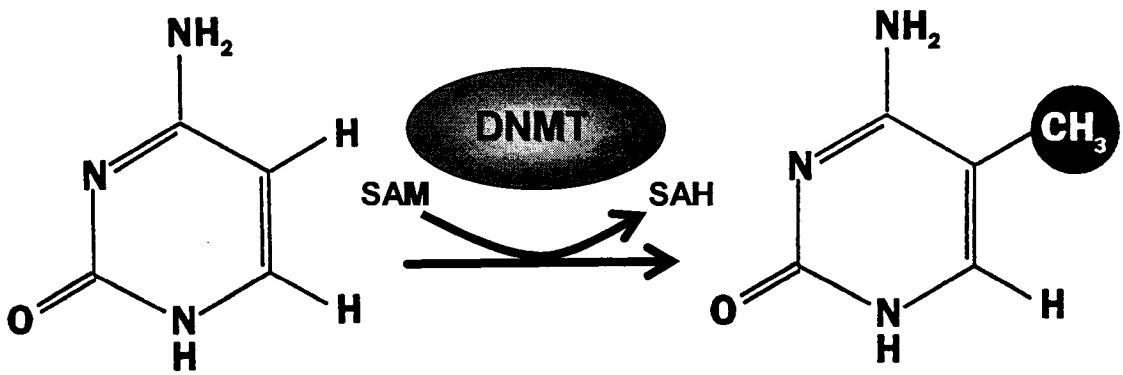

PRIMORDIAL GERM CELL
ENTERS GONAD
OOGONIUM
MITOSIS
DIPLOID OOGONIA PROLIFERATE BY MITOTIC CELL DIVISION INSIDE OVARY
PRIMARY OOCYTE
DIVISION I OF MEIOSIS BECOMES ARRESTED IN PROPHASE AS THE PRIMARY OOCYTE GROWS
MEIOTIC DIVISION I
FURTHER DEVELOPMENT OF PRIMARY OOCYTE
egg coat
cortical granules
MATURATION OF PRIMARY OOCYTE; COMPLETION OF DIVISION I OF MEIOSIS
first polar body
SECONDARY OOCYTE
DIVISION II OF MEIOSIS
MEIOTIC DIVISION II
second polar body
MATURE EGG

Figure 1.4 ***Schematic representation of the conversion of cytosine to 5-methylcytosine by DNA methyltransferases.*** DNMTs catalyzed the transfer of a methyl group from the cofactor S-adenosylmethionine (SAM) to position 5 of the cytosine ring, creating 5-methylcytosine and S-adenosylhomocysteine (SAH).



Cytosine

5-methylcytosine

Figure 1.5 ***Organization of known mammalian DNA methyltransferases.*** Specific motifs are represented by boxes; five of the important amino acid motifs involved in catalysis are illustrated to demonstrate homology in the catalytic domain. Sizes of DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L are in amino acids (aa) and are those of the murine proteins. Reprinted from La Salle and Trasler (2006b) with permission from Cambridge University Press.

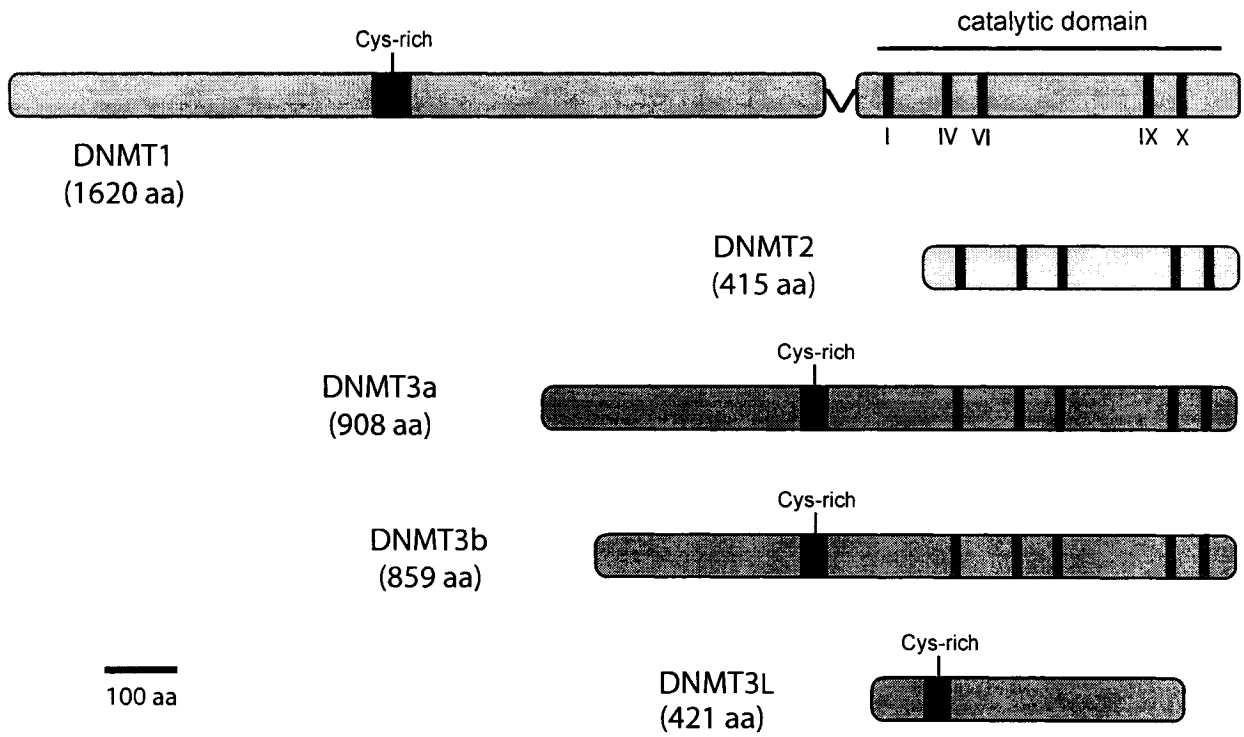
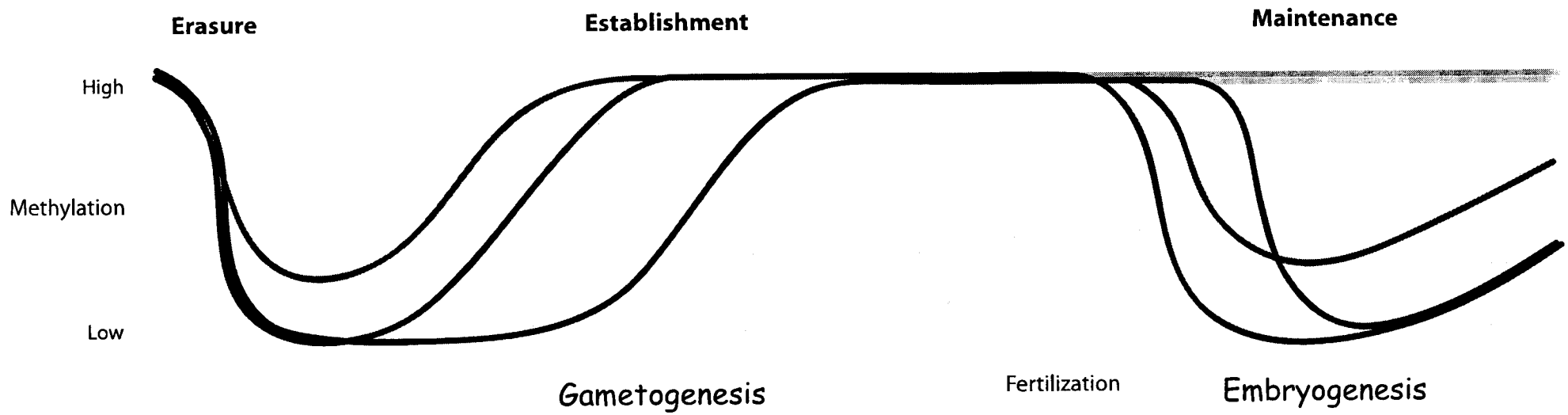

catalytic domain
Cys-rich
I
IV
VI
IX
X
DNMT1
(1620 aa)
DNMT2
(415 aa)
Cys-rich
DNMT3a
(908 aa)
Cys-rich
DNMT3b
(859 aa)
Cys-rich
100 aa
DNMT3L
(421 aa)

Figure 1.6 ***Methylation dynamics during germ cell and preimplantation embryo development.*** **A)** Methylation dynamics of maternal (red) and paternal (blue) genomes. During gametogenesis, non-imprinted genes acquire their methylation similarly to imprinted genes, however, after fertilization, both the maternal and paternal genomes become demethylated while imprinted genes retain their methylation status, as shown by the paler red and blue lines. Some repeat sequences (dark gray) appear to escape complete demethylation during gametogenesis and retain a high proportion of their initial methylation marking during preimplantation development. Methylation levels are not to scale. **B)** Progression of genomic methylation pattern acquisition during male germ cell development, as represented by the intensity of the blue shading. *De novo* and maintenance methylation events are indicated under the appropriate germ cell types. PGC, primordial germ cell; G, gonocyte; Spg, spermatogonia; PL, preleptotene; L/Z, leptotene/ zygotene; P, pachytene; D, diplotene; RS, round spermatid; ES, elongating spermatid; Sp, sperm; MI-MII, meiosis I-II. Reprinted from La Salle and Trasler (2006b) with permission from Cambridge University Press.

A)


Erasure
Establishment
Maintenance
High
Methylation
Low
Gametogenesis
Fertilization
Embryogenesis

B)

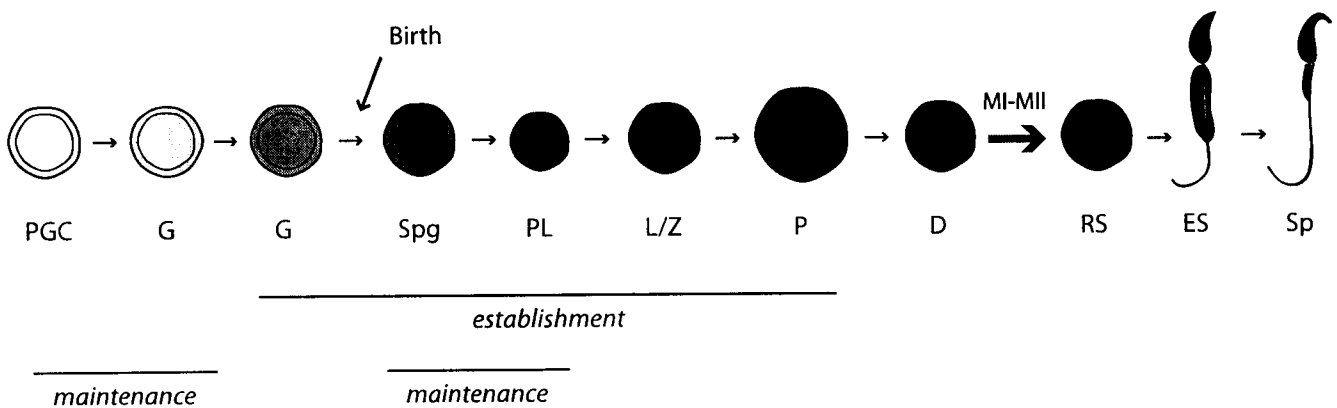
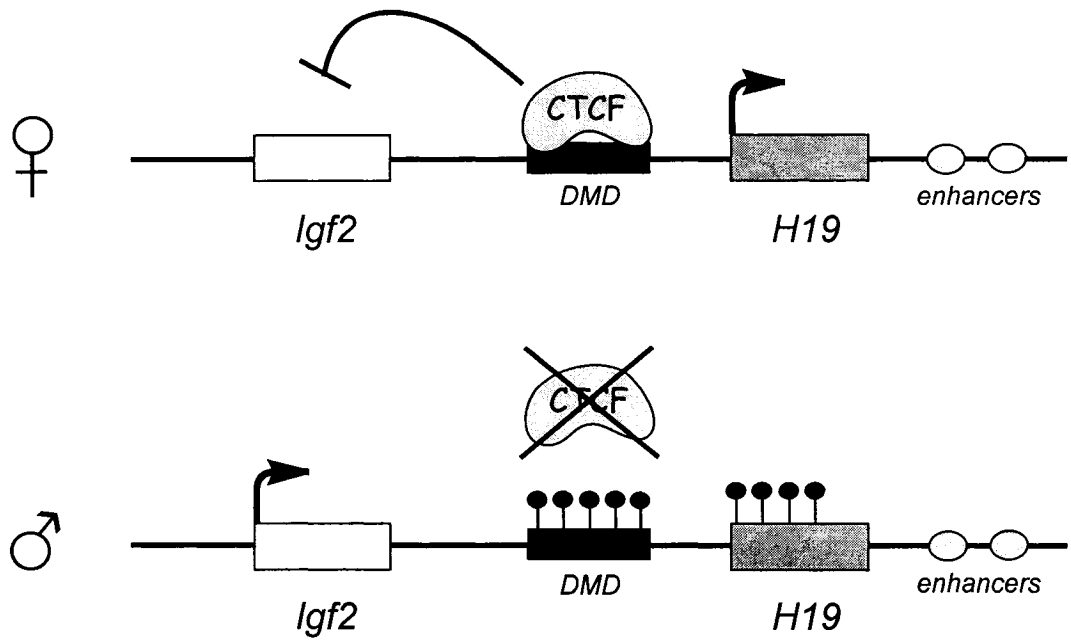

Birth
MI-MII
PGC
G
G
Spg
PL
L/Z
P
D
RS
ES
Sp
establishment
maintenance
maintenance

Figure 1.7 ***Imprinting regulation at the H19 subdomain.*** On the maternal alleles, binding of the insulator protein CTCF to the unmethylated differentially methylated domain (DMD) (black box) prevents access of *Igf2* (white box) to downstream enhancer elements, allowing for maternal expression of *H19* (gray box). However, paternal-specific methylation (filled circles) of the DMD prevents binding of CTCF, silencing *H19* and allowing expression of *Igf2.* Black arrows indicate transcriptional activity of a given gene. Reprinted from La Salle and Trasler (2006b) with permission from Cambridge University Press.


♀
CTCF
DMD
Igf2
H19
enhancers
♂
CTCF
DMD
Igf2
H19
enhancers

CHAPTER II

Windows for Sex-Specific Methylation Marked by DNA Methyltransferase Expression Profiles in Mouse Germ Cells

Sophie La Salle, Carmen Mertineit, Teruko Taketo, Peter B. Moens, Timothy H. Bestor and Jacquetta M. Trasler

Developmental Biology (2004) 268: 403-415.

ABSTRACT

The acquisition of genomic methylation in the male germ line is initiated prenatally in diploid gonocytes, while DNA methylation in the female germ line is initiated postnatally in growing oocytes. We compared the temporal expression patterns of the DNA methyltransferases, DNMT1, DNMT3a, DNMT3b, and Dnmt3L in the male and female germ lines. DNMT1 expression was examined by immunocytochemistry and Northerns with an emphasis on the prenatal period. In the female, there is a gradual down-regulation of DNMT1 protein in prenatal meiotic prophase I oocytes that is not associated with the production of an untranslated transcript, as it is in the male; these results suggest that the mechanism of meiotic down-regulation differs between the sexes. In the male, DNMT1 is unlikely to play a role in the prenatal acquisition of germ line methylation patterns since it is down-regulated in gonocytes between 14.5 and 18.5 days of gestation and is absent at the time of initiation of DNA methylation. To search for candidate DNMTs that could be involved in establishing methylation patterns in both germ lines, real-time RT-PCR was used to simultaneously study the expression profiles of the three DNMT3 enzymes in developing testes and ovaries; *Dnmt1* expression was included as a control. Expression profiles of *Dnmt3a* and *Dnmt3L* provide support for an interaction of the two proteins during prenatal germ cell development and *de novo* methylation in the male. DNMT3L is the predominant DNMT3 protein expressed at high levels in the postnatal female germ line at the time of acquisition of DNA methylation patterns. *Dnmt1* and *Dnmt3b* expression levels peak concomitantly, shortly after birth in the male, consistent with a role in the maintenance of methylation patterns in proliferating spermatogonia. Together, the results provide clues to specific roles for the different DNMT family members in *de novo* and maintenance methylation in the developing testis and ovary.

INTRODUCTION

Genomic methylation patterns are established and maintained by DNA (cytosine-5)-methyltransferases (DNMTs). In mammals, five DNMTs have been characterized and classified according to similarities found in their C-terminal catalytic domain: DNMT1, DNMT2, DNMT3a, DNMT3b, and Dnmt3L (reviewed by Bestor, 2000). Of these, only DNMT1, DNMT3a, and DNMT3b are known to be catalytically active in vivo. DNMT1, the major methyltransferase in somatic tissues, has a preference for hemimethylated DNA and is critical for the maintenance of methylation patterns during replication of DNA (Bestor, 1992; Lei *et al.*, 1996; Li *et al.*, 1992 and Yoder *et al.*, 1997a). DNMT3a and DNMT3b are encoded by essential genes (Okano *et al.*, 1999) that are expressed at high levels in mouse embryonic stem cells and during embryonic development and have been postulated to function predominantly in *de novo* methylation of DNA (Chen *et al.*, 2003; Okano *et al.*, 1998 and Okano *et al.*, 1999). Homozygous *Dnmt1*-deficient embryos have < 5% of the DNA methylation levels found in normal embryos and such embryos show bialleleic expression of most imprinted genes, inactivation of all X chromosomes, activation of retroposons, and apoptotic death before midgestation (Lei *et al.*, 1996; Li *et al.*, 1992; Panning and Jaenisch, 1996 and Walsh *et al.*, 1998). Gene-targeting studies in mice have also established the importance of *Dnmt3L*. Male mice deficient in *Dnmt3L* are infertile while females produce non-viable offspring due to aberrant acquisition of genomic methylation during oogenesis (Bourc'his *et al.*, 2001). Since DNMT3L has not been shown to possess DNA methyltransferase activity, it may be involved in the acquisition of germ cell methylation through interactions with other factors. An oocyte-specific isoform of DNMT1 (DNMT1o), which lacks 118 amino acids of the N-terminal domain of the somatic isoform of DNMT1, has also been described (Carlson *et al.*, 1992 and Mertineit *et al.*, 1998). However, depletion of DNMT1o shows that while it contributes to maintaining the methylation state of imprinted genes during embryogenesis, it does not play a role in the methylation of oocyte DNA (Howell *et al.*, 2001).

Genomic methylation patterns are acquired in the germ line and differ markedly for male and female gametes (Driscoll and Migeon, 1990; Monk *et al.*, 1987 and Sanford *et al.*, 1987; reviewed by Reik *et al.*, 2001). These methylation differences are especially striking at imprinted loci where they have important implications for allele-specific gene expression in the offspring (reviewed by Reik and Walter, 2001). Although further modification of methylation patterns occurs after fertilization (Howlett and Reik, 1991; Kafri *et al.*, 1992 and Monk *et al.*, 1987), some sequences, such as imprinted loci and IAP retroposons, retain their gamete-derived marking (Lane *et al.*, 2003; Olek and Walter, 1997; Shemer *et al.*, 1997 and Tremblay *et al.*, 1997). Accurate reprogramming is therefore required with every reproductive cycle to ensure proper erasure, acquisition, and maintenance of methylation marks. In both germ lines, DNA methylation patterns on most sequences appear to be erased around the time when primordial germ cells enter the gonad, at approximately days 10.5–12.5 of gestation in the mouse (Hajkova *et al.*, 2002; Kato *et al.*, 1999; Lee *et al.*, 2002; Szabo and Mann, 1995 and Szabo *et al.*, 2002). In female germ cells, methylation patterns are acquired postnatally, after the pachytene phase of meiosis is complete, during the oocyte growth phase (Brandeis *et al.*, 1993; Kono *et al.*, 1996; Lucifero *et al.*, 2002; Stoger *et al.*, 1993 and Walsh *et al.*, 1998). In contrast, in the male, genomic methylation begins to be acquired before birth in prospermatogonia and is complete after birth and before the end of pachytene (Davis *et al.*, 1999; Davis *et al.*, 2000; Kafri *et al.*, 1992; Lees-Murdock *et al.*, 2003; Ueda *et al.*, 2000 and Walsh *et al.*, 1998). No male germ cell-specific DNMT protein has been identified yet and, although DNMT3a and Dnmt3L are thought to play important roles during spermatogenesis, their exact contributions are still unknown (Bourc'his *et al.*, 2001 and Okano *et al.*, 1999). The role each DNA methylating enzyme plays in the genome-wide and sex-specific methylation events that take place in germ cells, as well as the mechanisms governing these events, still remain to be elucidated.

We report the expression patterns of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3L* during the times when genomic methylation patterns are acquired in the

male and female germ lines. We demonstrate that there are sex-specific differences in DNMT1 expression both before and shortly after birth, use anti-synaptonemal complex markers to determine the precise timing of events leading to DNMT1 down-regulation during prophase I in oocytes, and show the absence of DNMT1 expression at the time of acquisition of methylation in the prenatal male germ line. We identify candidate methyltransferases that are likely to be involved in establishing genomic methylation patterns in both germ lines as well as the dynamic relationship that exists between the enzymes during the putative windows of acquisition and maintenance of genomic methylation patterns.

MATERIALS AND METHODS

Isolation of gonads

CD-1 mice were purchased from Charles River Canada Inc. (St-Constant, QC, Canada). Noon of the day on which the vaginal plug was found was designated as embryonic day (E) 0.5 and the day of delivery as postnatal day (dpp) 0. Genital ridges were isolated from E11.5 mouse embryos; genetic sexing using primers specific for *Zfy1* and *Zfy2* was used to determine the sex of the embryo (Nagamine *et al.*, 1989). Ovaries and testes were distinguished in embryos collected at daily intervals from E12.5 to E18.5 by the presence of seminiferous cords in the testes. Pairs of urogenital complexes were dissected in Eagle Modified Essential Medium (MEM) containing Hank salts and 25 mM Hepes buffer, pH 7.3 (Gibco BRL, Grand Island, NY) and gonads were separated from adjacent mesonephric tissues. Fetal and postnatal testes and ovaries were decapsulated and rinsed in sterile DEPC-treated saline, pooled, and frozen in liquid nitrogen or between slabs of dry ice unless otherwise specified. All procedures were performed in accordance with the Canadian Council on Animal Care and approved by the McGill University Animal Care Committee.

Preparation of germ cells and gonads for immunocytochemistry

Intact E11.5 genital ridges were fixed by immersion in Ste Marie's fixative (Trasler *et al.*, 1996). Alternatively, genital ridges were pooled, washed briefly in Ca^{2+}–Mg^{2+}-free PBS, incubated in 0.2% EDTA-PBS solution for 20 min, transferred to MEM, and mechanically disrupted by pipetting to release primordial germ cells; cells were fixed for 15 min in 3.7% formaldehyde in PBS. Fetuses from E13.5 to 18.5 and postnatal mice were perfused through the heart with physiological saline followed by twice diluted (50%) Ste Marie's fixative; ovary and testis sections were prepared as previously described (Mertineit *et al.*, 1998). Oocytes in meiotic prophase were obtained from E15.5 to 17.5 ovaries as described previously (Amleh *et al.*, 2000). Briefly, ovaries were pooled, digested with collagenase, washed in MEM, and further digested with trypsin in Rinaldini

solution (Rinaldini, 1959). Ovaries were suspended in 10% fetal bovine serum in MEM, washed, transferred to Ca^{2+}–Mg^{2+}-free PBS, dissociated by pipetting, centrifuged, and resuspended in MEM. The cell suspension was applied to 0.5% NaCl droplets on glass multi-spot slides (Shandon Inc., Pittsburg, PA) and allowed to settle for 15 min. The slides were fixed in 2% paraformaldehyde, pH 8.2, rinsed in 0.4% Kodak Photo-Flo wetting agent (Eastman Kodak Co, Rochester, NY), pH 8.0, air-dried, and stored at −20°C.

Immunocytochemistry

All solutions for immunocytochemistry were prepared in PBS, pH 7.2 and procedures were carried out at room temperature, unless otherwise specified. The rabbit polyclonal anti-DNMT1 antibody PATH52, which recognizes epitopes in both the somatic (DNMT1s) and the oocyte-specific (DNMT1o) forms of DNMT1, has been described previously (Bestor, 1992 and Li *et al.*, 1992). The mouse monoclonal anti-stage-specific embryonic antigen-1 antibody (anti-SSEA-1; Solter and Knowles, 1978), which recognizes a cell surface glycoprotein on primordial germ cells, was a kind gift of Dr. T. Feizi (Northwick Park Institute for Medical Research, Harrow, UK). The mouse monoclonal anti-synaptonemal complex antibody (anti-SC), which labels the chromosomal components of germ cells in meiotic prophase, has been described previously (Dobson *et al.*, 1994 and Moens *et al.*, 1987). Sections were processed as previously described in Mertineit *et al.*(1998) until incubation with the secondary antibody; slides were incubated with a biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA), rinsed and incubated with the Vectastain ABC reagent (Vector Laboratories). Next, they were incubated with H_2O_2, diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and imidazole in 25 mM Tris-buffered saline, pH 7.6. Sections were counterstained with methylene blue and mounted with Permount (Sigma). Immunofluorescence was performed on E11.5 germ cell preparations and meiotic prophase oocytes, which were incubated with 1:500 PATH52 and either 1:1000 anti-SSEA-1 or 1:1000 anti-SC overnight, washed and then incubated with FITC and Texas Red secondary antibodies (Vector

Laboratories), as Moens *et al.*(1987) described. Slides were mounted with Prolong antifade reagent containing 0.4 μg/ml DAPI (Molecular Probes, Eugene, OR) and examined with a Zeiss Axiophot or a Zeiss LSM410 (Carl Zeiss Canada Ltd, Toronto, ON) confocal microscope as described (Laird *et al.*, 1995).

Northern blotting

Total RNA was extracted from the mesonephros, ovaries, and testes from prenatal and postnatal mice using TRIzol (Gibco BRL) as directed by the manufacturer and Northern Blotting was performed using 10 μg of RNA as previously described (Mertineit *et al.*, 1998).

Real-time RT-PCR

Total RNA was extracted from three separate collections of pooled prenatal and postnatal gonads of mice coming from different litters using the RNeasy extraction kit with DNaseI treatment (Qiagen Inc., Mississauga, ON, Canada) as described by the manufacturer. Real-time/quantitative RT-PCR (qRT-PCR) was performed on the Mx4000 qPCR system from Stratagene (Stratagene, La Jolla, CA) using the Quantitect SYBR Green RT-PCR kit (Qiagen). Gene-specific primers were used to determine the relative expression levels of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3L* according to the standard curve method (reviewed by Bustin, 2002). Primers (Table 2.1) were designed to span introns and pick up all known transcript variants of the various *Dnmts*, resulting in the detection of a single band on gels (data not shown). SYBR Green was used to detect the double-stranded DNA produced during the amplification reaction and 18S rRNA content to normalize for the input of RNA. Reactions were performed using approximately 10 ng or 100 pg of total RNA for the *Dnmts* and 18S, respectively. One-step RT-PCR reactions were performed in a 25-μl volume as directed by the manufacturer for 40 cycles. For each gene studied, a specific standard curve was established using single-use aliquots of the same stock of RNA (total RNA extracted simultaneously from multiple 6 dpp testes). In all cases, reactions were performed in triplicate on the same three independent

samples (i.e., same three separate pools) of ovary or testis RNA. PCR products were cloned and sequenced to confirm their identity before undertaking the study; specificity was assessed with the melting curve analysis and confirmed on a 3% agarose gel after each qRT-PCR experiment (data not shown). qRT-PCR results were normalized to their corresponding 18S rRNA content and calibrated accordingly to the lowest-expressing time point. Data are presented as mean ± SEM.

RESULTS

DNMT1 Localization in Fetal Germ Cells

We first examined the expression and localization of DNMT1 in primordial germ cells (PGCs) at E11.5 and then following sexual differentiation, from E12.5 to E18.5. The cell surface marker SSEA-1 was used to identify PGCs in the genital ridge of E11.5 embryos (Solter and Knowles, 1978). Genital ridges of both XX and XY embryos were double-labeled with anti-DNMT1 and anti-SSEA-1 antibodies; many cells, including SSEA-1-positive PGCs, showed strong expression of DNMT1 (Fig. 2.1A). SSEA-1 clearly marked the cell surface of isolated PGCs (Figs. 2.1C, D, F, and G), whereas DNMT1 was strongly expressed throughout these cells (Figs. 2.1B, D, E, and G). Z-sectioning through the entire depth of PGCs confirmed the presence of DNMT1 in the nucleus with some staining in the cytoplasm; the staining surrounded condensed mitotic chromosomes of germ (Fig. 2.1E) and somatic (Fig. 2.1H) cells.

Following sexual differentiation, marked changes in DNMT1 expression were observed in the developing fetal testis. Two ages, E13.5 and E18.5, representing times before and after the DNA of male gonocytes initially becomes methylated, are shown in Fig. 2.2. In contrast to the preimmune control (Fig. 2.2A), proliferating germ and somatic cells (Fig. 2.2B) showed strong expression of DNMT1. Mitotic gonocytes within the testicular cords revealed both intense DNMT1 nuclear staining, as well as staining around mitotic chromosomes (Fig. 2.2B). DNMT1 levels in E14.5 and E15.5 gonocytes gradually decreased (data not shown) and by E18.5, the enzyme was no longer detected although it was still abundant in the supporting cells along the base of the tubules (Fig. 2.2C). Noticeable changes were also detected in the fetal ovary. In the female mouse, germ cells actively proliferate by mitosis until E13.5–14.5 and then enter and reach the diplotene stage of meiotic prophase between E14.5 and birth. At E13.5, fetal ovaries incubated with rabbit preimmune serum remained negative (Fig. 2.2D), whereas sections incubated with PATH52 showed strong expression of the DNMT1 enzyme in germ cells (Fig. 2.2E). DNMT1 staining of germ cells

gradually decreased between E14.5 and E17.5 (data not shown) and by E18.5, when the majority of oocytes were at the pachytene or diplotene stages of meiotic prophase, they were devoid of DNMT1 while various supporting cells were positively stained (Fig. 2.2F).

DNMT1 Down-Regulation in Prenatal Meiotic Prophase Oocytes: Timing and Mechanisms

DNMT1 is expressed in preleptotene, leptotene, and zygotene spermatocytes before being down-regulated at pachytene (Jue *et al.*, 1995). Evidence from whole female prenatal ovary staining, shown in Fig. 2.2, suggested that down-regulation of DNMT1 expression was also occurring during female meiosis, but did not allow precise timing to be determined. To assess the meiotic cell type-specific timing of down-regulation during female meiosis, dissociated fetal ovary preparations were double-labeled with anti-DNMT1 and anti-synaptonemal complex antibodies; the latter served as a marker to identify oocytes in the various phases of meiotic prophase I according to the state of synapsis between homologous chromosomes (Dobson *et al.*, 1994 and Moens *et al.*, 1987; Fig. 2.3). At leptotene, the chromosome cores appeared as fine threads (Fig. 2.3A) and DNMT1 staining was intense and largely diffuse throughout the nucleus (Fig. 2.3B). Zygotene oocytes, characterized by sites of chromosome pairing (Fig. 2.3C), stained moderately for DNMT1 throughout the nucleoplasm (Fig. 2.3D). Pachytene oocytes were identified by complete formation of the synaptonemal complex between pairs of homologous chromosomes (Figs. 2.3E and 2.3G). In early pachytene oocytes, DNMT1 staining was weak (Fig. 2.3F), while no staining was observed in late pachytene oocytes (Fig. 2.3H). In contrast to germ cells, somatic cells at all stages of fetal ovary development were identified by their lack of staining for the synaptonemal complex (Fig. 2.3I) and many exhibited bright punctuate foci with diffuse nucleoplasmic staining for DNMT1 (Fig. 2.3J). Taken together, these data show a gradual down-regulation of DNMT1 during meiotic prophase, to reach complete disappearance of the protein at the pachytene stage.

In previous studies, we identified an untranslated 6.0 kb transcript of *Dnmt1* that is expressed during male meiosis and is associated with down-regulation of DNMT1 in postnatal pachytene spermatocytes (Mertineit *et al.*, 1998 and Trasler *et al.*, 1992). Northern Blot analysis was used here to determine if the same phenomenon was occurring during female pachynema. The ubiquitous 5.2-kb transcript, which gives rise to the full-length somatic form of DNMT1, was present at all times during development in both the ovary and the testis (Fig. 2.4A). In the postnatal ovary, the signal became especially strong compared to fetal mesonephros and ovaries (Fig. 2.4A, lanes 4 and 5) due to the expression of the 5.1 kb oocyte-specific transcript, undistinguishable on gel from the 5.2-kb transcript; the 5.1 kb transcript is absent before birth (Ratnam *et al.*, 2002). A third transcript of 6.0 kb is also detected in the testis; it results from the use of a different first exon (exon 1p) and remains untranslated, causing a loss of DNMT1 protein during male meiosis. To determine if ovaries containing pachytene oocytes also express the 6.0-kb transcript, we stripped and rehybridized the membrane with a probe to exon 1p (Mertineit *et al.*, 1998). Despite the fact that more than 30–50% of oocytes in E16.5–17.5 ovaries are at the pachytene stage of meiosis (McClellan *et al.*, 2003; O'Keeffe *et al.*, 1997 and Speed, 1982), we failed to detect the 6.0-kb transcript in these samples (Fig. 2.4B). In contrast, the transcript was present in testes that contain pachytene germ cells (20 and 70 dpp), but not in testes that are devoid of pachytene cells (E16.5 and 6 dpp).

