MECHANISMS OF TRANSLATIONAL REPRESSION OF THE SPERM MITOCHONDRIA-ASSOCIATED CYSTEINE RICH PROTEIN (*Smcp*) mRNA IN ROUND SPERMATIDS

A Dissertation Presented

By

DANIELLE L. CULLINANE

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ABSTRACT

MECHANISMS OF DEVELOPMENTAL TRANSLATIONAL REGULATION AND LOCALIZATION OF THE SPERM MITOCHONDRIA-ASSOCIATED CYSTEINE-RICH PROTEIN (*Smcp*) mRNA

December 2014

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Directed by Professor Kenneth Kleene

The sperm-mitochondria-associated cysteine-rich protein (SMCP) is a male germ cell-specific protein that localizes to the outer membranes of sperm mitochondria and increases sperm motility. The *Smcp* mRNA is transcribed in early spermatids, and stored in a translationally repressed state for ~7 days before translation is activated in late spermatids. Identifying the *cis*-elements and *trans*-factors that repress the *Smcp* mRNA in early spermatids is important because these factors and elements coordinate the translational activity of hundreds of mRNAs.

A mutation was studied in transgenic mice in which the 16 nucleotides downstream of the first poly(A) signal in the *Smcp* 3'UTR were replaced with the 17 nucleotides downstream of the poly(A) signal from the pEGFP plasmid 3'UTR.

Replacing this sequence of the *Smcp* 3'UTR eliminates two elements that are conserved in many mammalian *Smcp* mRNAs. My research using the GFP reporter and analysis of polysomal loading demonstrates that the mutation eliminates repression of a *Smcp-Gfp* transgenic mRNA in early spermatids.

Studies in our lab demonstrate that Y-box protein 2 (YBX2) binds the 3' termini of the protamine 1 (*Prm1*) and *Smcp* 3'UTRs, which have been demonstrated with mutations in transgenic mice to be necessary for repression in early spermatids. My research demonstrates that depletion of YBX2 in *Ybx2*-null mice eliminates the translational repression of the *Smcp* and *Prm1* mRNAs in early spermatids.

The localization of the *Smcp* mRNA in spermatids was studied using RNA-fluorescent *in situ* hybridization (RNA-FISH). The *Smcp* mRNA probe detected a signal in a germ cell-specific granule called the chromatoid body. It has been speculated that the chromatoid body stores repressed mRNAs in early spermatids. My RNA-FISH studies reveal that translationally repressed and translationally active mRNAs are concentrated in the chromatoid body implying that localization is independent of translational activity. A probe for the *Smcp* intron also localized to the chromatoid body suggesting that the *Smcp* pre-mRNA may be spliced in the chromatoid body. This is the first demonstration with RNA-FISH that translationally active mRNAs and introns localize to the chromatoid body. This research has permitted the formulation of a speculative model of translational repression of the *Smcp* mRNA.

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

LIST OF TABLES

CHAPTER 1

MECHANISMS OF TRANSLATIONAL CONTROL IN SPERMATOGENESIS

1.1 Introduction

Spermatogenesis is the process by which immature male germ cells undergo proliferation, differentiation and meiosis giving rise to haploid, male gametes, spermatozoa. This process takes place within the seminiferous tubules in the testis, and occurs in close association with Sertoli cells, the somatic cells of the seminiferous epithelium (Russell et al., 1990).

1.2 Spermatogenesis

Spermatogenesis is divided into three major phases: mitotic, meiotic, and the haploid differentiation phase, known as spermiogenesis (Russell et al., 1990; Cheng et al., 2009). The cells in each of these phases, respectively, have a different name, spermatogonia, spermatocytes and spermatids, and each of these cell types has a different amount of nuclear DNA and chromosome complement. The patterns of gene expression in spermatogenic cells are closely correlated with developmental changes in these different cell types. Thus, it is important to define the terminology that describes these developmental stages. In general, the stages of spermatogenic cells are recognized by the

position of the cells in the seminiferous tubules, and cellular size and morphology (Russell et al., 1990).

In mice, spermatogenesis is divided into 12 stages designated by Roman numerals which are based on morphology of spermatids and cell associations in the seminiferous tubules, and spermiogenesis is divided into 16 steps designated by Arabic numerals. The 12 stages of the seminiferous epithelium and 16 steps of spermiogenesis in the mouse are illustrated in (Figure 1.1).

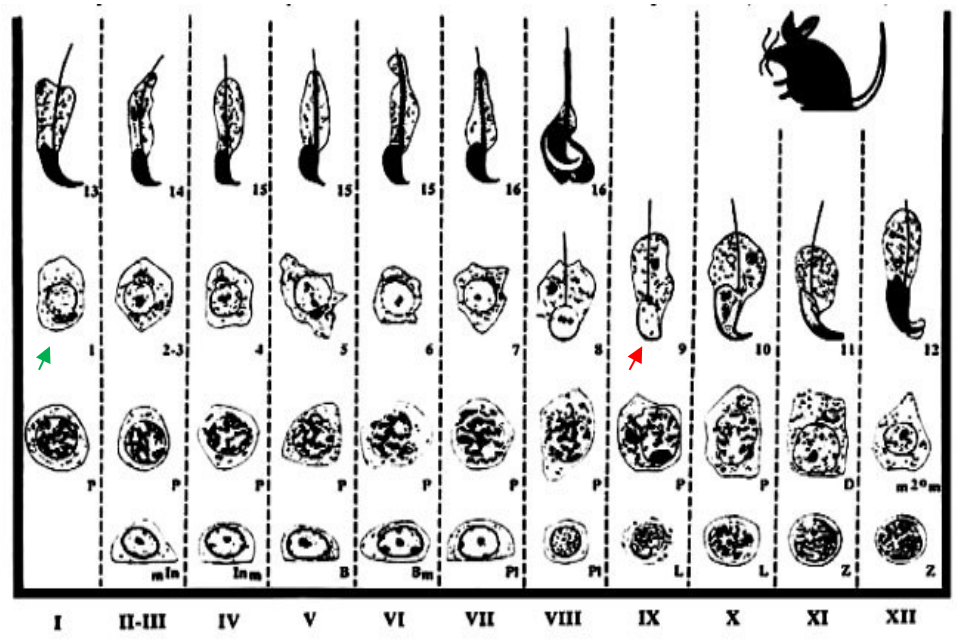

Fig. 1. Diagram of the 12 stages for the production of spermatozoa in the mouse seminiferous epithelium (Russell *et al*., 1990).

Figure 1.1 Diagram of the 12 stages for the production of spermatozoa in the mouse seminiferous epithelium. Columns are designated by Roman numerals and depict cell stages of mouse spermatogenesis. Developemetal progression of a spermatogenic cell is labeled horizontally from 1-16. The cycle ends with the completion of spermiation. Green arrow indicates when cells are haploid. Red arrow indicates when the nuclues starts to elongate. Adapted from (Russell et al., 1990).

The earliest proliferating cells, spermatogonia, are small, unspecialized cells located at the periphery of the tubules adjacent to the nuclei of the Sertoli cells. After a series of mitotic divisions, a subset of cells withdraw from the cell cycle and enter the pathway leading to meiosis, while the remaining cells continue to proliferate as stem cells. The cells that will pass through meiosis are located closer to the lumen. These meiotic cells, spermatocytes, have replicated their DNA, but have not divided; hence they are functionally tetraploid and genetically diploid. Spermatocytes progressively increase in size accompanied by changes in chromosome morphology corresponding to the stages of meiosis, leptotene, zygotene, pachytene and diplotene. Haploid spermatids are located close to the lumen and undergo striking changes in the morphology of all cellular organelles (Russell et al., 1990).

At the end of spermiogenesis, immature spermatozoa are released into the lumen of the seminiferous tubule, a process known as spermiation. Following spermiation, immature spermatozoa are exported through the *vas deferens* to the *epididymis*, where they undergo a series of maturational changes before becoming mature spermatozoa that are capable of fertilization (Russell et al., 1990). The positions of Sertoli cells, spermatogonia, spermatocytes and spermatids in seminiferous tubules are illustrated in (Figure 1.2).

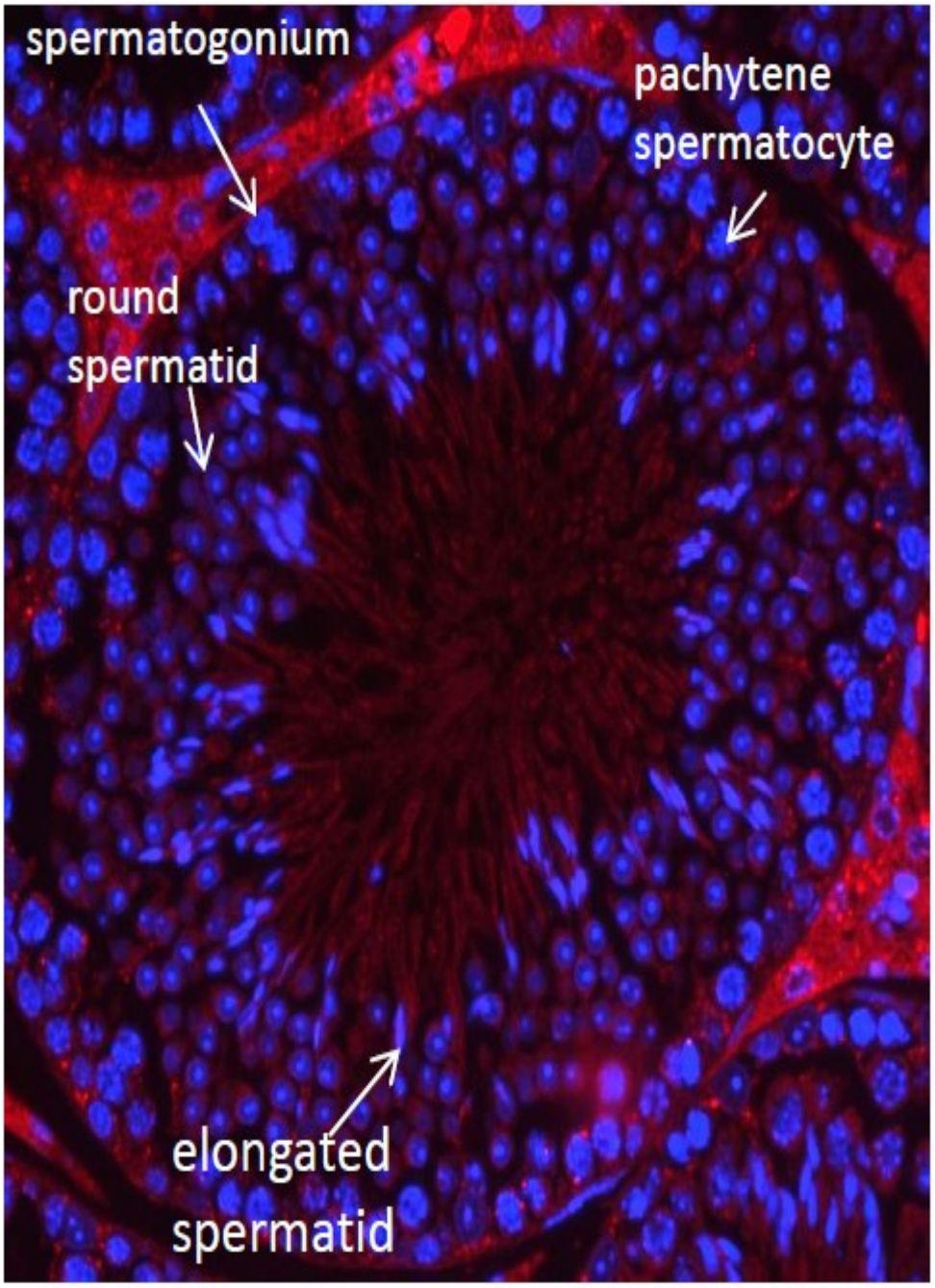

Figure 1.2 Cross-section of a seminiferous tubule. This picture displays the germ cells at different stages of maturation developing embedded in somatic Sertoli cells. Maturing sperm are shown in the lumen of the tubules. The stem cells and the pre-meiotic cells

(spermatogonia) are found at the base of the tubules, whereas the meiotic (spermatocytes) and the post-meiotic (round and elongating spermatids) cells are found organized in order of maturation towards the lumen.

Haploid round spermatids undergo a differentiation process, spermiogenesis, which transforms them into spermatozoa (O'Donnell et al., 2001; Martianov et al., 2005). This developmental process, diagrammed in (Figure 1.1), involves the formation and morphological changes of the acrosome, the sperm tail, chromatin remodeling and condensation, reshaping and elongation of the nucleus, and elimination of the cytoplasm. The acrosome is a sperm-specific secretory vesicle, which is located at the anterior tip of the sperm nucleus and contains hydrolytic enzymes that enable spermatozoa to penetrate the outer membranes of the egg during fertilization. The changes in the size and morphology of the acrosome are important in identifying stages of early spermatids (Figure 1.2). The differentiation of the sperm nucleus and tail are particularly important in my research and are described in some detail below.

The morphology of the nucleus is used to subdivide the 16 steps of spermatids into three types of cells known as round, elongating and elongated spermatids. Round spermatids, steps 1-8, have round transcriptionally active round nuclei, which later elongate in steps, 9-11, cells known as elongating spermatids. After completion of nuclear elongation, the structure of chromatin in the nucleus of the elongated spermatid, steps 12-16, has changed dramatically and is incapable of transcription (Meistrich et al., 2003). These changes involve histone hyperacetylation followed by replacement of the histones by transition proteins and protamines, which packages DNA into a condensed

spermatid nucleus (Meistrich et al., 2003; Martianov et al., 2005). Transition proteins, TNP1 and TNP2, replace histones and subsequently the transition proteins are replaced by protamines, PRM1 and PRM2 (Meistrich et al., 2003). The replacement of histones by transition proteins and protamines is accompanied by changes in the structure of chromatin from a nucleosomal supercoiled form to smooth non-supercoiled nucleoprotamine fibrils (Kierszenbaum et al., 1975; Meyer-Ficca et al., 2005). These changes in the structure of chromatin during elongating spermatids result in a drastic reduction in RNA synthesis during steps 9-11, and the total absence of detectable transcription in steps 12-16 of spermiogenesis (Kierszenbaum et al., 1975). To compensate for the absence of transcription in elongating and elongated spermatids, round spermatids transcribe high levels of mRNA that are subject to translational delay. These mRNAs remain translationally suppressed for several days to a week until translation is activated at the appropriate step in elongating or elongated spermatids (Chowdhury et al., 2012).

The differentiation of the sperm tail is a particularly complicated process involving elongation of the flagellum and the formation of three accessory structures, which are found in no other cell type in the mammalian body: the outer dense fibers, the fibrous sheath and the mitochondrial sheath. The accessory structures of the sperm tail in human are illustrated in (Figure 1.3).

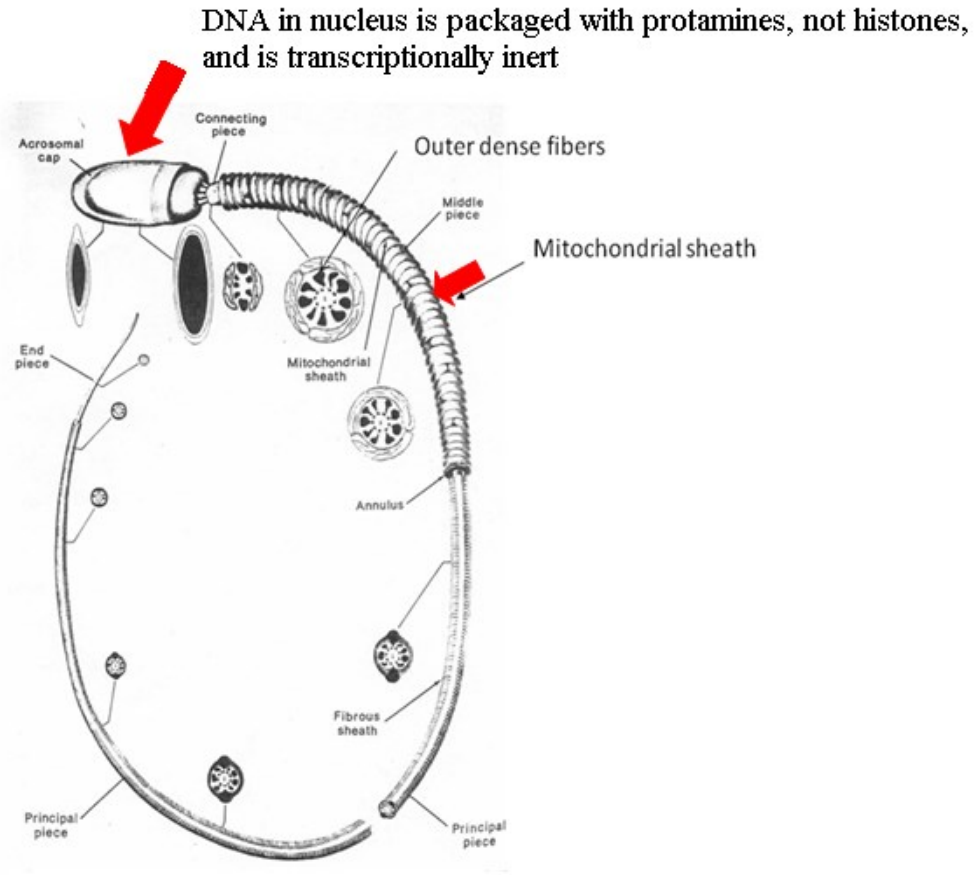

Figure 1.3 Depiction of a sperm cell. The male gamete has a head containing the nucleus and acrosome, a middle piece with the mitochondria, and a tail with the microtubule pattern. The nucleus consists of condensed chromatin and histone proteins. The acrosome contains hydrolytic enzymes capable of lysing the egg coats at fertilization. Actin molecules which aid in the interaction between sperm and egg are found in the area between the acrosome and nucleus. The mitochondria in the middle piece provide the energy necessary for the motility created by the tail. The tail has a central core, or axial filament, made up of nine double microtubules and two central tubules. Adapted from (Fawcett et al., 1975).

The outer dense fibers are electron dense rods associated with each tubulin dimer in the flagellar axoneme along the complete length of the sperm tail (Russell et al., 1990). The outer dense fibers are thought to increase the efficiency of flagellar beating in the viscous fluids of the female reproductive tract. The remainder of the sperm tail is divided

into two additional segments by the mitochondrial sheath, which surrounds the outer dense fibers proximal to the nucleus, and the fibrous sheath, which surrounds the outer dense fibers distal to the mitochondrial sheath. The fibrous sheath functions as a scaffold by binding glycolytic enzymes, which supply ATP for sperm motility. As well as signal transduction proteins, which function in the activation of sperm motility in fertilization (Eddy et al., 2010).

Mitochondria in spermatogenic cells undergo dramatic changes in number, size, distribution, and internal structure. In late pachytene spermatocytes and early spermatids, small round mitochondria with condensed matrices and a few dilated cristae are dispersed throughout the cytoplasm (Aihara et al., 2009). In late spermiogenesis beginning in steps 15, mitochondria migrate to the base of the tail and form a tightly packed spiral surrounding the outer dense fibers and flagellar axoneme in the sperm midpiece known as the mitochondrial sheath. These morphological modifications are accompanied by changes in the energy metabolism and protein components of mitochondria (Aihara et al., 2009). Since the vast majority of ATP for sperm motility is supplied by glycolysis instead of oxidative phosphorylation, the primary function of the sperm mitochondria is not energy-production (Aihara et al., 2009). Conceivably, the mitochondrial sheath functions in structural support for the base of the tail to increase motility.

The outer membrane of the mitochondria in the sperm mitochondrial sheath is toughened by the formation of a keratinous capsule (Ursini et al., 1999). The sperm mitochondrial capsule can be purified by sonification of sperm to release the mitochondria, sucrose gradient sedimentation to purify the mitochondria, and treatment with SDS. The resulting preparations contain a structure, known as the mitochondrial

capsule, which retains the size and shape of the outer surface of the sperm mitochondria (Urisini et al., 1999).

The mitochondrial capsule contains two major proteins that are covalently crosslinked in a stable structure. The first is known as the sperm-mitochondria-associated cysteine-rich protein (SMCP) localizes to the sperm mitochondrial capsule (Mairoino et al., 2005; Hawthorne et al., 2006). The knockout of the *Smcp* gene produces background dependent decreases in sperm motility and male fertility, probably by stabilizing the mitochondrial sheath (Hawthorne et al., 2006; Nayernia et al., 2006). It is known that the mRNA encoding SMCP undergoes delayed translational activation and this is a major focus of my thesis research (Bagarova et al., 2010; Hawthorne et al., 2006; Kleene et al., 1989).

1.3 Mechanisms of Translational Control in Eukaryotic Cells

The final step in gene expression is the translation of mRNA into protein. This process can be divided into three phases, initiation, elongation, and termination (Jackson et al., 2010). The rate of mRNA translation is regulated in all three phases, but initiation is by far the most common phase where translation is regulated. Therefore, the focus of the discussion below will be on the initiation phase. The initiation of translation in eukaryotic cells occurs by three mechanisms: cap-dependent scanning, ribosome shunt, and internal ribosome entry (Jackson et al., 2010). The cap-dependent scanning model will be described below because it is the mechanism by which the vast majority of

mRNA species, probably more than 97%, in eukaryotic cells initiate translation (Jackson et al., 2010).

Translation initiation is the process of assembly of translation-competent 80S ribosomes in which the anticodon of the Met-tRNAi in the ribosomal P-site of the 60S ribosomal subunit is base-paired with the initiation codon (Jackson et al., 2010). In general, the cap-dependent pathway requires the assembly of two complexes which are assembled independently. The first is eIF4F bound to the 5' cap of the mRNA, and the second is the 43S preinitiation complex containing the 40S ribosomal subunit, the ternary complex and initiation factors eIF3, eIF1, and eIF1a. The process by which each complex is assembled is described below and is depicted schematically in (Figure 1.4).

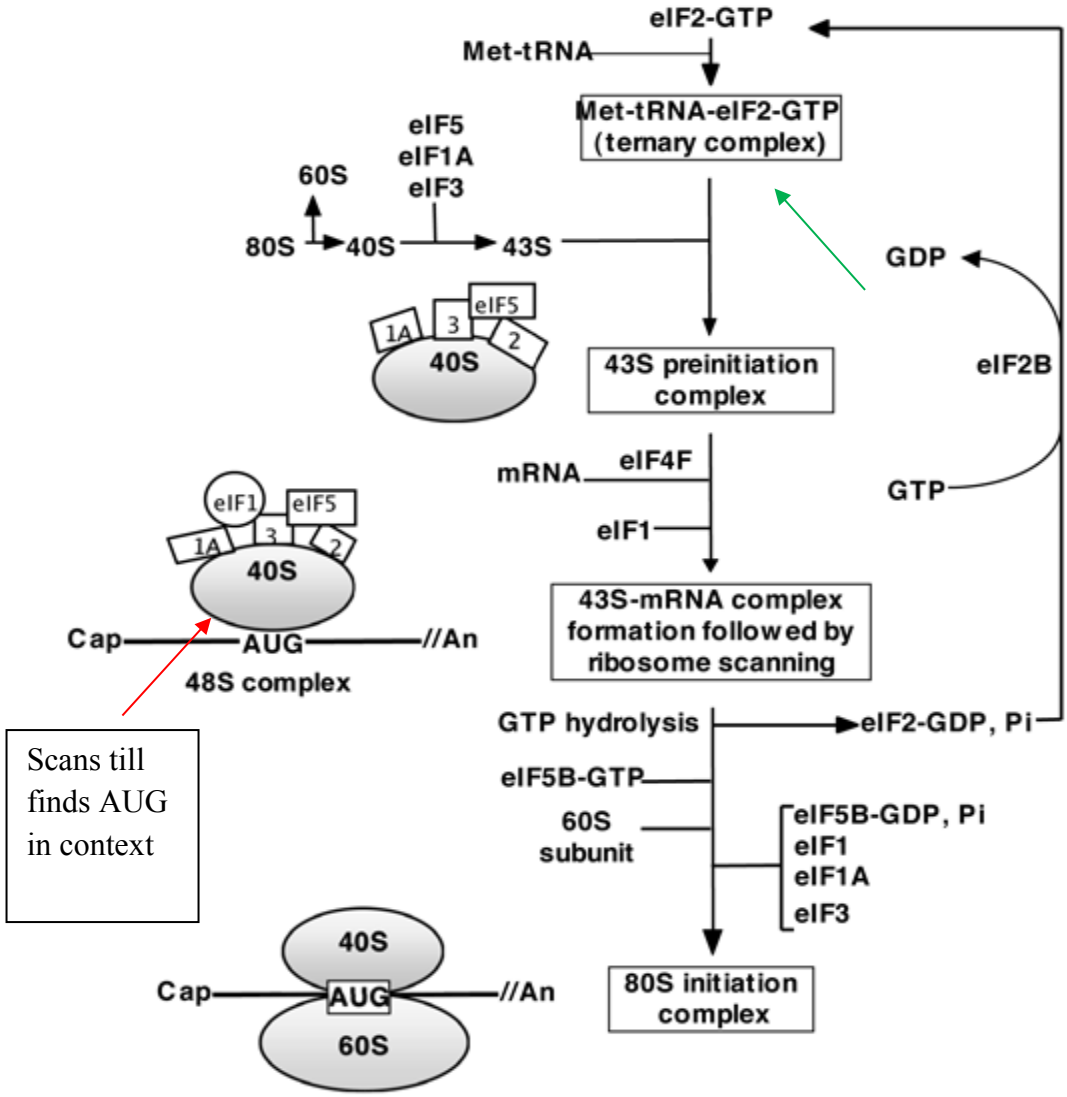

Figure 1.4 Schematic diagram of the cap-dependent scanning model for translational initiation. The vast majority of mRNAs initiate translation in five steps. (1) Translation initiation factor eIF4F (eIF4E, eIF4G and eIF4A) binds the m7guanosine 5' cap rendering the mRNA competent to initiation translation. (2) A recycled 40S ribosomal subunit becomes competent to initiation translation by binding eiF5, eIF1A, eIF3 and the ternary complex (Met-tRNAi-eIF2-GTP) forming the 43S preinitiation complex. (3) The activated mRNA binds the 43S preinitiation complex forming the 48S preinitiation complex. (4) The 40S ribosomal subunit with associated initiation factors scans in the 5'-3' direction until an AUG in a strong context is found. (5) The 48S initiation complex then recruits the 60S ribosomal subunit which together forms the 80S initiation complex. Protein synthesis may now proceed during

elongation. Red arrow indicates scanning complex and green arrow indicates ternary complex. Adapted from (Sonenberg et al., 2009).

One pathway assembles 43S preinitiation complexes which must occur before the 40S ribosomal unit can bind the 5'cap. Translation is a cyclical process; therefore ribosomal subunits that participate in initiation are derived from the recycling of post-termination ribosomal complexes composed an 80S ribosome bound to mRNA (Jackson et al., 2010). The post-termination complexes dissociate into the 40S and 60S ribosomal subunits. Once disassociated, initiation factors eIF3, eIF1, and eIF1a are recruited to the 40S ribosomal subunit which enables the ternary complex, composed of eIF2–GTP–Met-tRNAi, to bind the recycled 40S subunit. The entire complex containing the 40S ribosomal subunit, eIF3, eIF1 and eIF1A and the ternary complex, is referred to as the 43S preinitiation complex (Jackson et al., 2010). Each of the six factors in the preinitiation complex has a specific function. The Met-tRNAi is a specialized tRNA that binds eIF2 and functions in the initiation of translation. eIF1A and eIF1 are required for binding to the mRNA and migration of the 43S preinitiation complex in a 5' to 3' direction along the 5'UTR towards the initiation codon (Aitken et al., 2012). eIF1A enhances eIF4F-mediated binding of the 43S complexes to mRNA, while eIF1 promotes formation of the 48S preinitiation complex in which the initiator codon is base paired to the anticodon of the initiator Met-tRNAi (Aitken et al., 2012).

The second pathway prepares the mRNA for the initiation of translation by binding translation initiation factor eIF4F which is composed of the cap binding protein, eIF4E, the DEAD-box RNA helicase, eIF4A, and the scaffold protein, eIF4G. The 5'

termini of natural mRNAs often possess sufficient secondary structure formed by base-pairing interactions within the mRNA to inhibit the initiation of translation. Consequently, it is necessary for regions of double stranded RNA to be unwound before eIF4F can bind to the 5' cap. Unwinding the secondary structure proximal to the 5' cap is carried out by the cooperative actions eIF4A, an RNA helicase, and eIF4B or eIF4H, making it accessible to the 43S preinitiation complex (Jackson et al., 2010). It is thought that the binding of eIF4F to a 5'cap is an important regulatory event because that it commits that mRNA to translation into protein.

After the 43S preinitiation complex and the complex of mRNA and eIF4F have formed independently, the 43S preinitiation complex is recruited to the ^{7}methylguanosine cap at the 5' end of the mRNA forming the 48S preinitiation complex. eIF4G functions as a scaffold in assembling the 48S preinitiation complex by binding eIF4A and eIF4E which are bound to the 5' cap, and eIF3, which is bound to the 40S ribosomal subunit. The 43S preinitiation complex then scans the 5'UTR in a 5' to 3' direction for an AUG codon in a strong context for the initiation of translation. The scanning of the 43S preinitiation complex is also inhibited by 5'UTR secondary structure which forms naturally by base pairing within mRNAs. Scanning the 5'UTR is facilitated by the DEAD-box RNA helicase, eIF4A and the DEAH-box helicase DHX29, with the assistance of eIF2, eIF3, eIF1A, eIF1, eIF4B and eIF4H. The scanning phase ends when the Met-tRNAi anticodon recognizes an AUG codon in a strong context, usually the AUG codon closest to the 5' cap and bearing a purine in the -3 or a G in the +4 positions (the A of the AUG codon is defined as +1). Next, the 60S ribosomal subunit joins the 48S preinitiation complex forming an 80S initiation complex with an 80S ribosome

positioned at the initiator AUG codon in the mRNA and a Met-tRNAi in the ribosomal P-site. Identification of the AUG codon and subunit joining are mediated by eIF1, eIF1A, eIF5 and eIF5B. The first step in ribosomal subunit joining is hydrolysis of eIF2-bound GTP and release of eIF2-GDP from 48S complexes (Santiago et al., 2005). eIF5 causes hydrolysis of eIF2-bound GTP, resulting in release of the eIF2-GDP. In the absence of eIF1, eIF5 induces rapid hydrolysis of eIF2-bound GTP in 43S complexes. However, the presence of eIF1 in the 43S preinitiation complex inhibits eIF5-induced GTP hydrolysis (Aitken et al., 2012). Thus, eIF1 plays the role of a negative regulator that inhibits premature GTP hydrolysis and links codon-anticodon base pairing with hydrolysis of eIF2-bound GTP (Aitken et al., 2012). The formation of the 80S initiation completes the initiation of translation, the initiation factors and ternary complex are released from the 80S initiation complex, and the translation elongation phase begins.

It is important to note that eIF4G also binds the cytoplasmic poly(A) binding protein (PABP), which binds the poly(A) tail at the 3' end of the 3'UTR, and eIF4E which binds the 5' cap at the 5' end of the mRNA. These interactions cause circularization of the mRNA, which is referred to as the Closed Loop Model (Refer to Figure 1.5). This is critical because the majority of factors that regulate mRNA translation do so by binding to *cis*-elements in the 3'UTR (Jackson et al., 2010). The closed loop model provides a theoretical frame-work for understanding how elements at the 3' end of the mRNA regulate the initiation of translation at the 5' end of the mRNA. The closed loop is a stable structure which maximizes translational initiation and mRNA stability. The closed loop model is also versatile because the 3' UTR can promote, positive control, or inhibits, negative control, mRNA translation. It is thought that

translational repression happens in two ways. One is binding of a protein or small non-coding RNA (sncRNA) to an element in the 3'UTR, and the other is repression of translation by inhibiting the initiation of translation at the 5' end of the mRNA (Kleene et al., 2013). This is where the closed-loop model becomes important to my research because translational repression by the 3'UTR is normally mediated by blocking the formation of an active closed loop (Jackson et al., 2010). Hence, the closed loop is relevant to my work because the *Smcp* mRNA is translationally repressed in early spermatids by the 3'UTR (Bagarova et al., 2010; Hawthorne et al., 2006).

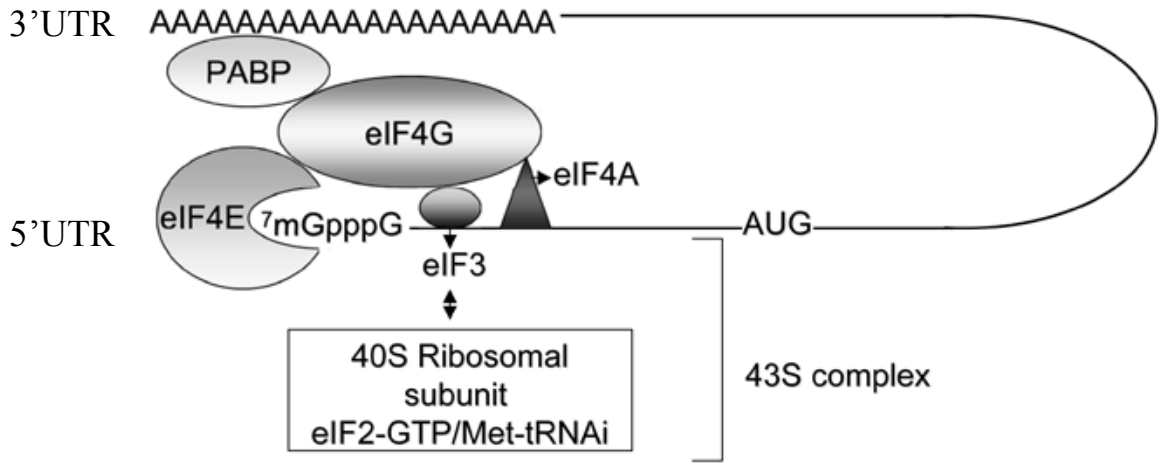

Figure 1.5 Schematic representation of the closed-loop model of translation initiation. In this model, the m^7G 5' cap, eIF4E, eIF4G and PABP and the poly(A) tail form a circular complex in which the 5' and 3' ends of the mRNA physically interact. eIF4G also recruits the 43S preinitiation complex via interaction with eIF3. For simplicity, other proteins have been omitted Adapted from (Lopez-Lastra et al., 2005).

The regulation of mRNA translation is used to modulate gene expression in a variety of biological situations. Translational regulation occurs during early embryonic development, cell differentiation and metabolic changes associated with changes in cell physiology such as changes in rate of cell growth, virus infection or stress (Mathews et

al., 2007). This process is used to fine-tune the rate of protein synthesis in time and space, and is used in many situations in which changes in protein levels mediated by transcription are too slow or impossible (Morris et al., 2004). Late spermatids are a well-known example of a cell-type in which transcription cannot be used to synthesize new mRNAs, because chromatin remodeling totally inactivates transcription (Kierszenbaum et al., 1975; Kleene et al., 1996; 2003).

Translational control can be divided into two broad categories, global and mRNA specific (Mathews et al., 2007). Global regulation affects all messages and usually involves modifications in the levels or phosphorylation of general initiation factors, while mRNA-specific translational regulation increases or decreases the rate of translation of specific mRNAs. However, these categories are not completely distinct because most global mechanisms do not affect all mRNAs and some mRNA-specific mechanisms affect thousands of mRNAs (Sonenberg et al., 2009; Morris et al., 2010). In addition, a class of RNA binding proteins known as Y-box proteins is thought to be capable of global repression of mRNA translation in vertebrate oocytes and early embryos (Matsumoto et al., 1998; Skabkin et al., 2006; Eliseeva et al., 2011).

Sucrose gradient analysis is a commonly used technique to examine the rate of mRNA translation. In this procedure, cytoplasmic extracts are sedimented on sucrose gradients, a procedure that separates particles by differences in size. The gradients are collected as fractions, the RNAs are extracted from each fraction, and the levels of specific mRNAs in each fraction are determined by Northern blots or quantitative real time reverse transcriptase PCR, RT-qPCR (Kleene et al., 2010). mRNAs that sediment slower than 80s single ribosomes are referred to as free-messenger RNA

ribonucleoprotein particles, free-mRNPs, which are translationally inactive because the mRNAs are not associated with ribosomes. The mRNAs sedimenting faster than 80s ribosomes are thought to be actively translated by polysomes (Kleene et al., 2010). The percentage of mRNAs that are associated with polysomes is often referred to as polysomal loading. Translationally repressed mRNAs usually exhibit high levels of free-mRNPs, implying that the translation is repressed by blocking the interaction of mRNA and the 43S preinitiation complex (Jackson et al., 2010; Groppo et al., 2009).

1.4 Translational Control in Mammalian Spermatogenesis

Spermatogenesis is a striking and well known system for regulation of mRNA translation. Translational control is known to be required because transcription ceases due to chromatin remodeling about midway through the 13-day long haploid phase in which spermatids differentiate into sperm (Kierszenbaum et al., 1975; Kleene et al., 1996). It has been reported that premature translation of the *Prm1* and *Tnp2* mRNAs in transgenic mice causes abnormal sperm development and male infertility (Lee et al., 1995; Tseden et al., 2007). This experiment demonstrates that translational regulation is used as a mechanism to prevent deleterious effects of expression of proteins at the wrong developmental stage in spermatogenic cells.

Polyadenylation is one mechanism of mRNA-specific translational control in developing male and female germs cells. The poly(A) tail is a stretch of RNA that has only adenine bases at the 3' ends of eukaryotic mRNAs (Lutz et al., 2008). In eukaryotes, polyadenylation is part of the process that produces mature mRNA for

translation. One form of polyadenylation requires two-cis elements in the 3' UTR of responding mRNAs, the U-rich cytoplasmic polyadenylation element (CPE), usually UUUUAU or UUUUUAU, and the hexanucleotide AAUAAA (Richter et al., 2001). The CPE is bound by the RNA recognition motif (RRM) and Cytoplasmic Polyadenylation Element Binding protein (CPEB) (Richter et al., 2001). This mechanism of regulation can activate or repress eukaryotic mRNAs by changing the length of their poly(A) tails in the cytoplasm. The poly(A) tail is important for the nuclear export, translation, and stability of mRNA. The tail is shortened over time, and, when it is short enough, the mRNA is enzymatically degraded. However, in oocytes and early embryos, mRNAs with short poly(A) tails are stored for later activation by re-polyadenylation in the cytoplasm (Richter et al., 2001). However, the CPE activates mRNA translation in early spermatogenic meiotic cells (Tay et al., 2001), and CPEs are absent from mRNAs that are translationally regulated in spermatids (Chowdhury et al., 2012).

Iguchi et al. used microarrays to analyze the proportions of 11,000 mRNA species in free-mRNP and polysome sucrose gradient fractions of adult and prepuberetal testes enriched in pachytene spermatocytes, round spermatids and elongated spermatids. They claim that 752 mRNAs undergo delayed translational activation in spermatids, but the accuracy of this number has been questioned on grounds of technical deficiencies and weak validation (Kleene et al., 2010; 2013). Nevertheless, it seems likely that many new proteins are synthesized in late spermatids to remodel chromatin and to construct the accessory structures of the sperm tail. Currently, about 14-20 mRNAs have been rigorously demonstrated to show developmental lags between the first detection of the mRNA and protein by *in situ* hybridization and immunocytochemistry (Kleene et al.,

1996; Chowdhury et al., 2012). However, only 6 of these mRNAs have been shown to be stored in free-mRNPs in early haploid cells and actively translated on polysomes in late haploid cells with sucrose gradient analysis (Chowdhury et al., 2012). Most mRNAs in mitotically dividing spermatogonia and testicular somatic cells show high polysomal loading, 85-90% (Kleene et al., 1996; 2001). Without exception, the >60 mRNAs in meiotic and haploid spermatogenic cells that have been analyzed with sucrose gradients exhibit lower levels of polysomal loading, 55% maximum and usually 33% or less, with the balance in free-mRNPs (Kleene et al., 1996; Schmidt et al., 1996). These findings indicate that mRNA translation is also globally repressed in meiotic and haploid spermatogenic cells (Kleene et al., 1996).