DNMT1 and the Maintenance of Methylation Patterns in Neonatal Spermatogonia

We assessed DNMT1 re-expression in the neonatal period shortly after birth, at the boundary of mitosis resumption, by immunostaining testes of newborn mice of zero, 3 and 6 dpp. The prospermatogonia arrested at the G1 phase of the cell cycle remained negative for DNMT1 in comparison to neighboring Sertoli and interstitial cells, in the testis of 0 dpp mice (Fig. 2.5A). Between 3 and 4 dpp, the germ cells resumed their mitotic activity and relocated to the basal compartment of the seminiferous epithelium; consistently, they

became highly reactive to PATH52 in 3 dpp testes (Fig. 2.5B). DNMT1 expression remained high in dividing spermatogonia in the 6 dpp testis, (Fig. 2.5C).

Expression of the DNMT3 Enzymes in the Developing Testis and Ovary

Previous studies (Howell *et al.*, 2001 and Sakai *et al.*, 2001) as well as the data presented here make it unlikely that DNMT1 is responsible for the genome-wide *de novo* methylation events that take place in the male germ line prenatally and in the female germ line postnatally. We examined the expression dynamics of the other DNA methyltransferase genes, *Dnmt3a* and *Dnmt3b*, as well as the DNA methyltransferase 3-Like gene *Dnmt3L*, using qRT-PCR in an effort to find candidate DNA methyltransferases responsible for the initial acquisition of DNA methylation patterns. Developmental stages before, during, and after genomic methylation patterns are first acquired in the male germ line were assayed using E13.5, E15.5, and E18.5 testes, respectively; the various populations of maturing germ cells in the postnatal testis were represented with 6, 16, 22, and 70 dpp testes (Bellvé *et al.*, 1977b). In the female, periods before and during, as well as the arrest in prophase I of meiosis were assayed with identical prenatal time points; the oocyte growth phase, the period during which methylation patterns are acquired in the female, was covered with the same postnatal time points as for the testis. Relative quantification using the standard curve method was used to determine the fold changes in expression according to the lowest expressing time point (calibrator) for a given gene in whole testes and ovaries; all other quantities were expressed as an *n*-fold difference relative to the calibrator. Because the same stock of RNA was used to prepare all standard curves, the relative quantities determined using this method could be compared across individual experiments.

We first compared the expression profiles of the enzymes known to be capable of methylating DNA in both the testis (Fig. 2.6A) and the ovary (Fig. 2.6B). Validation of the assay was assessed by determining the pattern of expression of *Dnmt1* in the male germ line, which is shown in Fig. 2.6A and is

consistent with previous reports (Benoit and Trasler, 1994 and Trasler *et al.*, 1992). Consistent with the Northern results in Fig. 2.4, Dnmt1 showed higher levels of variation in the ovary (up to 9-fold) than in the testis (up to 3-fold). In the ovary, the highest Dnmt1 levels were found early after birth, a time when folliculogenesis is active and DNMT1 may be required predominantly to maintain genomic methylation in dividing granulosa cells. *Dnmt3a* and *Dnmt3b* displayed unique developmental profiles that showed marked sex-specific differences. In the testis, *Dnmt3a* expression was prominent before and early after birth and gradually returned to basal levels in the postnatal testis (Fig. 2.6A). In contrast, in the female, *Dnmt3a* expression remained relatively constant throughout ovary development (Fig. 2.6B). In the male, Dnmt3b was present at low levels before birth, only increasing above day E18.5 levels after birth, most notably on days 6 and 16 (Fig. 2.6A). The developmental pattern of expression of *Dnmt3b* in the female (Fig. 2.6B) was similar to that in the male; however, in the ovary while expression peaked at 6 dpp, it decreased again by 16 dpp.

We subsequently analyzed the expression pattern of *Dnmt3L*, a member of the DNMT3 family known to lack DNA methyltransferase activity, but to nevertheless be essential for DNA methylation to occur. Dnmt3L transcripts were particularly abundant in both the testis and the ovary, varying up to 500- to 600-fold; however, peaks of expression were seen at very different developmental times in the two germ lines (Fig. 2.7). In the male, Dnmt3L was abundantly expressed before birth and was present at the highest levels at E15.5, just preceding the time methylation is known to be initiated in the male germ line. *Dnmt3L* expression in the testis dropped dramatically after birth; expression was approximately 470× lower at 22 dpp than at E15.5. A different pattern was seen in the ovary where Dnmt3L was present at low levels before birth with an up-regulation shortly after birth. In the female germ line, expression peaked at 16 dpp with more than 600-fold the levels at the lowest expressing time point (E18.5).

DISCUSSION

Although DNA methylation occurs at about 30 million sites in the mammalian genome (Bestor and Tycko, 1996), in a sex- and sequence-specific manner and is initiated in the germ line, little is known about the specific DNA methyltransferase enzymes involved, when they act developmentally and if and how the process differs between the sexes. Here, we provide evidence that the predominant DNA methylating enzyme, DNMT1, is present in prenatal gonocytes but is then down-regulated before and is absent at the time of acquisition of methylation in the male germ line, implicating other enzymes in the *de novo* methylation of DNA that is initiated in the prenatal period. Expression profiles showing concomitant peaks of Dnmt3a and Dnmt3L expression in the prenatal testis suggest that these two proteins may interact to help establish DNA methylation patterns in the male germ line. Dnmt1 and Dnmt3b expression levels peak in the early postnatal period in the male suggesting a role for these enzymes in the maintenance of methylation patterns in rapidly proliferating spermatogonia. In the female, DNMT1 is expressed in prenatal oogonia but is then down-regulated during meiotic prophase; a similar process occurs in the male in the postnatal period. Expression profiles identified DNMT3L as the predominant DNMT3 family member present in the postnatal ovary coincident with the timing of acquisition of methylation patterns in the female germ line.

DNMT1 Expression in the Nuclei of Male and Female Proliferating Primordial Germ Cells

Previous studies of DNMT1 expression in the germ line focused primarily on the postnatal events taking place during gametogenesis (Benoit and Trasler, 1994; Jue *et al.*, 1995; Mertineit *et al.*, 1998; Numata *et al.*, 1994 and Ratnam *et al.*, 2002). Here, we compared DNMT1 cellular expression patterns in the male and female prenatal gonad, using the same well-characterized anti-DNMT1 antibody (PATH52) that we had previously used to localize DNMT1 in the postnatal testis and ovary. We first showed that mitotically dividing E11.5 PGCs of both sexes contain high levels of DNMT1 and that the protein is present in the

nucleus where DNA methylation takes place; DNMT1 continues to be expressed in dividing male and female germ cells following sexual differentiation. Recent studies have examined the timing of methylation reprogramming events that take place between E10.5 and E12.5 in the PGCs colonizing the embryonic gonads. While the methylation at imprinted and single-copy loci is erased in E10.5 to E12.5 germ cells within about a 1-day window, it appears that some repetitive sequence elements, in particular the LTR of Intracisternal A Particle (IAP) elements, retain their methylation (Hajkova *et al.*, 2002; Lane *et al.*, 2003 and Lees-Murdock *et al.*, 2003). Consistent with our results, although sexing of the gonads was not carried out, Hajkova *et al.*(2002) showed that DNMT1 was expressed in E12.5 genital ridge germ cells. Thus, it is possible that DNMT1 is required in mitotic PGCs of both sexes to maintain the methylation of some repetitive elements, perhaps to prevent them from being expressed at a critical time when other sequences are losing their methylation.

Down-Regulation of DNMT1 in the Male and Female Fetal Gonads Following Sexual Differentiation and Re-Expression Postnatally

Our data also show that, following sexual differentiation, as germ cells of both sexes gradually entered a non-proliferative state, DNMT1 staining was lost. In the male, mitotic arrest is associated with maturation of germ cells into prospermatogonia at about E14.5 followed by genome-wide acquisition of methylation between E15.5 and E18.5 (Davis *et al.*, 1999; Davis *et al.*, 2000; Kafri *et al.*, 1992; Lees-Murdock *et al.*, 2003; Ueda *et al.*, 2000 and Walsh *et al.*, 1998). The current study shows that DNMT1 is not present in male germ cells during this critical period, although it is clearly present in surrounding somatic cells. Using a different anti-DNMT1 antibody, Sakai *et al.*(2001) found expression of DNMT1 in the prenatal testis to follow that of PCNA (a marker for proliferating cells), a finding consistent with our results. Together, the results indicate that DNMTs other that DNMT1 are responsible for the *de novo* acquisition of methylation patterns in male prenatal germ cells.

In the female germ line, DNMT1 was initially expressed in oogonia; expression then decreased between E13.5 and E18.5. However, unlike male germ cells that enter mitotic arrest during this time, female germ cells enter meiotic prophase I shortly after sexual differentiation in the embryo. Precise staging of meiotic prophase oocytes using a synaptonemal complex marker revealed a gradual decrease in nucleoplasmic DNMT1 staining as germ cells passed through meiotic prophase, such that by late pachytene, DNMT1 was undetectable in oocytes. We previously reported that DNMT1 protein levels were down-regulated at pachytene in the male mouse; this down-regulation is due to the production of a non-translated pachytene spermatocyte-specific 6.0 kb transcript derived from exon 1p of the *Dnmt1* gene (Jue *et al.*, 1995; Mertineit *et al.*, 1998 and Trasler *et al.*, 1992). We now report another example of DNMT1 down-regulation at pachytene, this time in the female germ line, in the absence of an alternative transcript, suggesting that the mechanism of meiotic down-regulation differs between the sexes. Several features of meiotic chromosomes render them vulnerable to *de novo* methylation during crossing over and DNMT1 preferentially targets DNA structures associated with recombination (Bestor and Tycko, 1996). Based on the results in the male, we proposed earlier (Bestor and Tycko, 1996 and Jue *et al.*, 1995) that germ cells may protect their meiotic DNA from inappropriate methylation by inactivating the expression of DNMT1. Our current results provide further evidence in support of this hypothesis.

Experiments in mouse 3T3 fibroblasts at S phase showed that DNMT1 localizes to discrete foci that are sites of DNA replication (Leonhardt *et al.*, 1992). We previously observed nuclear localization of DNMT1 foci in isolated type A spermatogonia from 8-day-old mice; as this cell type is mitotically active, we thought the foci most likely represented sites of DNA replication as observed in S-phase 3T3 fibroblasts (Jue *et al.*, 1995). However, we had not linked this observation to mitosis resumption early after birth, as germ cells exit their dormant state, relocate to the basement membrane, and recommence dividing. Here, we confirm that DNMT1 re-expression is synchronized with the cell cycle

and, consistent with its role as a replication factor, associates with actively dividing germ cells at 3 dpp but not with G1 phase gonocytes at 0 dpp.

Differential Expression of the DNMT3 Family Members and the Timing of Methylation in the Male and Female Germ Lines

De novo methylation begins in the male germ line before birth and is further consolidated after birth; once established, methylation patterns must be maintained during DNA replication that takes place in spermatogonia and preleptotene spermatocytes. In the female germ line, DNA methylation patterns are acquired during the oocyte growth phase after birth, but since there is no further DNA replication, there appears to be no need for maintenance methylation later in oogenesis. Recently, DNMT3a, DNMT3b, and Dnmt3L have been characterized and were shown to be expressed in multiple tissues, including the testis and the ovary; DNMT3a and DNMT3b are postulated to be involved in *de novo* methylation events (Aapola *et al.*, 2001; Chen *et al.*, 2002 and Okano *et al.*, 1998). Results of gene-targeting experiments indicate that *Dnmt3L* is dispensable for normal somatic development but is an important regulator of maternal imprint establishment as well as being essential for normal postnatal spermatogenesis (Bourc'his *et al.*, 2001).

We determined the time windows during which the DNMT3 family members are expressed (summarized in Fig. 2.8). In the male, Dnmt3a and Dnmt3L levels peaked at the same time before birth, during the critical time window of DNA methylation pattern initiation (E15.5–E18.5); the results support an interaction of the two proteins in the *de novo* methylation process taking place during prenatal male germ cell development. DNMT3L was recently shown to stimulate *de novo* methylation through DNMT3a, but not DNMT3b, at some imprinted gene loci in vitro (Chédin *et al.*, 2002) and we hypothesize it might be carrying out a similar function in the germ line in vivo.

In the female, particularly high levels of Dnmt3L coincided with the timing of oocyte growth in the postnatal ovary, further supporting the important role it is thought to play in regulating the establishment of maternal imprints. In the

current experiments, *Dnmt3a* expression did not vary greatly during development of the ovary. Nevertheless, Hata *et al.* (2002) demonstrated that embryos derived from transplanted *Dnmt3a*$^{-/-}$/*Dnmt3b*$^{+/-}$ ovaries have perturbed maternal imprints, suggesting a role for DNMT3a in methylation events occurring during oocyte growth.

There is growing evidence that DNMT family members interact (Hata *et al.*, 2002; Kim *et al.*, 2002 and Margot *et al.*, 2003). For instance, biochemical fractionation experiments have revealed an association between DNMT1 and DNMT3b (Datta *et al.*, 2003). Minor satellite repeats, found in the pericentromeric region of chromosomes, are thought to be specifically methylated by DNMT3b (Okano *et al.*, 1999) and ICF syndrome (immunodeficiency, centromere instability, and facial anomalies), a rare immune disease causing hypomethylation of centromeric repeats in humans, is caused by mutations in the *Dnmt3B* gene (Xu *et al.*, 1999). We show here that, in the male, DNMT3b is expressed at the highest levels postnatally, at times when the predominant cells are either mitotic spermatogonia (6 dpp) or spermatocytes entering meiotic differentiation (16 dpp). It is possible that DNMT3b plays a role at these early times during spermatogenesis in actively methylating centromeric regions to ensure proper pairing and recombination between homologous chromosomes. In addition, we suggest that DNMT1 and DNMT3b may cooperate in maintaining the integrity of the genome, since they show a similar pattern of expression in both germ lines.

Experiments over the last decade on DNMT1 in the germ line have uncovered several interesting features including highly regulated expression during male and female gametogenesis, the use of sex-specific first exons to produce translated and non-translated RNAs, as well as an important role in reproductive function due to an oocyte DNMT1 isoform essential for the maintenance of methylation in preimplantation embryos. The data presented here on the DNMT3 family members show striking gene-specific and germ line-specific patterns of expression, evidence that DNMT3a and Dnmt3L may interact in *de novo* methylation in the male germ line, a potential role of DNMT3b in

maintenance methylation in spermatogonia and high levels of Dnmt3L expression at the time of methylation acquisition in the female germ line. Our findings suggest that in-depth studies to understand the roles of each of the DNMT3 family members in male and female germ cell development and the acquisition of sequence-specific methylation patterns are warranted. As for DNMT1, such studies will likely require the identification and characterization of germ cell specific isoforms, detailed cell localization studies in isolated populations of germ cells, as well as the creation of germ cell-specific knockout models.

ACKNOWLEDGEMENTS

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TABLE 2.1 ***Details of primers used for Real-Time RT-PCR***

Gene	GenBank accession no.	Primers	Product size (bp)	Annealing temperature (°C)
Dnmt1	X14805	F: 5'-CCTAGTTCCGTGGCTACGAGGAGAA-3' R: 5'-TCTCTCTCCTCTGCAGCCGACTCA-3'	137	58
Dnmt3a	AF068625	F: 5'-GCCGAATTGTGTCTTGGTGGATGACA-3' R: 5'-CCTGGTGGAATGCACTGCAGAAGGA-3'	147	59
Dnmt3b	AF068626	F: 5'-TTCAGTGACCAGTCCTCAGACACGAA-3' R: 5'-TCAGAAGGCTGGAGACCTCCCTCTT-3'	145	59
Dnmt3L	AJ404467	F: 5'-GTGCGGGTACTGAGCCTTTTTAGA-3' R: 5'-CGACATTTGTGACATCTTCCACGTA-3'	120	63
18S	X00686	F: 5'-GCCCTGTAATTGGAATGAGTCCACTT-3' R: 5'-GTCCCCAAGATCCAACTACGAGCTTT-3'	149	59-63

Note. F, forward primer; R, reverse primer

Figure 2.1 ***Immunocytological localization of DNMT1 in the genital ridge.*** E11.5 genital ridge preparations were double-labeled with PATH52 to detect DNMT1 (green) and anti-SSEA-1 to identify primordial germ cells (PGCs; red), in addition to being counterstained with DAPI to visualize the nuclei (blue). (**A**) Longitudinal section of a male gonad. Examples of DNMT1-positive PGCs are indicated by open arrows. (**B-D**) High magnification of an isolated PGC imaged in the same z-plane via confocal microscopy. (**B**) DNMT1 immunoreactivity. (**C**) SSEA-1 immunoreactivity. (**D**) Superimposed confocal images of DNMT1 and SSEA-1 in the same germ cell. (**E-G**) High magnification of a PGC during the M-phase of the cell cycle imaged by confocal microscopy. (**E**) DNMT1 immunoreactivity surrounding condensed chromosomes. (**F**) SSEA-1 immunoreactivity. (**G**) Superimposed confocal images of DNMT1 and SSEA-1 staining. (**H-J**) High magnification of a somatic cell at metaphase photographed by conventional epifluorescence microscopy. (**H**) DNMT1 immunoreactivity. (**I**) DAPI staining of condensed chromosomes. (**J**) Double exposure of DNMT1 and DAPI.

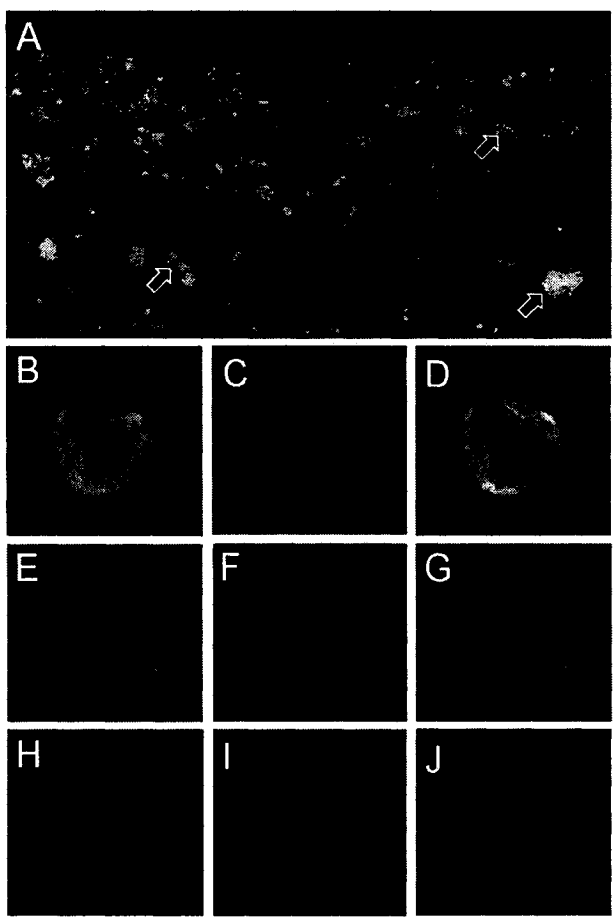

A
B
C
D
E
F
G
H
I
J

Figure 2.2 ***Immunohistological localization of DNMT1 in the fetal mouse testis and ovary.*** (**A-B**) E13.5 testis; (**C**) E18.5 testis; (**D-E**) E13.5 ovary; (**F**) E18.5 ovary. Parrafin-embedded fetal gonads were stained with immunoperoxidase (brown) using PATH52 to detect DNMT1 (B, C, E, F) or rabbit preimmune serum (A and D). Examples of dividing cells are indicated by thick, curved arrows. Representative cell types are indicated as follows: D, diplotene-stage oocyte; GC, germ cell; P, pachytene-stage oocyte; S, Sertoli cell.

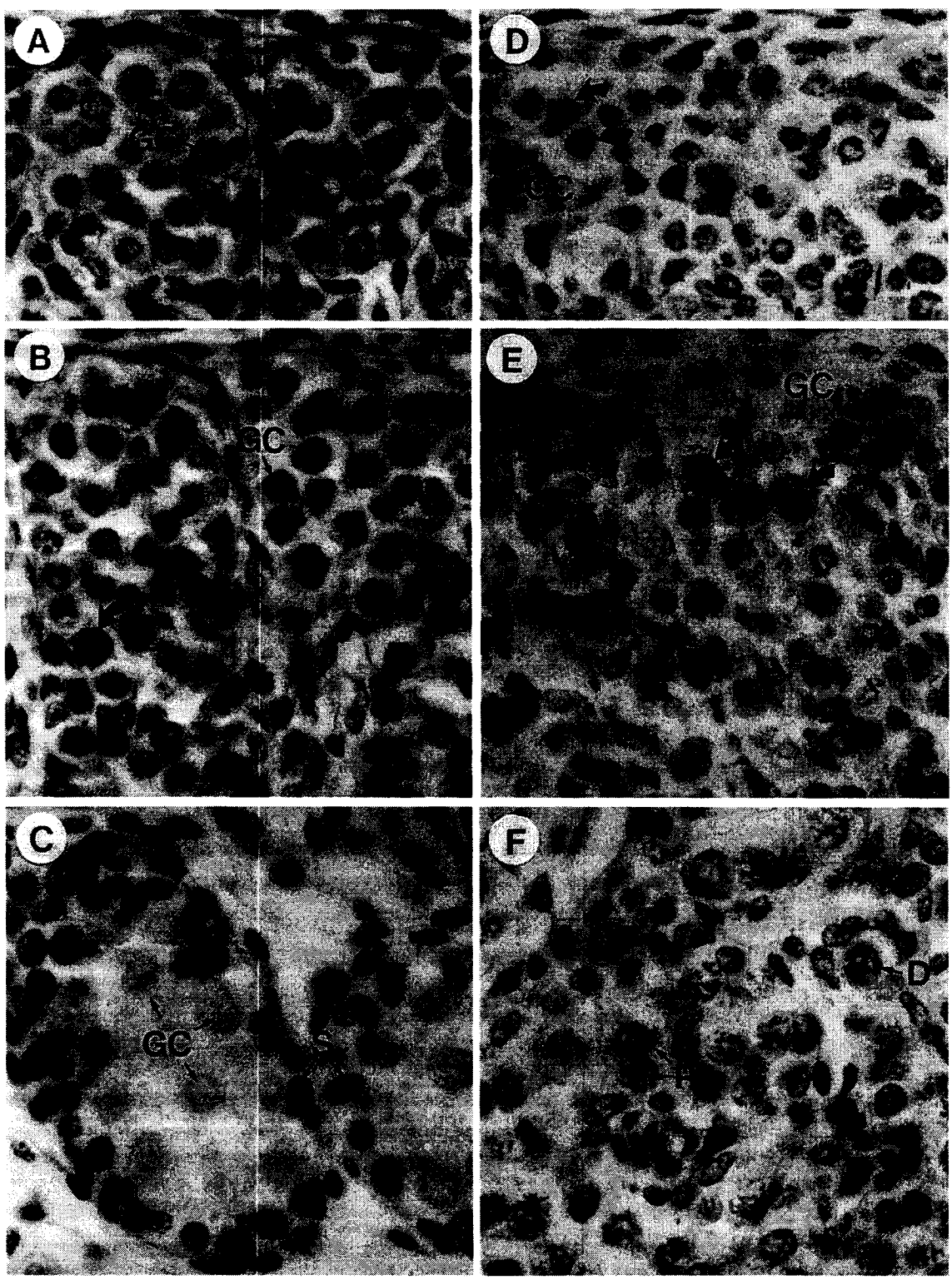

A
D
B
GC
E
GC
C
GC
F
D

Figure 2.3 ***DNMT1 expression in meiotic prophase oocytes.*** Fetal ovary preparations were double-labeled with PATH52 to detect DNMT1 (red) and anti-SC (green) to identify the various stages of meiotic prophase based on the state of synapsis between homologous chromosomes. (**A-B**) leptotene; (**C-D**) zygotene; (**E-F**) early pachytene and (**G-H**) late pachytene oocytes. (**I-J**) somatic cell. Note that DNMT1 staining is gradually down-regulated during meiotic prophase in the female mouse.

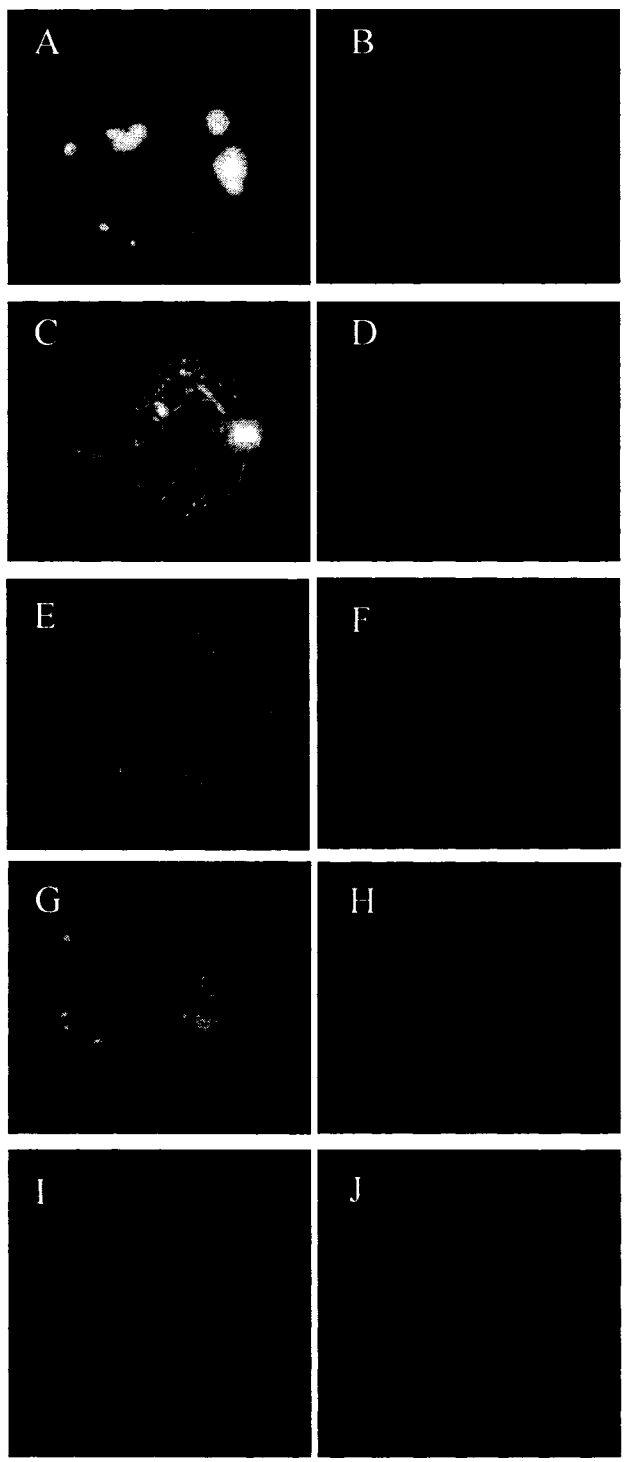

A
B
C
D
E
F
G
H
I
J

Figure 2.4 ***Northern Blot analysis of Dnmt1 mRNA in the developing mouse ovary and testis.*** Total RNA was extracted from E16.5 mesonephros (lane 1), E16.5 ovary (lane 2), E17.5 ovary (lane 3), 2 dpp ovary (lane 4), 6 dpp ovary (lane 5), 70 dpp ovary (lane 6), E16.5 testis (lane 7), 6 dpp testis (lane 8), 20 dpp testis (lane 9) and 70 dpp testis (lane 10). (**A**) Membrane probed with a ^{32}P-labeled Dnmt1 cDNA probe (pR5; Bestor *et al.*, 1988). (**B**) Membrane shown in (A) stripped and rehybridized with a ^{32}P-labeled cDNA probe to pachytene 1p 5' exon (Mertineit *et al.*, 1998). (**C**) Loading validated using a ^{32}P-labeled oligonucleotide complementary to 18S rRNA (Benoit and Trasler, 1994).

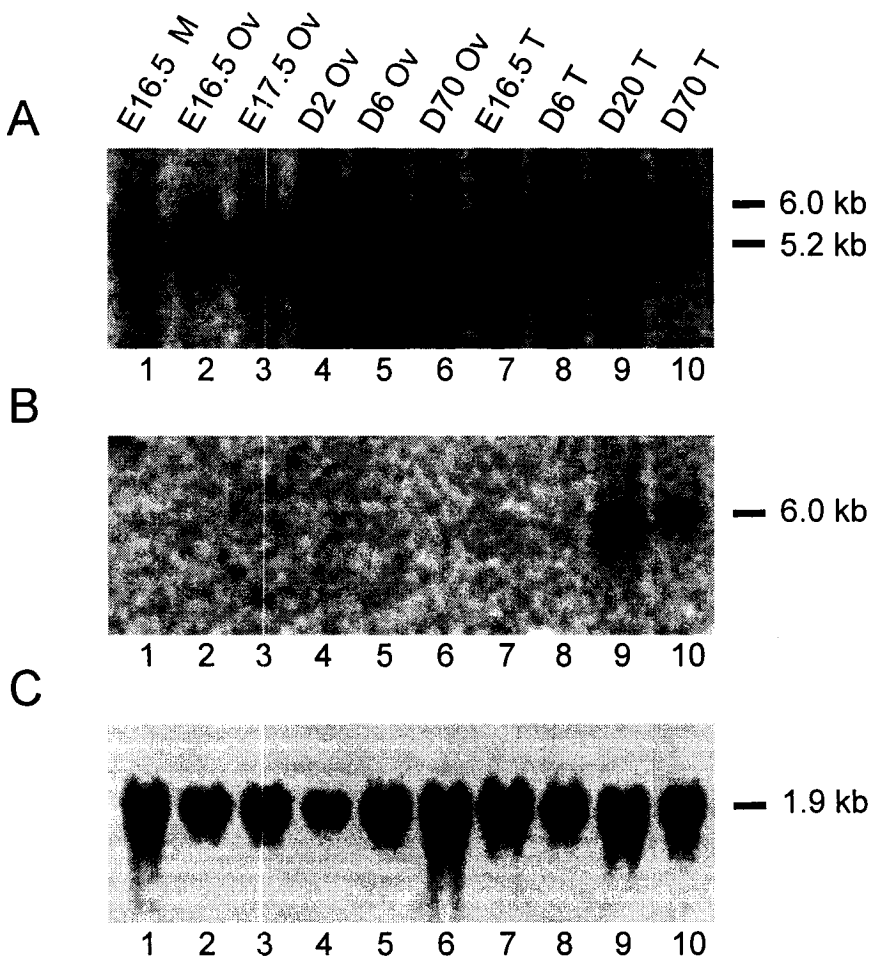

A
E16.5 M
E16.5 Ov
E17.5 Ov
D2 Ov
D6 Ov
D70 Ov
E16.5 T
D6 T
D20 T
D70 T
6.0 kb
5.2 kb
1 2 3 4 5 6 7 8 9 10
B
6.0 kb
1 2 3 4 5 6 7 8 9 10
C
1.9 kb
1 2 3 4 5 6 7 8 9 10

Figure 2.5 ***Immunohistological localization of DNMT1 in the postnatal mouse testis.*** Immunoperoxidase staining (brown) using the PATH52 antibody was used to detect the presence of DNMT1 in (**A**) 0 dpp testis, (**B**) 3 dpp testis and (**C**) 6 dpp testis. Examples of dividing germ cells intensely stained for DNMT1 are indicated by large arrows and positively stained interstitial cells by open arrows. Representative cell types are indicated as follows: GC, germ cell; S, Sertoli cell. Note that germ cells in the center of the tubules are unreactive at 0dpp but become highly reactive as they descend towards the basement membrane and resume mitosis.

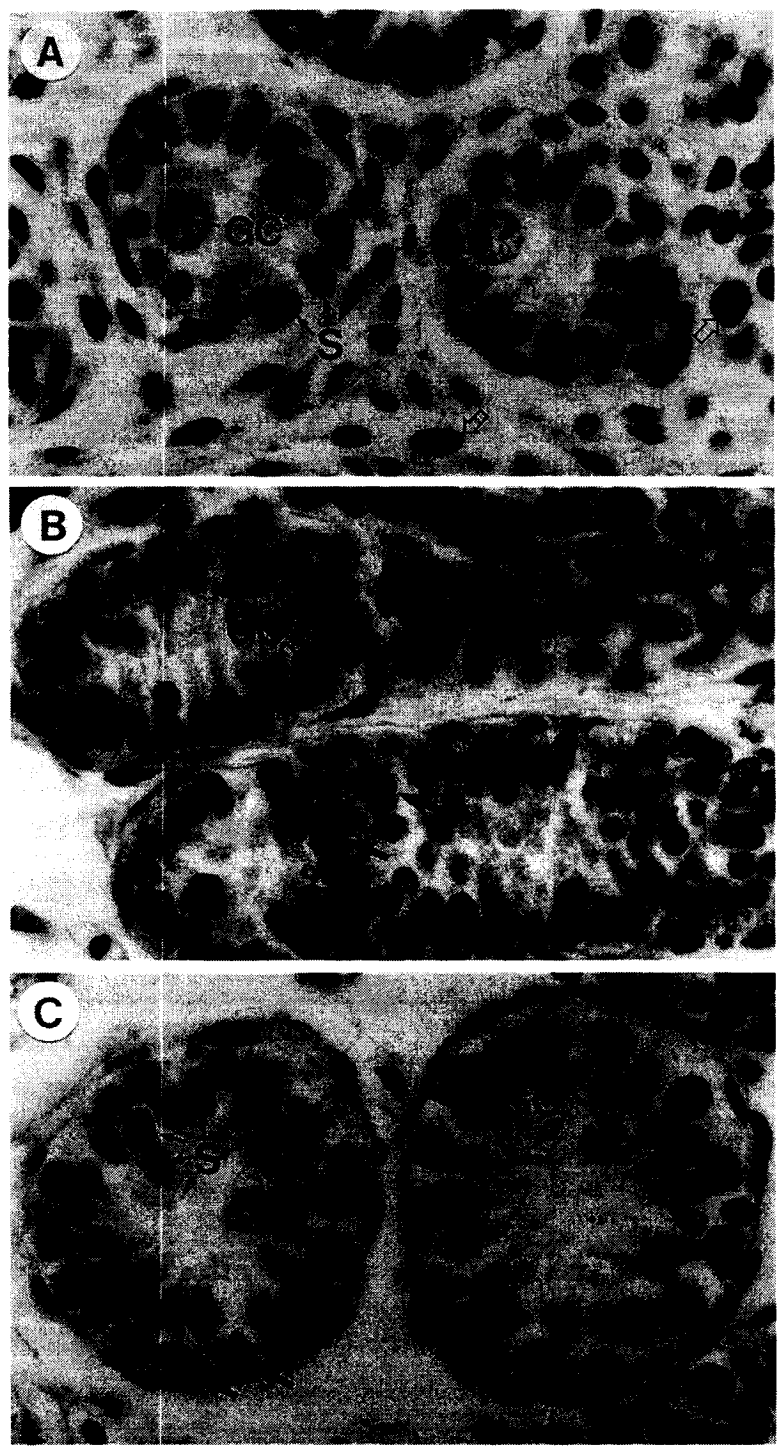

A
QC
S
B
C
S

Figure 2.6 ***Expression dynamics of DNA methyltransferases in the developing testis and ovary***. Relative quantification of Dnmt1 (black bar), Dnmt3a (gray bar) and Dnmt3b (white bar) in (**A**) testis and (**B**) ovary mRNA populations were done in triplicate on the same three individual samples of RNA extracted from pooled testes or ovaries at the indicated developmental stages via real-time RT-PCR. D= days. Mean ± SEM.

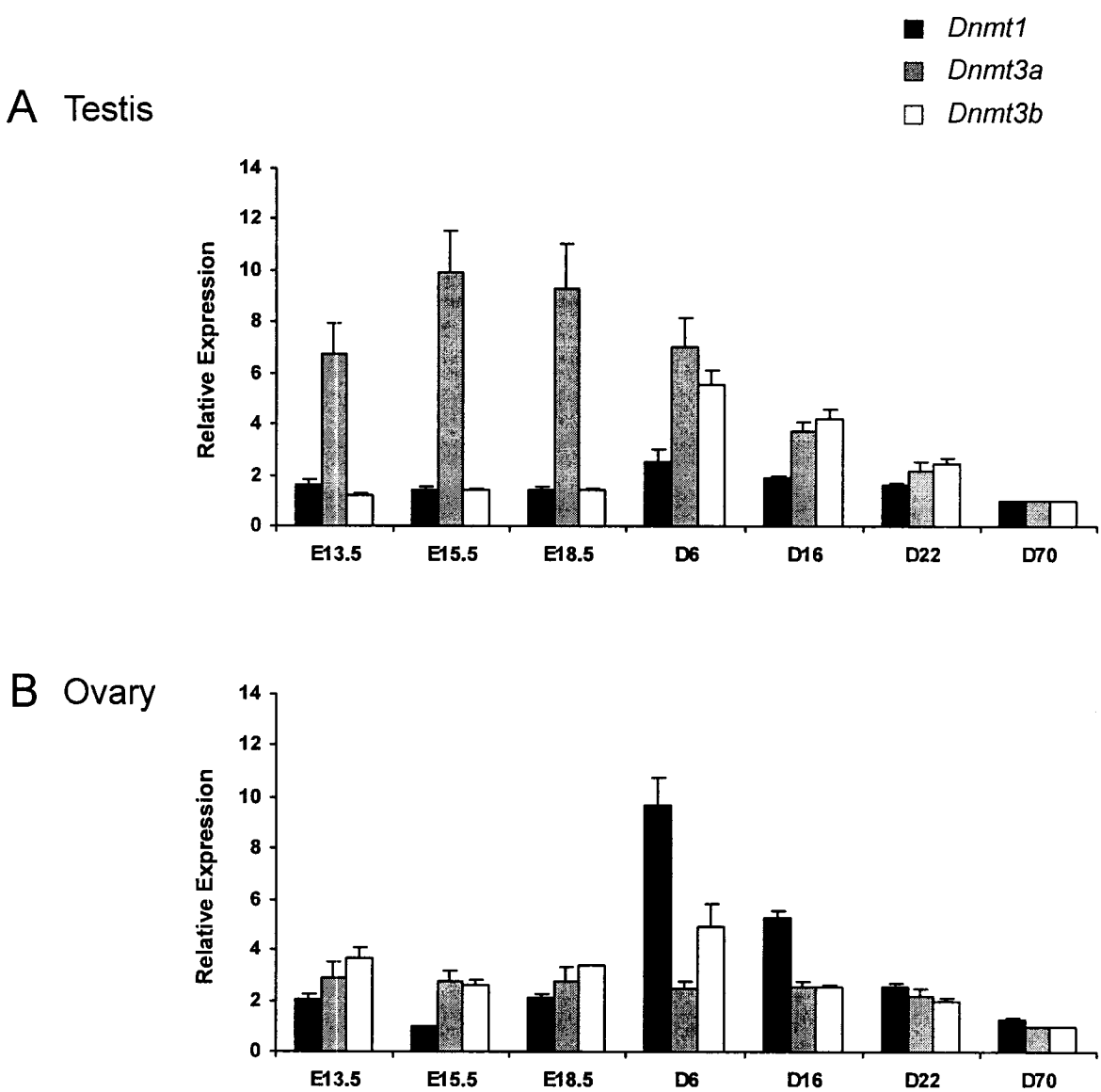

A Testis
Dnmt1
Dnmt3a
Dnmt3b
Relative Expression
0
2
4
6
8
10
12
14
E13.5
E15.5
E18.5
D6
D16
D22
D70
B Ovary
Relative Expression
0
2
4
6
8
10
12
14
E13.5
E15.5
E18.5
D6
D16
D22
D70

Figure 2.7 ***Expression dynamics of a DNA methyltransferase-like gene, Dnmt3L, in the developing testis (black bar) and ovary (white bar).*** Relative quantification of Dnmt3L mRNA population was done in triplicate on the same RNA samples via real-time RT-PCR. D= days. Mean ± SEM.

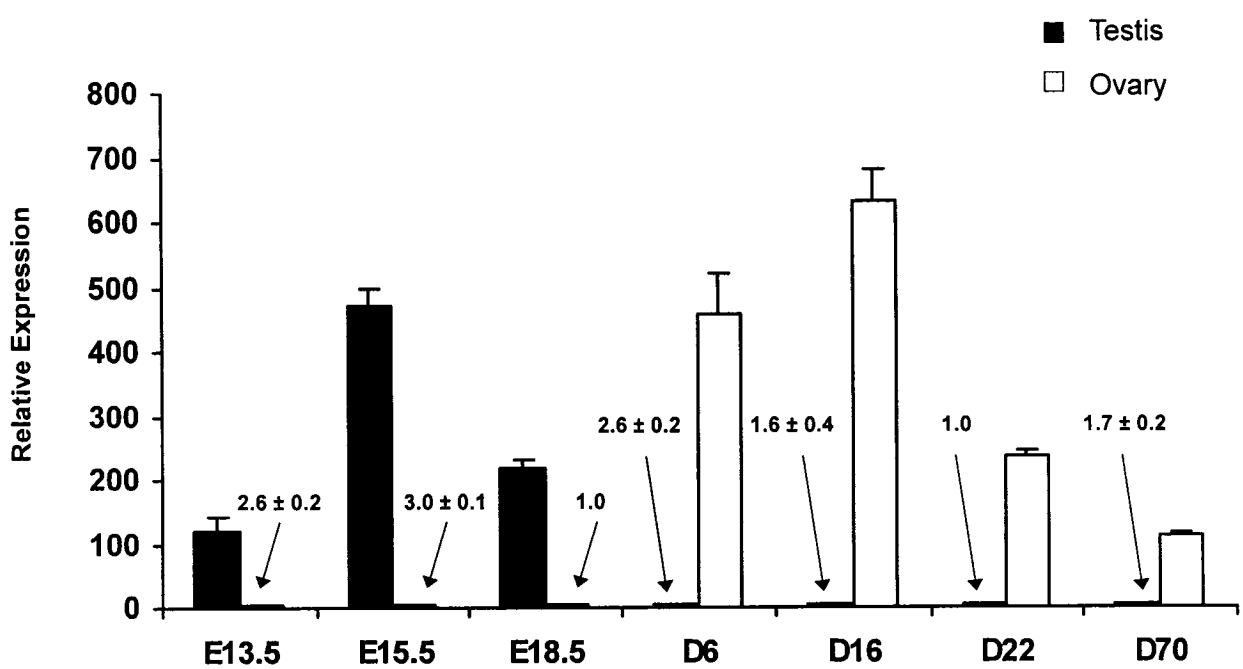
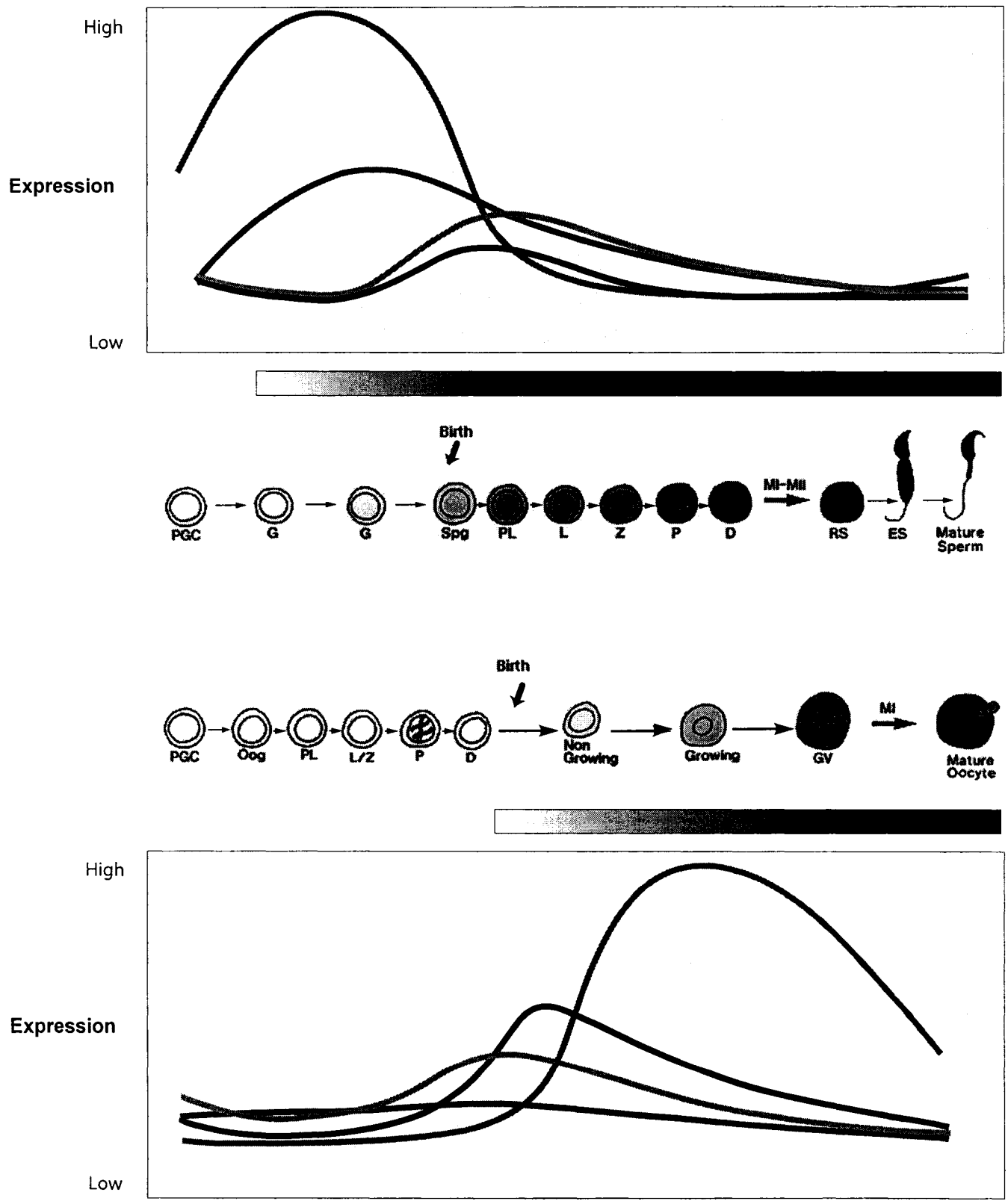

Testis
Ovary
Relative Expression
800
700
600
500
400
300
200
100
0
2.6 ± 0.2
3.0 ± 0.1
1.0
2.6 ± 0.2
1.6 ± 0.4
1.0
1.7 ± 0.2
E13.5
E15.5
E18.5
D6
D16
D22
D70

Figure 2.8 ***Schematic representation of DNA methyltransferase dynamics during gametogenesis.*** Progression of the establishment of methylation marks at imprinted and non-imprinted loci and relative levels of Dnmt1 (black), Dnmt3a (blue), Dnmt3b (green) and Dnmt3L (red) are presented as functions of spermatogenesis and oogenesis. Intensity of the shading in the boxes reflect the methylation status of the paternal (blue) and maternal (red) genomes. Dnmt3L levels are not to scale. Representative cell types are indicated as follows: PGC, primordial germ cell; G, gonocyte; Spg, spermatogonia; Oog, oogonia; PL, preleptotene; L, leptotene; Z, zygotene; P, pachytene; D, diplotene; GV, germinal vesicle; RS, round spermatid; ES, elongating spermatid. MI-MII, meiosis I-II. Modified from Lucifero *et al.*, 2002.


High
Expression
Low
Birth
PGC
G
G
Spg
PL
L
Z
P
D
MI-MII
RS
ES
Mature Sperm
Birth
PGC
Oog
PL
L/Z
P
D
Non Growing
Growing
GV
MI
Mature Oocyte
High
Expression
Low

CONNECTING TEXT

The studies described in Chapter II strongly suggest that DNMT1 is not the DNMT responsible for establishing methylation patterns during the initial phase of acquisition in the male, while characterizing the expression of the *Dnmt3* genes in both developing male and female gonads pointed to some interesting candidates that could be fulfilling that role. Follow up studies presented in Chapter III were designed to build on these observations as we decided to describe further the expression of the postulated *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* in male germ cells. Our choice to focus on the male germ line was guided by two factors: first, the most interesting modulations in *Dnmt* expression were observed in the developing testis; and second, both *de novo* and maintenance methylation events occur during spermatogenesis. In Chapter III, we present an in-depth analysis of the expression of the various transcript variants and protein isoforms of *Dnmt3a* and *Dnmt3b* in mitotic, meiotic and post-meiotic male germ cells.

CHAPTER III

Dynamic Expression of DNMT3a and DNMT3b Isoforms during Male Germ Cell Development in the Mouse

Sophie La Salle and Jacquetta M. Trasler

Developmental Biology (2006) 296: 71-82.

ABSTRACT

In the male germ line, sequence-specific methylation patterns are initially acquired prenatally in diploid gonocytes and are further consolidated after birth during spermatogenesis. It is still unclear how DNA methyltransferases are involved in establishing and/or maintaining these patterns in germ cells, or how their activity is regulated. We compared the temporal expression patterns of the postulated *de novo* DNA methyltransferases DNMT3a and DNMT3b in murine male germ cells. Mitotic, meiotic and post-meiotic male germ cells were isolated, and expression of various transcript variants and isoforms of *Dnmt3a* and *Dnmt3b* was examined using quantitative RT-PCR and Western blotting. We found that proliferating and differentiating male germ cells were marked by distinctive expression profiles. Dnmt3a2 and Dnmt3b transcripts were at their highest levels in type A spermatogonia, decreased dramatically in type B spermatogonia and preleptotene spermatocytes and rose again in leptotene/zygotene spermatocytes, while Dnmt3a expression was mostly constant, except in type B spermatogonia where it increased. In all cases, expression declined as pachynema progressed. At the protein level, DNMT3a was the predominant isoform in type B spermatogonia, while DNMT3a2, DNMT3b2, and DNMT3b3 were expressed throughout most of spermatogenesis, except in pachytene spermatocytes. We also detected DNMT3a2 and DNMT3b2 in round spermatids. Taken together, these data highlight the tightly regulated expression of these genes during spermatogenesis and provide evidence that DNMTs may be contributing differentially to the establishment and/or maintenance of methylation patterns in male germ cells.

INTRODUCTION

Methylation of genomic DNA is an epigenetic regulatory mechanism involved in controlling the transcriptional activity of genes and establishing higher order chromatin structures to preserve genome integrity (reviewed by Goll and Bestor, 2005). In mammals, DNA methylation patterns are initially reprogrammed during germ cell development. The patterns differ markedly between male and female gametes, especially at imprinted loci where methylation differences have important implications for allele-specific gene expression in the offspring (reviewed by Reik and Walter, 2001). In the mouse, a major demethylation event takes place in both germ lines between embryonic day (E) 10.5 and E12.5, around the time when primordial germ cells (PGCs) enter the gonads (Hajkova *et al.*, 2002, Kato *et al.*, 1999, Lane *et al.*, 2003, Lee *et al.*, 2002, Szabo and Mann, 1995 and Szabo *et al.*, 2002).

Following erasure, DNA methylation patterns are then reestablished in a sex- and sequence-specific manner during gametogenesis. In the male germ line, methylation acquisition begins before birth, in prospermatogonia (Davis *et al.*, 1999, Davis *et al.*, 2000, Kafri *et al.*, 1992, Lees-Murdock *et al.*, 2003, Li *et al.*, 2004, Ueda *et al.*, 2000 and Walsh *et al.*, 1998). Between E15 and E19 germ cells begin to stain strongly with an antibody directed against methylated cytosine, indicating the timing of increases in overall global methylation; further methylation changes occur in the few days after birth (Coffigny *et al.*, 1999). At the individual sequence level, the majority of methylated CpGs in the genome are found in repetitive DNA sequences. For repetitive elements such as intracisternal A particles (IAPs), long interspersed nuclear elements (LINEs) and satellite sequences, methylation acquisition is for the most part complete by E17.5 (Lees-Murdock *et al.*, 2003 and Walsh *et al.*, 1998). Remethylation of imprinted genes also begins around E15.5, but the process is only completed after birth (Davis *et al.*, 1999, Davis *et al.*, 2000 and Li *et al.*, 2004). Developmental studies have shown that the imprinted gene *H19* begins to acquire its methylation marks between E15.5 and E18.5, but only becomes fully methylated postnatally by

pachynema (Davis *et al.*, 1999 and Davis *et al.*, 2000). Similarly, Chaillet *et al.* (1991) have demonstrated that an imprinted transgene initially gains its methylation before birth, but the process is only completed postnatally. Assessing the methylation status of a few testis-specific genes has further substantiated methylation acquisition to be continual during spermatogenesis; while some genes are demethylated prior to their expression in the testis, others become *de novo* methylated (reviewed by Maclean and Wilkinson, 2005). A feature unique to the male germ line is that, in parallel to their establishment, methylation marks must be maintained during DNA replication in spermatogonia and preleptotene spermatocytes; these cells are therefore capable of *de novo* and maintenance methylation. Conversely in the female germ line, methylation patterns are acquired postnatally during the oocyte growth phase, after the pachytene phase of meiosis is completed and DNA has been replicated (Kono *et al.*, 1996, Lucifero *et al.*, 2002 and Walsh *et al.*, 1998).

Both *de novo* and maintenance DNA (cytosine-5)-methyltransferases (DNMTs) work in concert to create and propagate genomic methylation patterns. Currently, five DNMTs have been characterized and are classified according to similarities found in their C-terminal catalytic domain: DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L (reviewed by Goll and Bestor, 2005). Of these, only DNMT1, DNMT3a, and DNMT3b have been proven to have catalytic activity in vivo. DNMT1 is the major methyltransferase in somatic tissues; it has a preference for hemimethylated DNA and is critical for the maintenance of methylation patterns after DNA replication (Bestor, 1992, Li *et al.*, 1992, Lei *et al.*, 1996 and Yoder *et al.*, 1997a). Sex-specific exons control the expression of *Dnmt1* in the mouse germ line (Mertineit *et al.*, 1998). In the male, DNMT1 is not detected in prenatal gonocytes when methylation patterns are initially laid down, but it is detected in proliferating spermatogonia, as well as leptotene/zygotene spermatocytes (Jue *et al.*, 1995, La Salle *et al.*, 2004 and Sakai *et al.*, 2001). Complete down-regulation of DNMT1 during pachynema is associated with the expression of Dnmt1p, an untranslated pachytene-specific *Dnmt1* transcript (Jue *et al.*, 1995 and Mertineit *et al.*, 1998), whereas DNMT1 becomes reexpressed in

round spermatids (Jue *et al.*, 1995 and Trasler *et al.*, 1992). It is still unclear which role DNMT1 plays during meiotic prophase or in round spermatids in the absence of DNA replication.

In contrast, DNMT3a and DNMT3b have been postulated to function primarily as *de novo* DNA methyltransferases (Okano *et al.*, 1998). They are expressed at high levels in mouse embryonic stem (ES) cells and during embryonic development (Okano *et al.*, 1998, Okano *et al.*, 1999, Chen *et al.*, 2003 and Watanabe *et al.*, 2002). Expression of *Dnmt3a* is controlled by the use of alternate promoters to produce two different isoforms (Chen *et al.*, 2002). DNMT3a is expressed ubiquitously at low levels and localizes to heterochromatin, suggestive of a housekeeping role. In contrast, DNMT3a2 has been suggested to be more important to *de novo* methylation because it is expressed at high levels in embryonic stem cells and shows restricted expression in tissues known to undergo *de novo* methylation such as the testis and the ovary, in addition to localizing to euchromatin. All known isoforms of *Dnmt3b* result from alternative splicing of exons 11, 22, and/or 23 in various combinations (Chen *et al.*, 2002, Ishida *et al.*, 2003, Okano *et al.*, 1998 and Weisenberger *et al.*, 2004). Of these, only DNMT3b1 (full-length isoform) and DNMT3b2 (shorter isoform missing the amino acids encoded by exon 11) are capable of DNA methylation (Aoki *et al.*, 2001 and Okano *et al.*, 1998). Presumably, the other isoforms are incapable of methylating DNA since their catalytic domain is compromised by splicing of exons 22 and 23; however, they could act as regulators of DNA methylation (Aoki *et al.*, 2001, Chen *et al.*, 2002, Okano *et al.*, 1998 and Weisenberger *et al.*, 2004). Interestingly, DNMT3b1 and DNMT3b6 appear to be expressed only in ES cells, while DNMT3b2 and DNMT3b3 are expressed in a restricted manner in somatic tissues (Chen *et al.*, 2002 and Weisenberger *et al.*, 2004). We have previously shown that expression of *Dnmt3a* and *Dnmt3b* is highly modulated during testis development (La Salle *et al.*, 2004). Recently, Kaneda *et al.* (2004) have shown that early germ cell-specific inactivation of *Dnmt3a*, but not *Dnmt3b*, impairs the establishment of *de novo* methylation patterns in male germ cells, more specifically at paternally

imprinted loci, without affecting the methylation status of repeat sequences. Interestingly, inactivation of *Dnmt3L*, a member of the DNMT3 family that lacks DNA methyltransferase activity, produces a similar phenotype: deficient males are infertile, and their germ cells show abnormal DNA methylation acquisition of some repeat elements and imprinted loci (Bourc'his and Bestor, 2004, Bourc'his *et al.*, 2001, Hata *et al.*, 2002 and Webster *et al.*, 2005). Studies have started to look at the expression of these genes in germ cells (Lees-Murdock *et al.*, 2005, Sakai *et al.*, 2004 and Watanabe *et al.*, 2004), but detailed developmental studies monitoring expression of individual isoforms in key cell types and times when *de novo* and maintenance methylation are occurring during spermatogenesis are still lacking.

The role each DNA methylating enzyme plays in the genome-wide methylation events that take place throughout male germ cell development, as well as the mechanisms governing their expression, are still unclear. Spermatogenesis is a complex process during which diploid spermatogonia divide and mature into spermatocytes that undergo meiosis to produce haploid spermatids; spermatids go through a specialized maturation process termed spermiogenesis in order to become sperm. Establishment of DNA methylation patterns occurs in spermatogonia and in spermatocytes but is not thought to happen in spermatids. In parallel, maintenance methylation has always been thought to take place in the context of DNA replication, therefore in mitotically dividing spermatogonia and in preleptotene spermatocytes. Here, we explore how the expression of *Dnmt3a* and *Dnmt3b* is regulated during postnatal spermatogenesis. We report the expression profiles of *Dnmt3a* and *Dnmt3b* in spermatogonia, spermatocytes, and spermatids. We demonstrate that the expression of these genes is highly dynamic in isolated postnatal male germ cells, and we identify windows when the expression of these genes is downregulated. We show that specific germ cell types are marked by the expression of particular *Dnmt3a* transcript variants and isoforms. In addition, we demonstrate that these enzymes are present in spermatids, a cell type not suspected to require *de novo* or maintenance methylation capacities.

MATERIALS AND METHODS

Mice

Male CD-1 mice were purchased from Charles River Canada Inc. (St. Constant, QC, Canada); the day of birth was designated as postnatal day (dpp) 0. All procedures were performed in accordance with the Canadian Council on Animal Care and approved by the McGill University Animal Care Committee.

Isolation of Male Germ Cells

Purified populations of male germ cells were obtained from the testes of 8-, 17- and 70-dpp mice according to the sedimentation velocity cell separation method (Romrell *et al.*, 1976, Bellvé *et al.*, 1977a and Bellvé *et al.*, 1977b). Briefly, testes of a given age group are collected in RPMI media (Gibco BRL/Invitrogen, Burlington, ON, Canada), enzymatically digested and mechanically disrupted to produce a cell suspension; the cells are then allowed to separate by cellular sedimentation at unit gravity in a 2–4% BSA (bovine serum albumine) gradient generated with a STA-PUT apparatus (Johns Scientific, Toronto, ON, Canada). Cells are identified on the basis of morphological criteria and size. Populations of type A spermatogonia (average purity = 86%) and type B spermatogonia (average purity = 83%) were obtained from the testes of 8-dpp mice ($n = 2$ cell separations). Preleptotene spermatocytes (average purity = 85%), leptotene/zygotene spermatocytes (average purity = 87%) and prepubertal pachytene spermatocytes (average purity = 80%) were obtained from the testes of 17-dpp mice ($n = 2$ cell separations). Pachytene spermatocytes (average purity = 81%), round spermatids (average purity = 88%), and elongating spermatids mixed with residual bodies (average purity = 86%) were obtained from 70-dpp mice ($n = 2$ cell separations).

RNA Extraction and Real-Time, quantitative RT-PCR

Total RNA was extracted from snap-frozen pellets of isolated populations of male germ cells using the RNeasy extraction kit with DNaseI treatment and

was concentrated using the MinElute kit as described by the manufacturer (Qiagen Inc., Mississauga, ON, Canada). Samples were diluted to 10 ng/μl, dispensed in single-use aliquots and stored at −80°C. Quantitative RT-PCR (qRT-PCR) was performed on the Mx4000 qPCR system from Stratagene (La Jolla, CA) using the QuantiTect SYBR Green RT-PCR kit (Qiagen). Gene-specific primers (La Salle *et al.*, 2004) were used to determine the overall relative expression levels of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* according to the standard curve method (Bustin, 2002). Primers were designed to span introns and pick up all known transcript variants of the various *Dnmt* genes, resulting in the detection of a single band on a gel (data not shown). Transcript variant-specific primers were also designed to determine the relative expression levels of Dnmt3a and Dnmt3a2. Primer sequences were as follows: Dnmt3a forward 5′-CGACCCATGCCAAGACTCACCTTCCAG-3′ (Weisenberger *et al.*, 2002), Dnmt3a2 forward 5′-CCAGACGGGCAGCTATTTAC-3′, and Dnmt3a/3a2 reverse 5′-AGACTCTCCAGAGGCCTGGT-3′; annealing temperatures used were 64°C and 59°C for Dnmt3a and Dnmt3a2, respectively. SYBR Green was used to detect the double-stranded DNA produced during the amplification reaction. Reactions were performed using approximately 10 ng or 100 pg of total RNA for the Dnmts and 18S, respectively. One-step RT-PCR reactions were performed in a 25 μl volume as directed by the manufacturer for 40 cycles. For each product tested, a specific standard curve was established using single-use aliquots of the same stock of RNA (total RNA extracted simultaneously from multiple 6 dpp testes). In all cases, reactions were performed in triplicate on the same two independent sets of germ cells. Specificity was assessed by melting curve analysis and confirmed on a 3% agarose gel after each qRT-PCR experiment (data not shown). qRT-PCR results were normalized to their corresponding 18S rRNA content. Fold changes in expression for a given gene were determined in relation to the expression of that gene in pachytene spermatocytes (calibrator); all other quantities were expressed as an *n*-fold difference relative to the calibrator. Because the same stock of RNA was used to prepare all standard curves, the relative quantities determined for a given gene using this method could be

compared across individual experiments. Representative data for one set of germ cells are presented as mean ± SD.

RT-PCR

The same RNA used for qRT-PCR was used for RT-PCR. RT-PCR reactions were performed using the Qiagen One-step RT-PCR kit as described by the manufacturer. Primers used to discriminate between Dnmt3aα and Dnmt3aβ have been described elsewhere (Weisenberger *et al.*, 2002); primers that span exon 11 and exons 22–23 of the *Dnmt3b* gene were described by Weisenberger *et al.* (2004). RT-PCR reactions were performed using 20 ng of total RNA in a 25 μl volume. The products were run on a 2% low-melting point agarose gel and stained with ethidium bromide.

Protein Extraction and Immunoblotting

Proteins were extracted from the testes of 6- and 70-dpp mice or from freshly isolated male germ cells. Protein lysates were prepared by homogenization in 0.15 M NaCl, 0.05 M Tris–Cl (pH 7.4) and 0.01% protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada) according to the manufacturer's instructions. Total protein aliquots of 70 μg or 7 μg of whole testes or isolated germ cells, respectively, were heated at 65°C for 10 min in reducing sample buffer, electrophoresed on 8% SDS-polyacrylamide gels, and transferred to Hybond ECL nitrocellulose membranes (Amersham, Montreal, QC, Canada). Membranes were blocked in 5% non-fat dried milk and were incubated with one of the following primary antibodies diluted in blocking buffer: clone 64B1446, a monoclonal antibody (mAb) raised against recombinant mouse DNMT3a (1:400; Imgenex, San Diego, CA); NB 100–265, a polyclonal antibody (pAb) raised against amino acids 10–118 of human DNMT3A (1:2000; Novus Biologicals, Littleton, CO); clone 52A1018, a mAb raised against recombinant mouse DNMT3b (1:800; Imgenex); the specificity of these antibodies had previously been assessed by Chen *et al.* (2002), Beaulieu *et al.*

(2002) and Weisenberger *et al.* (2004), respectively. Membranes were then washed according to the manufacturer's instruction (Amersham), followed by incubation with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody or a HRP-conjugated goat anti-rabbit IgG antibody (1:7500; Molecular Probes/Invitrogen). After exposure to ECL Plus Western Blotting detection solution (Amersham), chemiluminescence was revealed on Hyperfilm ECL film (Amersham). Coomassie Brilliant Blue staining of gels electrophoresed under identical conditions was used to confirm that equals amounts of proteins were loaded on gel.

RESULTS

Expression of DNA Methyltransferases in Male Germ Cells

As DNA methylation pattern establishment and maintenance are ongoing during spermatogenesis, we examined the expression dynamics of *Dnmt3a* and *Dnmt3b* in isolated postnatal male germ cells using quantitative RT-PCR (qRT-PCR). Germ cells from dissociated testes were fractionated by unit gravity sedimentation on a gradient of BSA as described; eight different populations of male germ cells could be isolated using mice of different age groups. We first determined the overall expression levels of the genes in the various populations of male germ cells purified. Validation of the assay was assessed by determining the expression profile of *Dnmt1*, a DNA methyltransferase that has been well studied in the male germ line (Jue *et al.*, 1995 and Trasler *et al.*, 1992). In keeping with previous results obtained by Northern blotting (Jue *et al.*, 1995 and Trasler *et al.*, 1992), expression was highest in type A spermatogonia, somewhat lower in type B spermatogonia and preleptotene spermatocytes, and slightly increased in leptotene/zygotene spermatocytes; levels of transcripts decreased as pachynema progressed (Fig. 3.1A). *Dnmt1* was also expressed in round spermatids at levels comparable to type B spermatogonia but was almost undetectable in residual bodies/elongating spermatids. When global levels of Dnmt3a transcripts were measured, expression results similar to *Dnmt1* were obtained (Fig. 3.1B). *Dnmt3a* expression was high in type A spermatogonia, slightly decreased in type B spermatogonia and more so in preleptotene spermatocytes, but the decreases were not as marked as the ones observed for *Dnmt1* in the same germ cell types. Expression in leptotene/zygotene spermatocytes was about the same as in type A spermatogonia, only to decrease through pachynema. Again, Dnmt3a transcripts were detected in round spermatids but were at their lowest level in residual bodies/elongating spermatids. In contrast, *Dnmt3b* expression was a lot more dynamic during male germ cell development, increases and decreases in expression being more pronounced for this gene (Fig. 3.1C). *Dnmt3b* expression was highest in type A

spermatogonia, decreased dramatically in type B spermatogonia and preleptotene spermatocytes (by more than 4-fold), only to increase again by almost 2-fold in leptotene/zygotene spermatocytes. Once more, progression through pachynema was associated with a steady decrease in expression, while Dnmt3b transcripts were clearly detected in round spermatids. Dnmt3a and Dnmt3b transcripts can therefore be detected at all stages of male germ cell development tested, even at stages when *de novo* and maintenance methylation have not been shown to take place (see Supplementary Fig. 3.7 for a comparison of the relative expression levels of Dnmt3a, Dnmt3b, and Dnmt1 transcripts).