Mali et al. investigated the expression of mRNAs for a transition protein (*Tnp1*) and protamines (*Prm1* and *Prm2*) during rat and mouse spermiogenesis. Their results showed that the levels of mRNA for all three messages began to increase in step 7 spermatids at stage VII of the seminiferous cycle, and then was repressed from steps 8-9. The mRNA levels of all transcripts remained high during steps 8-13 in both species (Mali et al., 1989). In the mouse, *Tnp1* mRNA disappeared during step 13 (stage I). The *Prm1* mRNA level decreased before *Prm2* in step 14 (stage II), whereas *Prm2* was detected up to step 15 (stage V) (Mali et al., 1989). These results suggest that transcription of *Tnp1, Prm1*, and *Prm2* mRNAs starts at specifically defined times during spermiogenesis and that the temporal translational regulation of these mRNAs is different (Mali et al., 1989). *Prm1, Prm2, Smcp, Tnp1*, and *Tnp2* are known examples of a widespread phenomenon of developmental regulation of mRNA translation in which mRNAs are transcribed in early spermatids, stored as translationally inactive free-mRNPs for several days and then

translated on polysomes. This happens in late spermatids after the cessation of transcription caused by chromatin remodeling, midway through the 13 day haploid phase (Tekur et al., 1998). Certain Y-box proteins are also believed to play an important role in regulation of translation and are discussed in detail later in this section.

1.5 Identification of Cis-Regulatory Elements Through use of Transgenic Mice

The control of mRNA translation in spermatids is mRNA-specific. This is clearly demonstrated by reports that the ACEV2, PRM1, SMCP, PRM2 and ODF1 proteins are first detected respectively steps 9, 10, 11, 13 and 16 (Chowdhury et al., 2012), and different mRNAs are repressed to different extents in round spermatids. For example, the proteins encoded by the *Acr* and *Acrv2* mRNAs are first detected in round spermatids while the corresponding proteins are first detected in elongating or elongated spermatids (Kleene et al., 2013). The differences in rates of protein synthesis in round spermatids are supported by differences in polysomal loading for the small number of mRNAs for which this information is available: about 55% of the *Ldhc* mRNA is associated with polysomes in round spermatids, while at most a few percent of the *Prm1* and *Smcp* mRNAs are associated with polysomes in round spermatids (Kleene et al., 1989; Bagarova et al., 2010; Kleene et al., 2010).

mRNA-specific regulation of translation in spermatids is thought to be mediated by negative mechanisms which repress translation and positive mechanisms which activate translation (Braun et al., 2000; Kleene et al., 2013). In theory, the mRNA-specific repression in round spermatids could be achieved by differences in the extent of

repression by a single negative control factor, the combined effects of negative control factors, or antagonistic effects of negative and positive factors. The subsequent activation of translation of individual mRNA species at discrete stages in elongating and elongated spermatids could be achieved by decreases in the levels of the factors which repress translation in round spermatids, or positive factors that activate translation, or a combination of decreases in translational repression and positive regulation. The literature reviewed below reveals that little is known about these processes.

Factors that have been proposed to be translational repressors in round spermatids include hypophosphorylation of eIF4E and RPSP6, excess PABPC1, high levels of YBX3L/S, YBX2 and microRNAs (Yanagiya et al., 2010; Yang et al., 2005; Miyagi et al., 1995). For example, excess amounts of PAPBC1 bind to eIF4G, which is believed to block formation of the closed loop of mRNAs (Yanagiya et al., 2010). It has been reported that a regulatory mechanism involving PABPC1 and PABP-interacting protein 2a (PAIP2a) (Yanagiya et al., 2010). PAIB2a and PAIP2b are proteins that bind the site on PABPC1 that binds eIF4G. This binding blocks the association of PABPC with eIF4G, which blocks the formation of the closed loop and represses translation. Another example of a global repressor of mRNA translation is excess eIF4E. It is believed that high levels of eIF4E may bind to the site on eIF4G which normally binds eIF4E bound to the 5' cap, providing a second mechanism that potentially inhibits formation of the closed loop (Miyagi et al., 1995).

The *Prm1* mRNA is the best characterized example of mRNA-specific negative control during translation. Experiments performed by Braun et al. have shown that *Prm1*

mRNA contains a translational control element (TCE) in the 3'UTR. When the TCE is deleted or mutated, the *Prm1* mRNA is no longer repressed and expression can be detected as early as step 7 spermatids (Braun et al., 1989; Zhong et al., 2001). However, the factor, RBP or sncRNA that binds the TCE in the *Prm1* 3'UTR was unknown for thirteen years until our lab discovered it to be Y-box protein 2 (Cullinane et al., 2014).

The evidence that these factors have the stated effect on translation is incomplete. Some factors have not been demonstrated to affect the translation of any mRNAs in spermiogenesis, and their postulated effect is inferred from the functions of the same or similar factors in somatic cells. These inferences are complicated by the fact that RBPs affect multiple levels of post-transcriptional gene regulation and have been implicated by the phenotypes of gene knockouts. A general problem with the interpretation of gene knockouts is whether the effect of the knockout on post-transcriptional gene expression is direct or indirect. This is because RNA-binding proteins are expressed for prolonged periods during spermatogenesis, one to two weeks, and RBPs often interact with 10s to 1000s of mRNAs, some of which regulate gene expression. The combination of prolonged periods of expression and large numbers of targets creates questions whether the effect of a knockout on post-transcriptional gene expression in spermatogenic cells is direct or indirect. For example, in somatic cells the RBP ELAV1/HuR has targets that encode regulatory proteins. Thus, ELAV1/HuR has been described as a "regulator of regulators" (Mukherjee et al., 2011).

At the time I began working on this project, no factors had been identified which bind an element in its natural position and represses translation in early spermatids. This

is because the differentiation of mammalian spermatids cannot be studied in cell culture. As a result, elements must be identified by analyzing mutations in transgenic mice (Kleene et al., 2013). This approach is expensive and time-consuming and has been applied extensively only to the *Prm1* and *Smcp* mRNAs (Kleene et al., 2013). Therefore other approaches have been implemented to try to answer these questions.

Name of mRNA/ Species of Mammal[a]	Location of Protein[a]	mRNA[b]	Protein[c]	Sucrose Gradient[d]
Acev2 (M)	Angiotensin-converting enzyme, variant 2, membrane in midpiece	PS	Step 10	ND
Akap3 (M)	A kinase 3 activating protein, fibrous sheath	~Step 1	Step 4	ND
Akap4v2 (M)	A kinase 4 activating protein variant 2, fibrous sheath	~Step 1	Step 14	ND
Gapdhs (M)	Glyceraldehyde 3' phosphate dehydrogenase, spermatid-specific, fibrous sheath	Step 4	Step 12–13	ND
Odf1 (R)	Outer dense fiber 1, outer dense fiber	Step 6	Step 15	Yes
Prm1 (M)	Protamine 1, basic chromosomal	Step 7	Step 10	Yes
Prm2 (M)	Protamine 1, basic chromosomal	Step 7	Step 13	Yes
Prm3 (M)	Protamine 3, acidic, cytoplasmic	~Step 3	Step 9	ND
Smcp (M)	Sperm mitochondria-associated cysteine-rich protein, mitochondrial capsule	Step 3	Step 11	Yes
Spata18 (R)	Gap between the flagellar axoneme and outer dense fibers	Step 7	Step 15	ND
Tnp1 (M)	Transition protein 1, basic chromosomal	Step 7	Step 11	Yes
Tnp2 (M)	Transition protein 2, basic chromosomal	Step 7	Step 10	Yes

Table 1.1 mRNA species that undergo delayed translational activation in elongating and elongated spermatids. Name of mRNA, function and/or cellular location of protein, and species of mammal in which the stages of expression have been studied (M, mouse; R, rat). Step of spermiogenesis in which the mRNA is first detected, normally by *in situ* hybridization. The approximate stages of first detection of the Akap3 and Prm3 mRNAs were determined by northern blot analysis of RNAs extracted from testes of staged prepubertal mice. Step of spermiogenesis in which the protein is first detected by immunocytochemistry. mRNAs which have been analyzed with sucrose gradients are double underlined. The references for the cellular location each protein, the stage of detection of mRNAs and proteins, and sucrose gradient analyses are as follows: *Acev2* (Howard et al., 1990; Métayer et al., 2002; Langford et al., 1993); *Akap3* (Brown et al., 2003); *Akap4v2* (Brown et al., 2003); Gapdhs (Bunch et al.,1998; Welch et al., 1992, 1995); *Odf1* (Morales et al., 1994; Burmester & Hoyer-Fender, 1996); *Prm1* (Mali et al., 1989; Kleene, 1989, Yan et al., 2003); *Prm2* (Mali et al., 1989; Kleene, 1989, Yan et al., 2003); *Prm3* (Grzmil et al., 2008); Smcp (Kleene, 1989; Shih & Kleene, 1992; Cataldo et al., 1996; Hawthorne et al., 2008), *Spata18* (Iida et al., 2004, 2006), *Tnp1* (Mali et

al.,1989; Kleene, 1989; Yan et al., 2003) and *Tnp2* (Kleene, 1989; Shih & Kleene, 1992; Yan et al., 2003).

Almost all of the factors which have been implicated in translational control in spermatids have not been demonstrated to bind specific elements in putative target mRNAs. The failure to precisely define RNA elements prevents analysis of a small mutation in the target that inactivates binding of a factor. The ability to test the effect of targert mutations provides strong evidence that the factor interacts directly with the mRNA. Target mutations in the mRNAs enable experiments to determine what kind of effect a sequence has on translation of a specific mRNA in transgenic mice. The lack of this type of analysis in spermatids is a major deficiency.

A knockout that blocks sperm development before the stage at which an mRNA is usually activated in wild type mice infers that the knocked out factor directly regulates that particular mRNA. However it is known that there are many difficulties involving knockout mice in spermatogenesis. Knockouts often produce different abnormalities in diverse cells at the same stage in testes (Zhong et al., 1999; Dass et al., 2007). These phenotypes are the hallmarks of incomplete and variable expressivity and penetrance, indicative of modifying factors that compensate for or intensify the phenotype created by the absence of the factor. Studies of the over-expression of factors are even harder to connect with targets than knockouts because high levels of the factor have the potential to modify the expression of mRNAs that are not regulated at physiological levels (Giorgini et al., 2002; Chi et al., 2011).

As of now only two mRNAs have been studied with point mutations in transgenic mice, *Smcp* and *Prm1*. Braun et al. reported that the timing of *Prm1* mRNA translation in spermatids is mediated primarily by the 3' UTR. Subsequent studies of point and deletion mutations discovered two sequences in the *Prm1* 3'UTR that repress translation in early spermatids using transgenic mice. Mutations in both elements result in premature translation. The first is a highly conserved 17 nt translational control element (TCE), and the second is a 6-7 nt YRS that binds Y-box proteins YBX2 and YBX3 in a *Prm1* 3'UTR (Zhong et al., 2001; Giorgini et al., 2001). The finding that both of the cis-elements that repress *Prm1* mRNA translation bind Y-box proteins is relevant to my research because the factors that repress *Smcp* translation in early spermatids are potentially Y-box proteins (Bagarova et al., 2010; Chowdhury et al., 2012).

1.6 Translational Control of the *Smcp* mRNA

Sperm mitochondria-associated cysteine rich protein (SMCP) localizes to the capsule associated with the mitochondrial outer membranes and is thought to enhance sperm motility (Nayernia et al., 2006). Our lab studies the mechanisms of translational regulation of *Smcp* mRNA in transgenic mice. The *Smcp* mRNA exemplifies a widespread phenomenon of developmental regulation of mRNA translation in which mRNAs are transcribed in early spermatids, stored as translationally inactive free mRNPs for several days, and translated on polysomes in late spermatids after the cessation of transcription caused by chromatin remodeling midway through the 13 day haploid phase (Bagarova et al., 2010). It is known that in wildtype mice, *Smcp* mRNA can first be

detected in step 3 spermatids, but the protein is not detected until step 11 six days later (Shih et al., 1992; Cataldo et al., 1996). Sucrose and Nycodenz gradient analysis have shown that about 4% of the *Smcp* mRNA sediments with polysomes in 21 day old mouse testis, an age when the most advanced cells are step 4 spermatids, and about 35% of the *Smcp* is associated with polysomes in adult testis, which contain both early and late spermatids (Bagarova et al., 2010). These results indicate that *Smcp* mRNA is repressed in early spermatids in free-mRNPs and activated in late spermatids (Kleene et al., 1989; Hawthorne et al., 2006; Bagarova et al., 2010).

Our lab has constructed different transgenes that have produced varying extents of loss of translational control. All of the transgenes contain 518 nt of *Smcp* 5' flanking region and the *Gfp* coding region derived from the pEGFP plasmid. The promoter of the $S^5G^CG^3$ and $G^5G^CS^3$ transgenes directs expression of the *Gfp* mRNA in early spermatids at the same transcription start site and in the same cells as the natural Smcp mRNA (Baragova et al., 2010).

Table 1.2 summarizes the different constructs studied and the percentage of mRNA associated with free mRNPS or polysomes. Although relief of repression can be seen with individual 5' or 3'UTR alone, greatest amount release can be seen when both UTRs have sequences mutated.

		Polysomal loading (%)[a]			
		21 dpp testis		Adult testis	
mRNA[b]	First[c]	Nycodenz	Sucrose	Nycodenz	Sucrose
Smcp	st 11	4.2±2.7 (5)	4.9±2.9 (6)	33.0±2.6 (4)	35.1±4.5 (14)[d]
$G^5G^CG^3$	st 3	ND	ND	ND	32.0[e] (1)
$G^5G^CS^3$	st 9	3.4±0.9 (3)	55.3±7.9 (4)	23.7 (1)	37.3±4.3 (3)[d]
$G^5G^CS^3$-mut	st 4–6	10.4±4.8 (5)	9.0±0.91 (2)	29.8 (1)	48.4±7.6 (3)
$S^5G^CG^3$	st 5	7.8±0.1 (2)	16.7 (1)	14.5±1.5 (5)	21.8±4.3 (4)[d]
$S^5G^CG^3$-no-uORF1&2	st 3	28.9±0.8 (3)	32.8±3.7 (2)	26.2 (1)	40.7±6.0 (7)
$S^5G^CG^3$-no-uORF1	st 4	29.8±2.9 (2)	35.1 (1)	33.9±9.0 (2)	41.9±1.4 (2)
Ldhc	PS	34.6±2.9 (8)	52.7±8.5 (9)	32.6±3.7 (3)	56.8±2.4 (10)

Table 1.2 Quantification of polysomal loading of various mRNAs by sucrose and Nycodenz gradients. The polysomal loading of various mRNAs in Nycodenz and sucrose gradients was quantified with phosphorimage analysis of northern blots and RT-qPCR. The polysomal loading (%) is presented as mean and S.D. with the number of independent gradients in parentheses. b mRNA species.c The step of spermatids in which GFP or SMCP expression is first detected. The LDHC protein is first detected in mid-pachytene spermatocytes (references in Kleene (1996)). d These data contain a mixture of results obtained with RT-qPCR in the present study as well as phosphorimaging data obtained previously. Adapted from (Hawthorne et al., 2006a).

Two mutations in the 62 nt at the 3' terminus of the *Smcp* 3' UTR have been studied in transgenic mice. The wild type sequence of the 3' terminus of the *Smcp* 3' UTR which is present in $G^5G^CS^3$ transgene contains several features that are discussed below: (1) A YRS, bold underlined; (2) two canonical AAUAAA poly(A) signals, double underlined (3) a 40 nt conserved segment upstream of the first poly(A) signal, dotted underlined (4) GAGC flanked by 1-3 As between the poly(A) signals. (5) A poly(A) site, 3' terminal A, that was established by 3' RACE (Kleene, unpublished).

A transgene which is hypothesized to abrogate Y-box protein binding was examined. This transgene contains a segment 6-38 nt upstream of the first poly(A) signal was randomized in the 3'UTR, $G^5G^CS^3$-mut1 (Baragova et al., 2010). The $G^5G^CS^3$-mut1 lines exhibit partial loss of translational repression. GFP expression

was first detected in step 4 or 5 spermatids, instead of step 3 spermatids, and the levels of levels of polysomal mRNA (11%) were higher than those of the *Smcp* the $G^5G^CS^3$ mRNAs in sucrose gradients, 3-4% (Bagarova et al., 2010). The $G^5G^CS^3$-mut1 abrogates binding of Y-box proteins to a Y-box recognition sequence, bold underlined, a known translational repressor (Matsumoto et al., 1996; Giorgini et al., 2001). The functions of Y-box proteins in translational regulation in spermatogenic cells are discussed below.

The partial loss in translational repression caused by the $G^5G^CS^3$-mut1 implies the existence of additional negative translational control elements in the *Smcp* 3'UTR (Bagarova et al., 2010). A second transgene will be analyzed referred to as $G^5G^CS^3$-mut2. This transgene replaced the segment downstream of the first poly(A) signal containing the conserved GAGC and the downstream poly(A) signal with the 3'UTR and the 3' flanking sequence downstream of the pEGFP poly(A) signal, which does not delay translation (Bagarova et al., 2010).

Studies of mutations in the *Prm1* and *Smcp* mRNAs have reached complementary but different conclusions. Translational repression of the *Prm1* mRNA in early spermatids is thought to be totally mediated by the TCE in the 3'UTR, while translational repression of the *Smcp* mRNA is mediated by the combined effects of the uORFs in the 5'UTR, the YRS in the 3'UTR, and interactions between the 5'UTR and 3'UTR.

The following three sections describe the background for experiments designed to address the role of two interrelated factors in translational control in early spermatids:

storage of repressed mRNAs in the chromatoid body and translational repression by Y-box proteins.

1.7 Y-Box Proteins

The second focus of my research concerns the idea that many mRNAs in spermatids are regulated by a class of RNA binding proteins called Y-box proteins. The mammalian genome contains three genes encoding four isoforms of Y-box proteins. The members of the Y-box protein family are distinguished by an alanine- and proline-rich N-terminal segment, a central, highly conserved ~70 amino acid cold shock domain and a variable carboxy-terminal segment consisting of alternating ~30 amino acid clusters rich in basic-aromatic and acidic amino acids (Matsumoto et al., 1998; Mastrangelo et al., 2000; Skabkin et al., 2006; Eliseeva et al., 2011). The three genes are named *Ybx1*, *Ybx2*, and *Ybx3,* the last of which is expressed as two alternatively spliced mRNAs encoding two isoforms of different size. The various isoforms exhibit moderate amino acid differences in the N-terminal and C-terminal domains and relatively few differences in the cold shock domain. The two YBX3 isoforms differ in the number of clusters of basic-aromatic and acidic amino acids in the C-terminal domain (Mastrangelo et al., 2000).

The various Y-box proteins exhibit different levels of expression in different tissues. Western blots reveal that YBX1 is expressed at similar levels in the vast majority of adult tissues and all embryonic stages (Lu et al., 2006). YBX2 is only detectable in oocytes, and pachytene spermatocytes and spermatids in testis, and YBX3 is expressed in

pachytene spermatocytes, round spermatids and embryos (Oko et al., 1996; Davies et al., 2000; Giorgini et al., 2001; Lu et al., 2006).

YBX1 is the predominant Y-box protein isoform in most mammalian somatic cells and the best studied. YBX1 is a multi-functional protein which interacts with ssDNA and ssRNA and regulates mRNA transcription, splicing, translation and stability (Skabkin et al., 2006; Eliseeva et al., 2011). Interestingly, low YBX1 to mRNA ratios package mRNAs into "open" mRNPs which are accessible to ribosomes and actively translated, while slightly higher ratios of YBX1 package mRNAs into compact mRNPs that are inaccessible to ribosomes and translationally repressed (Skabin et al., 2006). Since YBX1 is thought to have an important role in determining the configuration of mRNPs (Skabkin et al., 2006), it likely also has secondary effects on the association of other RNA binding proteins and sncRNA with mRNA.

The binding of YBX1 and other Y-box proteins to mRNA is both sequence-specific and non-specific.YBX1 binds single stranded mRNA non-specifically and with moderate affinity through the clusters of basic-aromatic amino acids in the C-terminal domain. YBX1 and FRGY2, the *Xenopus laevis* orthologue of YBX2, also bind single-stranded mRNA sequences specifically with higher affinity through cooperative interactions of the cold shock domain and C-terminal domains (Bouvet et al., 1995; Manival et al., 2001; Skabkin et al., 2006). Since YBX1 binds strongly to mRNA, it is usually undetectable as a free protein. It is unclear what proportions of YBX1 are bound to mRNA by the sequence-specific and non-specific modes in living cells, although these questions could be addressed with UV-crosslinking in cells (Kishore et al., 2011; Ascano et al., 2012). The element to which YBX2 and YBX3 bind *in vitro* is described by the

consensus sequence, [ACU][CA]CA[UC]C[ACU], in which alternative bases in brackets exhibit similar affinity for RNA (Giorgini et al., 2001; Chowdhury et al., 2012). Most, but not all of the permutations of the degenerate bases exhibit strong binding to YBX2 in testis extracts (Chowdhury et al., 2012).

There are several reasons for believing that YBX2 and YBX3S/L repress translation of specific mRNAs in spermatids. First, Y-box proteins have been demonstrated to repress mRNA translation in mammalian somatic cells and *Xenopus* oocytes (Skabkin et al., 2006; Matsumoto et al., 1996; Giorgini et al., 2001; Eliseeva et al., 2011; Lyabin et al., 2011). Second, a YRS in the *Prm1* 3'UTR in an abnormal position represses translation in early spermatids and a mutation that abrogates binding releases the repression (Giorgini et al., 2001). Third, Western blots demonstrate that YBX2 and YBX3S/L sediment primarily with translationally inactive free-mRNPs in sucrose and Nycodenz gradient analyses of adult testis with little or no protein detectable in the free-protein and polysomal regions (Kwon et al., 1993; Herbert et al., 1999; Davies et al., 2000; Giorgini et al., 2002; Yang et al., 2007). Fourth, immunohistochemistry reveals that the levels of YBX2 and YBX3S/L are very high in late pachytene spermatocytes and early spermatids, and that the levels progressively decrease in elongating spermatids eventually becoming undetectable in step 14 elongated spermatids (Oko et al., 1996; Davies et al., 2000; Giorgini et al., 2001). The high levels of YBX2 and YBX3 in pachytene spermatocytes and round spermatids correlate with the period in which many mRNAs are translationally repressed, and the decreasing levels of Y-box proteins correlate with the delayed activation of translation of many mRNAs in elongating and elongated spermatids (examples in Table 1.1).

It is important to note that the dominant Y-box protein in spermatogenesis appears to be YBX2, because the knockout of the *Ybx2* gene results in male infertility and a variety of morphological abnormalities in elongating and elongated spermatids (Yang et al., 2005; 2007). In contrast, the knockout of the *Ybx3* gene produces no morphological abnormalities in sperm, although it does result in reduced sperm number due in part due to increased apoptosis in pachytene spermatocytes (Lu et al., 2006). Evidently, YBX3 is important in the expression of a small number of genes. The knockout of YBX1 is an embryonic lethal, so its importance in sperm development is unknown (Lu et al., 2006). The large effect of the YBX2-knockout on sperm cell development may be related to the very high levels of expression of this protein in testis, 0.7% of total protein in testis (Yang et al., 2005), but the levels of YBX2 are even higher in pachytene spermatocytes and round spermatids, because lower levels of YBX2 are present in other testicular cells.

There are two radically different ideas for the functions of Y-box proteins in developmental regulation of mRNA translation in spermatids. First, Robert E. Braun and his colleagues propose that Y-box proteins are sequence-specific RNA-binding proteins which bind mRNA in the cytoplasm and repress translation (Giorgini et al., 2001; 2002). These ideas are supported by analysis in transgenic mice demonstrating that a YRS in an abnormal position close to the poly(A) tail represses translation and that a mutation which abrogates protein binding releases translational repression (Giorgini et al., 2001). The idea that Y-box proteins repress translation in the cytoplasm by binding with high affinity to specific mRNA sequences is shared by many workers in the field (Bouvet et al., 1995; Matsumoto et al., 1996; Giorgini et al., 2001; 2002; Lyabin et al., 2011).

Second, Norman Hecht and his colleagues propose that Y-box proteins bind mRNAs non-specifically (Kwon et al., 1993; Yu et al., 2002). To account for the apparent mRNA-specific translational repression, Yang et al. propose that mRNAs that are repressed by Y-box proteins are transcribed from promoters that contain Y-box transcription elements, CTGATTGGC[TC]TAA, a dsDNA motif in the promoter of many genes specifically expressed in male germ cells (Yang et al., 2007). Although these ideas conflict sharply with work from the Braun lab described above, the low-affinity, non-specific binding of the C-terminal domain Y-box proteins to RNA is well documented (Skabkin et al., 2006). Furthermore, the idea that the association of RNA-binding proteins with pre-mRNA in the nucleus can have important effects on mRNA translation and stability in the cytoplasm is also well documented (Bouvet et al., 1994; Trcek et al., 2011; Lebedeva et al., 2011; Mukherjee et al., 2011). However, the proposal that the association of YBX2 with pre-mRNA in the nucleus represses translation in the cytoplasm in spermatids is based on correlation, without decisive evidence that mutation of the Y-box element in specific genes in transgenic mice abrogates translational repression of the corresponding mRNAs in round spermatids. Furthermore, findings that the *Ybx2* knockout does not decrease transcriptional activities of the *Tnp2* and *Acr* mRNAs measured with nuclear run-off assays undermines claims that the binding of YBX2 to Y-box promoter elements is necessary for transcription of these mRNAs (Yang et al., 2007). The *Smcp* mRNA is relevant to this controversy since it is translationally repressed in step 3-10 spermatids even though its 5'flanking region lacks a Y-box element (Kleene, unpublished).

Another important controversy concerns the consequences of depletion of YBX2 by the *Ybx2* knockout. Yang et al. report that depletion of YBX2 results in a drastic, ~20-fold reduction in the levels of mRNAs that are translationally dormant in pachytene spermatocytes and round spermatids. They suggest two potential explanations for the mRNA degradation. The depletion of YBX2, a major mRNA binding protein, leaves mRNAs exposed to ribonucleases resulting in degradation. Alternatively, depletion of YBX2, results in premature translational activation and early degradation by a pathway specific for translationally active mRNAs (Braun et al., 1989; Fajardo et al., 1997; Yang et al., 2007) seem to prefer the second idea, although they cite none of the previous studies that have documented coupling of mRNA degradation to mRNA translation in spermatogenic cells (Braun et al., 1989; Fajardo et al., 1997) and mammalian somatic cells (Chang et al., 2004). Although Yang et al. report sucrose gradient analyses demonstrating that the *Ybx2* knock-out results in premature translational activation, the only developmentally regulated mRNAs studied, the *Pgk2* mRNA, undergoes slight decay and undetectable translational activation in response to YBX2 depletion. The premature translational activity mRNAs that undergo strong decay (*Prm1*, *Prm2*, *Tnp1, and Tnp2*) were not studied with sucrose gradients analysis of 25 dpp prepubertal mice in which round spermatids are the most advanced cell type (Braun et al., 1989; Kleene unpublished).

The review of the literature above highlights unresolved controversies concerning the roles of YBX2 in post-transcriptional regulation in spermatogenic cells. While addressing all of these controversies is beyond the scope my thesis research, my

studies can address the most important question, whether the *Ybx2* knockout results in premature translation of the *Prm1* and *Smcp* mRNAs.

1.8 Localization of mRNA in the Chromatoid Body

The third focus of my research concerns the localization of mRNAs in the chromatoid body. Translationally repressed mRNAs in eukaryotic cells are often sequestered into microscopically visible cytoplasmic organelles, collectively referred to as RNP granules, which contain mRNAs, RNA, binding proteins and sometimes small non-coding RNAs (Anderson et al., 2009). RNP granules are given a variety of names in different cell types and organisms: processing bodies in yeast, processing-bodies and stress granules in mammalian tissue culture cells, neuronal granules in nerve cells, and germ cell granules and chromatoid bodies in germ cells and early embryos of many kinds of animals. Some RNP granules, such as processing bodies and stress granules; form and dissociate in response to environmental or metabolic stimuli, while germ cell granules are relatively stable. In general, RNP granules are never surrounded by phospholipid-bilayer membranes, and all lack 60S ribosomal subunits and 80S ribosomes, which mean that they cannot translate mRNAs into proteins. All RNP granules contain a diverse set of proteins which functions in promoting mRNA degradation, and the association and dissociation of proteins and sncRNAs with mRNA (Anderson et al., 2009).

Developing mammalian spermatogenic cells have two types of germ cell granules, the intermitochondrial cement in spermatocytes and the chromatoid body in spermatids. The term chromatoid body describes the fact that it is strongly stained by basic dyes similar to other nucleic acid-containing organelles such as chromosomes,

nuclei and nucleoli (Yokota et al., 2008). The chromatoid body is composed of thin filaments that are consolidated into branching strands of varying thickness that form dense irregular networks (Parvinen et al., 2005). The fibrous moiety of the chromatoid body in round spermatids, referred to as the stroma, is electron dense, and usually surrounds small less dense non-fibrous areas, referred to as lacunae, which appear to contain the same material as the surrounding cytoplasm (Yokota et al., 2008).

Current studies favor the idea that the chromatoid body first appears in late pachytene spermatocytes, as intermitochondrial cement (IMC) disperses during the meiotic divisions, and coalesces into its mature form post-meiotically in round spermatids (Parvinen et al., 2005; Yokota et al., 2008). In round spermatids, the chromatoid body moves dynamically between the nuclear pores and Golgi area of the cytoplasm suggesting that it transports RNA from the nucleus to the cytoplasm (Parvinen et al., 2005). In elongating spermatids, the chromatoid body migrates to the posterior end of the nucleus, forms a ring around the base of the flagellum, and then moves down the tail, decreasing dramatically in size, finally disappearing in elongated spermatids (Parvinen et al., 2005; Kotaja et al., 2007). Formation of the chromatoid body in part has been attributed to the Tudor domain containing proteins which constitute a conserved class of chromatoid body components. Tanaka et al. show that *tudor domain containing 7* (*Tdrd7*) is essential for haploid spermatid development and defines, in concert with *Tdrd6*, key biogenesis processes of chromatoid bodies. Single and double knockouts of *Tdrd7* and *Tdrd6* demonstrated that these spermatogenic tudor genes orchestrate developmental programs for ordered remodeling of chromatoid bodies (Tanaka et al., 2010).

The chromatoid body, like other RNP granules, is proposed to have an important function in post-transcriptional gene regulation (Yokota et al., 2008; Meikar et al., 2011). Much of the evidence that the chromatoid body plays a role in storage and degradation of mRNAs comes from immunocytochemical and biochemical evidence that many proteins which localize in the chromatoid body function in RNA metabolism. A study by Kotaja et al. showed the localization of the following proteins. Mouse Vasa Homolog (MVH) is an RNA helicase that is required for spermatogenesis and is known to be involved in RNA metabolism. MIWI is a RNA-binding protein of the PIWI/Argonaute family; shown to be crucial for progression through spermatogenesis (Kotaja et al., 2006). Ago subfamily proteins which are components of RISC in RNAi and miRNA pathways have also been shown to localize to the chromatoid body. Dcp1a and GW182 are both known important components of P-bodies that have been shown to localize to the chromatoid body. Dcp1a is a 5' decapping enzyme, and GW182 is a RNA binding protein that is essential for microRNA-mediated gene silencing in animal cells (Kotaja et al., 2006). Dicer and RNase III enzyme that plays a role in the RNAi pathway along with miRNAs have also been shown to localize to the chromatoid body.

Proteins that play a role in the transport of mRNAs have also been shown to localize in the chromatoid body. KIF17b is a testis-specific kinase motor protein. This protein binds to RNA-protein complexes that contain specific CREM-regulated mRNAs through an interaction with TB-RBP, and then transports these mRNAs between the nucleus and the cytoplasm (Kotaja et al., 2006). It is known that MIWI interacts with KIF17. Kimura et al. showed an association between MIWI and PABP2C. Both localize in the chromatoid body, and it is thought that PABP2C may also participate in mRNA

transport to the chromatoid body (Kimura et al., 2009). Tsai-Morris et al. claim that the RNA helicase (GRTH) is located in the chromatoid body and has been shown to transport messages from nucleus to cytoplasm in NIH3T3 cells. They believe that GRTH also plays a role in maintaining the integrity of functional components in chromatoid body (Tsai-Morris et al., 2009). Proteins that are currently known to localize to the chromatoid body are summarized in (Table 1.3).

Protein Group	Protein Names	Molecular Function
Heterogeneous nuclear ribonucleoproteins	hnRNPDL, hnRNPH, hnRNPM, hnRNPF, hnRNPA2/B1, hnRNPA3, hnRNPU	mRNA processing, splicing, RNA transport and localization
poly(rC)- and poly(A) binding proteins	PCBP1, PCBP2, PABP1	mRNA processing, splicing, translation, mRNA turnover
Splicing regulators	SRSF1, SRSF3, TRA2B, DDX5, SAM68/KHDR1, TDRD3	splicing, RNA export, regulation of translation, RNP assembly
mRNA binding proteins	YBOX2, ELAV1, DDX25, PIWL1/MIWI, PIWL2/MILI, IF2B3, ADAD1	regulation of translation, mRNA stability, RNA localization, RNA processing
Exon junction complex (EJC) proteins	RBM8A, CASC3, MAGOH, eIF4A3	regulation of translation, splicing, RNA transport and localization
Nonsense mediated RNA decay pathway	UPF1, SMG1, SMG6, RBM8A, CASC3, MAGOH, eIF4A3	RNA quality control, regulation of translation
Proteins involved in translation	eIF4A1, eIF4E, eEF1A1, RPL24, RPL11, RPL23A, RSP14, RSP8	translation initiation, elongation, ribosome assembly

Table 1.3 Abundant RNA binding proteins identified with mass spectrometry sequencing in purified chromatoid bodies from murine round spermatids. This table lists more than 40 RNA binding proteins that were identified in purified chromatoid bodies. These proteins can be divided into groups with related functions in pre-mRNA splicing, mRNA degradation, binding of small non-coding RNAs to mRNA, translational

repression and translational activation. Red arrows point towards proteins that function in splicing. Adapted from (Meikar et al., 2014).

The chromatoid body is hypothesized to have many functions in post-transcriptional gene regulation based on the functions of the proteins it contains. It is proposed as a site for storage and processing of reproductive cell-specific mRNAs in male germ cells (Kotaja et al., 2007). Another idea is that miRNA and RNA-decay pathways are coordinated by the chromatoid body, analogous to the functions of P-bodies in somatic cells and yeast (Kotaja et al., 2007). A third idea is that the chromatoid body is a remodeling center in which mRNPs emerge from the nucleus, and undergo changes in the set of RNA-binding proteins and sncRNAs that are associated with mRNA. At present there is no direct evidence that any mRNA is degraded, repressed or remodeled in the chromatoid body.

Despite the fact that the chromatoid body is generally agreed to have a critical function in post-transcriptional gene regulation, there is a striking paucity of evidence how much mRNA is actually contained in the chromatoid body (Kleene et al., 2011). At the outset, it can be safely assumed that translationally active mRNA is present in the general cytoplasm because ribosomes are present in the general cytoplasm and absent from the chromatoid body (Parvinen et al., 2005; Kotaja et al., 2006). The question becomes: What proportion of free-mRNPs is present in the general cytoplasm and chromatoid body? Evidence relevant to this question can be derived from biochemical studies of fractionated cells and *in situ* hybridization studies of intracellular localization of mRNA.

Evidence that translationally repressed mRNA is localized in the chromatoid body is derived from a single previous study of the transition protein 2 (*Tnp2*) mRNA in rat testis (Saunders et al., 1992). The *Tnp2* mRNA is expressed in step 7-12 spermatids, is translationally repressed in free-mRNPs in steps 7-9, and is translationally active beginning in step 10 (Yan et al., 2003; Meistrich et al., 2003). Saunders et al. noted that the *Tnp2* mRNA is strongly localized adjacent to the nucleus in step 7 using digoxigenin-based non-isotopic *in situ* hybridization, fixation by perfusion with Bouin's, and 2 μm polystyrene sections. Although immunological markers, which would reliably identify the chromatoid body, were not available at this time, the size and perinuclear location of the localized hybridization signal are consistent with the chromatoid body. The photographs also show less intense *in situ* hybridization signal throughout the cytoplasm in 5 μm paraffin sections of step 7 spermatids. The hybridization signal in steps 8 and 9 is stronger and is not localized in the general cytoplasm, even though the *Tnp2* mRNA is repressed in steps 8 and 9. However, it is difficult to assess by eye the proportions of localized and unlocalized *Tnp2* mRNA in step 7 spermatids. The possibility merits consideration that unlocalized mRNA predominates, because the chromatoid body occupies ~0.4% of the cytoplasmic volume, based on the relative diameters of the chromatoid body, ~1.5 μm, and round spermatid cells and nuclei, 10 μm and 5 μm (Parvinen et al., 2005). Recently, it has been reported through the use of RNA-FISH that the protamine2 (*Prm2*) mRNA transits through chromatoid bodies of round spermatids and localizes to cytosol of elongating spermatids for translation (Fukuda et al., 2013).

It would be reasonable to expect that the literature would contain many reports of mRNA localization in the chromatoid body, because the developmental expression of

many mRNAs in spermiogenesis has been analyzed with *in situ* hybridization. Most mRNAs should exhibit localization because each of the more than 50 mRNA species that are expressed in spermatids that have been analyzed with sucrose gradients exhibit high levels of translationally inactive free mRNPs, usually >50% (Kleene et al., 2003). However, useful information is limited to a relatively small group of studies which utilize non-isotopic hybridization and good fixation and present photographs in which the location of the hybridization signal can be visualized. Such studies typically show no localization (Weitzel et al., 2003; Iida et al., 2004). Morales et al. argue that the Prm1 and Tnp1 mRNAs are not localized in the chromatoid body. In these studies, the testes were fixed by perfusion with 2% glutaraldehyde and 4% paraformaldehyde, embedded in agar and sectioned at 100 μm. These thick sections were hybridized to anti-sense ^{3}H-riboprobes followed by washes, osmium staining, embedding in epon, thin sectioning and light and electron microscope autoradiography. These preparations beautifully preserve the ultra structure of the chromatoid body, and reveal that the *Prm1* and *Tnp1* mRNAs are uniformly distributed in the cytoplasm of step 7 to 9 spermatids.

Unfortunately, the use of glutaraldehyde fixation in these studies is known to cause artifacts *in situ* hybridization. Lawrence & Singer (1985) demonstrated that glutaraldehyde increases the background, and sharply decreases the specific *in situ* hybridization signal because it cross-links cytoplasmic proteins tightly, rendering mRNA inaccessible to the hybridization probe, and decreasing the efficiency of removal of non-hybridized probe by the washes. Both problems would likely be aggravated by performing *in situ* hybridization on 100 μm thick sections. This appears to be a problem because the *Prm1* and *Tnp1 in situ* hybridization signals are present over the nuclei and

cytoplasm of pachytene spermatocytes and step 1-6 spermatids, observations which conflict with studies demonstrating that these mRNAs are first detected in step 7 spermatids (Braun et al., 1989; Mali et al., 1989). The absence of hybridization signals in late spermatids and with sense strand negative controls may reflect low penetration of the probes deep into the tissue.

Recent studies have demonstrated the localization of RNAs and proteins in the chromatoid body with dried down preparations (Kotaja et al., 2006). In dried down preparations, mechanically dispersed cells from short pieces of seminiferous tubules are added to dilute Triton X-100 and paraformaldehyde and the cells are dried on microscope slides. Nguyen Chi et al. used dried-down preparations to demonstrate that the *Gcnf* and *Brd2* mRNAs undergo developmental changes in sequestration in the chromatoid body in dried-down preparations of round spermatids. They report that both mRNAs are strongly localized in the chromatoid body in step 1-5 spermatids and that both mRNAs are absent from the chromatoid body in step 6-9 spermatids. They further argue that the apparent developmental change in *Brd2* mRNA sequestration is correlated with a modest increase in polysome loading, 44% in 23 day testis, to 60% in adult testis. The failure to detect *Brd2* in the general cytoplasm does not support the inference that the *Brd2* is sequestered in a translationally repressed state in the chromatoid body in early round spermatids and exported to the general cytoplasm for translation in step 6-9 spermatids.

Not only is the localization of translationally repressed mRNA in the chromatoid body poorly documented, but factors that are associated with translationally repressed mRNAs also are not exclusively associated with the chromatoid body. The most convincing studies concern mouse Y-box proteins, YBX2 and YBX3, because western

blot analyses of sucrose gradients demonstrate that both proteins sediment primarily with free mRNPs with virtually no free protein sedimenting at the top of the gradient (Kwon et al., 1993; Davies et al., 2000; Giorgini et al., 2002; Yang et al., 2005). Light microscope immunocytochemistry of paraffin sections of adult testis with anti-FRGY2, the *Xenopus laevis* homologue of YBX2, and anti-YBX3 reveals that both proteins are distributed throughout the cytoplasm (Oko et al., 1996; Davies et al., 2000). In contrast, electron microscope immunogold studies were interpreted as evidence that YBX2 is concentrated in the lacunae and immediate vicinity of the chromatoid body, and at lower levels throughout the general cytoplasm.