Transcriptional Control of Dnmt3a Expression: Usage of Alternative First Exons and Promoters

Transcriptional regulation at the *Dnmt3a* locus is multifaceted (Fig. 3.2 and Fig. 3.3). We used qRT-PCR to tease out the discrepancies in expression between Dnmt3a and Dnmt3a2, the transcripts resulting in the production of the two main protein products (Fig. 3.2B). A forward primer unique to either Dnmt3a or Dnmt3a2 was used in combination with a reverse primer common to both products; the individual PCR products were amplified in separate reactions due to the type of detection method employed. Two distinct expression profiles were obtained for Dnmt3a and Dnmt3a2. Dnmt3a (Fig. 3.2B, top panel) was expressed relatively constantly until pachynema, even if its expression did seem to increase in type B spermatogonia, and was not significantly expressed in spermatids. Expression in spermatids and residual bodies could not be determined because the initial amount of Dnmt3a template was not sufficient in these samples to be distinguished above background and produce a Ct (threshold cycle) value that could be analyzed. Dnmt3a2 (Fig. 3.2B, bottom panel) presented a very dynamic expression pattern reminiscent of the one obtained for *Dnmt3b* (Fig. 3.1C). Expression of Dnmt3a2 was highest in type A spermatogonia, drastically decreased in type B spermatogonia and preleptotene spermatocytes, and increased again in leptotene/zygotene spermatocytes to levels almost as high as in type A spermatogonia. There was an abrupt drop in

expression in prepubertal pachytene spermatocytes that persisted in more mature pachytene spermatocytes. Contrary to Dnmt3a, Dnmt3a2 could be amplified in round spermatids and to a lower extent in residual bodies/elongating spermatids. Clear differences in expression could therefore be detected between Dnmt3a and Dnmt3a2, suggesting that their two protein products could be playing distinct roles during male germ cell development (see Supplementary Fig. 3.8 for a comparison of the relative expression levels of Dnmt3a and Dnmt3a2 transcripts).

Additional alternatively spliced variants of *Dnmt3a* have also been described and are illustrated in Fig. 3.3A (Weisenberger *et al.*, 2002). Dnmt3a can be produced from the use of alternate 5′ first exons (exons 1α and 1β) and transcripts that include intron 4 have also been detected. Transcripts containing exon 1α (Dnmt3aα) are usually detected in somatic cells, whereas exon1β-containing transcripts (Dnmt3aβ) are preferentially expressed in mouse ES cells (Weisenberger *et al.*, 2002). Having monitored the levels of Dnmt3a transcripts by qRT-PCR without being able to discriminate between Dnmt3aα and Dnmt3aβ, we wanted to determine if transcription went through both first exons in male germ cells. Contribution of the two first exons was evaluated using RT-PCR and was found to proceed through both first exons (Fig. 3.3B). Interestingly, we were able to detect Dnmt3a transcripts in round spermatids using this approach, albeit at very low levels, conversely to when qRT-PCR was used. We next tried to detect the presence of intron 4-containing transcripts using a similar RT-PCR strategy but were unable to detect any of these transcripts in male germ cells (data not shown). Overall, the data indicate that Dnmt3aα, Dnmt3aβ, and Dnmt3a2 are the main forms of *Dnmt3a* transcripts present in male germ cells and suggest that tight control of transcription takes place by use of different mechanisms (alternate promoter or first exon) throughout spermatogenesis.

Production of Multiple Dnmt3b Transcripts by Alternative Splicing in Male Germ Cells

The mouse *Dnmt3b* gene contains 24 exons, of which exons 11, 22, and/or 23 can be alternatively spliced to produce eight different transcripts (Fig. 3.4A; Ishida *et al.*, 2003 and Weisenberger *et al.*, 2004). Weisenberger *et al.* (2004) determined that exon 11-spliced transcripts are usually expressed in somatic cells, whereas unspliced products are mostly expressed in ES cells. Using primers that span the two splicing events, we established the occurrence of these events in male germ cells by means of RT-PCR. We could see that transcripts with a spliced exon 11 were usually more prevalent (Fig. 3.4B, top panel), and that at least three of the four possible splicing combinations at exons 22 and/or 23 (which affect the integrity of the catalytic domain) could be detected at all stages of male germ cell development tested (Fig. 3.4B, bottom panel). Since spliced-exon 11 mRNA species are more abundant, and that the strongest amplification was obtained for intact exons 22 and 23, we can predict that the predominant form of *Dnmt3b* mRNA is Dnmt3b2.

Immunoblot Analysis of DNMT3a and DNMT3b Expression during Male Gametogenesis

Having established the expression profiles of *Dnmt3a* and *Dnmt3b* at the mRNA level during male germ cell development, we undertook a similar analysis of protein expression. Lysates of purified germ cells were analyzed by immunoblot using commercially available antibodies. For *Dnmt3a*, two antibodies were used to detect the presence of DNMT3a and DNMT3a2 (see Fig. 3.5A for epitope mapping). Consistent with its epitope mapping to the N-terminus, antibody NB 100–265 detected a single band of ~130 kDa corresponding to DNMT3a only (Fig. 3.5B, top panel). DNMT3a was detected in type A and B spermatogonia, preleptotene, leptotene/zygotene, and prepubertal pachytene spermatocytes but was completely absent in pachytene spermatocytes. Most surprisingly, DNMT3a expression peaked in type B spermatogonia, consistent with the mRNA expression profile; it was not detected in either round spermatids

or in residual bodies/elongating spermatids. When mAb 64B1446 was used, an additional band of ~100 kDa corresponding to DNMT3a2 was detected in addition to the 130-kDa band corresponding to DNMT3a (Fig. 3.5B, middle panel). Albeit the bands were fainter most likely due to antibody titer, a profile similar to the one obtained with pAb NB 100–265 was detected for DNMT3a, expression peaking again in type B spermatogonia. The profile obtained for the 100-kDa band was very different: DNMT3a2 expression was highest in type A spermatogonia and leptotene/zygotene spermatocytes, was present at lower levels in type B spermatogonia and preleptotene spermatocytes, but decreased in prepubertal pachytene spermatocytes and became almost absent in pachytene spermatocytes. DNMT3a2 was then reexpressed in round spermatids but was not detected in residual bodies/elongating spermatids. Using a combination of antibodies, we were able to show distinctive expression profiles for DNMT3a and DNMT3a2 in germ cells.

For *Dnmt3b*, only one antibody capable of detecting all isoforms was used (Weisenberger *et al.*, 2004). The RT-PCR data presented here as well as a study done by Chen *et al.* (2002) on adult tissues including the testis suggested that Dnmt3b2 and Dnmt3b3 were the two major transcripts of the male germ line. Because DNMT3b isoforms have similar molecular weights, we compared the migration profiles obtained for the germ cell lysates to the one obtained for 6 and 70 dpp testis protein extracts (Fig. 3.5C). We could detect five bands in the 6 dpp extract, two bands being more prominent than the others. The highest molecular weight band was consistent with the weight expected for DNMT3b1 (~120 kDa) and is only very faintly expressed. The following, predominant two bands most likely corresponded to DNMT3b2 and DNMT3b3, since the most abundant mRNA species detected in the adult testis and in germ cells were ones containing and lacking exons 22/23, respectively (Fig. 3.4B; Chen *et al.*, 2002). The last two bands were of much smaller molecular weight and could correspond to DNMT3b4 or DNMT3b7, since transcripts lacking exon 22 were also detected in germ cells (Fig. 3.4B). We could only detect 3 bands in the 70 dpp extract, DNMT3b2 and DNMT3b3 most likely being the two predominant isoforms as

Chen *et al.* (2002) had suggested. When we looked at the migration profile obtained for germ cell lysates, we saw only two predominant bands that migrate at the same rate as the bands corresponding to DNMT3b2 and DNMT3b3 in the 6 and 70 dpp testis lanes (Fig. 3.5C). According to the RT-PCR results (Fig. 3.4B), other isoforms could be present in germ cells, but they are either expressed at levels too low to be detected in these conditions or these mRNA remain untranslated.

DISCUSSION

DNA methylation events taking place during spermatogenesis have important implications for gamete integrity and transmission of epigenetic information to the next generation (Bourc'his and Bestor, 2004 and Kaneda *et al.*, 2004). Recent studies have started to shed light on DNA methyltransferases that might be involved in these important processes (Bourc'his *et al.*, 2001, Hata *et al.*, 2002 and Kaneda *et al.*, 2004), but it is still unclear if all DNMTs contribute equally to the establishment and the maintenance of DNA methylation patterns throughout spermatogenesis. We provide evidence that the expression of the genes encoding the postulated *de novo* DNA methyltransferases is developmentally regulated during male germ cell development. We show that proliferating and differentiating male germ cells are marked by distinctive Dnmt3a and Dnmt3b expression profiles and that specific transcript variants as well as isoforms are expressed in particular cell types. We also demonstrate that *Dnmt3a* and *Dnmt3b* expression is downregulated both at the RNA and protein levels during pachynema, as it is the case for *Dnmt1*. Finally, we clearly show that DNMT3a and DNMT3b isoforms are detected in round spermatids.

There is growing evidence that DNMT family members directly interact and cooperate to establish and maintain DNA methylation patterns (Chen *et al.*, 2003, Datta *et al.*, 2003, Hata *et al.*, 2002, Kim *et al.*, 2002 and Margot *et al.*, 2003). Since DNMTs do not appear to have any sequence specificity beyond CpG dinucleotides, multiple mechanisms are proposed to explain how DNA methyltransferases can find their targets in the genome, including differential accessibility to chromosomal regions and recruitment of DNMTs to specific sequences by accessory factors (reviewed by Li, 2002). Regulation of the establishment of DNA methylation patterns has been suggested to be controlled by differential expression of *Dnmt3a* and *Dnmt3b* isoforms by Chen *et al.* (2003); different enzymes would have both common and preferred target sequences. In their system, minor satellite repeats were methylated by DNMT3b1, major satellite repeats were methylated by DNMT3a and DNMT3a2, while the 5′ region

of *H19* was only methylated by DNMT3a2. Consistent with that, gene-targeting studies clearly demonstrate that not all sequences are affected equally upon *Dnmt* inactivation (Bourc'his and Bestor, 2004, Bourc'his *et al.*, 2001, Hata *et al.*, 2002, Kaneda *et al.*, 2004, Li *et al.*, 1992, Okano *et al.*, 1999 and Webster *et al.*, 2005). DNMT3b-deficient mice show demethylation of minor satellite repeats, while inactivation of *Dnmt3L* leads to incomplete acquisition of methylation at the paternally imprinted loci *H19* and *Rasgrf1*, in addition to preventing methylation of LINE-1 and IAP repeat elements in the male germ line (Bourc'his and Bestor, 2004, Okano *et al.*, 1999 and Webster *et al.*, 2005). Of particular interest is a recent report by Kaneda *et al.* (2004) showing that acquisition of DNA methylation at some but not all imprinted loci is impaired when *Dnmt3a* is specifically inactivated in germ cells. Preference for different genomic sequences may simply reflect differences in chromatin accessibility, given that DNMT3a and DNMT3b localize to different subcellular compartments (Bachman *et al.*, 2001 and Chen *et al.*, 2002). Nonetheless, how sequence-specific methylation patterns are generated during gametogenesis escapes our understanding.

In the male germ line, acquisition of methylation begins prenatally in gonocytes and is further consolidated following birth until pachynema. In parallel, acquired marks have to be maintained as DNA is replicated in spermatogonia and preleptotene spermatocytes. We have previously demonstrated that expression of the *Dnmt* genes is highly dynamic during testis development and proposed that DNMT3a and DNMT3L might interact together to establish DNA methylation patterns in prenatal gonocytes, while DNMT3b and DNMT1 could be cooperating to maintain genome integrity after birth (La Salle *et al.*, 2004). Here, we further investigate these findings by exploring the expression pattern of the active *de novo* DNA methyltransferases in isolated postnatal male germ cells (summarized in Fig. 3.6). Several lines of evidence indicate that the use of isolated germ cells along with qRT-PCR is a reasonable approach for studying the expression of the different *Dnmt* genes during postnatal spermatogenesis. In our previous studies, *Dnmt1* expression patterns were examined using Northern and Western blotting and immunocytochemistry on isolated cells as well as

immunocytochemistry on testicular tissue (Jue *et al.*, 1995 and Mertineit *et al.*, 1998). Here, the *Dnmt1* qRT-PCR profile fits closely with our previously published DNMT1 protein expression and immunocytochemistry data. In the current study, the *Dnmt3a* and *Dnmt3b* qRT-PCR germ cell expression data were similar to the protein expression profiles. Furthermore, in keeping with our results, Watanabe *et al.* (2004) used immunocytochemistry on early postnatal testicular tissue and reported expression of DNMT3a in type B spermatogonia and preleptotene spermatocytes and DNMT3b in type A spermatogonia.

In the current study, when expression of *Dnmt3a* and *Dnmt3b* is probed and compared to that of *Dnmt1*, a similar pattern of expression is identified for all three genes albeit with small differences. Dnmt1 and Dnmt3b expression is highest in type A spermatogonia but diminished in type B spermatogonia and preleptotene spermatocytes; Dnmt3a is expressed similarly but expression is not reduced as extensively. Progression through meiosis is also associated with a decrease in expression of all three genes, but expression is turned on again in round spermatids. Based on these results, we identify two developmental windows during which transcription of these genes has to be downregulated during spermatogenesis: (1) differentiation of spermatogonia into spermatocytes and (2) pachynema. These data are the first indication of how tightly regulated the expression of *Dnmt3a* and *Dnmt3b* is during spermatogenesis. We postulate that the down regulation of the DNMTs at two times during spermatogenesis could be related either to the need to express testis-specific genes or to changes in chromatin structure. Although there are a number of examples of testis-specific genes that show a correlation between hypomethylation and germ cell expression, more studies are needed, and it is presently unresolved whether alterations in methylation are a cause or consequence of events associated with gene transcription (for review, see Maclean and Wilkinson, 2005). At the chromatin level, distinct differences in core histone acetylation have been found in spermatogonia and spermatocytes (Hazzouri *et al.*, 2000) and Coffigny *et al.* (1999) have hypothesized that genome-wide changes in 5-methylcytosine

staining in germ cells in the perinatal testis might be associated with functional genomic reorganization related to cellular differentiation.

Assessing the mechanisms that control the transcriptional activity of *Dnmt3a* provides further support for the tight regulation of the *Dnmt3s* during spermatogenesis. Alternate promoter usage at the *Dnmt3a* locus gives rise to two transcripts: Dnmt3a and Dnmt3a2. Evaluating the relative amounts of these transcripts in male germ cells exposed, surprisingly, two distinct expression profiles. While the profile obtained for Dnmt3a2 resembles that of Dnmt1 and Dnmt3b, the one obtained for Dnmt3a is strikingly different. Instead of being downregulated in type B spermatogonia and preleptotene spermatocytes, expression of Dnmt3a increases in these cells. These data differ markedly from what Lees-Murdock *et al.* (2005) found, where they did not detect any significant difference in expression between Dnmt3a and Dnmt3a2 in the testis. Because non-quantitative RT-PCR was conducted on whole gonads at only three times during development, expression differences would most likely not have been detected. Since the ratio of somatic cells to germ cells as well as the proportion of different germ cells types change drastically during the first wave of spermatogenesis, expression at multiple times during testis development or in isolated populations of male germ cells has to be considered. Immunoblotting analyses confirm the patterns we find for Dnmt3a and Dnmt3a2 at the protein level and clearly demonstrate DNMT3a to be the major *Dnmt3a* isoform in type B spermatogonia. Consistent with our findings is an immunofluorescence study conducted by Watanabe *et al.* (2004) on adult testis sections, where another antibody that recognizes DNMT3a detected this isoform only at stages where type B spermatogonia and preleptotene spermatocytes are found; however, their antibody did not allow them to examine DNMT3a2 expression. Our analyses also reveal that both DNMT3a and DNMT3a2 are absent in pachytene spermatocytes, but that DNMT3a2 is present in round spermatids, results reminiscent of DNMT1 (Jue *et al.*, 1995 and Trasler *et al.*, 1992).

A different regulatory mechanism, namely alternative splicing, gives rise to the DNMT3b isoforms. While DNMT3b1 and DNMT3b2 are postulated to

function as *de novo* DNA methyltransferases, the role of the other isoforms beyond being hypothetical regulators of DNA methylation remains elusive. Although Kaneda *et al.* (2004) have suggested that *Dnmt3b* might not be required for spermatogenesis, a role for this enzyme in the male germ line cannot be excluded since the details concerning this conditional knock-out model have not been reported yet. Compensatory mechanisms could be activated in the absence of DNMT3b, attenuating the appearance of a phenotype. We find that DNMT3b1 is unlikely to be involved in *de novo* methylation in the male germ line, as the corresponding transcript is presumably not expressed since unspliced-exon 11 transcripts are rare, and we are unable to detect the protein by immunoblotting. Our data show that the main transcript detected in male germ cells that can produce a catalytically active protein and carry out *de novo* methylation is Dnmt3b2. Consistent with these results, we detect DNMT3b2 by immunoblotting at high levels in type A spermatogonia and in leptotene/zygotene spermatocytes, cell types where *de novo* methylation is believed to take place. Previous studies have also shown that transcripts lacking exon 22 and exons 22/23 can be amplified at different stages of postnatal testis development (Chen *et al.*, 2002 and Lees-Murdock *et al.*, 2005). However, because whole gonads were used, it was impossible to conclude if these transcripts are specifically expressed in germ cells. Here, we show that transcripts lacking exon 22 and exons 22/23 are present in isolated postnatal male germ cells; however, only the DNMT3b3 isoform can be detected in these same germ cells. Taken together, these results suggest that DNMT3b2 and DNMT3b3 are the two major *Dnmt3b* isoforms in male germ cells. DNMT3b3 could serve as a positive regulator of DNMT3b2 or of another DNMT similarly to DNMT3L, or it could target DNMTs to loci destined to be methylated (Chédin *et al.*, 2002 and Weisenberger *et al.*, 2004). On the flip side, DNMT3b3 could act as a dominant negative regulator of DNA methylation by competing for target sites (Saito *et al.*, 2002).

Interestingly, DNMT3a and DNMT3b, like DNMT1, are reexpressed in round spermatids. One role for DNMTs late in spermatogenesis could be for the remethylation of testis-specific genes. For instance, phosphoglycerate kinase-2

is remethylated during transit through the epididymis (Ariel *et al.*, 1994 and Geyer *et al.*, 2004). Another possible role for DNMTs during spermiogenesis is for DNA repair, more specifically for the restoration of epigenetic information. A recent report by Mortusewicz *et al.* (2005) shows that in somatic cells, DNMT1 is recruited to sites of DNA damage induced by microirradiation, suggesting a role for DNMTs in repair.

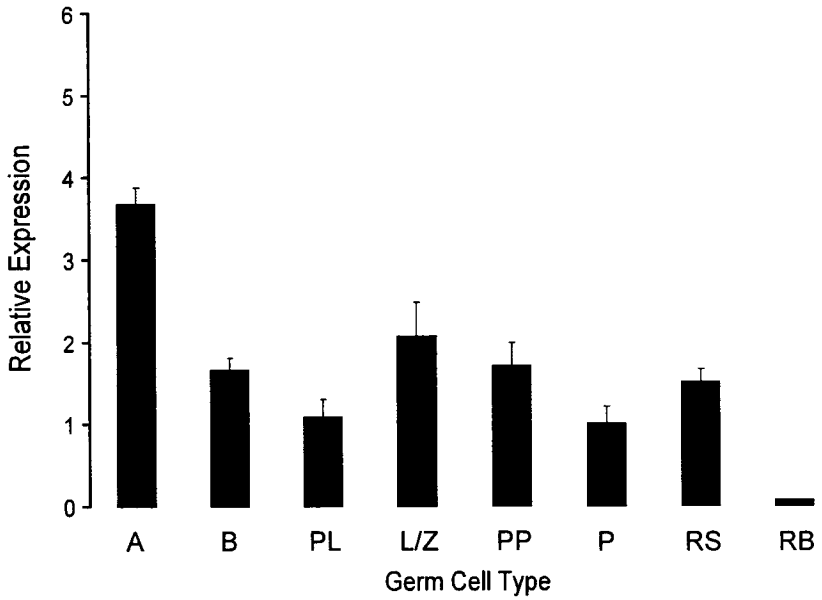
Several interesting features pertaining to DNMT1 in the germ line have been exposed during the last decade, including highly regulated expression during gametogenesis and the use of sex-specific exons to control the production of translated and non-translated RNAs. Nonetheless, issues related to the role of DNMT1 in the male germ line during meiosis and in round spermatids, as well as the relevance of its down-regulation during pachynema, remain unresolved. The findings presented here further support a common regulatory theme for the expression of DNA methyltransferases during key periods of spermatogenesis. Our data clearly demonstrate that all of the DNMT3a and DNMT3b isoforms are downregulated during pachynema, emphasizing the need to restrict expression of DNMTs during this period of male germ cell development. We also show that DNMT3a is the predominant DNMT in differentiating spermatogonia, suggestive of a specific role for this isoform in these cells. In light of our understanding of DNA methylation patterning in the male germ line, our data strongly indicate that DNMT3a2, DNMT3b2, and DNMT3b3 could be involved in *de novo* methylation in spermatogonia and spermatocytes, in addition to repair-associated *de novo* or maintenance DNA methylation in round spermatids. In conclusion, the data presented here set the stage for in-depth gene-targeting studies by emphasizing the need to inactivate specific transcript variants or isoforms of the DNA methyltransferase genes at different times during male germ cell development.

ACKNOWLEDGEMENTS

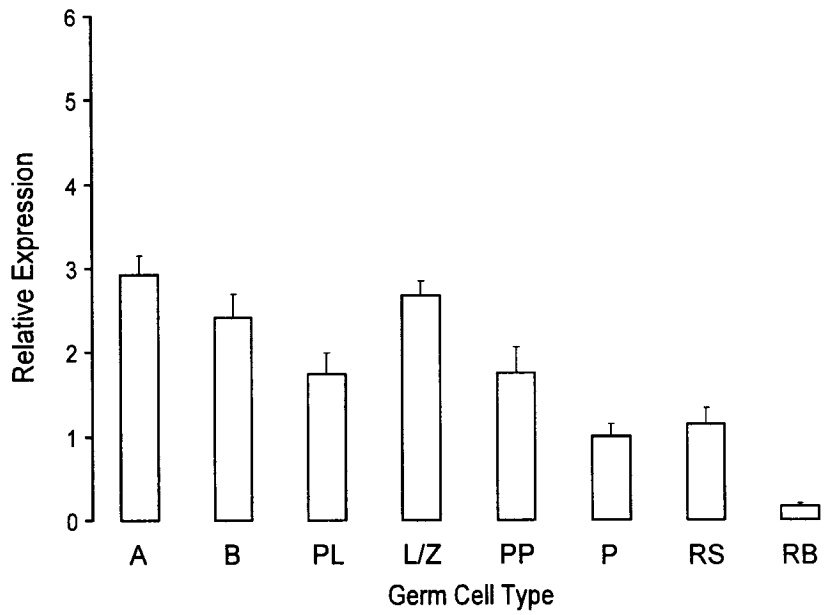
This work was supported by grants from the Canadian Institutes of Health Research (CIHR) to J.M.T. S.L. is a recipient of a CIHR Doctoral Research Award. J.M.T. is a William Dawson Scholar of McGill University and a Scholar of the Fonds de la Recherche en Santé du Québec (FRSQ).

Figure 3.1 ***Expression dynamics of DNA methyltransferases during male germ cell development.*** Relative quantification of **A**) *Dnmt1*, **B**) *Dnmt3a* and **C**) *Dnmt3b* expression in isolated populations of male germ cells. Real-time RT-PCR was used to determine the expression levels of the three *Dnmt* genes in total RNA extracted from type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/ zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/ elongating spermatids (RB). Expression of each gene was determined in triplicate in each of the two series of germ cells; shown here are the mean expression results obtained for one series. Mean ± SD.

A *Dnmt1*



B *Dnmt3a*



C *Dnmt3b*

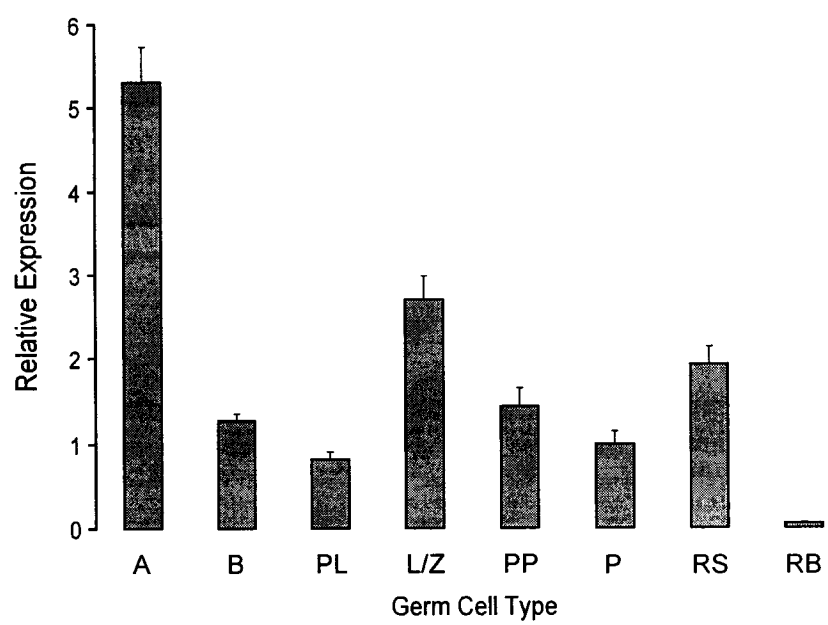


Figure 3.2 ***Differential expression of Dnmt3a and Dnmt3a2 in male germ cells***. **A**) Structure of the mouse *Dnmt3a* locus (top) and the two main mRNA species produced from it (bottom). The Dnmt3a2-unique exon is marked by an asterisk. Exons are shown as black bars (exon sizes and positions are not to scale; adapted from Chen *et al.*, 2002). AUG, the translation initiation codon. **B**) Relative expression of Dnmt3a (top) and Dnmt3a2 (bottom) in purified populations of male germ cells. qRT-PCR was used to determine the expression levels of the two transcripts in total RNA extracted from type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/ zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/ elongating spermatids (RB). Expression of each transcript was determined in triplicate in each of the two series of germ cells; shown here are the mean expression results obtained for one series. Mean ± SD.

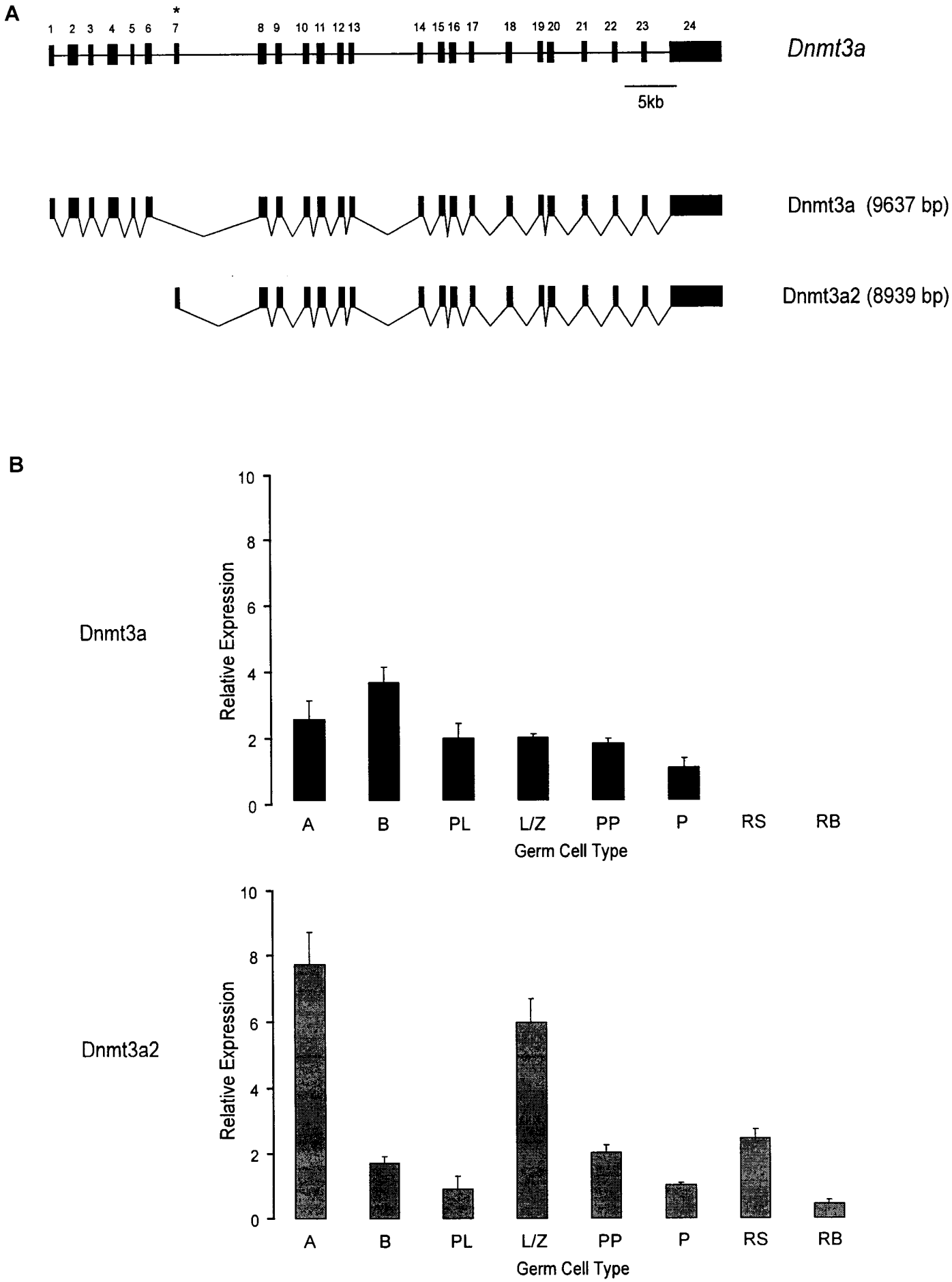
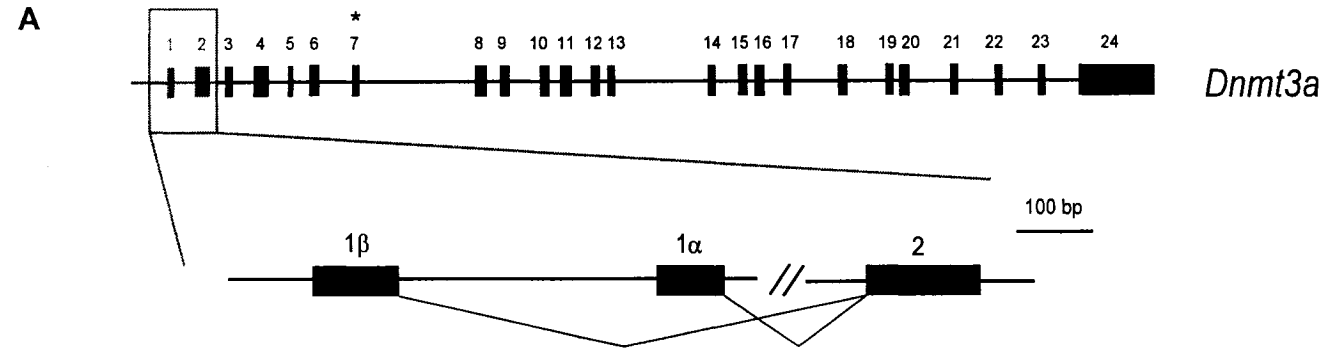

A
1 2 3 4 5 6 * 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Dnmt3a
5kb
Dnmt3a (9637 bp)
Dnmt3a2 (8939 bp)
B
Dnmt3a
Relative Expression
0 2 4 6 8 10
A B PL L/Z PP P RS RB
Germ Cell Type
Dnmt3a2
Relative Expression
0 2 4 6 8 10
A B PL L/Z PP P RS RB
Germ Cell Type

Figure 3.3 ***Expression of Dnmt3aα and Dnmt3aβ in mouse germ cells.*** **A)** Schematic representation of the 5' region of the mouse *Dnmt3a* gene. The shaded area enlarged below shows an additional first exon (exon 1β) situated 5' of the first exon that was initially identified (exon 1α). Splicing of exon 1α to exon 2 produces the Dnmt3aα transcript, while splicing of exon 1β to exon 2 gives rise to the Dnmt3aβ transcript (both splicing events are depicted here). Exons are represented as black bars (adapted from Weisenberger *et al.*, 2002). **B)** Expression of both Dnmt3aα and Dnmt3aβ in isolated male germ cells. RT-PCR was used to establish the incidence of transcription through exons 1α and 1β in total RNA extracted from type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/ zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/ elongating spermatids (RB). –RT, negative control lacking reverse transcriptase.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Dnmt3a
100 bp
1β
1α
2