Cell fractionation yields another striking contradiction with the idea that translationally inactive free mRNPs are sequestered in the chromatoid body. The chromatoid body is a rather large structure, which pellets during centrifugation at 500-1000 x G for 10 min (Meikar et al., 2011). Thus, the chromatoid body would be expected to sediment with nuclei in preparing cytoplasmic extracts for sucrose gradient analysis, 13,000 x G for two min. However, using two different methods of RNA extraction, 8.8 ± 4.3% (mean and S.D. of four experiments) of the *Smcp* mRNA pellets with nuclei in adult testis (Kleene, unpublished), whereas ~65% sediments as free mRNPs near the top of sucrose gradients after centrifugation at 125,000 x G for 80 min (Bagarova et al., 2010). These findings suggest either that *Smcp* free mRNPs are not localized in the chromatoid body in intact cells, or that free mRNPs are released into the cytoplasm during cell fractionation. Studies in yeast also indicate that repressed mRNAs that are sequestered in P-bodies in intact cells sediment as free mRNPs in sucrose gradients (Kedersha et al.,

2006). P-bodies and stress granules are remarkably fragile and these organelles have never been isolated.

Meikar et al. purified the chromatoid body from paraformaldehyde fixed cells from adult testis with differential centrifugation and immunoprecipitation with antibody to mouse vasa homologue (MVH), a major constituent of the chromatoid body. After reversal of the formaldehyde cross-linking and RNA extraction, the levels of *Odf1*, *Prm1* and *Tnp2* mRNAs were analyzed in all fractions with RT-PCR. Sucrose gradient analysis demonstrates that ~75% of all three mRNAs are present in free mRNPs in adult testis and purified elongated spermatids (Cataldo et al., 1996; Kleene et al., 1989; Cataldo et al., 1999), all of which would be expected to co-purify with the chromatoid body if it stores dormant mRNAs for later translation. In contrast, (Meikar et al., 2011) observed that the vast majority of all three mRNAs are in the supernatant after the initial centrifugation, and that negligible amounts are present in the initial pellet and anti-MVH pellet. The high levels of these mRNAs in the supernatant may represent polysomal mRNAs and free mRNPs in the general cytoplasm, or free mRNPs that exit the chromatoid body after cell lysis, but the very small fraction of free mRNPs that co-purifies with the chromatoid body does not support the idea that the free mRNPs are stored in that organelle.

Given the controversies concerning the proportion of repressed mRNA that is stored in the chromatoid body, I have worked on developing RNA fluorescent *in situ* hybridization techniques that can be used to determine the localization of the *Smcp* mRNA. I have been able to show that probe sets of fluorophore tagged tiled 20 nucleotide-long "Stellaris" probes from Biosearch Technologies give an unprecedented

strong hybridization to the Smcp mRNA in the chromatoid body in dried down preparations. I describe how this technique can potentially also be used to analyze developmental changes in the intracellular localization of the *Smcp* mRNA, the developmental regulation of *Smcp* mRNA transcription, and the sharing of gene transcripts through cytoplasmic bridges connecting syncytial round spermatids.

1.9 Objectives

I am interested in understanding mRNA activity and localization throughout a brief period of translational regulation during spermatogenesis. Specifically, my research examines the mechanisms that control the timing of translational activity of the sperm mitochondria-associated cysteine-rich protein (SMCP) in haploid spermatogenic cells. I would like to identify the regulatory elements in the *Smcp* 3'UTR that repress translation in early haploid spermatogenic cells. At this point, in time it is well known that interaction between the 5' and 3'UTR is necessary for full repression of the *Smcp* message, and that the 3'UTR plays a more important role in this function in spermatids. However, the cis-elements or trans-acting factor that account for translational repression of the *Smcp* remained to be identified. The transgenic line I have studied in mice provides the first insight as to where the cis-element in the Smcp 3'UTR may be located and what sequence is necessary for repression of the message, and this work is discussed in Chapter 2.

My second major interest, which will be discussed in Chapter 3, is YBX2/MSY2, an RNA binding protein that is known to play a key role in repression of specific spermatogenic messages. I have had the opportunity to obtain *Ybx2* null mice and have investigated the percentages of *Prm1* and Smcp mRNA that are associated with polysomes at sexually immature *Ybx2*- null males at an age when both mRNAs are translationally repressed. This experiment is designed to test the hypothesis is that YBX2/MSY2 may be the major trans-acting factor that represses binds the *Smcp* 3'UTR and represses *Smcp* translation. If YBX2 is indeed the critical factor, then the *Ybx2*-null mutation produce observable relief of repression of *Smcp* mRNA translational repression. Results from Real-Time PCR quantification are supporting this theory. I also found YBX2 is localized in the chromatoid body, suggesting that YBX2 associates with the *Prm1* and *Smcp* mRNAs in the chromatoid body.

Lastly in chapter 4, the intracellular localization of the *Smcp* mRNA in early spermatids was studied with RNA-fluorescent *in situ* hybridization (RNA-FISH). The *Smcp* mRNA probe detected intense concentration of the *Smcp* mRNA in a male germ cell-specific granule called the chromatoid body. It has long been speculated that the chromatoid body stores repressed mRNAs in early spermatids because it is devoid of ribosomes. However, my RNA-FISH studies reveal that translationally repressed and translationally active mRNAs are strongly concentrated in the chromatoid body implying that localization is independent of translational activity. Unexpectedly, a probe for the *Smcp* intron also localized to the chromatoid body suggesting that the *Smcp* pre-mRNA may be spliced in the chromatoid body. This is the first report that translationally active

mRNAs and introns localize to the chromatoid body. This research has permitted the formulation of a speculative model of translational repression of the *Smcp* mRNA.

CHAPTER 2

ANALYSIS OF *CIS*-ELEMENTS THAT CONTROL THE DEVELOPMENTAL EXPRESSION OF THE SPERM MITOCHONDRIA-ASSOCIATED CYSTEINE-RICH PROTEIN mRNA TRANSLATION IN TRANSGENIC MICE

2.1 Abstract

The sperm mitochondria-associated cysteine-rich protein mRNA is translationally repressed in early spermatids and translationally active in late spermatids. Previous studies in transgenic mice have demonstrated that the *Smcp* 5' and 3' UTRs alone account for partial repression, and that both *Smcp* 5' and 3' UTRs are required for full translational repression. Previous studies of a 34 nt mutation in the *Smcp* 3'UTR upstream of the first poly(A) signal resulted in a small release of translational repression, indicating that critical cis-elements remain to be identified. The studies described below demonstrate the requirement of the 16 nt downstream of the first AAUAAA polyadenylation signal for translational repression.When these sequences are replaced with the 17 nt downstream of the early pEGFP polyadenylation signal, it totally eliminates the translational repression by the *Smcp* 3' UTR.

2.2 Introduction

Translational regulation is important in controlling gene expression during spermatogenesis, as transcription in late spermatids ceases due to chromatin remodeling (Meistrich et al., 2003; Kleene et al., 2013). In the absence of transcription, delayed activation of mRNA translation is utilized to synthesize the sperm mitochondria-associated cysteine-rich protein (SMCP) in elongating and elongated spermatids (Chowdhury et al., 2012). The *Smcp* mRNA is transcribed in early spermatids, stored as translationally inactive messenger ribonucleoprotein particles (free-mRNPs) for several days to a week before translation is activated in transcriptionally inactive late haploid cells, elongated spermatids (Kleene et al., 1989; 2003; 2013). Repression of mRNA translation in round spermatids is necessary for normal sperm development because premature activation of translation of many mRNAs in round spermatids in transgenic mice decreases male fertility (Lee et al., 1995; Tseden et al., 2007).

mRNA-specific translational regulation typically involves *cis*-elements which bind *trans*-factors, either RNA binding proteins (RBPs) or small non-coding RNAs, which activate or repress translation. Many studies utilizing knockout mice or overexpression of specific RBPs have implicated a variety of RBPs and microRNAs in translational regulation (Kleene et al., 2013; Kotaja et al., 2014). However, defining precisely whether the effect of these factors on translation of specific target mRNAs is direct or indirect is difficult (Kleene et al., 2013). Therefore, it is necessary to study translational regulation in spermatids by analyzing mutations in *cis*-elements in transgenic mice.

This study uses transgenic mice to analyze the developmental regulation of the *Smcp* mRNA. SMCP is a structural protein in the keratinous capsule surrounding mammalian sperm mitochondria (Cataldo et al., 1996; Ursini et al., 1999). The evolutionary origin of the *Smcp* mRNA differs from those of the protamine and transition protein mRNAs which are commonly used in studies of translational regulation in spermatids (Hawthorne et al., 2006a). Thus, studies of the *Smcp* mRNA address the question whether all the mRNAs in spermatogenesis are regulated by the same set of *cis*-elements and *trans*-factors.

The *Smcp* mRNA is synthesized in step 3 spermatids, and is stored as a translationally inactive free-mRNP for about 6 days before the mRNA is recruited onto polysomes in step 11 spermatids as demonstrated by the appearance of the SMCP protein (Kleene et al., 1989; Shih et al., 1992; Cataldo et al., 1996). Previous studies using the EGFP reporter in transgenic mice reveal that the *Smcp* mRNA is regulated by multiple mechanisms involving both the 5'UTR and the 3'UTR (Hawthorne et al., 2006; Bagarova et al., 2010). However, the *Smcp* 5' UTR alone delays GFP expression until step 5, the *Smcp* 3'UTR alone delays GFP expression until step 9, and a mutation in the *Smcp* 3'UTR results in a small release of translational repression (Bagarova et al., 2010). Clearly, these studies have not identified critical elements that repress the *Smcp* mRNA until step 11 elongating spermatids.

This study continues our goals of identifying the *cis*-elements and *trans*-factors that are necessary and sufficient for translational repression of the *Smcp* mRNA from step 3 to step 11 spermatids. We have analyzed a transgene that replaces a highly conserved segment in the *Smcp* 3'UTR downstream of the first AAUAAA polyadenylation signal to

search for elements that strongly repress translation in round spermatids. We chose this region based on evidence that elements that repress *Prm1* mRNA translation in round spermatids are located at the 3' terminus of the 3' UTR (Zhong et al., 2001). We also use RNA affinity chromatography and mass spectrophotometry sequencing to demonstrate specific proteins that bind the 3' terminus of the *Smcp* 3'UTR.

2.3 Materials and Methods

2.3.1 Construction of the $S^5G^CS^3$ and $G^5G^CS^3$-mut 2 transgenes

The $S^5G^CS^3$ transgene was constructed from $G^5G^CS^3$ and $S^5G^3G^3$ transgenes constructed previously (Hawthorne et al., 2006). Briefly, plasmids containing the $G^5G^CS^3$ and $S^5G^3G^3$ transgenes were digested with *Bsrg* I and *Afl II*, and the large $S^5G^3G^3$ and small $G^5G^CS^3$ fragments were purified by agarose gel electrophoresis and a Gene Clean II kit (Bio101), and the small $G^5G^CS^3$ fragment was ligated into the large $S^5G^3G^3$ fragment.

The $G^5G^CS^3$-mut2 transgene was constructed from the $G^5G^CS^3$ and $G^5G^CG^3$ transgenes in several steps. A *Swa* I site was inserted overlapping the upstream *Smcp* poly(A) signal with overlap extension PCR in the $G^5G^CS^3$ transgene (Higuchi et al., 1988). The Swa I-Afl II fragment from the $G^5G^CG^3$ transgene was inserted into the Swa I-Afl II sites of the $G^5G^CS^3$. Finally, the Swa I site was reversed to that of the original *Smcp* 3'UTR with a second round of overlap extension PCR.

The plasmids were electroporated into *E. coli* DH5α, plated on LB agar

containing 50 µg/ ml kanamycin, and the sequence of the transgene was verified by sequencing on both strands, the small *Xho* I and *Afl* II fragment containing the transgene is purified with agarose gel electrophoresis, extracted with a NucleoTrap kit (Clontech), filtered, and adjusted to 50 ng/µl in 0.1 mM EDTA, 5 mM Tris-HCl (pH 7.4). One-cell C57BL/6 X SJL F2 embryos are injected and implanted into pseudopregnant females at the University of Massachusetts Medical Center Transgenic Core facility and tail-biopsies were analyzed to determine which pups contain the transgene. After weaning, the founders are transferred to the UMass Boston Animal Care Facility. Transgenic founders are bred to C57BL/6 X SJL mice of the opposite sex to produce lines. To identify transgenic mice, 5 mm is excised from the end of the tail of 10-21 day old pups in accord with NIH guidelines for genotyping transgenic mice, and the DNA is purified with a DNAeasy Blood and Tissue kit (Qiagen). The presence of transgenes is assayed by PCR using *Gfp*-specific primers (Hawthorne et al., 2006).

2.3.2 Analysis of GFP fluorescence in squashes of seminiferous tubules

The stage of GFP expression was analyzed in living spermatogenic cells as described previously (Bagarova et al., 2010) and is based on techniques described by (Kotaja et al., 2004). Briefly, adult mice were sacrificed with CO_2 hypoxia, the testes were dissected out and the *tunica albuginea* was removed. The seminiferous tubules were teased apart in phosphate buffered saline (PBS) and visualized with a dissecting microscope and transillumination to identify tubule segments of potential interest (Kotaja et al., 2014). The stages of spermatids were identified in one cell thick squashes of 0.5

mm tubule segments with phase contrast microscopy at 100X using an Olympus BX51 microscope equipped with a Plan Fluorite 100X phase objective (NA 1.3), 100 W mercury burner, and SPOT XPLORER monochrome camera, SPOT image processing software (Diagonistic Instruments, Sterling Heights, MI, USA). EGFP fluorescence was excited at 470 nm and emitted light was captured at 525 nm and photographed at a manual setting of 3 sec and γ=1, and is depicted as the grayscale images that were actually recorded by the camera. ImageJ (downloaded from NIH) was used to quantify the pixel intensity with GFP fluorescence associated with various cell types.

2.3.3 Sucrose and Nycodenz Gradient Analysis

Cytoplasmic extracts of 21/25 dpp and adult testes were prepared by dissecting testes (1 testis for adult mouse and 2 testes for 21/25 day mice), removing the *tunica albuginea* and homogenizing the testes in 300 µl HNM buffer (20 mM HEPES, pH 7.4, 0.1 M NaCl, 3 mM $MgCl_2$) containing 0.5% Triton X100 and 1 unit/µl RNasin Plus (Promega Biotech). The nuclei were pelleted by centrifugation at 13,000 X g for 2 minutes, and 250µ l of the supernatant was layered on either a 3.8 ml linear 15-40% sucrose gradient in HNM buffer (w/w) or a 3.8 ml 20-60% (w/v) Nycodenz gradient prepared by layering 760 ml of 60, 50, 40, 30, and 20% Nycodenz (Accurate Scientific Chemical Corporation, Westbury, NY, USA) in HNM (w/v) in polyallomer centrifuge tubes for the Beckman SW60 rotor. Sucrose gradients were centrifuged for 80 min at 35,000 rpm at 4°C, and ~0.4 ml fractions were collected onto 0.3 g guanine thiocyanate, and RNA was extracted as described previously (Kleene et al., 2010). Nycodenz gradients were centrifuged for 24 hr at 37,000 rpm at 4°C, and 0.2 ml fractions were

collected, and RNA was extracted as for sucrose gradients with adjustments for the smaller volume of fractions. RNA was extracted from each fraction of sucrose or Nycodenz gradients with techniques that recover of equal amounts of RNA from each fraction (Kleene et al., 2010).

2.3.4 Quantitative Reverse Transcriptase real-time PCR

Quantitative Reverse Transcriptase real-time PCR (RT-qPCR) was carried out as described by (Bagarova et al., 2010).

2.3.5 UV-crosslinking RNA binding assays

UV-crosslinking RNA binding assays were carried out as described by (Chowdhury et al., 2012). Plus and minus strands oligonucleotides corresponding to various segments of *Smcp* 3'UTR were purchased from Life Technologies (Grand Island, NY). The oligos were annealed, digested with *Eco* RI and *Hind* III, and ligated into the *Eco*RI and *Hind* III sites of pGEM3 (Promega-Biotec) downstream of the T7 promoter. The sequence of the insert was verified through sequencing at Massachusetts General Hospital DNA Sequencing Facility (Cambridge, MA). The plasmid was linearized with *Hind* III and probes were synthesized with the T7 bacteriophage RNA polymerase (New England Biolabs, Beverely MA) and α-[^{32}P]-rUTP (Perkin Elmer, Boston MA). Probes were extracted twice with phenol:chloroform, chromatographed on a Biogel P6 column (Bio-Rad), ethanol-precipitated, and dissolved in DEPC-treated H_2O. The cpm of each probe was determined by scintillation counting, and 10^5 cpm was used in each reaction.

RNA probes were combined with 3 µl DEPC-treated H_2O and 5 µl 2X Binding Buffer (40 mM HEPES, 6 mM $MgCl_2$, 80 mM KCl and 1 mM DTT, pH7.6) denatured by heating at 70°C for 5 mins, renatured by slow cooling to room temperature. Following renaturation, sequence specific complexes were created by the following incubations at room temperature, ~25^{o}C: (1) incubating the samples with 1 µl of cytoplasmic extract of adult testis (25-50 µg/µl) and *E. coli* tRNA (5 mg/ml) for 20 min, (2) digestion with RNase T1 (5U) for 10 min, (3) treatment with 1 µl heparin (50 mg) for 10 min. The samples were irradiated with UV using two Sylvania G15T8 germicidal bulbs at a distance of 8 cm for 8 min on ice, and mixed with 12 µl 2X SDS sample loading buffer, boiled for 4 min and resolved on SDS-polyacrylamide gels containing a 3 cm 5% stacking gel and a 20 cm 10% separating gel. Gels were fixed in methanol: H_2O: acetic acid (5:4:1), dried, and autoradiographed at -80°C with an intensifier screen.

2.3.6 RNA affinity chromatography- Performed by Tamzid Chowdhury

5'-biotinylated RNA probes were purchased from Sigma-Aldrich Corp. (ST Louis, MO). 20 µg of biotinylated RNA probes were mixed with 400 µl binding buffer (20 mM HEPES, 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, 1 mM DTT, pH 7.6), heated to 70°C for 5 minutes and slow cooled to room temperature before incubating with 1 mg total cytoplasmic testis extract and 5 µg of tRNA for 30 min. The samples were then treated with 2 µl of heparin (200 mg/ml) for 10 min, incubated with pre-washed streptavidin agarose (Pierce 20347, Rockford IL) on a rotating disc for 2 hr at 4°C. After five 1 ml washes with 1X Binding Buffer (with protease inhibitor), bound proteins were released by boiling in 2X SDS sample buffer, resolved by 10% SDS-PAGE and

visualized by silver staining. Protein bands of interest were excised from the gel, and identified with mass spectrometry sequencing at the Taplin Mass Spectrometric Facility (Boston, MA).

2.4 Results

2.4.1 Design of *Smcp-Gfp* Transgenes

Previous studies demonstrate that 518 nt of the *Smcp* 5' flanking region directs transcription of the *Gfp* mRNA in round spermatids at the same start site and in the same cells as the natural *Smcp* mRNA, and that the timing of translational expression of GFP is regulated by the 5' UTR and 3'UTR (Hawthorne et al., 2006; Bagarova et al., 2010).

Randomization of a conserved sequence 6-38 nt upstream of the first *Smcp* poly(A) signal in the $G^5G^CS^3$-mut1 transgene resulted in a small increase in polysomal mRNA in round spermatids (Bagarova et al., 2010) implying that the major *cis*-element(s) that strongly repress translation lie elsewhere in the *Smcp* 3' UTR. Here we studied a mutation in the segment of the *Smcp* 3'UTR downstream of the upstream poly(A) signal which contains two sequences that are conserved in many species of mammals, a second downstream AAUAAA poly(A) signal and a GAGC motif between the two poly(A) signals (Chowdhury et al., 2012). We therefore replaced the sequence of the *Smcp* 3'UTR downstream of the first poly(A) signal with the corresponding sequence in the pEGFP plasmid, because the pEGFP 3'UTR results in loss of translational repression in round spermatids (Figure 2.1) (Hawthorne et al., 2006; Bagarova et al., 2010). The hypothesis behind the $G^5G^CS^3$-mut2 transgene is that this mutation abrogates the binding of a factor that represses translation.

Figure 2.1 Sequence of the 3' terminus of the natural and mutant *Smcp* 3'UTRs in transgenes. The FRGY2 YRS sequence is highlighted yellow, AAUAAA canonical poly(A) signals are highlighted grey, and poly(A) addition sites determined with 3'RACE are highlighted red (Chowdhury et al., 2012). The underlined sequence in the $G^5G^CS^3$-mut1 transgene is randomized and eliminates the CAUC element that is essential for binding YBX2 (Bagarova et al. 2010). The double underlined sequence in the $G^5G^CS^3$-mut2 transgene is derived from pEGFP plasmid (Kessler et al., 1986). Adapted from (Cullinane et al., 2014).

As noted above, transgenes containing the full-length EGFP 3'UTR, $G^5G^CG^3$ and $S^5G^CG^3$, respectively result in GFP expression after little or no delay demonstrating that the eGFP 3'UTR does not contain negative control elements that repress translation in round spermatids. The assumption underlying the $G^5G^CS^3$-mut2 transgene is that this mutation will abrogate the binding of a factor that represses translation. The plasmid was originally derived from the early SV40 tumor virus polyadenylation signal (Kessler et al., 1986). We assumed that this sequence would lack *cis*-elements because there are very few reports of *cis*-elements in the short 15-30 nt segments of 3'UTRs between the poly(A) signal and the polyadenylation site (Tian et al., 2005) , and a literature search found no reports of protein binding and effects of the early SV40 signal on post-transcriptional gene expression.

2.4.2 Developmental expression of GFP fluorescence in $S^5G^CS^3$ –mut2 testes

The developmental expression of GFP fluorescence was determined with phase contrast and fluorescence microscopy of single-cell layer squashes of short segments of living seminiferous tubules (Kotaja et al., 2004). GFP-expressing cells were identified initially by enhancing the brightness and contrast in ImageJ (Bagarova et al., 2010). In general, GFP-positive cells exhibited fluorescence throughout their nuclei and cytoplasm while the fluorescence of GFP-negative cells, pachytene spermatocytes and Sertoli cells, was not greater than background in cell-free areas.

The developmental expression of GFP fluorescence in $G^5G^CS^3$-mut2 transgenic lines was most easily analyzed in 25 and 28 dpp testes which lack intensely fluorescent elongated spermatids that can overwhelm weak fluorescence in round spermatids. GFP fluorescence was not detected in pachytene spermatocytes and was first detected in step 1 $G^5G^CS^3$-mut2 (Figure 2.2) spermatids which are distinguished by the absence of acrosomes. The levels of GFP fluorescence are noticeably higher in step 3 spermatids which are characterized by a circular acrosome with a central, dark acrosomal granule. GFP fluorescence is excluded from the acrosomes, demonstrating that the EGFP-protein is present in the general cytoplasm of step 3 spermatids.

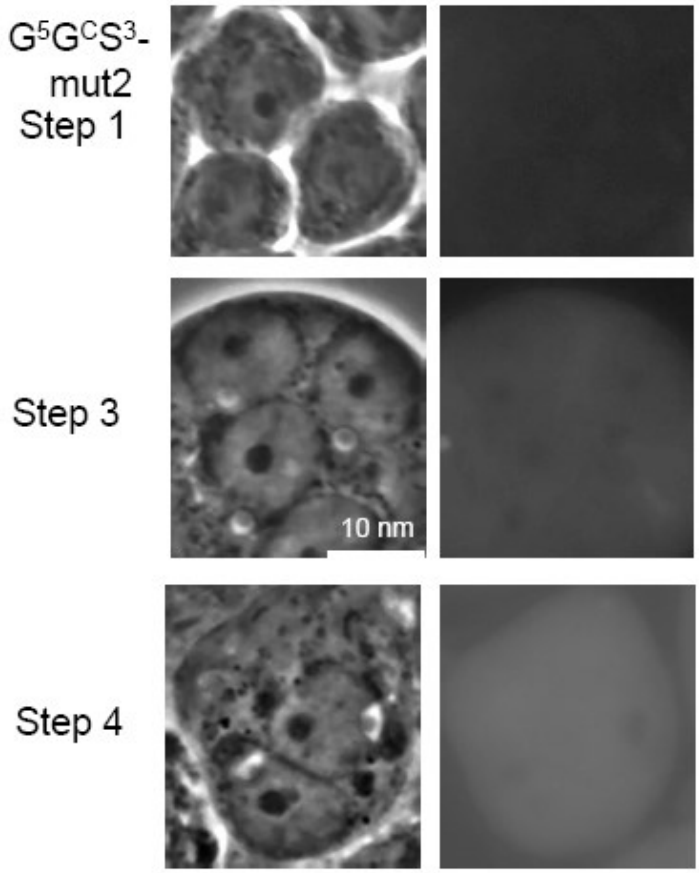

Figure 2.2 Stage of first detection of GFP fluorescence in $G^5G^CS^3$-mut2 transgenes in round spermatids. Squashes of 0.5 mm microdissected segments of seminiferous tubules were visualized with phase contrast microscopy to identify cell types and fluorescence microscopy to detect GFP expression. The contrast and brightness were enhanced to facilitate the visualization of GFP fluorescence. Step 1 spermatids are identified by the absence of an acrosome and step 3 spermatids are identified by a circular acrosome with a dark central acrosomal granule. Note the dark spots in the fluorescent images of step 3 spermatids corresponding to acrosomes. The exclusion of GFP fluorescence from the acrosome demonstrates that GFP is present in the round spermatid cytoplasm. Contrast and brightness was uniformly enhanced across all panels.

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The developmental expression of GFP fluorescence in $G^5G^CS^3$-mut2 transgenic lines was analyzed in 25 and 28 dpp testes which lack intensely fluorescent elongated spermatids that can overwhelm weak fluorescence in round spermatids. GFP fluorescence was not detected in step 1 $G^5G^CS^3$-mut2 (Figure 2.2) spermatids which are distinguished by the absence of acrosomes, and was first detected in step 2-3 spermatids which are characterized by a circular acrosome with a central, dark acrosomal granule. GFP fluorescence is excluded from the acrosomes, demonstrating that the EGFP-protein is present in the general cytoplasm of step 2-3 and 4 spermatids.

The average pixel intensities of GFP fluorescence in 10 cells were quantified with ImageJ in which phase contrast images identify the exact steps of spermatids that showed fluorescence. Student's two sided unpaired t-test was used to compare the pixel intensities of the fluorescent spermatids and the background fluorescence in pachytene spermatocytes and cell-free areas. The pixel intensities of pachytene spermatocytes were indistinguishable from those in cell free- areas. The pixel intensities of step 1 spermatids were about 10% greater than those in pachytene spermatocytes, while the pixel intensities in step 3 spermatids were about 1.9 fold greater than those of pachytene spermatocytes.

We suggest that translational repression of the $G^5G^CS^3$-mut2 mRNA in step 1 spermatids is leaky and that translation of the mRNAs is activated in step 3 spermatids.

The initial detection of GFP-fluorescence in step 3 $G^5G^CS^3$-mut2 spermatids was observed in three independent lines.

Transgene[1]	Spermatid or spermatocyte[2]	Pixel intensity, Mean ±SD[3]	Background: cell-free or spermatocyte[2]	Pixel Intensity Mean ± SD[3]	Ratio[4]	P-value[5]
Ln59 25 dpp	pachytene	21.6 ± 1.9	cell-free	21.3 ± 2.3	1.01	0.3
Ln59 25 dpp	step 1	21.1 + 1.7	pachytene	18.8 ± 1.7	1.12	0.0004
Ln59 25dpp	step 3	44.4 ± 4.8	pachytene	23.4 ± 1.9	1.89	0.0001
Ln78 28 dpp	pachytene	10.6 ± 1.5	cell-free	11.1 ± 1.3	0.95	0.29
Ln78 28 dpp	step 1	13.2 ± 1.5	pachytene	10.0 ± 1.2	1.3	0.0001
Ln78 28 dpp	step 3	16.2 ± 2.2	pachytene	8.2 ± 0.5	1.95	0.0001
Ln117 found.	pachytene	11.6 ± 1.5	cell-free	11.2 ± 1.5	1.04	0.84
Ln117 found.	step 1	11.4 ± 1.6	cell-free	9.9 ± 0.5	1.15	0.16
Ln117 found.	step 3	23.2 ± 1.6	pachytene	12.4 ± 1.3	1.87	0.0001

Table 2.1 Quantification of GFP fluorescence in $G^5G^CS^3$-mut2 spermatids and spermatocytes. Pixel intensities of adjacent spermatids, spermatocytes and cell-free areas were measured with ImageJ. The pixel intensities of various stages of meiotic cells (zygotene, pachytene, diplotene and secondary spermatocytes) and cell-free areas were virtually identical, and were assumed to be background. [1]Transgene and specific line or founder. All testes were from adult males except for two sexually immature 25 dpp and 28 dpp testes. [2]Stage of spermatid or spermatocyte measured. [3]Average and standard deviation of pixel intensities of 10 cells or cell free-areas. [4]Ratio of average pixel intensity in spermatocytes and spermatids in column 2 to that of background in column 5. [5]P-value calculated using Student's two-sided paired t-test for samples of spermatids, spermatocytes and/or cell-free areas. All lines are the $G^5G^CS^3$-mut2 transgene.

2.4.3 Sucrose and Nycodenz gradient analysis of translational activity

To determine whether the differences in developmental expression of GFP-fluorescence the of $G^5G^CS^3$-mut2 transgene represents differences in translational activity, the proportion of $G^5G^CS^3$-mut2 mRNA was analyzed by sedimentation of cytoplasmic extracts from 21 dpp mice on sucrose and Nycodenz gradients. Sucrose gradients separate free-mRNPs and polysomes by differences in sedimentation velocity

determined primarily by the number of ribosomes bound to the coding region (Arava et al., 2003; Kleene et al., 2010; Mathews et al., 2007). Nycodenz gradient analysis is a technique that separates free-mRNPs and polysomes by differences in buoyant density (Kleene et al., 2010). Analyzing translational activity with both sucrose and Nycodenz gradients is more rigorous than with either procedure alone, because different artifacts affect sedimentation velocity and buoyant density.

The gradients were collected as fractions, and quantified through RT-qPCR. The distribution of the transgenic mRNAs in each gradient was compared with those of two control mRNAs, the *Smcp* mRNA and the *Ldhc* mRNA encoding the testis-specific isoform of lactate dehydrogenase. The *Ldhc* mRNA is a control for mRNA recovery and polysome integrity because it exhibits constant polysome loading in prepubertal and adult testes (Bagarova et al., 2010; Kleene et al., 2010). In contrast, the *Smcp* mRNA sediments is almost exclusively present in free-mRNPs in round spermatids, and shows modest levels in polysomal mRNA (~35%) in adult testis (Hawthorne et al., 2006; Bagarova et al., 2010).

The $G^5G^CS^3$-mut2 mRNA exhibits high polysomal loading in 21 dpp testes in sucrose and Nycodenz gradients consistent with active translation and GFP expression (Figure 2.3). 21 dpp mice were chosen to analyze because this is a time point in spermatogenesis when endogenous *Smcp* mRNA is known to be highly repressed in the most advanced cells, step 4 spermatids (Bagarova et al., 2010; Kleene et al., 2010). Fractions 2-4 in the sucrose gradient and fractions 2-6 in the Nycodenz gradient contain substantial proportions of polysomal mRNA in 21 dpp transgenic mice, compared to endogenous *Smcp* mRNA which shows very little polysomal loading in the same

fractions (Figure 2.3). These findings support the analysis of GFP fluorescence indicating that the $G^5G^CS^3$-mut2 mRNA is prematurely active in step 3 and 4 spermatids. .

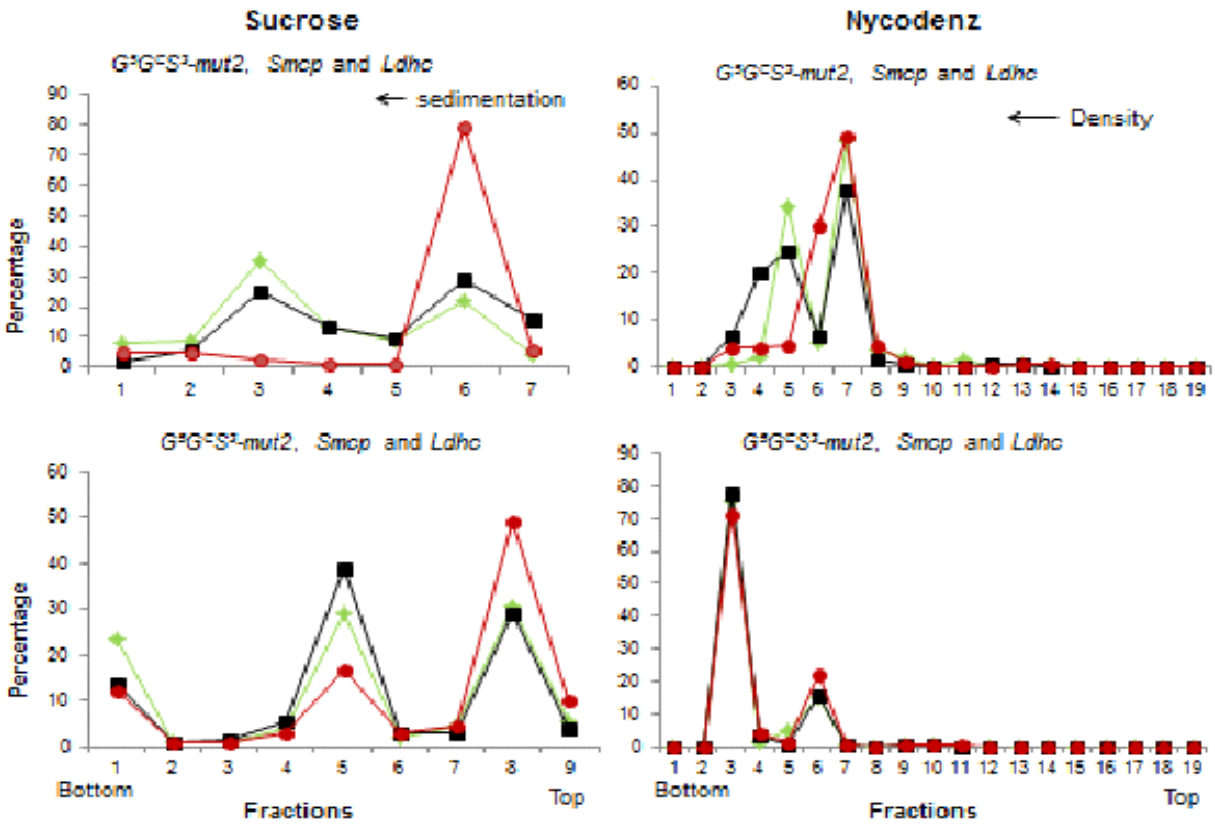


Figure 2.3 Quanitative analysis of the distribution of the $G^5G^CS^3$-mut2, *Smcp* and *Ldhc* mRNAs in the free mRNP and polysome regions of Nycodenz and sucrose gradients from 21 day old and adult $G^5G^CS^3$-mut 2 transgenic mice. Cytoplasmic extracts were sedimented on Nycodenz and sucrose gradients fractions were collected from the bottom, RNAs were extracted using techniques that recover virtually identical proportions of RNA from each fraction (Kleene et al., 2010). The results are depicted as graphs of the percentage of total RNA on the gradient in each fraction. Green lines and parallelograms depict the $G^5G^CS^3$-mut2 mRNA, red circles and lines depict the *Smcp* mRNA, and black lines and squares depict the *Ldhc* mRNA. Top gradients are 21dpp transgenic mice and bottom gradients are adult mice.

2.4.4 Proteins binding to the 3' termini of the *Prm1*, *Smcp* and $G^5G^CS^3$-mut2 3'UTRs

To figure out the trans-factors that bind the sequences we disrupted in the $G^5G^CS^3$-mut2 transgene; we utilized RNA affinity chromatography followed by mass spec sequence analysis on proteins that bound the 3' termini of endogenous *Smcp* mRNA and the $G^5G^CS^3$-mut2 construct. 5' biotinylated RNA probes were incubated with testis protein extracts, treated with heparin to reduce non-specific binding by electrostatic interactions, and protein-RNA complexes were captured with streptavidin-agarose resin (Figure 2.4).

The segment of the *Smcp* 3'UTR in lane 4 (Figure 2.4) contains a Y-box recognition sequence (YRS) so it is not that surprising that YBX2 would be one of the major proteins found in the complex. However, YBX2 binds to the 3'end of the *Smcp* 3'UTR, which is unexpected because this portion of the 3'UTR does not contain an identifiable YRS (Figure 2.4, lane6). The probe for the 3' end of the $G^5G^CS^3$-mut2 3' termini in the 3'UTR (3T3U) binds two bands at ~51 and ~52 kDa (lane 5). By mass spectrometry analysis the most abundant proteins in the ~52 kDa band are YBX2 and YBX3L, but YBX2 is only 2.8-fold more abundant than YBX3L. The most abundant protein in the ~51 kDa band is the mouse homolog of the Lupus antigen (NP_001103615.1) which binds oligo (U) sequences (Alfano et al., 2004). It is possible that the Lupus antigen protein is binding the U-rich sequence inserted into the $G^5G^CS^3$-mut2 3'UTR. YBX2 also was shown to bind a YRS in the translational control element (TCE) in the *Prm1* mRNA, a strongly regulated message (lane 2). The YRS in the TCE of *Prm1* was previously unidentified and this is the first time in 13 years that the trans-

factor for the TCE cis-element has been identified. Lane 3 displays loss of YBX2 binding with mutated YRS in the TCE for the *Prm1* mRNA.

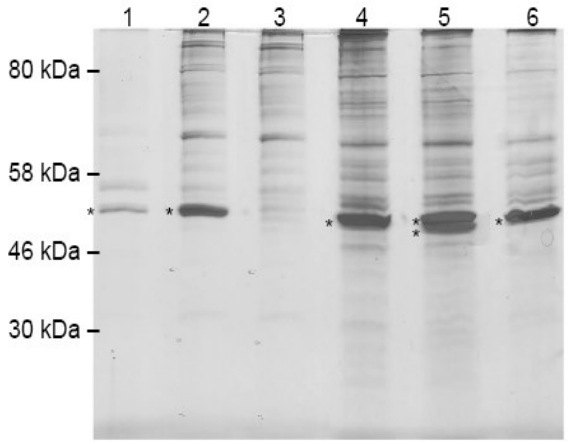


Figure 2.4 Identification of proteins binding to *Prm1*, *Smcp* and $G^5G^CS^3$-mut2 3T3Us. Using RNA affinity chromatography and mass spectrometry sequencing. Total testis cytoplasmic extract was reacted with 5'-biotinylated RNA probes with heparin, bound to streptavidin-agarose and eluted with SDS sample buffer. Proteins were resolved by SDS-PAGE, visualized with silver staining, and bands marked with asterisk were analyzed by mass spec. Red underlined sequences have been mutated. (Performed by Tamzid Chowdhury).

Lane 1= Protein extract only

Lane 2= wildtype *Prm1* TCE and poly(A) signal,
GAACAAUGCCACCUGUCAAUAAAU

Lane 3= mutated *Prm1* TCE and poly(A) signal,
GAACAAUGACGACUGUCAAUAAAU

Lane 4= *Smcp* 3'UTR YRS, AAAGGAUAGAAACAUCUUGUCUAGUGAUCCUG

Lane 5= $G^5G^CS^3$-mut2 3T3U poly(A)
UGACAUUUAGAUAGCAAAGAAAUAAAGCAUUUUUUUCACUGC

Lane 6= *Smcp* 3T3U poly(A)
UGACAUUUAGAUAGCAAAGAAAUAAAAGAGCAAAUAAA

2.5 Discussion

To date only two mRNAs that have been extensively studied with point and deletion mutations in transgenic mice to identify the *cis*-elements that mediate the initial translational repression in round spermatids. The mutant *Smcp* transgenes our lab analyzed were based on the evidence that *Prm1* negative control elements that repress translation in early spermatids only function when the elements are at the 3' terminus of the 3'UTR (Fajardo et al., 1997; Giorgini et al., 2001; Zhong et al., 2001; Soundararajan et al., 2010). There is also evidence from comparative genomics suggesting that the distal end of the *Smcp* 3'UTR is highly conserved (Kleene et al., 2006; Chowdhury et al., 2012).