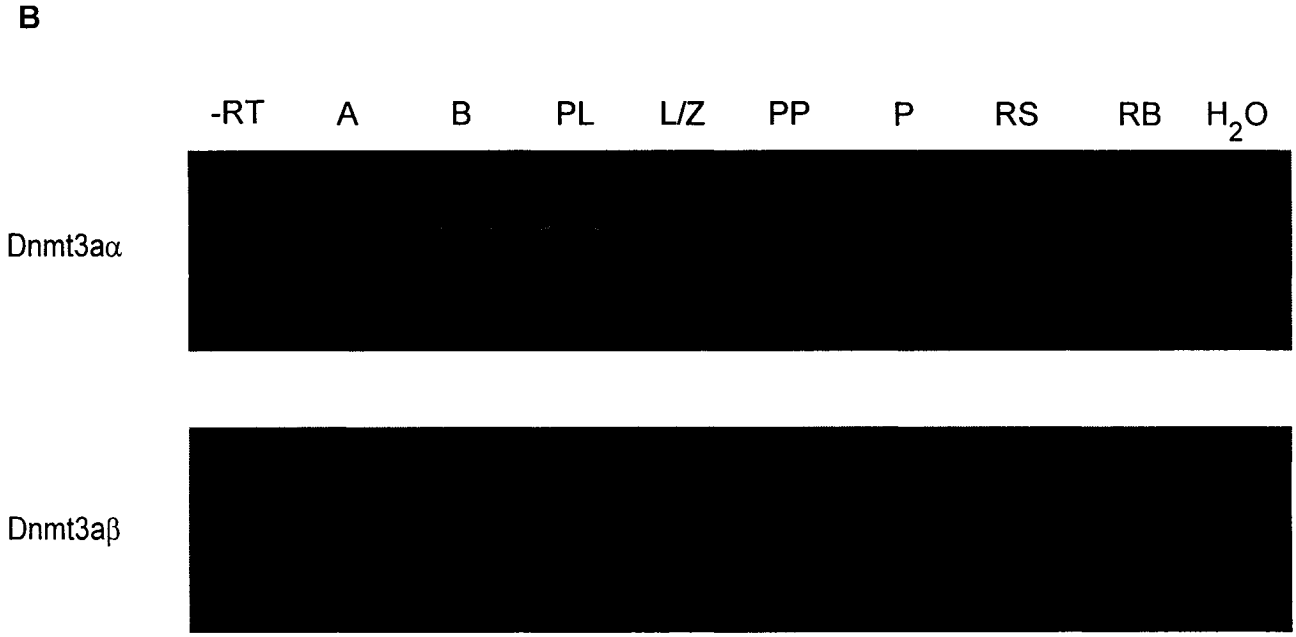
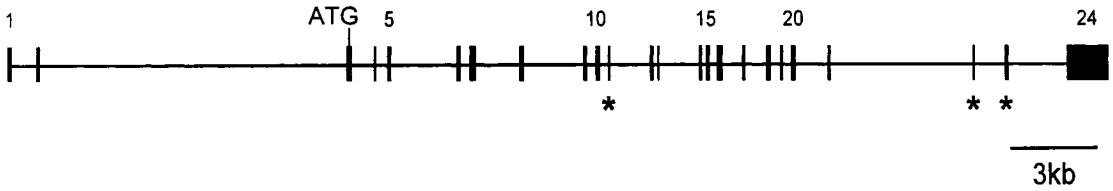
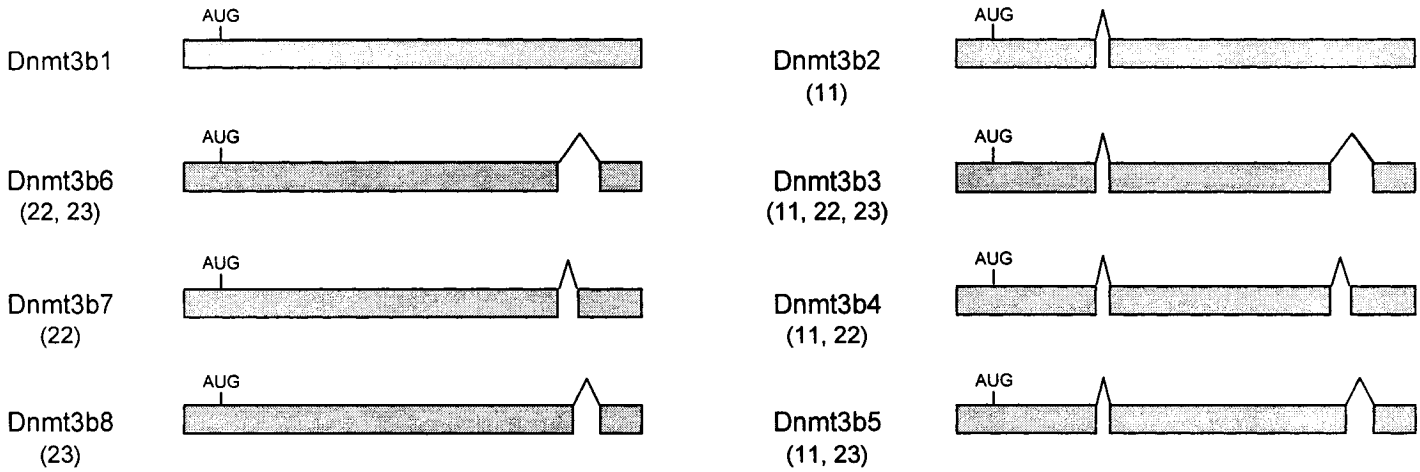

B
-RT
A
B
PL
L/Z
PP
P
RS
RB
H2O
Dnmt3aα
Dnmt3aβ

Figure 3.4 ***Expression of multiple Dnmt3b transcripts in male germ cells.*** **A**) Genomic organization of the mouse *Dnmt3b* gene. (Top) Structural organization of the gene. Exons that can be spliced are marked by asterisks. Exons are shown by filled boxes; exon size and position are to scale. ATG, the translation initiation codon. (Bottom) Representation of the eight possible mRNA species created by alternative splicing of exons 11, 22 and 23. Transcripts are depicted as grey boxes with open areas where splicing occurs. Spliced exons are shown in parentheses. (Adapted from Ishida *et al.*, 2003; Weisenberger *et al.*, 2004). **B**) Analysis of the occurrence of the two alternative splicing events: exon 11 and exons 22 and/or 23. Prevalence of the splicing events was determined by RT-PCR in type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/ zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/ elongating spermatids (RB). + or -, unspliced or spliced exon, respectively.

A


1
ATG
5
10
15
20
24
Dnmt3b
3kb


AUG
Dnmt3b1
Dnmt3b2
(11)
Dnmt3b6
(22, 23)
Dnmt3b3
(11, 22, 23)
Dnmt3b7
(22)
Dnmt3b4
(11, 22)
Dnmt3b8
(23)
Dnmt3b5
(11, 23)

B

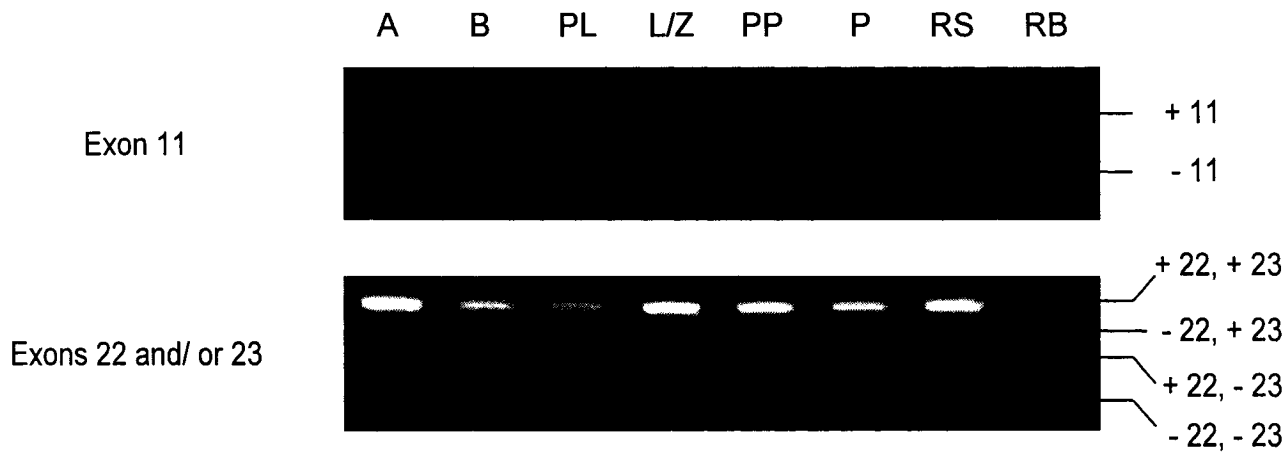

A
B
PL
L/Z
PP
P
RS
RB
Exon 11
+ 11
- 11
Exons 22 and/ or 23
+ 22, + 23
- 22, + 23
+ 22, - 23
- 22, - 23

Figure 3.5 ***Immunoblot detection of DNMT3a and DNMT3b in purified populations of male germ cells***. **A**) Schematic representation of DNMT3a and DNMT3a2. The conserved PWWP and PHD domains, and methyltransferase motifs (I, IV, VI, IX, X) are depicted. The location of the different epitopes is underlined. **B**) Expression of DNMT3a and DNMT3a2 in male germ cells. *Top panel:* Detection of DNMT3a in protein lysates obtained from type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/ zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/ elongating spermatids (RB) using the NB 100-265 antibody. *Middle panel*: Expression of DNMT3a and DNMT3a2 in the same germ cell populations using the 64B1446 antibody. *Bottom panel*: Gel electrophoresed under identical conditions but stained with Coomassie Brilliant blue dye demonstrating equal protein loading in each lane. **C**) Detection of multiple DNMT3b isoforms in male germ cells. *Upper panel*: Western Blot showing the presence of several DNMT3b isoforms in total protein extracted from various populations of male germ cells and from 6- (D6) and 70- (D70) dpp testes using the 52A1018 antibody (lane abbreviations are the same as in B for germ cells). *Lower panel*: Gel electrophoresed under identical conditions but stained with Coomassie Brilliant blue dye demonstrating equal protein loading in each lane.

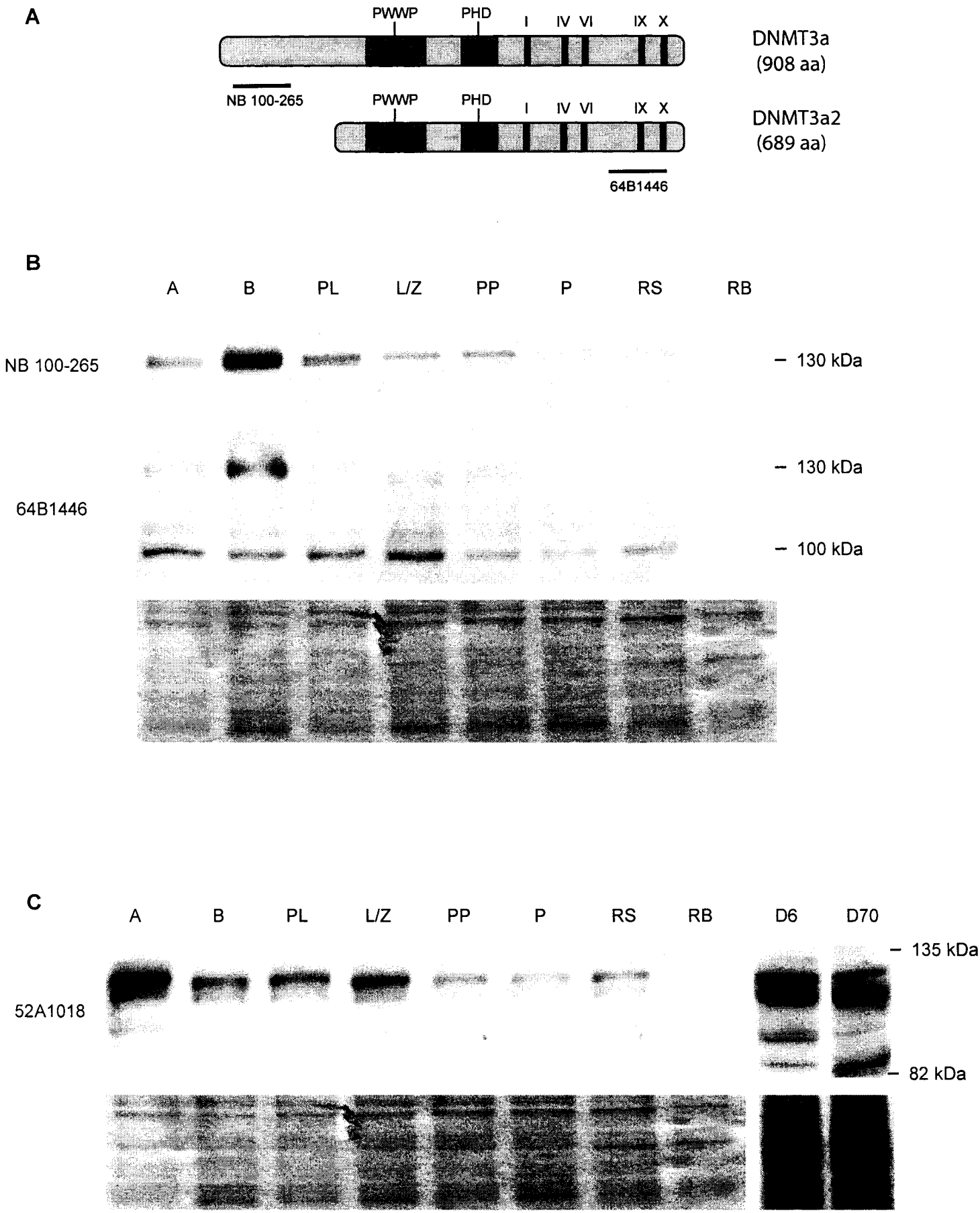
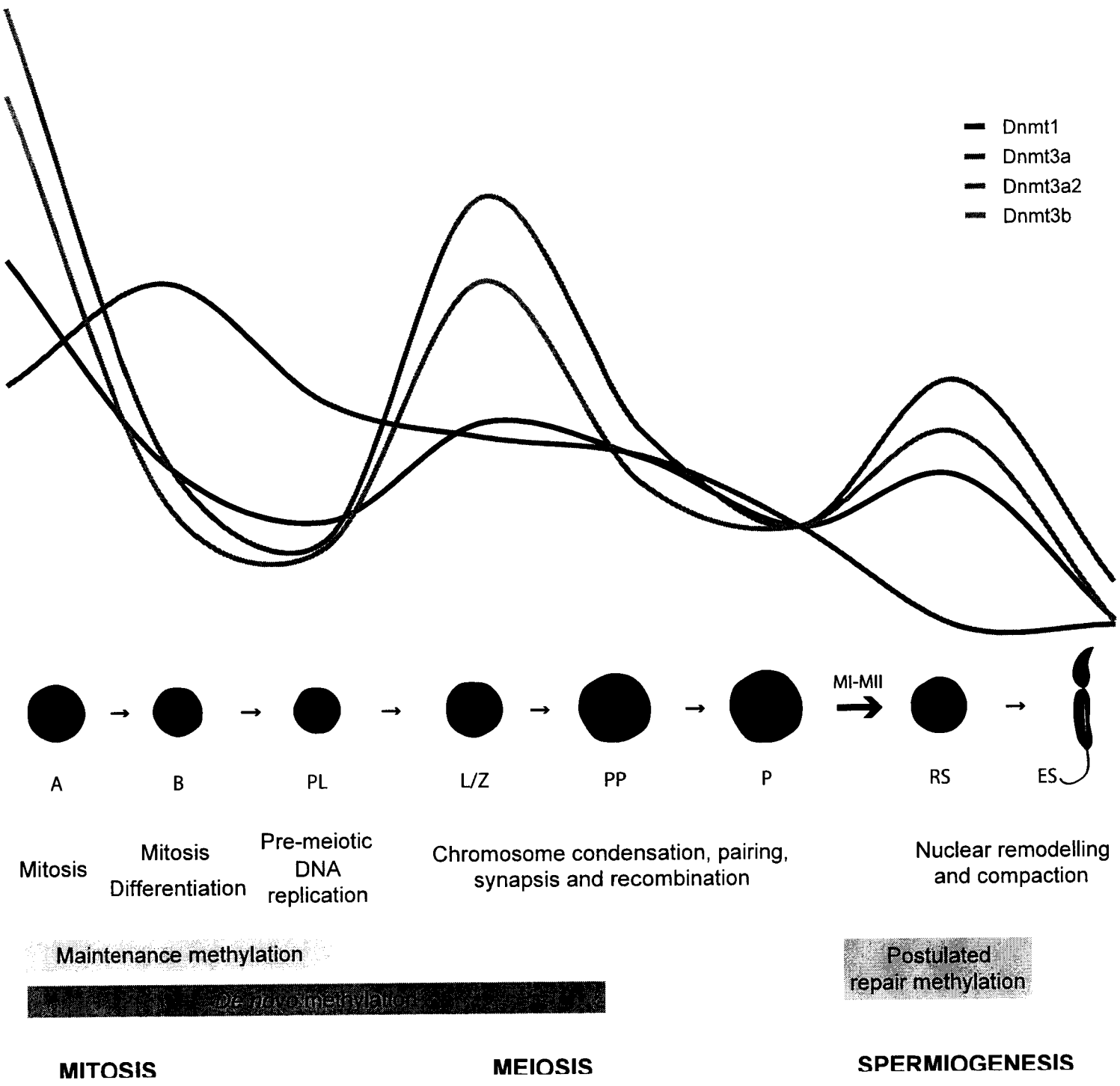
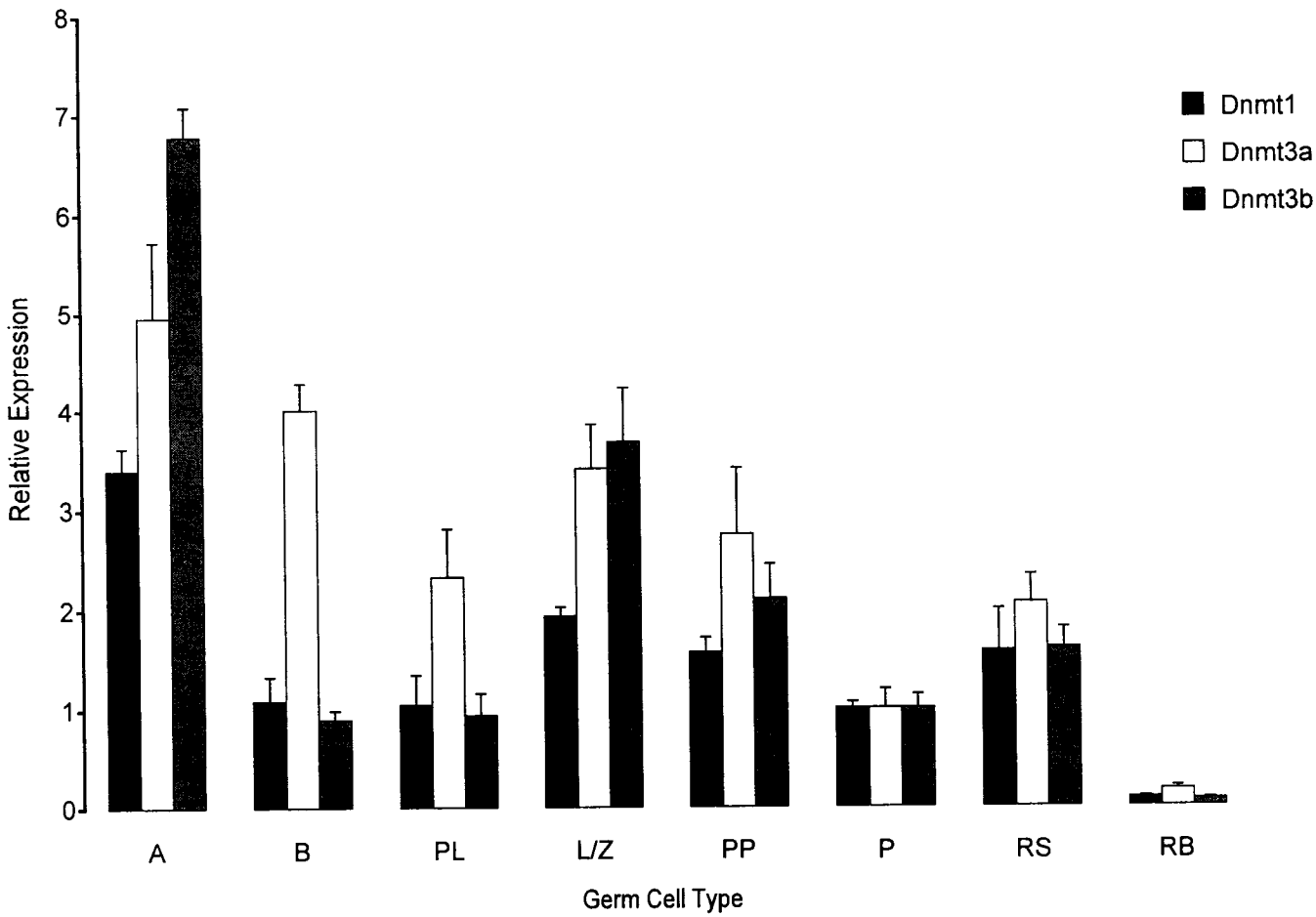

A
PWWP
PHD
I
IV
VI
IX
X
DNMT3a
(908 aa)
NB 100-265
PWWP
PHD
I
IV
VI
IX
X
DNMT3a2
(689 aa)
64B1446
B
A
B
PL
L/Z
PP
P
RS
RB
NB 100-265
– 130 kDa
– 130 kDa
64B1446
– 100 kDa
C
A
B
PL
L/Z
PP
P
RS
RB
D6
D70
– 135 kDa
52A1018
– 82 kDa

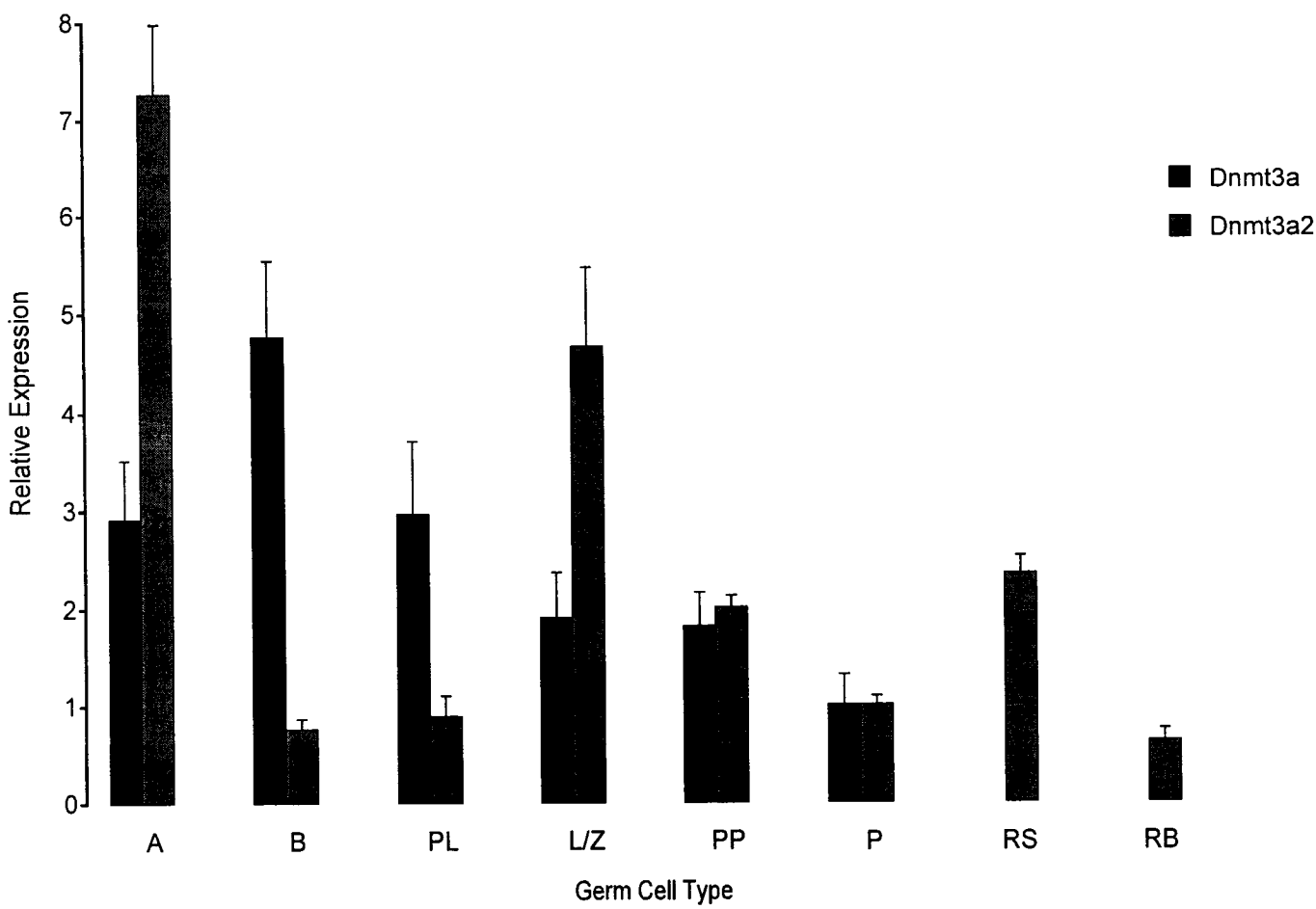
Figure 3.6 ***Schematic representation of DNA methyltransferase dynamics during spermatogenesis***. Progression of methylation pattern establishment and maintenance, and expression of Dnmt1 (black), Dnmt3a (red), Dnmt3a2 (blue) and Dnmt3b (green) are presented as functions of male germ cell development. The intensity of the blue shading in germ cells reflects the methylation status of the paternal genome. Some of the most characteristic biological processes associated with mitosis, meiosis and spermiogenesis are also depicted. Representative cell types are indicated as follows: A, type A spermatogonia; B, type B spermatogonia; PL, preleptotene spermatocyte, L/Z, leptotene/ zygotene spermatocyte; PP, prepubertal pachytene spermatocyte; P, pachytene spermatocyte; RS, round spermatid; ES, elongating spermatids. MI-MII, meiosis I – II.


Dnmt1
Dnmt3a
Dnmt3a2
Dnmt3b
MI-MII
A
B
PL
L/Z
PP
P
RS
ES
Mitosis
Mitosis
Differentiation
Pre-meiotic
DNA
replication
Chromosome condensation, pairing,
synapsis and recombination
Nuclear remodelling
and compaction
Maintenance methylation
Postulated
repair methylation
MITOSIS
MEIOSIS
SPERMIOGENESIS

Supplementary Figure 3.7 ***Comparative expression analysis of Dnmt1, Dnmt3a and Dnmt3b***. Comparison of the relative expression levels of *Dnmt1*, *Dnmt3a* and *Dnmt3b* in the second series of isolated male germ cells. Real-time RT-PCR was used to determine the expression levels of the three *Dnmt* genes in total RNA extracted from type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/ zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/ elongating spermatids (RB) from the second series of germ cells. Expression of each gene was determined in triplicate. Mean ± SD.


Relative Expression
8
7
6
5
4
3
2
1
0
Dnmt1
Dnmt3a
Dnmt3b
A
B
PL
L/Z
PP
P
RS
RB
Germ Cell Type

Supplementary Figure 3.8 ***Comparative expression analysis of Dnmt3a and Dnmt3a2.*** Comparison of the relative expression levels of Dnmt3a and Dnmt3a2 in the second series of isolated male germ cells. Real-time RT-PCR was used to determine the expression levels of the two transcripts in total RNA extracted from type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/ zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/ elongating spermatids (RB) from the second series of germ cells. Expression of each gene was determined in triplicate. Mean ± SD.


Relative Expression
8
7
6
5
4
3
2
1
0
A
B
PL
L/Z
PP
P
RS
RB
Germ Cell Type
Dnmt3a
Dnmt3a2

CONNECTING TEXT

The primary objective of the work described in Chapter II and III was to characterize the expression of DNA methyltransferases in the germ line to establish which of these enzymes could be involved in methylation pattern acquisition in male germ cells. While the work presented in this thesis was taking place, gene-targeting experiments conducted in other laboratories revealed the importance of *Dnmt3L* to the male germ line. To understand how a protein that does not possess any detectable methyltransferase activity could be crucial to spermatogenesis, experiments in Chapter IV were designed to further characterize the expression and the role of DNMT3L in the male germ line. Using a *Dnmt3L* mutant mouse model, we explored the histological defect behind DNMT3L-deficiency and found new sequences targeted for methylation by this protein.

CHAPTER IV

Genome-wide DNA methylation defect in DNMT3L-deficient male germ cells

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Submitted for revision to Human Molecular Genetics

ABSTRACT

Formation of haploid spermatozoa capable of fertilization requires passage through mitosis and meiosis, histone-to-protamine exchange, as well as proper programming of epigenetic information. How each DNA methyltransferase (DNMT) contributes to DNA methylation pattern acquisition in prenatal gonocytes and during spermatogenesis remains unclear. We determined the temporal expression pattern of *Dnmt3L*, a postulated regulator of DNMT activity, in prenatal and postnatal male germ cells using qRT-PCR. Expression was highest in gonocytes but was also detected and developmentally regulated during spermatogenesis. We compared the expression of other known *Dnmts* in gonocytes; whereas both transcript variants of *Dnmt3a* were present, expression of *Dnmt3b* remained low. Next, we used a *Dnmt3L* mutant mouse model to better understand the role of this protein in the male germ line. A detailed developmental study covering the first 12 days following birth allowed us to detect lower germ cell numbers and a delay in entry into meiosis in *Dnmt3L*$^{-/-}$ males, revealing a mitotic defect. We used a restriction enzyme qPCR assay (qAMP) to conduct DNA methylation analyses on isolated primitive type A spermatogonia lacking DNMT3L. In addition to paternally imprinted genes and retroviral elements, unique non-repetitive, non-CpG island sequences were hypomethylated in DNMT3L-deficient germ cells. Most importantly, we observed severe hypomethylation across multiple sites (~30 different loci/ chromosome) along chromosomes 4 and X in *Dnmt3L*$^{-/-}$ spermatogonia, suggesting that many loci throughout the genome are marked for methylation by DNMT3L. Taken together, these data suggest that DNMT3L plays a more global role in genomic methylation patterning in the male germ line than was previously suspected.

INTRODUCTION

DNA methylation is central to epigenetic control of the genome, playing roles in the transcriptional regulation of genes and the establishment of higher order chromatin structures (Bestor, 2000). Targeted inactivation of DNA methyltransferase genes causes lethality at early embryonic or postnatal stages, emphasizing the importance of DNA methylation in supporting mammalian development (Li *et al.*, 1992; Okano *et al.*, 1999). Germ cell development is the first developmental window during which DNA methylation patterns are programmed in mammals. Marked differences are observed between male and female gametes, especially at imprinted loci where allele-specific gene expression in the offspring is dependent on methylation differences (reviewed by Reik and Walter, 2001). In the mouse, a major demethylation event takes place in both germ lines between embryonic days (E) 10.5 to 12.5 as primordial germ cells (PGCs) enter the gonads, following which point DNA methylation patterns are reestablished in a sex- and sequence-specific manner as gametogenesis progresses (reviewed by Reik *et al.*, 2001 and Trasler, 2006).

Most information concerning the dynamics of DNA methylation comes from studies done on the control region of imprinted genes and repeat sequences (Davis *et al.*, 1999; Davis *et al.*, 2000; Kafri *et al.*, 1992; Kono *et al.*, 1996; Lees-Murdock *et al.*, 2003; Li *et al.*, 2004; Lucifero *et al.*, 2002; Ueda *et al.*, 2000; Walsh *et al.*, 1998). In the male germ line, methylation acquisition begins before birth, between E15.5 and E18.5. Repetitive elements such as intracisternal A particles (IAPs), long interspersed nuclear elements (LINEs) and satellite sequences have acquired most of their methylation by E17.5 (Lees-Murdock *et al.*, 2003; Walsh *et al.*, 1998). Although the remethylation of imprinted genes also begins around E15.5, the process is only completed after birth (Davis *et al.*, 1999; Li *et al.*, 2004). A developmental study done on the imprinted gene *H19* shows that it begins acquiring its methylation between E15.5 and E18.5, but only becomes fully methylated postnatally by pachynema (Davis *et al.*, 1999). Importantly, since male germ cells continue to undergo DNA replication after birth, they are also capable of maintenance methylation to ensure proper

propagation of the patterns already acquired. In contrast, in the female germ line methylation patterns are acquired postnatally during the oocyte growth phase, once pachynema is completed and DNA has been replicated (Kono *et al.*, 1996; Lucifero *et al.*, 2002; Walsh *et al.*, 1998).

DNA methylation patterns are created and propagated through the activity of both *de novo* and maintenance DNA (cytosine-5)-methyltransferases (DNMTs). A number of DNMTs have been characterized and are classified according to similarities found in their catalytic domain (reviewed by Goll and Bestor, 2005). However, we are only starting to understand how these enzymes interact in the germ line to establish and maintain methylation patterns. We have previously shown that the expression of *Dnmt1*, *Dnmt3a*, *Dnmt3b* and *Dnmt3L* marks windows of sex-specific methylation in both germ lines, and more recently that the expression of *Dnmt3a* and *Dnmt3b* is tightly regulated in developing male germ cells (La Salle *et al.*, 2004; La Salle and Trasler, 2006a). Germ cell-specific inactivation of *Dnmt3a*, but not *Dnmt3b*, impairs the establishment of *de novo* methylation patterns in germ cells, more specifically at imprinted loci, without affecting the methylation status of repeat sequences (Kaneda *et al.*, 2004).

Targeted inactivation of the DNA methyltransferase 3-Like gene, *Dnmt3L*, also results in methylation defects at imprinted loci and, additionally, at repeat elements such as IAPs and LINEs, but only in male germ cells (Bourc'his *et al.*, 2001; Bourc'his and Bestor, 2004; Hata *et al.*, 2002; Kaneda *et al.*, 2004; Webster *et al.*, 2005). Spermatogenesis is impaired in *Dnmt3L* mutant males due to abnormal synapses between homologous chromosomes resulting in meiotic failure (Bourc'his and Bestor, 2004; Webster *et al.*, 2005). Even if DNMT3L shares common motifs with DNMT3a and DNMT3b, it lacks the ability to transfer methyl groups to DNA. As such, this protein has been postulated to work in complex with another DNA methyltransferase, possibly DNMT3a or its isoform DNMT3a2, to accomplish its function (Chédin *et al.*, 2002; Hata *et al.*, 2002; Kareta *et al.*, 2006). Although concomitant expression of *Dnmt3a* and *Dnmt3L* in gonocytes has previously been reported (Lees-Murdock *et al.*, 2005; Sakai *et al.*, 2004) and *Dnmt3L* mutant mouse models have clearly established the

importance of this protein to the germ line (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Webster *et al.*, 2005), the action of DNMT3L - alone or in combination with another DNMT - is still not fully understood.