The early transgenes designed by this lab identified elements in the *Smcp* 3' UTR that repress translation in early spermatids (Baragova et al., 2010). This transgene contained a randomized 39 segment 28-61 nt upstream of the poly(A) site, ($G^5G^CS^3$-mut1), a position similar to that of the *Prm1* TCE 3'UTR that repress translation in early spermatids (Giorgini et al., 2001).The $G^5G^CS^3$-mut1 produced a small release of translational repression and GFP expression could first be detected in step 4 spermatids. Levels of polysomal $G^5G^CS^3$-mut1 mRNA in sucrose and Nycodenz gradients in 21 day testes were ~10%, which is intermediate between those of the repressed *Smcp* mRNA, ~4.5%, and the translationally active $S^5G^CG^3$-no-uORF1&2 and $S^5G^CG^3$-no-uORF1,

~31%, $S^5G^CS^3$ mRNAs. Evidently, translational repression by the *Smcp* 3'UTR is primarily mediated by other segments of the *Smcp* 3'UTR (Baragova et al., 2010; Hawthorne et al., 2006; Chowdhury et al., 2012).

Here we studied a transgene, $G^5G^CS^3$-mut2, in which the 16 nt downstream of the first AAUAAA poly(A) signal in the *Smcp* 3'UTR is replaced by the 17 nt downstream of the pEGFP poly(A) signal (Figure 2.1). The segment of the *Smcp* 3' UTR we replaced, downstream of the first poly(A) signal, contains two of the most conserved sequences in the *Smcp* 3'UTR, a second AAUAAA poly(A) signal and a highly conserved GAGC sequence (Chowdhury et al., 2012).

$G^5G^CS^3$-mut2 completely abolishes the regulation of *Gfp* mRNA based on first detection of GFP in step 3 spermatids (Figure 2.2) and high levels of polysomal mRNA in sucrose and Nycodenz gradient analysis of 21 dpp testes (Figure 2.3). Note that the proportions of polysomal mRNA for *Smcp*, $G^5G^CS^3$-mut2, and *Ldhc* mRNAs in 21dpp gradients never approach the levels expected for fully active mRNAs in somatic mammalian cells, $\geq$85%, indicative of global translational repression (Kleene et al.,1998; 2013; Schmidt et al.,1999). This is especially significant in the case of the $G^5G^CS^3$-mut2 mRNA associated with polysomes in 21 dpp testis in sucrose and Nycodenz gradients, in which the mutation releases the strong mRNA-specific repression in round spermatids. However, the 50% of the $G^5G^CS^3$-mut2 mRNA in polysomes indicates that the $G^5G^CS^3$-mut2 mRNA is still partially repressed by global mechanisms (Kleene et al., 1996; 2003; 2013).

The design of the $G^5G^CS^3$-mut2 mutation was based on the evidence that the 17 nt extending from the polyadenylation signal to the poly(A) site in the EGFP plasmid is derived from the early SV40 polyadenylation signal that binds the 160 kDa subunit of the cleavage and polyadenylation stimulation factor, CPSF160, and no other proteins in somatic mammalian cells (Murthy et al., 1995). CPSF160 is the protein that binds AAUAAA poly(A) signals and has a key role in specifying the poly(A) site in all cells (Lutz et al., 2011).

The interpretation of the loss of translational repression by the $G^5G^CS^3$-mut2 transgene is influenced by studies of protein binding to the 3' termini of the *Smcp* and $G^5G^CS^3$-mut2 mRNAs through RNA affinity chromatography and mass spectrometry sequencing. This demonstrated that the 3' terminus of the *Smcp* 3'UTR binds YBX2 (Chowdhury et al., 2012). This is consistent with the hypothesis that YBX2 represses *Prm1* and *Smcp* mRNA translation based partly on evidence that depletion of YBX2 with the *Ybx2*-null mutation releases translational repression of the *Prm1* and *Smcp* mRNAs in round spermatids described in Chapter 3.

At the time we believed this data was enough to support the assumption that replacing the segment of the *Smcp* 3'UTR downstream of the first poly(A) signal with the early SV40 polyadenylation segment was only removing cis elements in the *Smcp* 3' UTR and replacing with EGFP plasmid would not allow for binding of new trans-factors. Surprisingly, we found that the early SV40 polyadenylation unit binds at least two major proteins in testis extracts. It follows that the $G^5G^CS^3$-mut2 releases repression in round spermatids is subject to multiple interpretations. Some of these proteins may be translational activators such as Lupus antigen protein and ELAV1/HuR. The La protein is

a multifunctional RNA binding protein that is necessary for early embryonic development and binds pyrimidine-rich sequences (Alfano et al., 2004) and is expressed at high levels in testis (Carter et al., 2000). The binding of the La protein to the *Smcp* 3'terminus of the 3'UTR (3T3U) raises questions whether the release of repression in the $G^5G^CS^3$-mut2 transgene results from diminished binding of YBX2 or stimulation of translation by a pathway involving La or HuR, another known translational activator.

This leaves open the possibility that SV40 polyadenylation segment may abrogate translational repression of the $G^5G^CS^3$-mut2 mRNA in round spermatids because it binds translational activators and/or eliminates translational repressors that bind the *Smcp* 3T3U. To distinguish between these possibilities would require additional RNA binding assays and mutant transgenes.

UV-crosslinking assays and RNA-pulldowns identified YBX2 as a protein that binds the 3T3U of the *Smcp* 3'UTR. This was unexpected because the *Smcp* 3T3U lacks a known YRS. However, we do know that YRSs are ill defined and other data has proven known YRSs to have degenerate sites (Giorgini, et al., 2001; Chowdhury et al., 2012). For this reason it is possible that YBX2 binds an unidentified YRS in the 3T3U of *Smcp*. Translational repression by YBX2 seems to require position near the 3' end of the mRNA based on findings that the *Prm1* YRS and the *Prm1* TCE repress translation close to the poly(A) signal (Giorgini et al., 2001; Zhong et al., 2001). In contrast, YRSs in the *Smcp* and *Prm1* 5'UTRs and 3'UTRs >34 nt upstream of the poly(A) signal do not repress translation (Giorgini et al., 2001; Zhong et al., 2001; Bagarova et al., 2010; Soundajaram et al., 2010). This positional-dependence of YRSs in the 3'UTR implies that strong repression by YBX2 and YRSs requires interactions with unidentified additional factor(s)

which potentially bind the 3' poly(A) tail, canonical or non-canonical AAUAAA polyadenylation signals or an unrecognized short, degenerate element.

It will be important in futures studies of translational repression in spermatids to identify additional YRSs and validate the functions of these YRSs by analyzing the effects of mutations that drastically reduce YBX2 binding by quantifying the duration and strength of translational repression in transgenic mice (Kleene et al., 2010; Kleene et al., 2013). Precise determination of the duration d strength of translational repression will be necessary to establish whether a mutated-YRS results in partial or complete release of repression, thereby indicating whether strong repression requires additional *cis*-elements and factors (Bagarova et al., 2010). Future experiments with the *Smcp* mRNA will need to begin with the identification of 3T3U YRSs that bind YBX2, since this segment of the 3' UTR lacks an element that conforms to the degenerate YRS, [ACGU][AC]CA[UC]C[ACU] (Giorgini et al., 2001; Chowdhury et al., 2012).

CHAPTER 3

YBX2 IS THE TRANS-ACTING FACTOR THAT BINDS THE CIS-ELEMENT IN SPERM MITOCHONDRIA-ASSOCIATED CYSTEINE-RICH PROTEIN (*Smcp*) mRNA AND REGULATES TRANSLATION

3.1 Abstract

The protamine (*Prm1*) and sperm mitochondria-associated cysteine-rich protein (*Smcp*) mRNAs exemplify a widespread phenomenon of mRNA specific developmental regulation in post meiotic spermatogenic cells. The *Prm1*and *Smcp* mRNAs are transcribed and initially stored in free-mRNPs in round spermatids and translated on polysomes in elongating and elongated spermatids. Previous work in our lab demonstrates with RNA affinity chromatography and mass spectrometry that Y-box protein 2 (YBX2/YBX2) as the major protein that interacts with the translational control element in the *Prm1* 3'UTR and the 3' terminus of the *Smcp* 3'UTR. Here we show that depletion of YBX2 protein in *Ybx2*-null mice results in premature activation of translation of the *Prm1* and *Smcp* mRNAs in round spermatids. Immunofluorescence demonstrates the localization pattern of YBX2 revealing high expression correlates with stages in spermiogenesis where many mRNAs are under strong repression, indicating that YBX2 is potentially a major repressor of the *Prm1, Smcp* and other mRNAs. Furthermore we demonstrate with the use of *in situ* hybridization that *Smcp* mRNA displays an intense

signal in the chromatoid in *Ybx2*-null mice. The chromatoid body is a germ cell specific nuage suggested to play a role in mRNA repression during spermatogenesis. Our findings suggest that the chromatoid body is not a site of repression due to the fact that in the absence of YBX2, a major repressor of the *Smcp* mRNA, the message still localizes to this granule.

3.2 Introduction

Translational regulation of specific mRNAs is an important mechanism for controlling protein expression during mammalian spermatogenesis. Transcription in late spermatids ceases due to chromatin remodeling. Therefore it is necessary for translationally regulated mRNAs to be transcribed in early spermatids and stored for a given period of time until proper activation of translation (Kleene et al., 2013; Meistrich et al., 2003). The protamine 1 (*Prm1*) and sperm-mitochondria cysteine-rich protein (*Smcp*) mRNAs clearly illustrate this phenomenon: the *Smcp* mRNA is transcribed in early spermatids, stored as translationally inactive messenger ribonucleoprotein particles (free-mRNPs) for about 6 days, before translation begins in elongating and elongated spermatids (Kleene et al., 1989).

mRNA-specific translational regulation usually involves *cis*-elements within the transcript which bind trans-factors, such as RNA binding proteins (RBPs) or small-non coding RNAs, which can either activate or repress translation (Jackson et al., 2010). The majority of RBPs and small non-coding RNAs that have been implicated as translational

regulators have been done so through the use of knockout or over expression studies. The problem with these experimental approaches is that they do not demonstrate if the factor produces a direct or indirect effect on the target mRNA (Kleene et al., 2013). Many knockouts that produce blocks in early spermatogenesis are difficult to pinpoint the specific defect in post-transcriptional regulation results from knockouts in spermatogenic cells. This is because of problems in distinguishing between mRNA targets that are regulated directly and indirectly by a given factor. Many factors regulate groups of target mRNAs directly, but some of the direct mRNA targets encode factors that regulate secondary targets, creating confusion as to whether the effect of the knockout is direct or indirect. This problem is amplified by the interactions of factors with very large numbers of mRNAs and functions in multiple post-transcriptional processes (Kleene et al., 2013). The functions of *trans*-factors must be identified using transgenic mice that have specific mutations in the target transcript that abrogate binding of the RBP; only then can it be determined as a direct effect. Previous studies using the GFP reporter indicate that both the 5' and 3'UTRs are necessary for the complete 6 day repression of *Smcp* mRNA (Hawthorne et al., 2006; Bagarova et al., 2010). The critical *cis*-elements and corresponding factors that repress *Smcp* mRNA until step 11 spermatids have yet to be identified.

Translational repression of many mRNAs in spermatids has been proposed to be imposed a family of RNA binding proteins called Y-box proteins (Tafuri et al., 1993; Kwon et al., 1993; Yang et al., 2005; Giorgini et al., 2002). The mammalian genome contains three genes encoding four isoforms of Y-box proteins (Mastrangelo et al., 2000;

Kleene et al., 2013). The members of the Y-box protein family are distinguished by an alanine- and proline-rich N-terminal segment, a central, highly conserved ~70 amino acid cold shock domain and a variable carboxy-terminal segment consisting of alternating ~30 amino acid clusters rich in basic-aromatic and acidic amino acids (reviewed in Matsumoto et al., 1998; Skabkin et al., 2006 ; Eliseeva et al., 2011). The three genes are named *Ybx1*, *Ybx2*, and *Ybx3,* the last of which is expressed as two alternatively spliced mRNAs encoding two isoforms of different size (Mastrangelo et al., 2000; Davies et al., 2000) . All four Y-box protein isoforms are expressed in mouse spermatids.

There are several reasons for believing that YBX2 represses mRNA translation in spermatids. First, Y-box proteins have been demonstrated to repress mRNA translation in somatic mammalian cells and *Xenopus* oocytes (Skabkin et al., 2006; Matsumoto et al., 1996; Eliseeva et al., 2011; Lyabin et al., 2011; Giorgini et al. 2001; 2002). Second, a Y-box recognition sequence (YRS) in the *Prm1* 3'UTR in an abnormal position represses translation in round spermatids and a mutation that abrogates binding releases the repression (Giorgini et al., 2001). Recently our lab has identified a YRS in the *Smcp* 3'UTR, that weakly represses translation in round spermatids (Bagarova et al., 2010). Here we demonstrate that this YBX2 binding has an effect *in vivo* through the use of analyzing sucrose gradients on *Ybx2*$^{-/-}$ mice. We also show that the *Smcp* mRNA localizes to the chromatoid body in the absence of YBX2. The chromatoid body is composed of thin filaments that are consolidated into branching strands of varying thickness that form dense irregular networks (Parvinen et al., 2005). The fibrous moiety of the chromatoid body in round spermatids, referred to as the stroma, is electron dense,

and usually surrounds small less dense non-fibrous areas, referred to as lacunae, which appear to contain the same material as the surrounding cytoplasm (Oko et al., 1996;Yokota et al., 2008). The observation that the *Smcp* mRNA localizes to the chromatoid body in the absence of YBX2 is important because it conflicts with the prevailing idea that the chromatoid body is a site of prolonged storage of repressed mRNAs (Parvinen et al., 2005; Kotaja et al., 2006; Meikar et al., 2011) .

3.3 Material and Methods

3.3.1 Construction of *Msy2* null mice

We obtained two female *Ybx2*$^{+/-}$ heterozygotes from Richard Schultz at the University of Pennsylvania Medical School, which we bred to produce *Ybx2*$^{-/-}$ knockout males. A detailed description of generation of the construct can be found in (Yang et al., 2005). Briefly, the *Ybx2* $^{-/-}$ targeting construct was produced by using a 129S6/SvEv mouse genomic library that is isogenic with the AB2.2 ES cells used for electroporation. ES cell clones were electroporated, selected, and screened by Southern blotting.

3.3.2 Genotyping

DNA from tail biopsies was purified with a DNAeasy Blood and Tissue kit (Qiagen). The presence of wide type and knockout alleles was assayed by PCR using specific primers. Program: 94°C for 2 min, 94°C-50 sec, 57°C-50 sec, 7°2C-50 sec, (27 cycles), 72°C-7 min.

One set of primers detects wild-type *Ybx2* alleles in wildtype (+/+) and heterozygote (+/):

Forward: 5'-GGA GGG AGA AGG GGA CAT T-3'

Reverse: 5'-GCA GAA CAG GAT GGG TTG TT-3'

A second set of primers detects knockout Ybx2 alleles in heterozygous (+/-) and knockouts (-/-):

Forward: 5'-TTT GTA CTT TAG AAA TGT CAG TTG CT-3'

Reverse: 5'-GCA GAA CAG GAT GGG TTG TT-3'

PCR products corresponding to wildtype and knock-out *Ybx2* alleles, respectively ~350 and 650 nt were distinguished with agarose gel electrophoresis.

3.3.3 Sucrose Gradient Analysis

Cytoplasmic extracts of 21/25 dpp and adult testes were prepared by dissecting testes (1 testis for adult mouse and 2 testes for 25 day mice), removing the *tunica albuginea* and homogenizing the testes in 300 µl HNM buffer (20 mM HEPES, pH 7.4, 0.1 M NaCl, 3 mM $MgCl_2$) containing 0.5% Triton X100 and 1 unit/µl RNasin Plus (Promega Biotech). The nuclei were pelleted by centrifugation at 13,000 X g for 2 minutes, and 250 µl of the supernatant was layered on a 3.8 ml linear 15-40% sucrose gradient in HNM buffer (w/w) in polyallomer centrifuge tubes for the Beckman SW60 rotor. Sucrose gradients were centrifuged for 80 min at 35,000 rpm at 4°C, and ~0.4 ml fractions were collected onto 0.3 g guanine thiocyanate, and RNA was extracted as described previously (Kleene et al., 2010).

3.3.4 RNA affinity chromatography of proteins binding to the 3' terminus of the *Smcp* 3'UTR

To identify proteins that bind the 3'termini of the *Smcp* mRNA 3'UTR, 5' biotinylated RNA probes were incubated with testis protein extracts, treated with heparin to reduce non-specific binding by electrostatic interactions, and protein-RNA complexes were captured with streptavidin –agarose resin. After extensive washing, the bound proteins were eluted in SDS sample buffer, resolved by SDS-PAGE, and unique bands were identified by mass spec sequencing.

3.3.5 Immunocytochemistry and RNA-FISH

Stage 2-6 seminiferous tubule segments were identified with transillumination and dissected in DEPC-treated PBS, mechanically dispersed in DEPC-treated 100mM sucrose and fixed and spread as dried down preparations on slides dipped in 0.05% Triton-X-100 and freshly prepared 4% formaldehyde (EM Sciences, Hatfield, PA, USA) (Kotaja et al., 2004). For RNA-FISH, 20nt oligo probe sets for *Smcp* coding region were selected with the Stellaris Probe Designer at the Biosearch Technologies (Petaluma, CA) website. The probe set consists of 24-48 oligos, were tagged with Quasar 570. Cells were permeabilized with 70% ethanol at room temperature (RT) for 1 hour, rinsed twice in wash buffer (2X SSC, 10% deionized formamide (Ambion AM 9342, Austin TX, USA) 5min at 37° C with a 1:50 dilution in hybridization buffer (10% dextran sulfate (Sigma-Aldrich D8906) in wash buffer). After hybridization slides were washed 3X for 30min at 37 °C in wash buffer, rinsed with DEPC-treated PBS and mounted in Prolong Gold Antifade reagent with DAPI (Life Technologies P36931, Bedford, MA).

For immunocytochemistry , dried down preparations were rehydrated in PBS, blocked with 10% normal goat serum (S-1000, Vector Laboratories, Youngtown, OH) for 20min at RT, washed with DEPC PBS, and incubated with rabbit polyclonal antibody to mouse vasa homologue MVH (Abcam, Eugene, OR, ab13840, 1:200, overnight at 4° C), or affinity purified rabbit polyclonal to Y-box protein 2, YBX2 (Yu et al., 2003)(1:200, 1 hr, RT), washed with PBS, reacted with goat anti-rabbit secondary antibody (Alexa Fluor 488, A11008) or 594 (A11037) (1:500, 30 min, RT), washed in PBS, and mounted and counterstained with DAPI as described above. Cells were photographed with an Olympus BX51 microscope 100X panfluorite objective equipped with Olympus filters, U-N31000 (excitation 360 emission 460) and U-N31004 (excitation 560 emission 630), or scanned at 0.8μm Zeiss LSM 510 confocal microscope equipped with Zeiss Planapo 63x NA oil objective. The wavelengths (nm) used for the confocal excitation and emissions of the fluors in this study follow: DAPI, 405 and 460; Quasar 570, 547 and 570; Quasar 670, 644 and 670; Alexa Fluor488, 488 and 525; Alexa Fluor 594, 594 and 617.

3.4 Results

3.4.1 Stage Specific Expression of YBX2

The developmental expression of YBX2 has been previously analyzed with immunohistochemistry using an antibody to the *Xenopus laevis* orthologue of YBX2, known as P48/52 or FRGY2 (Oko et al., 1996). Since that antibody is no longer available, and an affinity-purified polyclonal antibody to recombinant mouse YBX2 has been prepared (Yu et al., 2002) , we re-examined the developmental expression of YBX2 in formaldehyde-fixed, paraffin embedded adult mouse testis.

Immunohistochemistry reveals that the levels of YBX2 are very high in the cytoplasm of late pachytene spermatocytes and round spermatids, and that the levels progressively decrease in elongating spermatids eventually becoming undetectable in step 14 elongated spermatids (Figures 1.1A-C). YBX2 is virtually undetectable in spermatogonia at the periphery of the tubules and interstitial cells between the tubules (Figure 1.1A). YBX2 is uniformly distributed without localization n the cytoplasm of pachytene spermatocytes and round spermatids and the levels are low or undetectable levels in the nuclei of pachytene spermatocytes and round spermatids. All of these findings agree with the previous study of (Oko et al., 1996). The high levels of YBX2 in pachytene spermatocytes and round spermatids correlate with the period in which many mRNAs are translationally repressed, and the decreasing levels of Y-box proteins correlate with the delayed activation of translation of many mRNAs in elongating and elongated spermatids (Chowdhury et al., 2012). This data is consistent with the idea that YBX2 is a major repressor of *Prm1* and *Smcp* mRNA translation. As documented below, the specificity of (Yu et al., 2002)YBX2 antibody is further validated by evidence that the *Ybx2*-null mutation abrogates YBX2 detection.