Spermatogenesis is a complex process by which haploid male germ cells are created. Sperm integrity not only depends on unique processes such as specialized transcription, meiosis and histone-to-protamine replacement, but also on various epigenetic events including DNA methylation reprogramming (reviewed by La Salle and Trasler, 2006b and Rousseaux *et al.*, 2005). Treating male mice with the cytosine analogue 5'-aza-2'-deoxycytidine, an agent that causes genomic hypomethylation, results in testicular abnormalities with decreased sperm counts and fertility (Kelly *et al.*, 2003). In addition, *Dnmt3a* and *Dnmt3L* mutant males are sterile due to unsuccessful meiosis (Bourc'his *et al.*, 2001; Bourc'his and Bestor, 2004; Hata *et al.*, 2002; Kaneda *et al.*, 2004; Webster *et al.*, 2005). Although little is known about the functional requirement(s) of methylation at single-copy or repeat sequences on germ cell proliferation and differentiation, these results suggest a crucial role for DNA methylation in male germ cell development. Methylation of DNA may be required for successful passage through spermatogenesis.

Our analysis of *Dnmt3L* function in the male germ line revealed a more prominent and developmentally earlier role for this protein during spermatogenesis than was previously reported. A thorough developmental study exploring the expression of *Dnmt3L* in both prenatal and postnatal male germ cells showed that, although Dnmt3L expression peaked in gonocytes, it was also detected in spermatogonia, spermatocytes and spermatids. Using a *Dnmt3L* knockout mouse model (Bourc'his *et al.*, 2001), we determine that histological abnormalities were already occurring during the first week of postnatal development. Lower germ cell counts and delayed entry into meiosis were observed in *Dnmt3L*$^{-/-}$ males. Finally, DNA methylation analysis of whole chromosomal domains revealed that DNMT3L was crucial to the establishment of global DNA methylation patterns in the male germ line.

MATERIALS AND METHODS

Mice

CD-1 mice were purchased from Charles River Canada Inc. (St-Constant, QC, Canada). *Dnmt3L* mutant mice (Bourc'his *et al.*, 2001) and GOF18/deltaPE-Oct-4/GFP transgenic mice (Yoshimizu *et al.*, 1999) have been described elsewhere. Noon of the day on which the vaginal plug was found was designated as embryonic day (E) 0.5, while the day of birth was designated as postpartum day (dpp) 0. All procedures were performed in accordance with the Canadian Council on Animal Care and approved by the McGill University Animal Care Committee.

Isolation of Male Germ Cells by Flow Cytometry

Timed-pregnancies were established between CD-1 females and GOF18/deltaPE-Oct-4/GFP males. Testes were collected from male embryos at E13.5, E15.5 and E18.5 or from male pups at 6 dpp and rinsed twice in sterile phosphate buffered saline (PBS). Decapsulated testes were digested in 0.25% trypsin-EDTA (Gibco-BRL/ Invitrogen, Burlington, ON, Canada) for 10 minutes at 37°C, dispersed and digested further for 10 minutes. The cell suspension obtained was washed twice and resuspended in PBS. GFP-positive gonocytes and primitive type A spermatogonia were collected by flow cytometry using a MoFlow cell sorter (Cytomation Inc., Ft. Collins, CO).

Isolation of Male Germ Cells by Sedimentation Velocity

Purified populations of male germ cells were obtained from the testes of 8-, 17- and 70-dpp CD-1 mice according to the sedimentation velocity cell separation method as described previously (La Salle and Trasler, 2006a; Bellvé, 1993). Cells were identified on the basis of morphological criteria and size. Populations of type A spermatogonia (average purity= 86%) and type B spermatogonia (average purity= 83%) were obtained from the testes of 8dpp mice (n=2 cell separations); preleptotene spermatocytes (average purity= 85%), leptotene/zygotene spermatocytes (average purity= 87%) and prepubertal

pachytene spermatocytes (average purity= 80%) were obtained from the testes of 17dpp mice (n=2 cell separations); finally, pachytene spermatocytes (average purity= 81%), round spermatids (average purity= 88%) and elongating spermatids mixed with residual bodies (average purity= 86%) were obtained from 70dpp mice (n=2 cell separations).

Quantitative RT-PCR

Total RNA was extracted from snap-frozen pellets of male germ cells using the RNeasy Mini kit with DNaseI treatment according to the manufacturer's protocol (Qiagen Inc., Mississauga, ON, Canada). Real-Time or quantitative RT-PCR (qRT-PCR) was performed using the Mx4000 qPCR system from Stratagene (La Jolla, CA) using the QuantiTect™ SYBR® Green RT-PCR kit (Qiagen) as described previously (La Salle *et al.*, 2004). The gene-specific or transcript-specific primers used to determine the relative expression levels of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Dnmt3L*, *Dnmt3a* and *Dnmt3a2* according to the standard curve method (Bustin, 2002) have been described elsewhere (La Salle *et al.*, 2004; La Salle and Trasler, 2006a). In all cases, reactions were performed in triplicate on the same two independent sets of germ cells. Fold changes in expression for a given gene were determined in relation to the expression of that gene in E13.5 gonocytes for prenatal germ cells or in pachytene spermatocytes for postnatal germ cells; all other quantities are expressed as *n*-fold differences relative to the expression of that gene in these respective cells types. Representative data for one set of germ cells are presented as mean ± SD.

Histology

For histological examination, testes were immersed in Bouin's fixative (BDH Inc, Toronto, ON, Canada) for 4 hours, dehydrated, and embedded in paraffin. Sections (5 μm) were cut, mounted on glass slides, deparaffinized with xylene, and stained with hematoxylin and eosin. A Zeiss AxioImager Z1 microscope was used to view the slides and pictures were taken using a digital camera and the AxioVision 4.5 software (Carl Zeiss Canada Ltd, Toronto, ON).

Germ Cell Counts

The paraffin-embedded testes used for histological analysis were cut into serial sections, with every fifth section used for germ cell quantification. The monoclonal germ-cell nuclear antigen 1 antibody GCNA1 was used to identify germ cells (Enders and May, 1994). Briefly, rehydrated sections were incubated with undiluted primary antibody at 37°C for one hour, rinsed in PBS and incubated with a biotinylated anti-rat IgG secondary antibody (Vector Laboratories) for 30 minutes at room temperature; slides were then incubated with the Vectastain Elite ABC reagent for one hour (Vector Laboratories), couterstained with Hematoxylin QS (Vector Laboratories) and mounted with Permount (Fisher Scientific, Fairlawn, NJ). The slides were viewed using a Zeiss AxioImager Z1 microscope (Carl Zeiss); germ cells were counted by an individual blinded to the slide identities and are reported per 2000 Sertoli cells as described by Nadler and Braun (2000). Results are presented as means ± SEM.

Immunofluorescence

Testes were fixed for four hours in Ste Marie's fixative as previously described (Trasler *et al.*, 1996) and were embedded in paraffin. Five-micrometer sections were rehydrated, blocked in 3% bovine serum albumin, 0.1% PBS-Tween 20 (blocking buffer) and incubated with the monoclonal mouse anti-phospho-histone-H2A.X primary antibody diluted in blocking buffer for one hour at 37°C (1:500; Upstate Biotechnologies Inc., Charlottesville, VA). Rinsed sections were then incubated with a biotinylated anti-mouse IgG secondary antibody diluted in blocking buffer (1:500, Vector Laboratories) for 30 minutes at room temperature, followed by incubation with Avidin-AlexaFluor488 (Molecular Probes/ Invitrogen) for one hour at room temperature in the dark. Slides were mounted with Vectashield containing DAPI (Vector Laboratories) and analyzed using a Zeiss AxioImager Z1 microscope (Carl Zeiss). Tubules containing at least one positive gamma-H2A.X cell, or positive tubules, were counted by an individual blinded to the slide identities and were reported as incidence of positive tubules per 100 tubules. Results are presented as means ± SEM.

Restriction Landmark Genomic Scanning

Genomic DNA was isolated from whole testes of 10 dpp wild type, heterozygous and homozygous *Dnmt3L* mice, as well as sperm and liver from adult wild type mice using proteinase K followed by phenol extraction. Restriction landmark genomic scanning (RLGS) analysis was performed as described by Okazaki and colleagues (1995). Briefly, two-dimensional spot profiles were produced by digesting genomic DNA with the methylation-sensitive restriction enzyme *Not*I followed by radioactive end-labeling. RLGS gels were exposed to a phosphorimager screen (Kodak, Rochester, NY) and were analyzed using the ImageQuant v5.1 software from GE Healthcare (Piscataway, NJ). Densitometry values were obtained by comparing spot density values of a spot of interest to approximately 10-15 surrounding spots of unchanged intensity.

Isolation of Dnmt3L mutant germ cells and DNA Methylation Analysis

Heterozygous *Dnmt3L* females were crossed with GOF18/deltaPE-Oct-4/GFP males to obtain [*Dnmt3L*$^{+/-}$, GFP^{+}] mice. Males and females with the proper genotype were crossed to obtain GFP^{+} males with the three possible *Dnmt3L* genotypes; paired testes were collected at 6 dpp and were processed as mentioned above to allow for the isolation of *Dnmt3L* wild type, heterozygous and homozygous GFP^{+} primitive type A spermatogonia by flow cytometry. RNA and DNA were simultaneously extracted using the AllPrep DNA/RNA Mini kit according to the manufacturer's protocol (Qiagen); the RNA was used in qRT-PCR analyses as described above, while DNA methylation analyses were carried out on the DNA. Quantitative analysis of DNA methylation using real-time PCR, or qAMP, was conducted to analyze the DNA methylation status of a number of sequences as described by Oakes *et al.* (2006a). Briefly, the DNA was either mock digested (sham group), or digested with methylation-sensitive restriction enzymes (*Not*I, *Hpa*II or *Hha*I), which cleave DNA if the restriction site(s) are unmethylated, or with a methylation-dependent restriction enzyme (McrBC), which cleaves DNA only if it is methylated. For a given sequence, primers were designed to flank the restriction sites of interest and real-time PCR was

performed on the different digested templates using the QuantiTect™ SYBR® Green PCR kit (Qiagen) according to the manufacturer's suggested conditions for use of the Mx3000P PCR machine (Stratagene). Primer sequences for germ cell-specific spots, *H19* and *U2af1-rs1* are the same as in Oakes *et al.* (2006a), primers for the 31 sites analyzed on chromosome 4 are from Oakes *et al.* (2006b), and primers for the 30 sites analyzed along chromosome X and Dlk1-Gtl2 are from Oakes *et al.* (2006c). The cycle threshold (C_t) values obtained for the different digested templates are expressed relative to the sham digested group; any differences in C_t values (ΔC_t) are indicative of differences in the methylation status of the given sequence of interest. Since each successive round of PCR amplification results in approximately a 2-fold increase in the amount of amplicon, a percentage methylation value can be extracted from the ΔC_t values. Thus, a ΔC_t of 1.0 is indicative of 50% template cleavage; 2.0, of 75% template cleavage, and so on. For methylation-sensitive restriction enzymes, the following formula can be used to described the relationship of ΔC_t to percent methylation: $\%Me=100(e^{-0.7(\Delta Ct)})$; the relationship follows the inverse function for methylation-dependent restriction enzymes: $\%Me=100(1-e^{-0.7(\Delta Ct)})$.

RESULTS

Expression of Dnmt3L in male germ cells

To investigate the role of *Dnmt3L* in male germ cell development, our first aim was to clarify the expression of this gene during spermatogenesis. A number of groups have reported exclusive expression of Dnmt3L in pre- and peri- natal gonocytes (Bourc'his *et al.*, 2001; Bourc'his and Bestor, 2004; Sakai *et al.*, 2004; Webster *et al.*, 2005), while others have also detected Dnmt3L in more mature cell types (Hata *et al.*, 2002). We have previously used quantitative RT-PCR (qRT-PCR) to determine the expression profiles of *Dnmt3a* and *Dnmt3b* during spermatogenesis. We used the same approach to investigate the expression of *Dnmt3L* in postnatal male germ cells obtained by sedimentation at unit gravity and flow cytometry; nine different populations of germ cells could be isolated using mice of different ages. Dnmt3L could be detected in all cell types tested, albeit at different levels (Fig. 4.1). Expression was highest in primitive type A spermatogonia and remained elevated in type A spermatogonia, but decreased drastically in type B spermatogonia and was at its lowest in preleptotene spermatocytes. Dnmt3L was found in leptotene/ zygotene spermatocytes at levels similar to those seen in type A spermatogonia, but expression decreased steadily as meiosis progressed. *Dnmt3L* expression was also detected in round spermatids and residual bodies/ elongating spermatids, but at low levels.

Next, we assessed *Dnmt3L* expression before, during and after the initial period of DNA methylation acquisition in the male germ line to get a complete picture of *Dnmt3L* expression in male germ cells. In an earlier study done on the expression of *Dnmts* in whole gonads composed of germ cells and somatic cells, we had shown that *Dnmt3L* expression peaks in E15.5 prenatal gonads (La Salle *et al.*, 2004). Here, we isolated pure germ cells, i.e., gonocytes, by flow cytometry at the same time points we had studied earlier, E13.5, E15.5 and E18.5, and used qRT-PCR to analyze the expression of *Dnmt3L* in these cells. Results were very similar to those obtained when using whole gonads (Fig. 4.2A). Expression peaked in E15.5 gonocytes, being ~800 times higher than that in E13.5 gonocytes. Dnmt3L was still present at high levels in E18.5 gonocytes, but by

this developmental stage, expression was ~8-fold lower than that seen at E15.5. For comparison purposes, the expression result obtained for primitive type A spermatogonia was reanalyzed in the context of the prenatal expression results. The recalibrated value was in agreement with our previous expression data in the 6 dpp testis, being ~800 times lower than that seen in E15.5 gonocytes. The expression profiles presented here demonstrate that, although Dnmt3L expression is at its highest before birth, it is still detectable throughout postnatal male germ cell development.

Expression of other DNA methyltransferases in prenatal male germ cells

Additional studies have also examined transcript levels of other *Dnmts* during the prenatal period, but those studies were done using whole gonads or semi-quantitative approaches (Lees-Murdock *et al.*, 2005; Sakai *et al.*, 2001 and 2004). Using qRT-PCR, we evaluated the expression of *Dnmt3a*, *Dnmt3b* and *Dnmt1* in the same populations of gonocytes used to examine *Dnmt3L* expression. Again, expression profiles in isolated gonocytes were consistent with those obtained in whole gonads (La Salle *et al.*, 2004). For *Dnmt3a,* expression was elevated in E15.5 and E18.5 gonocytes (Fig. 4.2B). *Dnmt3b* expression was consistently lower than that of *Dnmt3a*, although increasing by 4-fold between E13.5 and E18.5 (Fig. 4.2C). *Dnmt1* remained at basal levels throughout the prenatal period (Fig. 4.2D). Expression was also evaluated in primitive type A spermatogonia. While Dnmt3a levels were ~6 times lower than those observed in E18.5 gonocytes, Dnmt3b levels were at their highest (Figs. 4.2B and C). Once more, *Dnmt1* expression remained low (Fig. 4.2D). *Dnmt3a* appeared to be the main *de novo* DNA methyltransferase expressed in gonocytes, although Dnmt3b could also be detected.

Two main transcripts variants can be produced via usage of alternate promoters at the *Dnmt3a* locus: Dnmt3a and Dnmt3a2 (Chen *et al.*, 2002). To determine which of these two transcripts are expressed in gonocytes, we performed qRT-PCR using forward primers unique to either Dnmt3a or Dnmt3a2 in combination with a reverse primer common to both transcripts. Although two

distinct expression profiles were obtained, both transcripts could be detected in gonocytes at all time points (Figs. 4.3A and B). For Dnmt3a, expression increased steadily until E18.5, while Dnmt3a2 levels increased by more than 15-fold between E13.5 and E15.5 and remained as elevated in E18.5 gonocytes. Our results demonstrate that both Dnmt3a and Dnmt3a2 are expressed during the initial period of methylation acquisition.

Abnormal testicular histology, germ cell counts and onset of meiosis in absence of DNMT3L

Three mutant mouse models have been created to study the role of *Dnmt3L* in the germ line (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Webster *et al.*, 2005). All studies have reported the appearance of clear histological defects starting at two weeks after birth, including abnormal synapsis of homologous chromosomes and accumulation of ribosomal particles in spermatocytes, as well as decreased proliferation and loss of germ cells by apoptosis (Bourc'his and Bestor, 2004; Hata *et al.*, 2006; Webster *et al.*, 2005). We were intrigued that the consequences of depriving germ cells of a protein that is so highly expressed before birth would only appear after cells have resumed mitosis, differentiation, and have entered meiotic prophase. In hopes of delineating the timing of the defect, we conducted a detailed histological study of developing *Dnmt3L* mutant testes using the model created by Bourc'his and colleagues (2001). Testes were collected at two-day intervals, starting from 4 dpp until 14 dpp, the time at which defects have been reported to appear. Between 2 dpp and 4 dpp, germ cells reenter mitosis and relocate to the basement membrane. Prospermatogonia become primitive type A spermatogonia by day 6, after which point they mature into type A spermatogonia. Some spermatogonia are set aside as spermatogonial stem cells, while the rest enter the first wave of meiosis. Between 7 dpp and 8 dpp, the more mature dividing type A spermatogonia engage in the differentiation process leading to formation of type B spermatogonia, the last cell type to mitotically divide before entering meiosis.

Cells first engage in meiosis between 9 dpp and 10 dpp, as DNA is replicated one last time in absence of cell division (Bellvé *et al.*, 1977b).

Histological examination of *Dnmt3L*$^{-/-}$ testes at the light microscopy level revealed no detectable morphological differences at 4, 6 and 8 days postpartum when compared to *Dnmt3L* wild type and heterozygous testes (data not shown). Germ cells had relocated to the basal compartment and dividing cells were present in both control and homozygous testes. While testicular histology of wild type and heterozygous males remained the same at all time points examined, perceptible changes in the organization of the cells within the tubules first became visible by 10 dpp in the homozygous males, although there was no definite defect (data not shown). By 12 dpp, clear differences between *Dnmt3L* control and homozygous testes could be noted: fewer cells appeared to enter meiosis and almost no cells with condensed nuclei were visible. As exemplified in Fig. 4.4A, very few tubules display cells engaged in meiosis in the absence of DNMT3L at 12 dpp.

The histological observations prompted us to quantify the number of germ cells. We used the germ cell-specific antibody GCNA1 to determine the number of germ cells in control and DNMT3L-deficient testes. GCNA1 can be detected as early as E11.5 and is expressed in all germ cells until the zygotene stage in postnatal testes (Enders and May, 1994). First, we established that there were no differences in germ cell counts between wild type and heterozygous Dnmt3L males at 10 dpp (data not shown). From then on, solely heterozygous males were used as control. Staining of *Dnmt3L*$^{+/-}$ testes at 10 dpp revealed numerous cells, but there were consistently fewer cells stained in *Dnmt3L*$^{-/-}$ testes (Fig. 4.4B – top panels); the same was observed at 8 dpp (Fig. 4.4B – bottom panels). In fact, there was a clear difference in germ cell counts as early as day 6 (Fig. 4.4C). A trend to decreased counts could also be noted at 4 dpp, but there were only two males per group available for analysis. Clearly, depletion of Dnmt3L results in an early mitotic defect that results in a lower pool of germ cells able to engage in spermatogenesis.

Our histological analysis revealed that fewer cells appeared to be engaged in meiosis at 12 days postpartum (Fig. 4.4A). To analyze progression to meiosis, we examined the presence of phosphorylated histone H2A.X (gamma-H2A.X) positive cells in homozygous *Dnmt3L* males. Gamma-H2A.X is recruited to sites of double-stranded breaks created to permit homologous chromosome pairing and synapsis during meiosis, and as such, serves as an early marker of entry into meiosis (Mahadevaiah *et al.*, 2001). Since the first meiotic cells become visible between 9-10 dpp (Bellvé *et al.*, 1977b), we counted the number of tubules that contained at least one positive gamma-H2A.X cell in 10 dpp control and homozygous *Dnmt3L* testes. As shown in Figure 4.5A, numerous tubules containing at least one positive cell (usually ≥5 cells/ tubule) were observable in heterozygous males, while only a few tubules presented positive cells (usually ≤ 5 cells/ tubule) in homozygous males. The incidence of positive tubules, i.e. tubules presenting at least one gamma-H2A.X positive cell, was calculated per 100 tubules for both genotypes (Fig. 4.5B). There were 74.9 ± 4.0 positive tubules in *Dnmt3L*$^{+/-}$ males compared to only 28.2 ± 2.6 in *Dnmt3L*$^{-/-}$ males. Although there are fewer germ cells to begin with, these results also suggest that there is a delay in the onset of meiosis in Dnmt3L-deficient males.

Expression of DNA methyltransferases in Dnmt3L KO germ cells

In addition to characterizing the early morphological defects observed in the testis of DNMT3L-deficient males, we set out to investigate whether the expression of other *Dnmts* changed in response to Dnmt3L depletion. In an earlier study, we had observed an up-regulation in expression of *Dnmt3b* and the oocyte-specific form of *Dnmt1*, Dnmt1o, in 15 dpp *Dnmt3L*$^{-/-}$ oocytes (Lucifero *et al.*, 2006). To obtain pure populations of *Dnmt3L* mutant cells, we crossed *Dnmt3L* mice with mice expressing GFP in the germ line (Yoshimizu *et al.*, 1999). By crossing [GFP^{+}, *Dnmt3L*$^{+/-}$] males and females together, primitive type A spermatogonia could isolated by flow cytometry at 6 dpp from *Dnmt3L* wild type, heterozygous and homozygous GFP^{+} males. We chose to isolate cells at this time to obtain a maximal number of germ cells, as spermatogonia are actively

dividing at this stage and GFP is still expressed highly in these cells. Real-Time RT-PCR was used to examine the levels of Dnmt3a, Dnmt3a2, Dnmt3b and Dnmt1 in *Dnmt3L* (+/+), (+/-) and (-/-) germ cells. First, we confirmed the purity of our germ cell preparations by examining Dnmt3L expression in RNA extracted from these cells (Fig. 4.6A). As anticipated, Dnmt3L transcripts could not be detected in homozygous cells, validating the purity of our germ cell sample. Interestingly, expression in heterozygous cells was similar to that in wild type cells. Next, we determined the levels of the other *Dnmts* (Fig. 4.6B). There were no observable differences in expression of any of the *Dnmts* or their transcript variants in absence of Dnmt3L, independently of genotype.

DNA methylation analysis of Dnmt3L mutant testis

Thus far, *Dnmt3L* has been implicated in the methylation of paternally imprinted genes, unique nonpericentric heterochromatic sequences and repeat elements in male germ cells (Bourc'his and Bestor, 2004; Kaneda *et al.*, 2004; Webster *et al.*, 2005). Another study has also revealed a number of changes in gene expression in whole testes lacking Dnmt3L, suggesting this protein could be involved in methylation of other sequences (Hata *et al.*, 2006). Very few techniques allow for the analysis of methylation differences at the whole genome level in a quantitative manner. Restriction landmark genomic scanning (RLGS) allows us to scan and quantify the methylation status of approximately 2600 genomic *Not*I restriction sites. These sites are mostly found within CpG islands but are also located in non-coding unique and repetitive sequences outside of CpG islands. Presence or absence of a spot on an RLGS profile is indicative of the methylation state of that site. If the restriction site is unmethylated, *Not*I will be able to cleave the DNA template, resulting in appearance of a spot on the scan; conversely, no spot will be visible if the site is methylated.

In an attempt to find additional targets of DNMT3L, RLGS was performed on whole testes collected from 10 dpp *Dnmt3L* wild type, heterozygous and homozygous males. We first compared the methylation profiles of *Dnmt3L* wild type and heterozygous testes and found them to be identical (data not shown).

Next, we compared the heterozygous profile to the one obtained for *Dnmt3L* homozygous testis and found striking differences between genotypes, as shown in figure 4.7A (top panels). The intensity of a number of spots appeared to decrease in the absence of DNMT3L, suggesting that these sites were becoming methylated. To identify the spots that were changing between Dnmt3L control and homozygous testes, we compared these profiles to the ones obtained for adult sperm and liver (Fig. 4.7A – bottom panels). We have previously identified a number of differentially methylated sites between mature sperm and adult somatic tissues, including liver (Oakes *et al.*, 2006b). All the spots that were changing in *Dnmt3L*$^{-/-}$ testis were the germ cell-specific ones identified in mature sperm. This observation prompted us to question the legitimacy of the methylation differences observed in whole testis of homozygous males. A testis at day 10 after birth is made up of approximately 40% germ cells, the rest being somatic cells (Bellvé *et al.*, 1977b). Measuring the density of the five changing spots labeled in Fig. 4.7A gave an average density value of 44 and 16 for *Dnmt3L* (+/-) and (-/-) testes, respectively (Fig. 4.7B). After considering the proportion of germ cells present in a testis at 10 dpp and the decrease in germ cell counts observed at this point in *Dnmt3L*$^{-/-}$ testes (Fig. 4.4C), it became obvious that the mean density values corresponded roughly to the amount of germ cells present in these tissues. Therefore, changes in spot density observed in the *Dnmt3L*$^{-/-}$ testis were due to the loss of germ cells in the absence of DNMT3L, not to changes in DNA methylation state. These results render the use of whole testes for gene expression or DNA methylation analyses invalid and further emphasize the need to use isolated populations of male germ cells.

Requirement of DNMT3L for correct establishment of genome-wide DNA methylation patterns

We pursued our search of new candidate sequences targeted by DNMT3L for methylation by conducting DNA methylation analyses on the pure populations of primitive type A spermatogonia isolated by flow cytometry from *Dnmt3L* wild type, heterozygous and homozygous GFP^{+} males. Quantitative analysis of DNA

methylation using real-time PCR, or qAMP, was used to evaluate the methylation state of a number of sequences, including germ cell-specific spots identified by RLGS, imprinted genes and whole chromosomes. The qAMP assay approximates the percentage of DNA methylation present in a target sequence by combining the power of real-time PCR with the use of methylation-sensitive and methylation-dependent restriction enzymes (Oakes *et al.*, 2006a).

Since our initial study conducted on whole testes did not allow us to investigate the status of germ cell-specific spots previously identified in sperm (Oakes *et al.*, 2006b), we performed qAMP with primers specific to these sequences. The status of 11 loci chosen specifically because they were either hyper- or hypo- methylated in sperm was analyzed (Fig. 4.8A). Selected loci found in genes were non-5' and not in the vicinity of a CpG island, and all loci, including intergenic sequences, were non-repetitive. Sites that were consistently hypomethylated in *Dnmt3L* (+/+) or (+/-) germ cells remained so in *Dnmt3L* (-/-) germ cells, independently of the enzymatic site tested, suggesting that hypomethylated sites did not gain atypical methylation in the absence of DNMT3L. However, all sequences that were fairly to completely methylated presented lower methylation levels of at least one site tested in Dnmt3L (-/-) germ cells. Thus, the seven loci specifically hypermethylated in germ cells are also sequences targeted by DNMT3L.

Next, we used qAMP to confirm that paternally imprinted genes were not acquiring methylation marks properly in *Dnmt3L* KO cells. Two paternally imprinted genes (*H19* and *Dlk1-Gtl2*) and one maternally imprinted gene (*U2af1-rs1*) were chosen for analysis. Primers were designed to the established differentially methylated region (DMR) of these genes (Shibata *et al.*, 1996; Takada *et al.*, 2002; Tremblay *et al.*, 1995). Two somatic tissues serving as positive controls were analyzed in parallel with Dnmt3L mutant germ cells. For all three imprinted genes tested, we obtained an average of 50% methylation in both liver and brain samples, as expected for somatic tissues (Fig. 4.8B). In *Dnmt3L* (+/+) and (+/-) germ cells, methylation levels of *H19* and *Dlk1-Gtl2* neared 100% and 90%, respectively, as expected for paternally imprinted genes, while

methylation of the maternally imprinted gene *U2af1-rs1* was almost undetectable (Fig. 4.8B). In *Dnmt3L* (-/-) germ cells, *U2af1-rs1* methylation levels remained low, again showing that hypomethylated sequences do not gain abnormal methylation marks in absence of DNMT3L. However, we did observe methylation differences for *H19* and *Dlk1-Gtl2* in these same deficient germ cells. For *H19* (Fig. 4.8B – top left), the HhaI site had lost ~85 % of its methylation, while the McrBC sites were demethylated by about 40%; for *Dlk1-Gtl2* (Fig. 4.8B – top right), methylation was down to less than 10% at all cut sites tested. Our results for *H19* are in agreement with the mosaic pattern obtained by bisulphite sequencing reported by others (Bourc'his and Bestor, 2004; Webster *et al.*, 2005), while hypomethylation of *Dlk1-Gtl2* differs from previous findings (Bourc'his and Bestor, 2004; Kaneda *et al.*, 2004). Notwithstanding, we find that paternally imprinted genes are not acquiring their methylation marks properly in cells lacking DNMT3L.

Staining DNA with ethidium bromide shows that the genome of *Dnmt3L*-deficient germ cells is demethylated, an adverse outcome largely attributed to lack of methylation acquisition at repeat loci (Bourc'his and Bestor, 2004). We extended our analysis of DNA methylation levels in *Dnmt3L* mutant germ cells to multiple sites along chromosomes 4 and X in order to determine if there could be other factors contributing to genome hypomethylation. An autosome and a sex chromosome were chosen for analysis using qAMP. Primers were designed to flank HhaI, HpaII or McrBC restriction sites and were placed at roughly five Mb intervals along each chromosome; loci were chosen solely on the basis of the sequence not being in proximity of a known 5' region of a gene, a CpG island or within a repetitive sequence. Approximately 30 small regions were surveyed along each chromosome. As illustrated in Fig. 4.9, striking differences in DNA methylation levels could be observed for both chromosomes between *Dnmt3L* (+/+) & (+/-) cells and the two *Dnmt3L* (-/-) samples. Although the methylation status of a few hypermethylated loci on each chromosome did not change, most sites presented decreased DNA methylation levels, albeit to different extents. On each chromosome, hypomethylated loci remained unmethylated in the absence

of DNMT3L. The data presented here clearly establish that the role of DNMT3L is not restricted to specific classes of sequences such as imprinted genes or repeat elements but extends to the whole genome.

DISCUSSION

The ability of male gametes to support life is dependent on a number of interconnected events taking place during spermatogenesis, including epigenetic programming. As such, proper establishment and propagation of DNA methylation patterns by DNA methyltransferases during male germ cell development are crucial to transmission of epigenetic information to the next generation. The nature of the mechanisms governing the creation of these patterns has started to emerge as recent studies have pointed to DNMTs that could be involved in this process (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Kaneda *et al.*, 2004). A number of gene targeting experiments have revealed the importance of *Dnmt3L* to spermatogenesis: paternally imprinted genes and repeat elements are abnormally methylated in deficient germ cells and these cells are unable to progress through meiosis (Bourc'his and Bestor, 2004; Kaneda *et al.*, 2004; Webster *et al.*, 2005). However, how and why a protein incapable of directly methylating DNA causes such a drastic effect on germ cell integrity is still unclear. Here, we present a comprehensive developmental expression profile of *Dnmt3L* in isolated male germ cells and show that this gene is still expressed during postnatal spermatogenesis. Using purified germ cells, we also examine the expression profiles of other Dnmt genes during the initial period of methylation acquisition. Perhaps of most significance, we reveal the presence of a mitotic defect in *Dnmt3L* mutant males, as germ cell counts are already significantly lower at 6 days after birth, in addition to showing that the rate of entry into meiosis is markedly reduced in *Dnmt3L*$^{-/-}$ germ cells. Most importantly, we provide evidence that DNMT3L is involved in broader DNA methylation events.

Expression dynamics of Dnmt3L during male germ cell development

Acquisition of methylation patterns during male germ cell development is multifaceted: the process begins prenatally in gonocytes and continues after birth up until the end of prophase I of meiosis. As well, acquired marks are maintained as DNA replication takes place in spermatogonia and preleptotene spermatocytes. We recently demonstrated that expression of

Dnmt3a and *Dnmt3b,* the postulated *de novo* DNA methyltransferases, is tightly regulated during spermatogenesis (La Salle and Trasler, 2006a). Here, we investigated expression of the DNA methyltransferase 3-Like gene, *Dnmt3L*, during both prenatal and postnatal male germ cell development. Our analyses conducted on pure populations of prenatal gonocytes revealed strikingly elevated levels of this transcript during the initial period of methylation acquisition (Bourc'his *et al.*, 2001; Bourc'his and Bestor, 2004; La Salle *et al.*, 2004; Webster *et al.*, 2005). In agreement with previous findings by Hata and colleagues (2002), we also found expression of Dnmt3L during spermatogenesis. Although the magnitude in expression of *Dnmt3L* in postnatal male germ cells was not comparable to the one observed before birth, probing expression of this gene revealed a profile very reminiscent of the one obtained for *Dnmt1*, *Dnmt3a* and *Dnmt3b* in isolated populations of male germ cells (La Salle and Trasler, 2006a). The data presented in Fig. 4.1 show that *Dnmt3L* expression is down-regulated during the same two developmental windows identified for other *Dnmts*, which are differentiation of spermatogonia into spermatocytes and pachynema. These results are the first indication of regulated expression of *Dnmt3L* during postnatal male germ cell development. DNMT3L could be involved in the establishment or the maintenance of methylation patterns during postnatal male germ cell development, in addition to the role it plays in gonocytes.

In addition to exploring *Dnmt3L* expression, we also determined the expression dynamics of other *Dnmt* genes in prenatal germ cells. Although Dnmt1 and Dnmt3b are not present at very high levels in gonocytes, both Dnmt3a and Dnmt3a2 can be detected at significant levels in these cells (Fig. 4.2). In fact, Dnmt3a2 is highly expressed at the same time as Dnmt3L, but expression of Dnmt3a seems to increase as Dnmt3L levels start to come down. A previous study demonstrated co-expression of DNMT3a2 and DNMT3L in gonocytes using immunohistochemistry, corroborating our results (Sakai *et al.*, 2004). Since DNMT3L does not possess the required motifs allowing transfer of methyl groups to DNA, it has been hypothesized that it acts by stimulating the activity of other DNMTs (Chédin *et al.*, 2002; Hata *et al.*, 2002). A recent report

suggests that DNMT3L participates in *de novo* methylation by reorganizing DNMT3a2 subunits, forming a complex with enhanced DNA methyltransferase activity (Kareta *et al.*, 2006). Although the existence of such a complex remains to be demonstrated in germ cells, the data presented here is in line with such a hypothesis.

Mitotic defect and delayed entry into meiosis in absence of DNMT3L

Detailed histological analysis of early testicular development in *Dnmt3L* mutant males has allowed a better definition of the timing of the histopathological defects observed in mice lacking DNMT3L. Although testis histology appears normal, *Dnmt3L*$^{-/-}$ males do present abnormalities at one week of age: germ cell counts are down by more than 50% at 6 dpp. Not surprisingly, this defect has gone unnoticed by others since testis histology appears normal up until ~10 dpp (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Hata *et al.*, 2006; Webster *et al.*, 2005). Hata and colleagues (2006) recently demonstrated that, at 3 weeks of age, germ cells from *Dnmt3L*$^{-/-}$ males proliferate at about half the rate wild type germ cells do. Proliferation rates could be affected from the beginning, explaining why germ cell counts are already affected at 4 dpp – the time at which most germ cells resume mitosis. In turn, a lower incidence of spermatogonia to begin with could account for the complete depletion of germ cells observed early on at adulthood.

In addition to lower germ cell counts, we also observed a delay in entry into meiosis, as illustrated by the lower frequency of tubules containing gamma-H2A.X positive cells in 10 day old DNMT3L-deficient male mice. Commitment of spermatogonia to meiosis is an important step in germinal differentiation upon which point dedifferentiation becomes impossible. Germ cells have to express the proper set of genes and present the appropriate chromatin structure that will allow for their passage through meiosis (Rousseaux *et al.*, 2005). Delay in onset of meiosis in *Dnmt3L* mutant males could be caused by failure of germ cells to undergo the necessary steps required to proceed to the next developmental stage. As DNA methylation and histone modifications are interconnected, a genome-wide DNA methylation defect could cause chromatin structure

abnormalities, retarding entry into meiosis. In fact, atypical chromatin structures have been reported in *Dnmt3L*$^{-/-}$ germ cells from the intermediate spermatogonia stage all the way through zygonema (Webster *et al.*, 2005). Transcriptional reactivation of retroviral elements and their subsequent random reintegration could also contribute to delaying entry in meiosis by compromising the integrity of the genome of *Dnmt3L*$^{-/-}$ germ cells through *de novo* mutations or ectopic gene expression (Bourc'his and Bestor, 2004; Webster *et al.*, 2005).

Genome-wide demethylation observed in germ cells lacking Dnmt3L

Our initial attempt to find new targets of DNMT3L proved unsuccessful, as the use of *Dnmt3L* mutant whole testis presented a major caveat: the proportion of germ cells was not sufficient to allow for the detection of methylation changes in germ cells per se. Any differences in DNA methylation levels were simply due to shifting somatic-to-germ cell ratios. However, we were able to gain insight on novel sequences targeted by DNMT3L by isolating primitive type A spermatogonia from Dnmt3L mutant males and performing qAMP. Using this approach, we were able to test a number of sequences.

When the methylation status of paternally imprinted genes was assessed, we observed loss of methylation at the *H19* DMR similarly to what has been published (Bourc'his and Bestor, 2004; Webster *et al.*, 2005). However, hypomethylation of the *Dlk1-Gtl2* locus was in disagreement with previous findings where normal methylation of this locus at been observed (Bourc'his and Bestor, 2004; Kaneda *et al.*, 2004). Discrepancies in the results can be accounted for by differences in timing of sample collection and techniques employed to isolate the cells. The analyses presented here were carried out on pure preparations of primitive type A spermatogonia isolated at 6 dpp, the earliest time point tested for DNA methylation differences in a *Dnmt3L* mutant mouse model, while all other studies have been conducted on mixed populations of germ cells isolated at later time points (Bourc'his and Bestor, 2004; Kaneda *et al.*, 2004; Webster *et al.*, 2005). Stochastic imprinting in the progeny of *Dnmt3L*$^{-/-}$ females has recently been reported, indicating that methylation of DMRs can still

occur in the absence of DNMT3L (Arnaud *et al.*, 2006). Although the molecular mechanisms underlying these observations are still unclear, a similar phenomenon could be taking place in *Dnmt3L*$^{-/-}$ male germ cells, partly accounting for the methylation differences observed. Additionally, we have previously reported that expression of other *Dnmts* is up-regulated in absence of DNMT3L in oocytes collected at 15 dpp, the time of imprint establishment in the female (Lucifero *et al.*, 2006). While we did not detect any differences in *Dnmt* expression in *Dnmt3L*$^{-/-}$ male germ cells, compensation mechanisms could still be activated at an earlier time in an attempt to preserve genomic integrity. Lack of Dnmt up-regulation could also suggest that the regulatory mechanisms governing expression in the male and female germ lines are different. Evidence of this is seen for *Dnmt1*, as different mechanisms appear to control DNMT1 down-regulation during male and female meiosis (La Salle *et al.*, 2004).

When sequences specifically hypo- or hyper- methylated in sperm (Oakes *et al.*, 2006b) were surveyed in germ cells lacking DNMT3L, sequences that were hypermethylated in control cells had lost their methylation, albeit to different degrees, in *Dnmt3L*-deficient cells, while sequences that were hypomethylated remained so. This indicated to us that methylation patterns were being erased normally in primordial germ cells but were not being reacquired properly in the absence of DNMT3L. The methylation state of multiple loci across chromosomes 4 and X was also analyzed using qAMP. Numerous sites on both an autosome and a sex chromosome were severely demethylated in *Dnmt3L*$^{-/-}$ germ cells. Again, hypomethylated sequences remained so in cells lacking DNMT3L, but interestingly, a few sequences were methylated properly. Either these sequences are not targets of DNMT3L and are methylated by another DNMT that does not require DNMT3L stimulation, or a permissive chromatin structure allows for proper methylation of these sequences even in the absence of DNMT3L.

To our knowledge, this is the first indication that unique, non-CpG island, non-repetitive sequences are also targets of DNMT3L but, most importantly, that the action of DNMT3L extends to multiple sites in the genome that are known to become methylated during spermatogenesis.

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Figure 4.1 ***Dynamic expression of Dnmt3L in postnatal male germ cells.*** Relative quantification of *Dnmt3L* expression in isolated populations of male germ cells. Real-time RT-PCR was used to measure levels of Dnmt3L in total RNA extracted from primitive type A (PA), type A (A) and type B (B) spermatogonia, preleptotene (PL), leptotene/ zygotene (L/Z), prepubertal pachytene (PP) and pachytene (P) spermatocytes, as well as round spermatids (RS) and residual bodies/ elongating spermatids (RB). Expression was determined in triplicate in each of the two series of germ cells; shown here are the mean expression results obtained for one series. Mean ± SD.

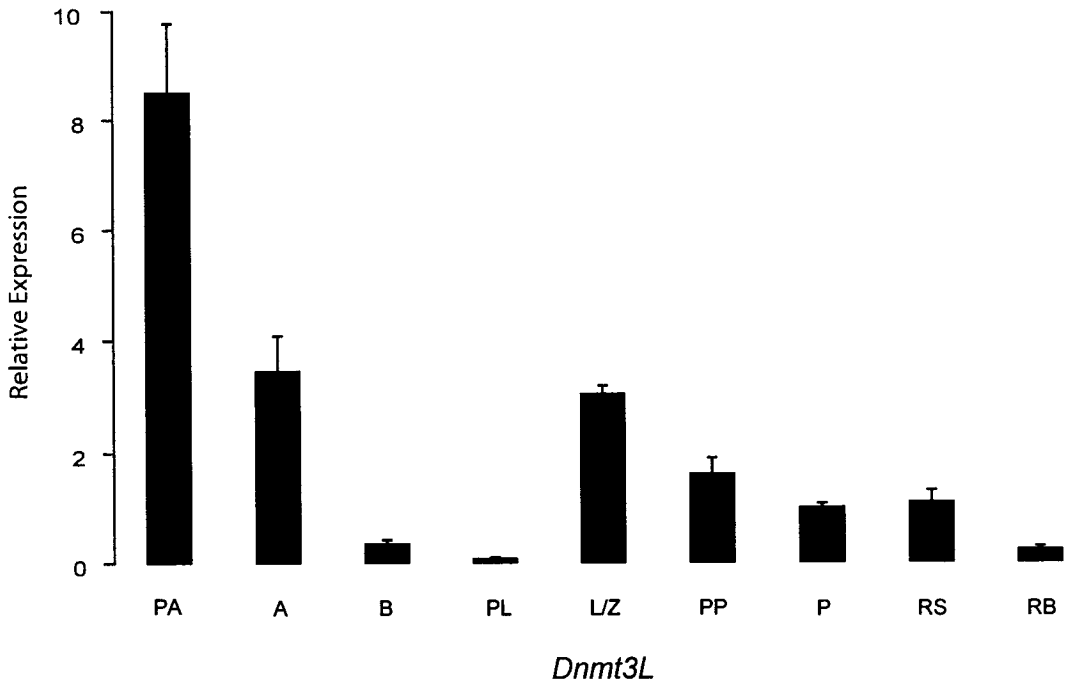

10
8
6
4
2
0
Relative Expression
PA
A
B
PL
L/Z
PP
P
RS
RB
Dnmt3L

Figure 4.2 ***Expression dynamics of DNA methyltransferases in prenatal male germ cells.*** Relative quantification of **A**) *Dnmt3L,* **B**) *Dnmt3a,* **C**) *Dnmt3b* and **D**) *Dnmt1* expression in purified populations of male germ cells. Quantitative RT-PCR was used to determine the global expression levels of these genes in total RNA extracted from E13.5, E15.5 and E18.5 prospermatogonia and 6 dpp primitive type A spermatogonia (PA). Note the difference in scale magnitude between A) and B), C), D). Expression of each gene was determined in triplicate in each of the two series of germ cells; shown here are the mean expression results obtained for one series. Mean ± SD.

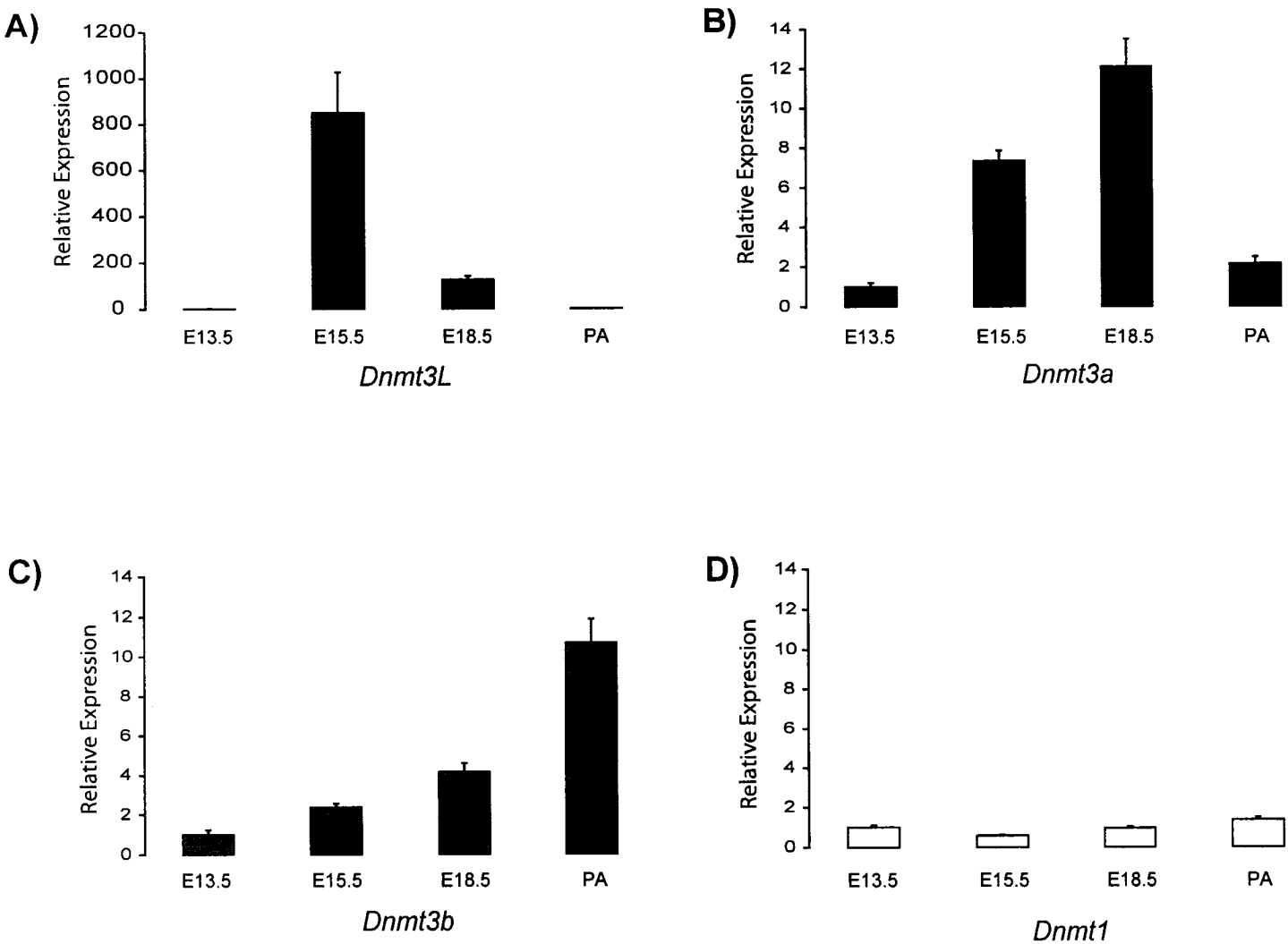
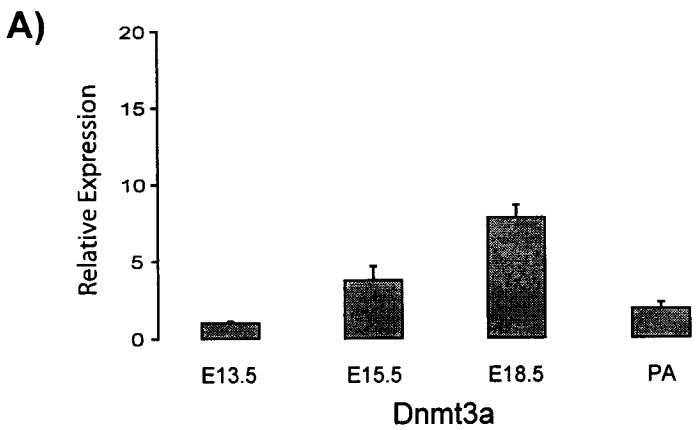

A)
Relative Expression
1200
1000
800
600
400
200
0
E13.5
E15.5
E18.5
PA
Dnmt3L
B)
Relative Expression
14
12
10
8
6
4
2
0
E13.5
E15.5
E18.5
PA
Dnmt3a
C)
Relative Expression
14
12
10
8
6
4
2
0
E13.5
E15.5
E18.5
PA
Dnmt3b
D)
Relative Expression
14
12
10
8
6
4
2
0
E13.5
E15.5
E18.5
PA
Dnmt1

Figure 4.3 ***Differential expressions of Dnmt3a and Dnmt3a2 in prenatal male germ cells.*** Relative expression of Dnmt3a (top) and Dnmt3a2 (bottom) in purified populations of male germ cells. Real-time RT-PCR was used to determine the expression levels of the two transcripts in total RNA extracted from E13.5, E15.5 and E18.5 prospermatogonia and 6 dpp primitive type A spermatogonia (PA). Expression of each transcript was determined in triplicate in each of the two series of germ cells; shown here are the mean expression results obtained for one series. Mean ± SD.


A)
Relative Expression
20
15
10
5
0
E13.5
E15.5
E18.5
PA
Dnmt3a

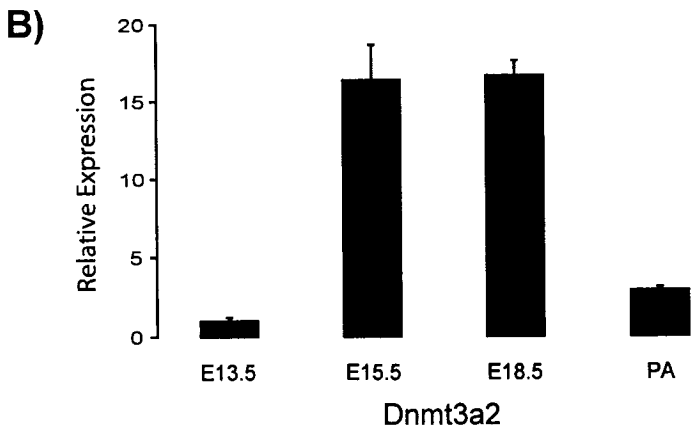

B)
Relative Expression
20
15
10
5
0
E13.5
E15.5
E18.5
PA
Dnmt3a2

Figure 4.4 ***Examples of histological abnormalities in the Dnmt3L$^{-/-}$ testis.*** **A)** Hematoxylin and eosin staining of cross-sections of testes from 12 dpp *Dnmt3L* +/- (left) and -/- (right) mice. Germ cells do not appear to be entering meiosis in absence of DNMT3L (right panel). **B)** Immunoperoxidase staining of germ cells with the GCNA1 antibody (brown) in testis sections of 10 dpp (top) and 8 dpp (bottom) heterozygous and homozygous *Dnmt3L* mice. **C)** Graphical representation of germ cell counts in *Dnmt3L* (+/-) and (-/-) testes. A difference in germ cell count is observed as early as 4 dpp in absence of DNMT3L. By 10 dpp, mutant males have approximately a quarter of the number of germ cells their heterozygous littermates have. For each time point, GCNA-positive germ cells were counted per 2000 Sertoli cells; two to three males were examined per genotype. Results are presented as means ± SEM; mean counts for individual males are presented as dots when only two animals were analyzed.

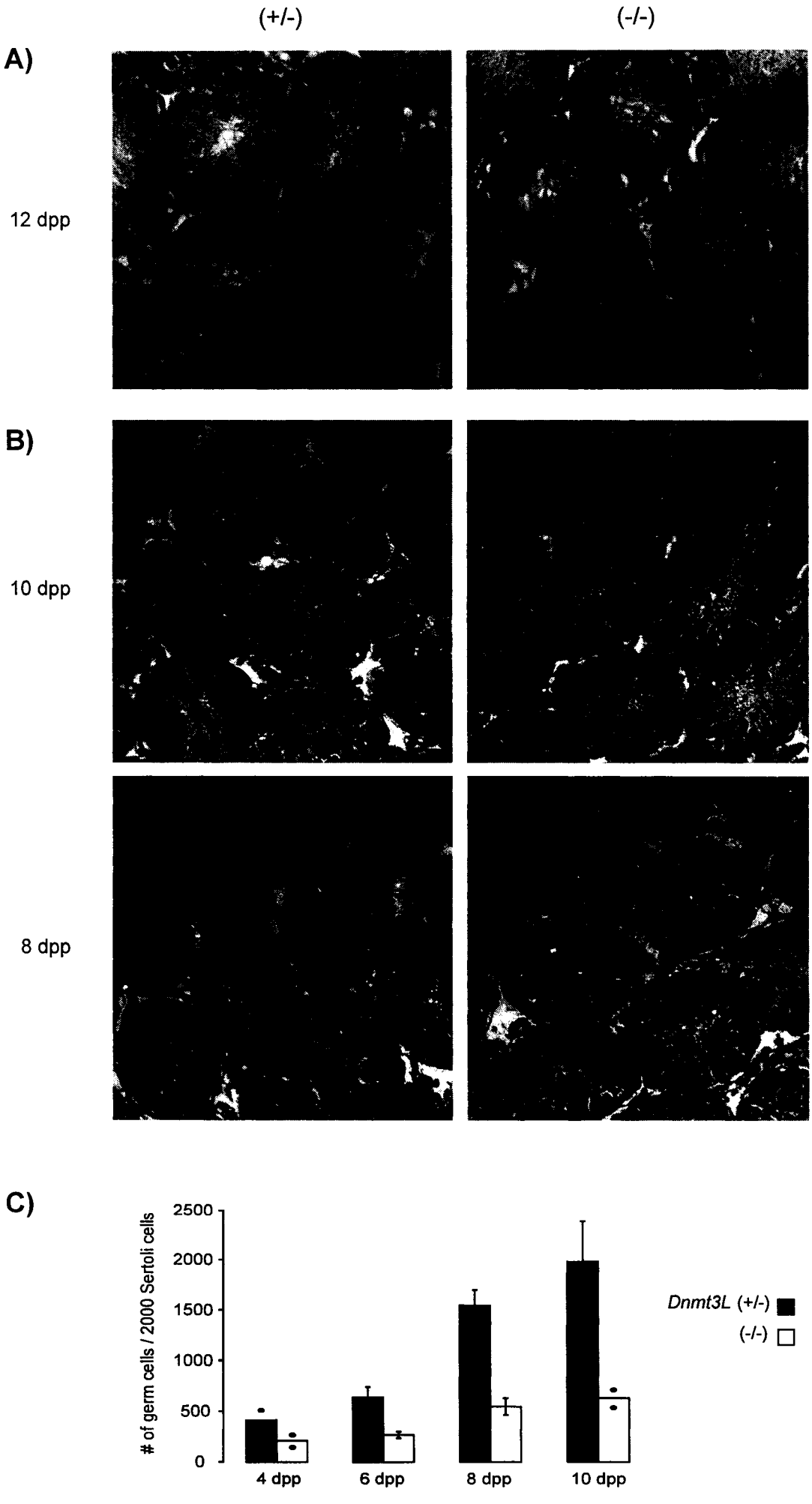
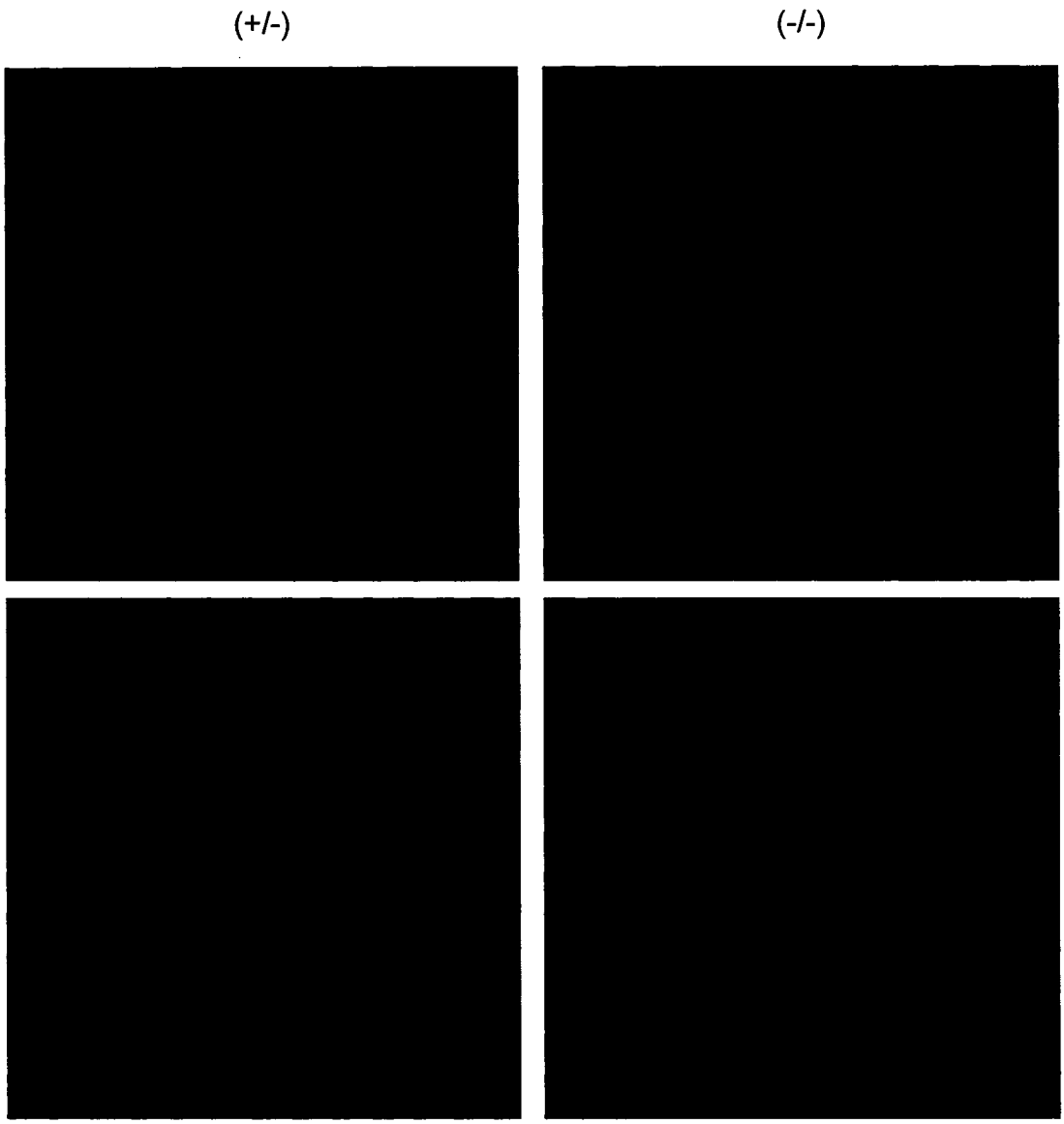

(+/-)
(-/-)
A)
12 dpp
B)
10 dpp
8 dpp
C)
# of germ cells / 2000 Sertoli cells
2500
2000
1500
1000
500
0
4 dpp
6 dpp
8 dpp
10 dpp
Dnmt3L (+/-)
(-/-)

Figure 4.5 ***Delayed onset of meiosis in the absence of DNMT3L.*** Immunofluorescence analysis of testis cross-section from 10 dpp *Dnmt3L* (+/-) and (-/-) mice using an antibody directed against gamma-H2A.X (green). **A)** (Top panel – left) Representative staining pattern obtained in *Dnmt3L*$^{+/-}$ sections: a majority of tubules contain gamma-H2A.X positive cells. (Top panel- right) Only a few tubules containing positive cells can be detected in *Dnmt3L*$^{-/-}$ sections. Below each panel are the corresponding DAPI (blue) counterstained images. **B)** Quantification of the number of tubules containing at least one positive gamma-H2A.X cell in heterozygous and homozygous testes. The incidence of positive tubules per 100 tubules was determined from six to nine sections per male, with three males per genotype group. Results are presented as means ± SEM.

A)



B)

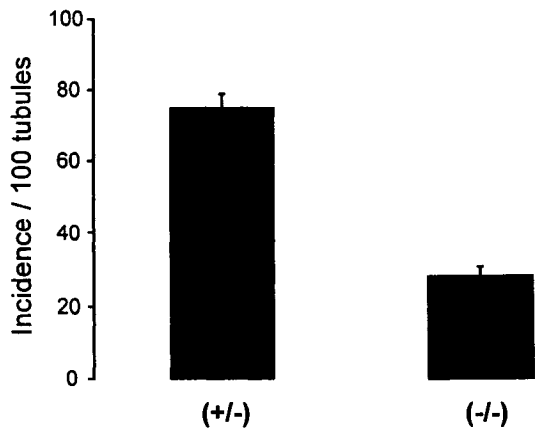


Figure 4.6 ***DNA methyltransferase expression in Dnmt3L$^{-/-}$ male germ cells.*** QRT-PCR was used to evaluate the relative expression of *Dnmt* genes in isolated *Dnmt3L* mutant 6 dpp primitive type A spermatogonia. Expression of a given Dnmt was analyzed in triplicate and normalized to 18S expression for each genotype; the normalized value was calibrated to the expression observed in homozygous cells. **A)** Illustration of *Dnmt3L* expression in wild type, heterozygous and homozygous male germ cells. Dnmt3L could not be detected in the homozygous sample, confirming the purity of our preparation. Only the normalized values are presented in this case, calibrating the data to the homozygous sample being impossible. ND, not detectable. **B)** Expression of *Dnmt3a*, *Dnmt3a2*, *Dnmt3b* and *Dnmt1* in wild type (black bars), heterozygous (grey bars) and homozygous (white bars) germ cells. No observable differences in DNA methyltransferase expression could be detected in Dnmt3L-deficient germ cells at 6 dpp. Results from one series of germ cells are presented as mean ± SD.

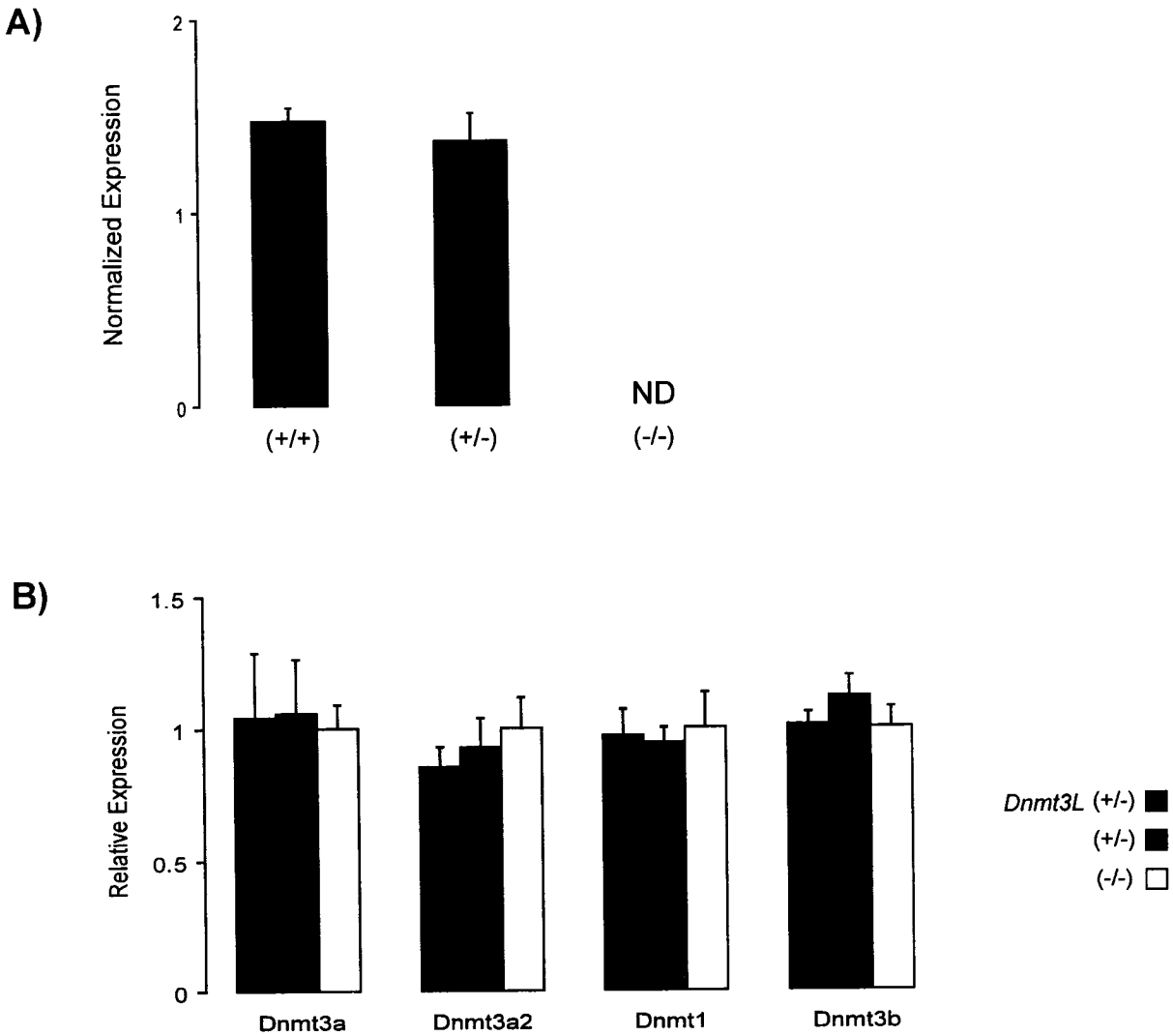
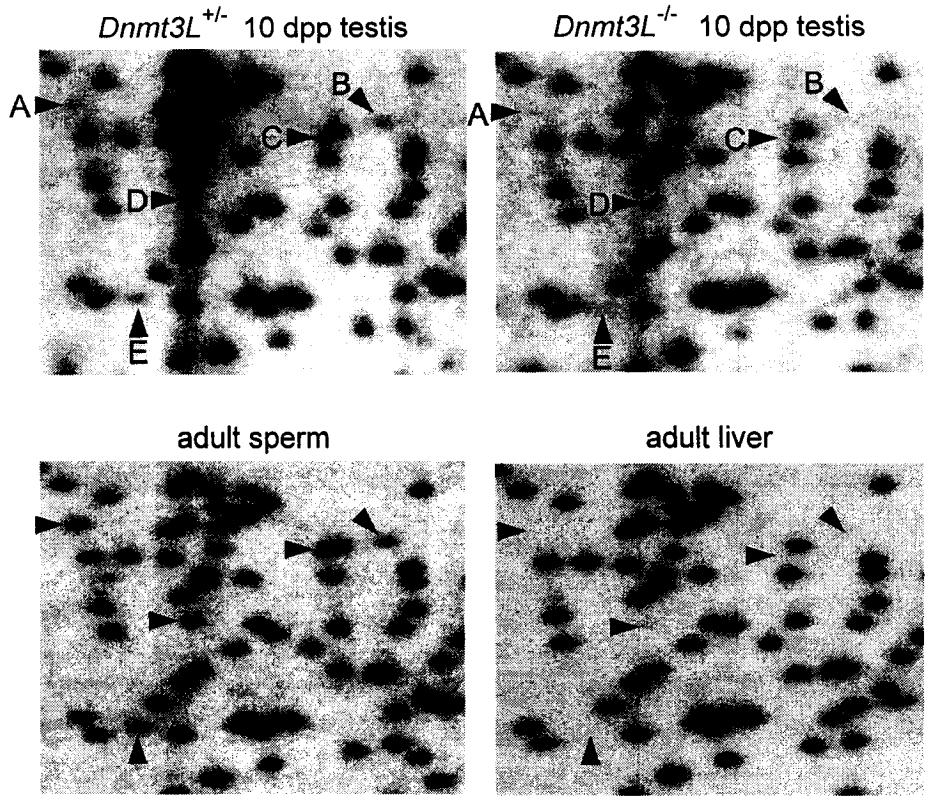

A)
Normalized Expression
2
1
0
(+/+)
(+/-)
ND
(-/-)
B)
Relative Expression
1.5
1
0.5
0
Dnmt3a
Dnmt3a2
Dnmt1
Dnmt3b
Dnmt3L (+/-)
(+/-)
(-/-)

Figure 4.7 ***RLGS analysis of Dnmt3L-deficient testis.*** Restriction landmark genomic scanning was used to investigate global methylation levels of *Dnmt3L* mutant testis. The following tissues were used for analysis: 10 dpp whole testes from heterozygous and homozygous *Dnmt3L* males, and sperm and liver from wild type adult males. **A)** Shown here are enlargements of autoradiographs obtained for these tissues. Arrowheads point to five pre-identified germ cell-specific spots present in adult sperm but absent in liver (Oakes *et al.*, 2006b). **B)** Densitometry analysis of the five germ cell-specific spots in *Dnmt3L* heterozygous and mutant testes. Once the somatic-to-germ cell ratio of a 10 dpp testis is considered and the lower germ cell counts in *Dnmt3L*$^{-/-}$ testes are accounted for, the mean density values obtained for *Dnmt3L* heterozygous and homozygous 10 dpp testes are reflective of the proportion of germ cells present in these tissues, not of changes in methylation state. Potential changes in DNA methylation levels in *Dnmt3L* homozygous testes fall under the limit of detection.

A)



B)

	Dnmt3L testis	
Spot	**(+/-)**	**(-/-)**
A	44	13
B	40	6
C	47	15
D	53	29
E	36	17
mean density	**44**	**16**

Figure 4.8 ***Abnormal DNA methylation levels in Dnmt3L$^{-/-}$ germ cells.*** DNA extracted from the purified populations of *Dnmt3L* germ cells used in Fig. 6 was analyzed via a quantitative restriction enzyme assay, quantitative analysis of methylation PCR or qAMP (Oakes *et al.*, 2006a). **A)** Methylation levels of 11 sites identified as being specifically hypo- or hyper- methylated in sperm (Oakes *et al.*, 2006b) were examined in *Dnmt3L* wild type, heterozygous or homozygous primitive type A spermatogonia. Sites that were consistently hypomethylated in (+/+) and (+/-) germ cells remained so in (-/-) cells; sites that were methylated in (+/+) and (+/-) germ cells showed a lower degree of methylation of at least one site in (-/-) cells (changes are in bold). -, cut site not present in the sequence. **B)** Analysis of established DMR regions of two known paternally methylated imprinted genes, *H19* (top left) and *Dlk1-Gtl2* (top right), and one known maternally methylated imprinted gene, *U2af1-rs1* (bottom). At least one site of *H19* and *Dlk1-Gtl2* is affected in the two (-/-) samples analyzed, whereas *U2af1-rs1* methylation remains unchanged. Three different enzymes were used in qAMP: *Hha*I (white), McrBC (black) and *Not*I (grey).

A)

location (mm7)	gene	NotI			HhaI			McrBC		
		(+/+)	(+/-)	(-/-)	(+/+)	(+/-)	(-/-)	(+/+)	(+/-)	(-/-)
chr12:89165537	*4933401N24Rik*	100	100	100	96	100	**61**	93	91	90
chr16:10316903	*AK016529*	100	100	100	100	100	**43**	99	99	99
chr16:34906165	*Adcy5*	100	100	**78**	75	94	64	99	97	96
chr4:130167229	*Matn1*	100	95	**57**	92	82	**36**	98	98	**54**
chr8:118899998	*CJ052809*	84	91	**44**	-	-	-	86	80	**62**
chr18:47871042	*AK045080*	69	76	**22**	-	-	-	78	73	**28**
chr15:85761755	intergenic	45	27	**2**	65	43	**6**	62	48	**32**
chr4:52511113	intergenic	24	25	22	2	2	3	16	18	9
chr2:74130834	*S76657*	3	3	4	-	-	-	13	15	28
chr14:94881621	intergenic	5	9	7	-	-	-	17	14	3
chr1:135992032	*LO6234*	4	4	3	-	-	-	19	20	10

B)

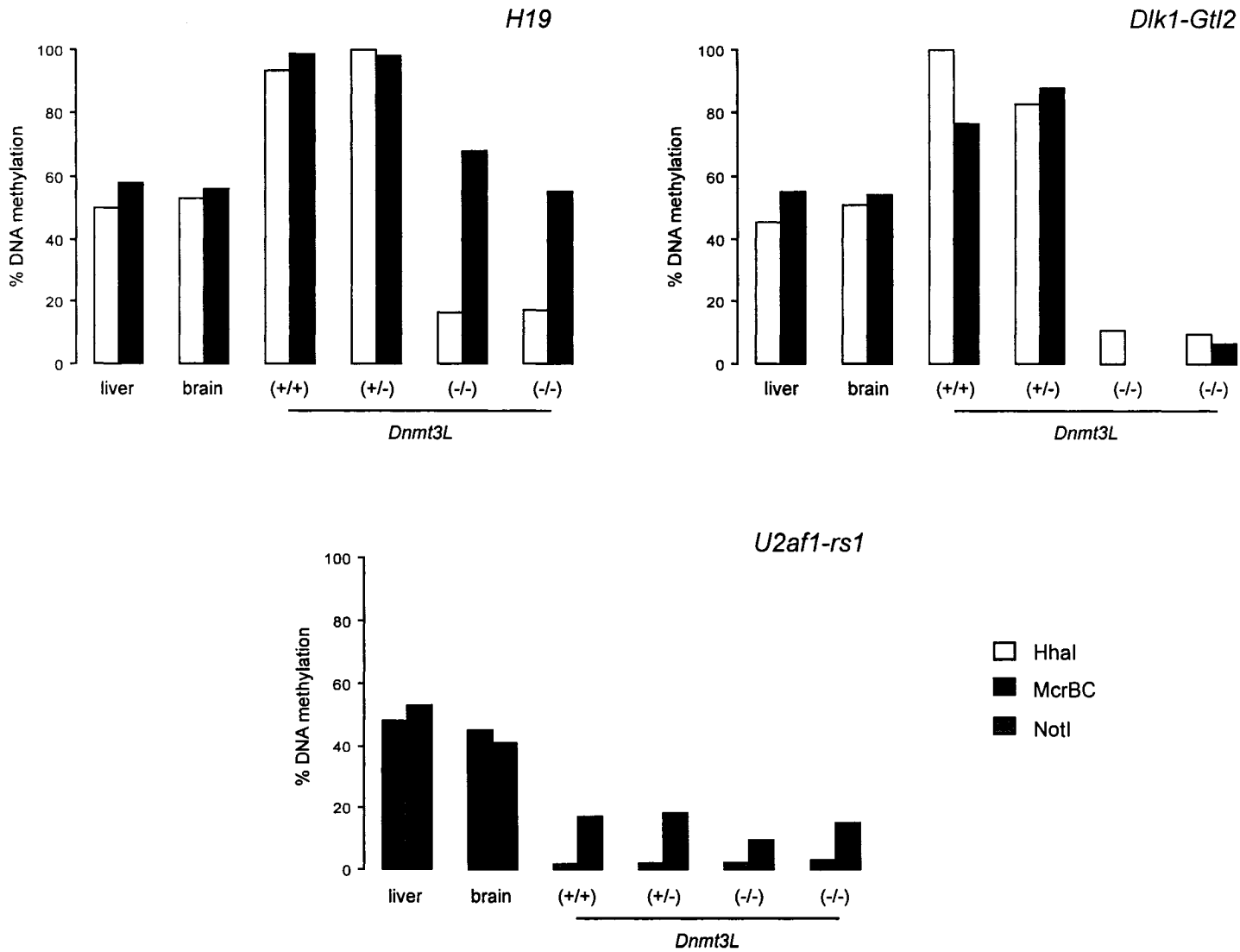
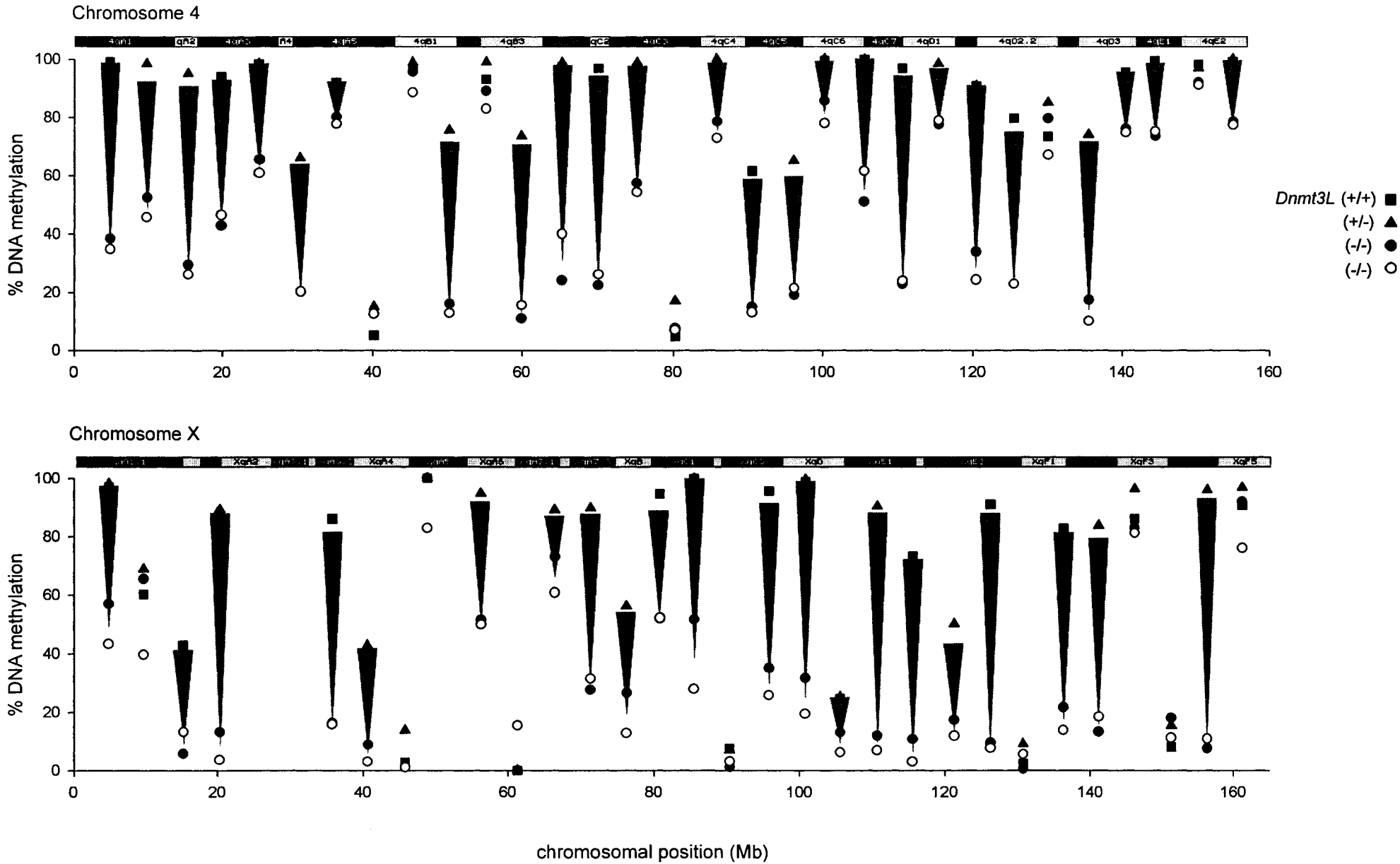


Figure 4.9 ***Decreased levels of DNA methylation on chromosomes 4 and X in Dnmt3L$^{-/-}$ germ cells.*** DNA methylation analysis of non-CpG island DNA on chromosomes 4 (top) and X (bottom) using qAMP. The percent of DNA methylation of each amplified region is shown for wild type (solid square), heterozygous (solid triangle) or homozygous (open and solid circles) *Dnmt3L* male germ cells; differences in methylation are shown by blue arrows. Chromosomes 4 and X ideograms (obtained from the UCSC genome browser) are illustrated in the context of chromosomal positioning. For both chromosomes, most sites examined showed lower levels of DNA methylation in the absence of DNMT3L.


Chromosome 4
% DNA methylation
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
Dnmt3L (+/+)
(+/-)
(-/-)
(-/-)
Chromosome X
% DNA methylation
chromosomal position (Mb)
Changes in % DNA methylation between the means of Dnmt3L (+/+) & (+/-) vs. (-/-) & (-/-)

CHAPTER V

Discussion

The overall purpose of the work described in this thesis was to gain a better understanding of the action of DNA methyltransferases in male germ cells. This discussion will provide a brief overview of the experimental findings exposed in Chapters II through IV. In addition, experiments that could be conducted in the future to address unanswered questions raised by the results presented here will be proposed.

5.1 Overview of Major Findings

Initial experiments described in *Chapter II* provided the basis of our future investigations on DNA methyltransferase expression in the male germ line. In this study, immunohistochemistry analyses revealed that DNMT1, initially the best-characterized DNA methyltransferase of the germ line, was simply not present at the time of DNA methylation acquisition in gonocytes. This prompted us to search for other DNMTs that could be responsible for fulfilling this role. Performing Real-Time RT-PCR studies on developing prenatal gonads identified *Dnmt3L* and *Dnmt3a* as likely candidates in the *de novo* establishment of methylation marks in male germ cells, while the timing of the postnatal expression of *Dnmt3b* and *Dnmt1* suggested that these enzymes could be involved in maintaining genomic methylation patterns at times of DNA replication during spermatogenesis (La Salle *et al.*, 2004). We also examined expression of DNA methyltransferases in the female germ line. Consistent with what has been observed during meiosis in the male (Jue *et al.*, 1995), we also observed down-regulation of DNMT1 during meiosis in prenatal ovaries. However, loss of DNMT1 was not associated with the expression of the untranslated transcript of *Dnmt1*, Dnmt1p, as it is the case in pachytene spermatocytes (Jue *et al.*, 1995; Mertineit *et al.*, 1998). qRT-PCR was also used to assess expression of *Dnmts* in developing ovaries. Except for high levels of Dnmt3L detected at times of methylation pattern acquisition in the female, modulation in expression of the other Dnmts was not suggestive of any specific role (La Salle *et al.*, 2004).

Because the most interesting modulations in *Dnmt* expression were observed in the testis and both events of *de novo* and maintenance methylation take place during spermatogenesis, we opted to focus on the male germ line as a model system to better our understanding of DNMT expression in germ cells. In *Chapter III*, we compared the temporal expression patterns of the postulated *de novo* DNA methyltransferases, *Dnmt3a* and *Dnmt3b*, in different populations of male germ cells (La Salle and Trasler, 2006a). In all, eight different populations of mitotic, meiotic and post-meiotic germ cells could be isolated using mice of different age groups, giving us a sampling of the major cellular stages occurring during spermatogenesis. We found that proliferating and differentiating male germ cells are marked by distinctive expression profiles, and identified the transition from spermatogonium to spermatocyte to be a developmental window during which the expression of DNA methyltransferases is down-regulated. We also observed that expression of *Dnmt3a* and *Dnmt3b* rises again as germ cells engage in meiosis, only to decrease steadily at the approach of pachynema, identifying a second developmental window of *Dnmt* down-regulation. By conducting Western Blots, we determined that a specific isoform of *Dnmt3a*, DNMT3a, is highly expressed in type B spermatogonia, while DNMT3a2, as well as two isoforms of Dnmt3b, DNMT3b2 and DNMT3b3, are expressed mostly throughout spermatogenesis, except in pachytene spermatocytes. All of these results are reminiscent of previously published observations on DNMT1 expression in male germ cells (Jue *et al.*, 1995; Trasler *et al.*, 1992), and suggest a common regulatory theme for DNA methyltransferases in the male germ line. Taken together, these data highlight that DNMTs may be contributing differentially to the establishment and/ or the maintenance of methylation patterns in male germ cells and emphasize the need for transcript-specific inactivation studies at different times during male germ cell development.

Germ cell-specific inactivation of *Dnmt3b* has previously been reported to produce no adverse consequences on DNA methylation pattern establishment during male germ cell development (Kaneda *et al.*, 2004). In light of the expression results presented in Chapter III, one might wonder how this can be

possible. Since no detailed characterization of this mouse model has been published, one can only speculate that compensation by another DNMT is accounting for the lack of defect. Regardless, more studies are warranted before the participation of *Dnmt3b* in methylation patterns establishment and/ or maintenance can be invalidated.

A number of mouse models have been created to study the role of DNMT3L in the germ line (Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Webster *et al.*, 2005). All ensuing reports have suggested that this protein is mainly responsible for methylation of imprinted genes and repeat sequences in male germ cells, and have implied that adverse consequences of *Dnmt3L* inactivation only appear during meiosis (Bourc'his and Bestor, 2004; Hata *et al.*, 2006; Kaneda *et al.*, 2004; Webster *et al.*, 2005). Considering previous findings, the role of DNMT3L was assessed during early male germ cell development in *Chapter IV.* First, we produced a comprehensive expression profile of *Dnmt3L* in both prenatal and postnatal male germ cells. Although expression of Dnmt3L was highest in prenatal gonocytes, it could also be detected throughout spermatogenesis albeit at much lower levels than in gonocytes. In fact, the postnatal expression profile of *Dnmt3L* is very similar to the one obtained for *Dnmt3a*, *Dnmt3b* and *Dnmt1*.

As the first sign of histological defects in mice lacking Dnmt3L have been reported to appear around two weeks of age, we conducted a detailed histological analysis of *Dnmt3L* mutant males over the first twelve days of life using the model by Bourc'his *et al.* (2001). We demonstrated the presence of a mitotic defect in *Dnmt3L* mutant males, as germ cell counts were already significantly lower shortly after birth and entry into meiosis was delayed. Crossing *Dnmt3L* mutant mice with GFP-transgenic mice allowed us to isolate highly pure germ cells at an earlier stage than carried out before. Primitive type A spermatogonia were isolated from wild type, heterozygous and homozygous males and qAMP, an assay permitting detection of DNA methylation differences developed in our lab, was applied to find additional sequences targeted for methylation by DNMT3L. A number of unique, non-imprinted, non-repetitive sequences were affected by *Dnmt3L* deficiency, suggesting the role of DNMT3L

is not restricted to specific classes of sequences. Most of all, multiple sites along chromosomes 4 and X were hypomethylated in germ cells lacking DNMT3L, implying that this protein is involved in broader DNA methylation events than previously suggested.

5.2 Future Directions

Localization of DNA Methyltransferases in Germ Cells

There have been a number of reports on the unique localization properties of DNMT3a and DNMT3b1 to heterochromatin and of DNMT3a2 to euchromatin (Bachman et at., 2001; Chen *et al.*, 2002; Kim *et al.*, 2002), and on the trafficking of DNMT3L from the cytoplasm to the nucleus in presence of DNMT3a and DNMT3b (Hata *et al.*, 2002), nonetheless all of these studies have relied on expression of tagged versions of these proteins in cultured cells. The expression patterns described in Chapters III and IV have provided a good indication of the cellular expression of *Dnmt3a*, *Dnmt3b* and *Dnmt3L*. However, a previous study done on the localization of DNMT1 in male germ cells revealed interesting features that could not have been identified simply by doing Western Blots, including specific nuclear localization to foci in leptotene/ zygotene spermatocytes (Jue *et al.*, 1995). Consequently, *in situ* hybridization and localization studies on tissue sections are required to improve our knowledge of localization and possible interactions between DNMTs. The BSA cell separation method employed in the studies in Chapters III and IV has certain limitations: due to the biology of spermatogenesis, mice of different age groups have to be utilized to isolate different types of cells with significant purities. Thorough understanding of the DNMT enzymes in their biological context therefore requires a detailed analysis of their cellular localization in both isolated germ cells and developing testis sections. While confocal image analysis of isolated germ cells provides information on location to specific cellular compartments and co-localization of these proteins, *in situ* hybridization and immunohistochemistry

supply information on the expression of these enzymes in the context of all the cellular associations intrinsic to spermatogenesis.

Advances in this field are hindered by the lack of more sensitive antibodies allowing for the detection of specific isoforms of DNMT3a and DNMT3b or the lack of an antibody per se in the case of DNMT3L. Although there have been reports of existing DNMT3L antibodies, the quality of these antibodies remains to be determined as they either have not been tested in *Dnmt3L*$^{-/-}$ cells or have not been published on (Sakai *et al.*, 2004; Webster *et al.*, 2005). In the case of DNMT1, finding experimental conditions suitable for immunohistochemistry was only made possible by testing the antibody on isolated cells first (Jue *et al.*, 1995; Mertineit *et al.*, 1998). One can only suspect that a similar scenario can be envisaged for DNMT3a, DNMT3b and DNMT3L.

Biological Function of DNMT Down-Regulation at Pachynema

The results in Chapter III show that down-regulation of DNMT proteins at pachynema is a common regulatory theme amongst DNA methyltransferases. Bestor and Tycko (1996) have suggested that several features of meiotic chromosomes render them vulnerable to *de novo* methylation during crossing over and that DNA structures associated with recombination are targeted by DNA methyltransferases. We have previously proposed that germ cells may protect their DNA from ectopic methylation by neutralizing the expression of DNMT1 via the expression of the untranslated transcript Dnmt1p at pachynema (Jue *et al.*, 1995; Mertineit *et al.*, 1998). Determining the adverse consequences of DNMT1 expression during pachynema could confirm this hypothesis. Since the control elements leading to the transcription of Dnmt1p via use of the alternate first exon 1p remain unknown, an over-expression study using these elements to drive expression of Dnmt1s (transcript giving rise to DNMT1) cannot be undertaken. However, insertion of first exon 1s in exon 1p could be employed to trigger expression of DNMT1 under the control of the elements that drive expression in spermatocytes. Successful use of a similar strategy has previously been reported: insertion of exon 1s in the oocyte-specific exon 1o allowed for

expression of DNMT1 at times when it is usually not detected in oocytes and embryos (Ratnam *et al.*, 2002).

Dnmt3L transcript variants expressed specifically in pachytene spermatocytes and round spermatids have recently been uncovered and have been proposed to mediate DNMT3L down-regulation during pachynema similarly to what is observed for DNMT1 (Shovlin *et al.*, 2006). Identification of the regulatory sequences leading to the expression of these transcripts as well as their individual inactivation will be required to comprehend the role they play in the male germ line. As existence of pachytene-specific transcripts of *Dnmt3a* or *Dnmt3b* has not been reported yet, other factors such as mRNA stability, differences in protein localization or post-translation modification of these enzymes marking them for degradation may be responsible for down-regulation. Determining the localization of these proteins in spermatocytes or their propensity to be modified by the ubiquitin system may explain how their access to DNA can be limited.

Genome-Wide DNA Methylation Dynamics in Prenatal Germ Cells

Studies done over the course of the last decade have investigated the methylation status of very few sequences in the male germ line, mainly focusing on imprinted genes, repeat elements and testis-specific genes (reviewed in La Salle and Trasler, 2006b). Staining of gonocytes using an antibody directed against 5-methylcytosine revealed a hypermethylation event taking place around E16, indicating the timing of increase in global methylation of the genome in the male germ line (Coffigny *et al.*, 1999). However, a clear picture of genome-wide methylation dynamics in male germ cells is still lacking. Development of the qAMP assay has given us the power to analyze a vast number of sequences in very little time (Oakes *et al.*, 2006a). Establishing the timing of genome-wide methylation events taking place during the initial phase of methylation acquisition in the male could be done by combining the malleability of the qAMP assay to the convenience of flow sorting GFP-expressing gonocytes. In addition to chromosomes 4 and X, primers to chromosomes 7, 10 and 17 have already been

designed; designing primers to other chromosomes, or to germ cell-specific loci identified via RLGS analysis of sperm (Oakes *et al.*, 2006b) would provide a comprehensive overview of the genome. Using this approach, notion of genome-wide methylation dynamics in highly pure populations of developing gonocytes could be achieved. Conversely, tiling arrays could be employed to determine genome-wide DNA methylation patterns in male germ cells. Recent advances in increasing the sensitivity of these arrays make them an interesting alternative as they will become less costly to operate with increased usage of this technology.

Once an understanding of DNA methylation dynamics has been gained, changes in chromatin structures accompanying these modifications in DNA methylation content could be examined using chromatin immunopurification assay (ChIP). ChIP involves immunoprecipitating the proteins bound to DNA using antibodies directed against modified histones for example, and analyzing the nature of the DNA pulled down with the antibody using tiling arrays or PCR. In the present case, primers already designed for qAMP could be used to identify the sequences. ChIP analyses in the germ line have been limited by the number of cells required for such experiments. O'Neill *et al.* (2006) have recently carried out ChIP with as little as 100 cells using a modified protocol that includes carrier chromatin. Use of ChIP could be extended to study epigenetic modifications taking place during spermatogenesis per se with the development of GFP expressing-transgenic mouse models that would allow for isolation of cell types other than type A spermatogonia, as is the case with the mouse model used in the studies described in Chapter IV.

In parallel to understanding epigenetic changes taking place during male germ cells development, expression of other epigenetic players could be analyzed in order to get a better mechanistic view of these events. Assessing the developmental expression of methyl-CpG-binding proteins, histone modification enzymes and Polycomb group proteins using the qRT-PCR approach described in Chapters III and IV may allow identifying candidates involved in epigenetic reprogramming of the male genome. The role of these factors could then be

followed up using strategies similar to the ones described to assess the roles of DNA methyltransferases in the germ line.

Assessment of the Role of DNA Methyltransferases in Male Germ Cells

The data presented in Chapter III imply differential roles for the transcript variants Dnmt3a and Dnmt3a2. Transcript-specific inactivation of each transcript in germ cells would allow determination of their individual roles in male germ cell development. The structural organization of *Dnmt3a* makes it possible to individually inactivate the transcripts (Chen *et al.*, 2002). The role of DNMT1o, the oocyte-specific form of *Dnmt1*, has previously been elucidated using a similar strategy, as the exon responsible for giving rise to DNMT1o could be specifically targeted for inactivation (Howell *et al.*, 2001). By using germ cell-specific promoters to drive expression of Cre-recombinase, transcripts could be inactivated individually or in combination in given germ cell types. Based on the expression data obtained, two different sets of experiments should be conducted. First, inactivation of Dnmt3a and Dnmt3a2, individually and in combination, in prenatal gonocytes would shed light on their respective role in methylation pattern acquisition. Secondly, inactivation of Dnmt3a specifically in type B spermatogonia would determine the biological importance of this protein to these cells.

A number of germ cell-specific gene-targeting studies can be conducted to study the mitotic, meiotic and post-meiotic roles of each DNMT in the male germ line. Additionally, a number of strategies can be employed to inactivate or modulate the expression of these genes. Recently, Rao *et al.* (2006) have successfully employed RNA interference (RNAi) to down-regulate expression of Wilm's tumor 1 (WT1) in Sertoli cells and demonstrated the feasibility of creating tissue-specific RNAi transgenic mouse models. Unfortunately, regardless of the strategy employed, lack of germ cell type -specific promoters prevents the realization of most of these studies for the time being.

Timing and Nature of the Defect in Dnmt3L-Deficient Males

The work presented in Chapter IV underlines the prominent role of DNMT3L in methylation pattern acquisition in the male by establishing that the methylation status of numerous sequences is affected in 6 dpp *Dnmt3L*$^{-/-}$ primitive type A spermatogonia. However, a number of questions remain unanswered. *First, when does the methylation defect initially arise?* To answer this question, *Dnmt3L* mutant germ cells at different stages of development, starting with early gonocytes (E13.5) and ending with perinatal gonocytes (4 dpp), could be isolated as described in Chapter IV, and a similar qAMP strategy could be applied to study methylation differences between genotypes. This detailed developmental analysis would most likely identify the period during which the methylation defect initially occurs and would also provide information on the prenatal role of DNMT3L. In addition to methylation studies, a developmental expression analysis of other *Dnmt* genes should also be conducted to determine if their expression is affected at a different developmental time. Compensation mechanisms may be triggered early on to overcome the loss of DNMT3L in prenatal gonocytes but may be down-regulated postnatally when Dnmt3L levels are normally much lower, explaining why no expression differences were detected at 6 dpp. In this set of experiments, the technical challenge would lie in obtaining sufficient numbers of embryos to obtain adequate numbers of cells of each genotype.

With *Dnmt3L* being highly expressed in gonocytes, but still detected during spermatogenesis, another question surfaces: *is postnatal expression of Dnmt3L biologically relevant?* According to the expression profile obtained, inactivation of *Dnmt3L* at different times during spermatogenesis would be necessary to delineate the role of this gene during postnatal male germ cell development. Mice expressing *Cre-recombinase* under the control of mitotic-, meiotic- and post-meiotic –specific promoters could be used to specifically knockout *Dnmt3L* in type A spermatogonia, leptotene/ zygotene spermatocytes and round spermatids. For example, a post-meiotic -specific Cre-recombinase transgenic mouse model is available; the *protamine 1* promoter is utilized to drive expression in spermatids

(O'Gorman *et al.*, 1997). As more Cre models are developed, these experiments will be possible.

Another way to sort out this question is to attempt to rescue the defect observed in *Dnmt3L*$^{-/-}$ males. In the past, a rescue strategy has successfully been used to restore fertility in hormone-sensitive lipase (*HSL*)-deficient males by breeding the mutant mice with transgenic mice over-expressing human *HSL* (Wang *et al.*, 2004). Creating a transgenic mouse model over-expressing the human *DNMT3L* gene in spermatogonia and breeding these mice with *Dnmt3L*-deficient mice may be sufficient to rescue fertility by restoring methylation patterns in spermatogonia and allowing male germ cells to progress through spermatogenesis. Such a result would infer that postnatal expression of Dnmt3L is relevant to the integrity of the male genome.

5.3 Concluding Remarks

It has become increasingly apparent that the birth of a healthy child is both related to the genetic and the epigenetic contributions of the parents. Incidence of genomic imprinting diseases associated with alterations in DNA methylation amongst children born of ARTs has become a growing concern. Mounting interest in the use of demethylating agents as therapeutic tools has further underlined the need to understand the basic molecular mechanisms behind DNA methylation pattern establishment in germ cells in order to prevent adverse consequences to progeny outcome. The fundamental aim of the work presented in this thesis was to gain a better understanding of the events leading to the creation of the epigenetic program necessary to the production of male gametes capable of supporting development. The results presented in this thesis set the stage for the in-depth gene-targeting experiments required to thoroughly appreciate the role each DNA methyltransferase plays in the elaboration of this complex program.

Original Contributions

1. DNMT1 is absent at the time of initial methylation pattern acquisition in the male germ line, suggesting other DNMTs are responsible.

2. DNMT1 expression is down-regulated as meiosis progresses in the female, but a mechanism different than the one used during male meiosis seems to be involved. The nature of this mechanism remains unidentified.

3. Expression of *Dnmt3a*, *Dnmt3b*, *Dnmt3L* and *Dnmt1* is highly dynamic in developing male and female gonads and corresponds to the windows of sex-specific methylation marking in both germ lines. Also, *Dnmt3L* is expressed in a sexually dimorphic manner in the testis and the ovary.

4. Expression of the *de novo* DNA methyltransferases *Dnmt3a* and *Dnmt3b* is tightly regulated in developing male germ cells. There are two developmental windows during which expression of these genes is down-regulated: 1) differentiation of spermatogonia into spermatocytes and 2) pachynema.

5. The two major transcript variants and protein isoforms of *Dnmt3a* are differentially expressed during spermatogenesis. While DNMT3a2 expression is highly dynamic throughout male germ cell development, expression of DNMT3a is predominant in type B spermatogonia.

6. A number of transcript variants produced by alternative splicing of *Dnmt3b* are detected in male germ cells, but two protein isoforms prevail: DNMT3b2 and DNMT3b3.

7. *Dnmt3L* is highly expressed in prenatal gonocytes but is also expressed during spermatogenesis in a manner reminiscent of *Dnmt3a*, *Dnmt3b* and *Dnmt1*.

8. DNMT3L deficiency results in loss of germ cells soon after birth and delays entry into meiosis, suggesting a yet unreported mitotic defect in germ cells lacking *Dnmt3L*.

9. Expression of the other *Dnmt* genes is not affected in DNMT3L-depleted primitive type A spermatogonia, contrary to what is observed in the female, indicating that different regulatory mechanisms control the expression of *Dnmt* genes between the two germ lines.

10. DNMT3L also targets non-CpG island, non-repetitive sequences for DNA methylation and is required for the proper methylation of multiple sites throughout the genome, signifying that DNMT3L plays a much larger role than has been previously reported in the genome-wide DNA methylation events that take place during male germ cell development.

CHAPTER VI

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Volume: 296
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A quick response regarding this matter would be greatly appreciated.

Thank you for your time,

Sophie La Salle

****

Sophie La Salle

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