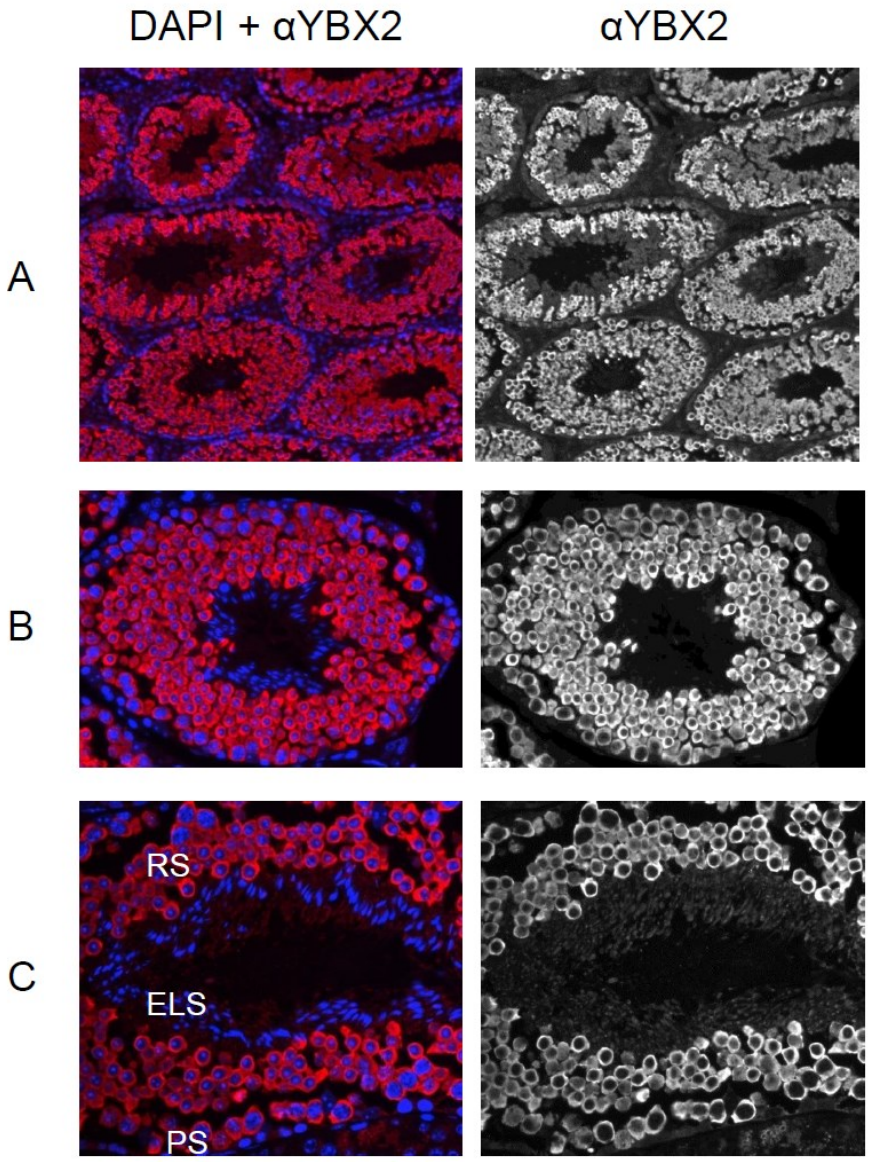


Figure 3.1 Expression of YBX2 in adult testis. Formalin-fixed, paraffin-embedded mouse testis was stained with an antibody against YBX2. YBX2 is expressed at high levels in the cytoplasm of pachytene spermatocytes (PS) and round spermatids (TRS) and the levels decrease progressively as elongating and elongated spermatids (ELS) move closer to the lumen.YBX2 is not detectable in interstitial cells, spermatogonia, or in very late elongated spermatids. The preparations were counterstained with DAPI to visualize nuclei. Panel A, 100 X magnifications, panels B & C, 400 X magnifications.

An analysis was also performed on the developmental expression of YBX1, a Y-box protein isoform that is known to be expressed in testis from northern and western blots (Tafuri et al., 1993; Mastrangelo et al., 2000; Lu et al., 2006). However, the levels of expression of YBX1 in various cell types in testis have never been analyzed in testis. Figure 3.2 shows that YBX1 is highly expressed in interstitial cells, and is expressed at lower, constant levels in all stages of spermatogenic cells. In all somatic and male germ cells, the levels of YBX1 are highest in the cytoplasm, but faint staining of nuclei also seems apparent. Clearly, the patterns of expression of YBX2 and YBX1 in testis differ, and the constant levels of YBX1 expression in all spermatogenic cells reduce its potential significance in the developmental regulation of mRNA translation in spermatocytes and spermatids.

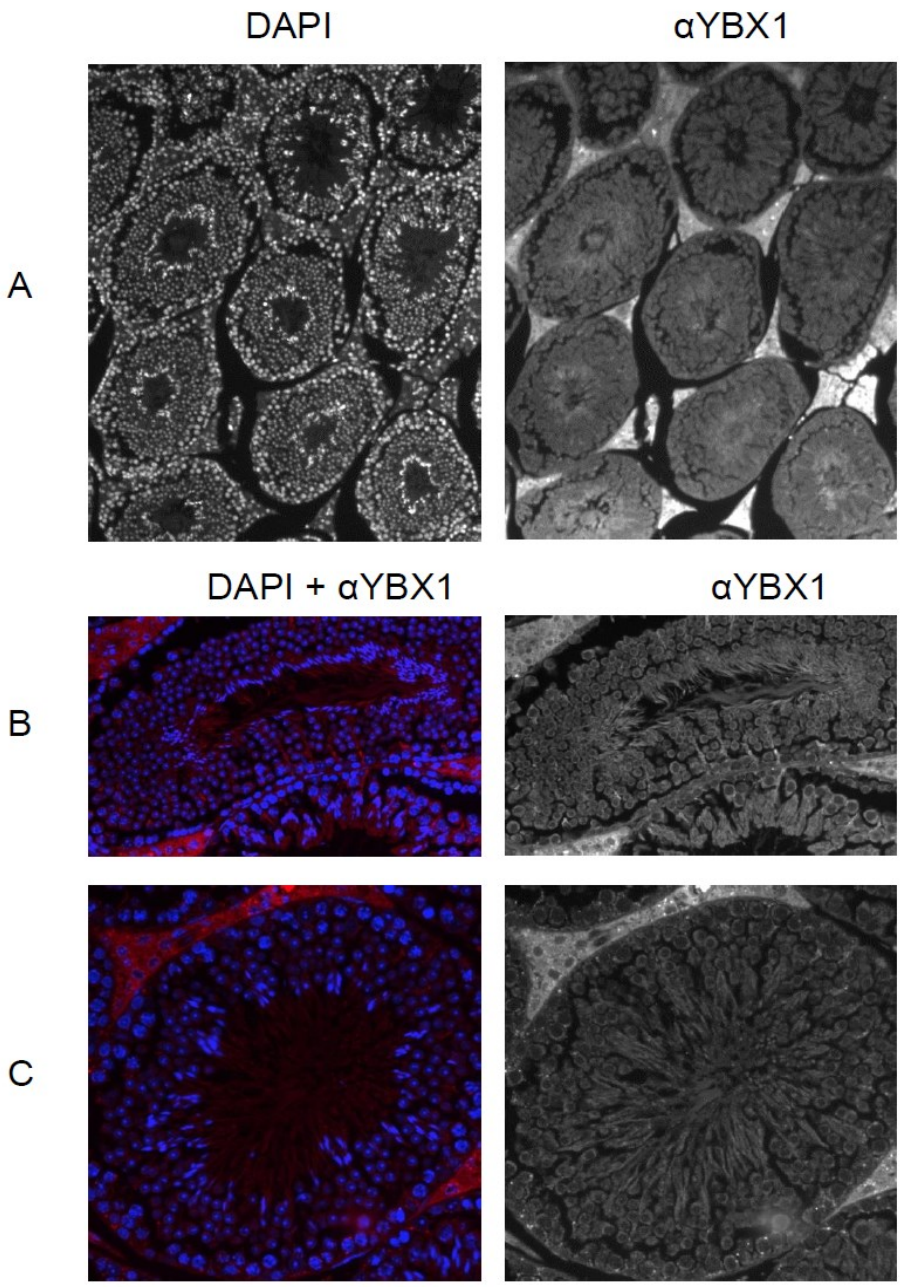


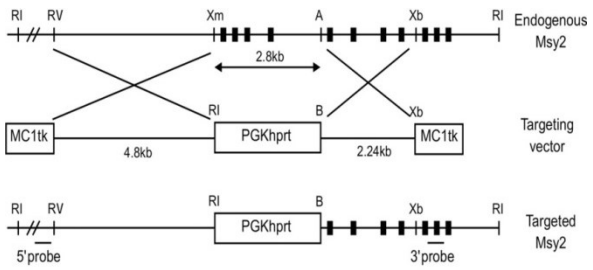
Figure 3.2 Expression of YBX1 in adult testis. Formalin-fixed, paraffin-embedded mouse testis was stained with an antibody against YBX1. The shape and size of nuclei were established by counter staining with DAPI.YBX1 is expressed at high levels in the

cytoplasm of interstitial cells and at lower levels in the cytoplasm of all stages of spermatogenic cells. Panel A, 100X magnification, panels B & C, 400 X magnifications.

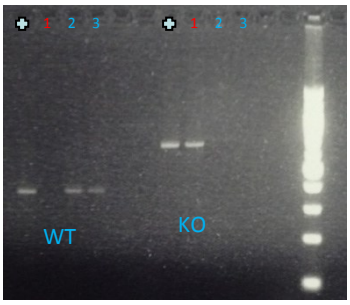
3.4.2 Construction and identification of YBX2 null mice

To determine the role of YBX2 in the repression of translation of the *Prm1 Smcp* mRNAs, we obtained *Ybx2*$^{+/-}$ female mice from Richard Schultz at the University of Pennsylvania medical school (Figure 3.3A). For a complete description on how the *Ybx2*-knockout mice were created please refer to (Yang et al., 2005). Heterozygous female were bred to produce *Ybx2*-null males. The gel shown in (Figure 3.3B) displays two PCR reactions that were analyzed on one agarose gel. The left right side of the gel in (Figure 3.3B) uses primers to identify the wildtype allele which is present in *Ybx2*$^{+/+}$ and *Ybx2* $^{+/-}$ mice. The second lane, titled lane 1 displays no band when targeting the *Ybx2* gene in this male mouse. The left side of the gel in (Figure 3.3B) is the same mouse run with the knockout primers displays a strong band at ~650bp, indicating that this mouse is null for the *YBX2* gene. Using a rabbit polyclonal antibody for YBX2 we were able to ensure loss of protein in the YBX2-null mice in round spermatids (Figure3.3C).

3A.



3B.



3C.

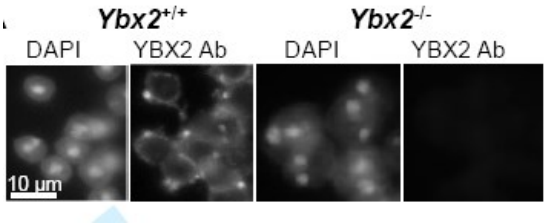


Figure 3.3 Creation and identification of *Ybx2*$^{-/-}$ mice. Figure 3A. Strategy for targeted disruption of the *Ybx2* gene. Exons are represented by vertical bars, and introns are represented by intervening horizontal lines. Exons 1, 2, 2′, and 3 and flanking region were replaced by homologous recombination with a hypoxanthine phosphoribosyl transferase (*Hprt*) gene driven by the *Pgk* promoter. Restriction sites: A, *Apa*I; B, *Bam*HI; RI, *Eco*RI; RV, *Eco*RV; Xb, *Xba*I; and Xm, *Xma*I Adapted from (Yang et al., 2005 PNAS). Figure 3B Identification of a male *YBX2* null Adapted from (Yang et al., 2005). This gel displays DNA from 3 male mice, the right side of the gel depicts the results observed with the wildtype primers ~350-400bp. Lane 1 control, Lane 2 mouse 1, Lane 3 mouse 2, Lane 4 mouse 3. The left side of the gel depicts the results observed with the knockout primers ~650 bp. Lane1 control, Lane 2 mouse 1, Lane 3 mouse 2, Lane 4 mouse 3. Note the absence of a wildtype band for mouse 1 on the right side of the gel and the presence of the knockout band on the left side of the gel for mouse 1. Figure 3C displays staining with a rabbit polyclonal antibody YBX2 in round spermatids on dried down preparations. The left panel is YBX2 on wildtype mice and the right displays loss of signal on *Ybx2*$^{-/-}$ mice.

3.4.3 Depletion of YBX2 results in premature recruitment of *Smcp* and *Prm1* mRNAs onto polysomes in round spermatids

Findings that that YBX2 is the predominant protein that binds the *Prm1* TCE and *Smcp* 3T3U (Chowdhury and Kleene, unpublished), suggest that depletion of this protein may lead to premature activation of translation of *Smcp* mRNA in round spermatids. The translational activity of *Prm1* and *Smcp* mRNAs was analyzed with sucrose gradients in testis extracts from 25dpp *Yb x2*$^{+/+}$, and *Ybx2*$^{-/-}$ prepubertal mice. This is an age of male mice when the most advanced cells in the testis are step 9 spermatids, a time point when *Prm1* and *Smcp* mRNAs *are* still highly repressed (Braun et al., 1989; Fajardo et al., 1997; Kleene et al., 2010). (Figure 3.4) reveals the absence of peaks of the *Prm1* and *Smcp* mRNAs in polysomes in *Ybx2*$^{+/+}$ 25 dpp testis, this finding is consistent with strong repression of both messenger RNAs. The *Prm1* and *Smcp* mRNAs in the *Ybx2*$^{-/-}$ testes display a clear peak in the polysomal fractions in sucrose gradient, indicating an activation of translation in the absence of YBX2 (Figure 3.4). The high level of polysomal *Ldhc* mRNA in both gradients validates the integrity of the polysomes and suggests that YBX2 is not an important repressor of the *Ldhc* mRNA. The *Ybx2*$^{+/-}$ testis show a smaller peak in the polysomal fraction with *Smcp* mRNA, suggesting that that there is a slight increase in activation of translation when levels of YBX2 are halved (Figure 3.4).

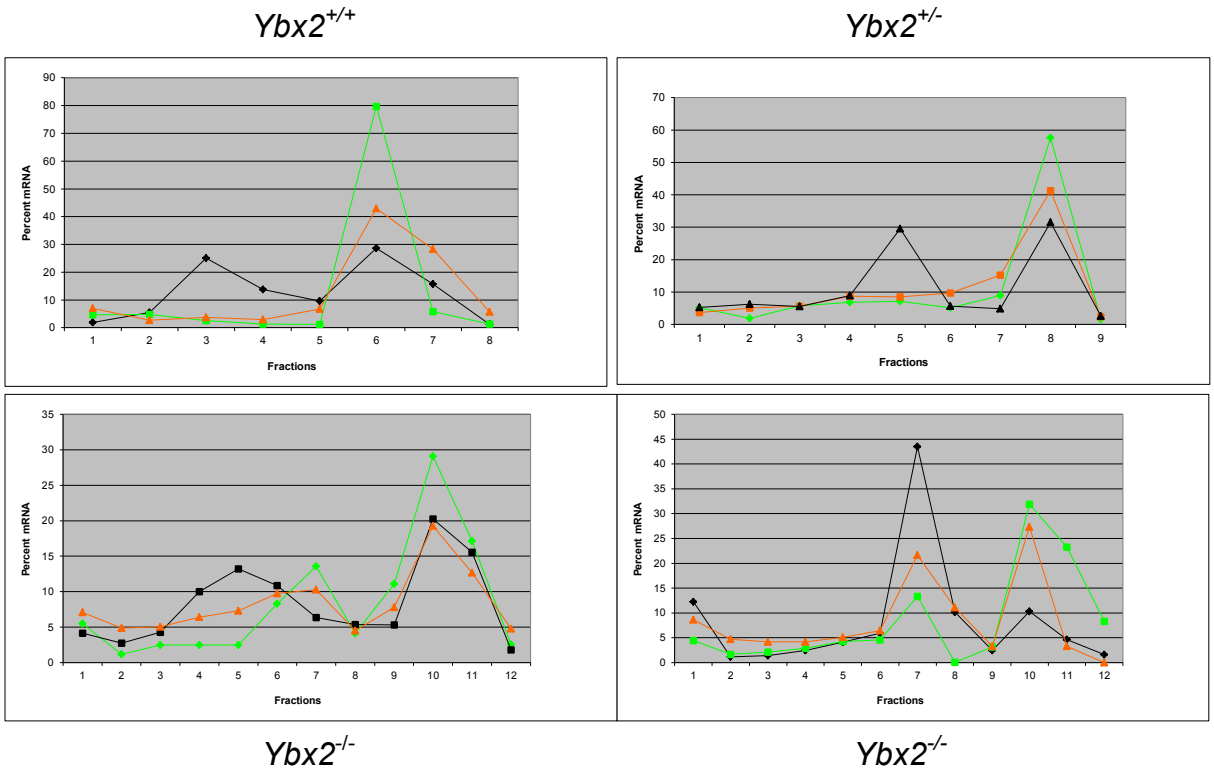


Figure 3.4 Sucrose gradient analysis of the *Prm1*, *Smcp* and *Ldhc* mRNAs in 25 dpp *Ybx2*$^{+/+}$, *Ybx2*$^{+/-}$ and *Ybx2*$^{-/-}$ testes. Cytoplasmic extracts of *YBX2*$^{+/+}$ and *YBX2*$^{-/-}$ 25dpp testes were sedimented on sucrose gradients, the gradients were fractionated and the relative levels of *Prm1*, *Smcp* and *Ldhc* mRNAs in each fraction were analyzed with RT-qPCR, and the percent total mRNA in each gradient was calculated. Both mRNAs exhibit bimodal distribution: free-mRNPs sediment in fractions ~8-12 and polysomal mRNPs sediment in fractions ~2-6 near the bottom.

3.4.4 *Smcp* mRNA localizes to the chromatoid body in the absence of YBX2

The chromatoid body, like other RNP granules, is proposed to have an important function in post-transcriptional gene regulation (Yokota et al., 2008; Meikar et al., 2011). Much of the evidence that the chromatoid body plays a role in storage and degradation of mRNAs comes from immunocytochemical and mass spectrometry sequencing evidence that many proteins which localize in the chromatoid body function in RNA metabolism

(Kotaja et al., 2006; Tanaka et al., 2011; Meikar et al., 2014). This proposed mechanism of storage in the chromatoid body lead to the investigation of *Smcp* mRNA localization to this form of nuage in the absence of YBX2. Previous data from this lab observed *Smcp* mRNA localizing to the chromatoid body as well as YBX2 in wildtype mice (Kleene et al., 2011) (Figure 3.5C). To investigate if YBX2 was binding the *Smcp* mRNA and sequestering the transcript in the chromatoid body away from ribosomes until proper activation of translation, we followed an *in situ* hybridization protocol in which 27 20 nt oligonucleotides, each labeled with a fluorochrome, tiled along *Smcp* coding region. This protocol allows for high sensitivity by virtue of excellent probe penetration and extensive target coverage (Raj et al., 2008). Confocal microscopy detected intense *in situ* hybridization signals in the chromatoid body in dried down preparations from stages II-VI seminiferous tubules in $YBX2^{+/+}$ and $YBX2^{-/-}$ mice (Figure 3.5). The *Smcp* mRNA RNA-FISH colocalizes with immunocytochemical staining of mouse vasa homolog, MVH/DDX4 (Figure 3.5A and B) an established marker for the chromatoid body (Kotaja et al., 2006; Onohara et al., 2010; Meikar et al., 2014).

We were able to show that *Smcp* mRNA does localize to the chromatoid body in the absence of YBX2. This data indicates that the chromatoid body is independent of translational activity, and that YBX2 is not repressing the *Smcp* mRNA inside the chromatoid body because regardless of whether YBX2 is absent or present *Smcp* mRNA still localizes to the chromatoid body.

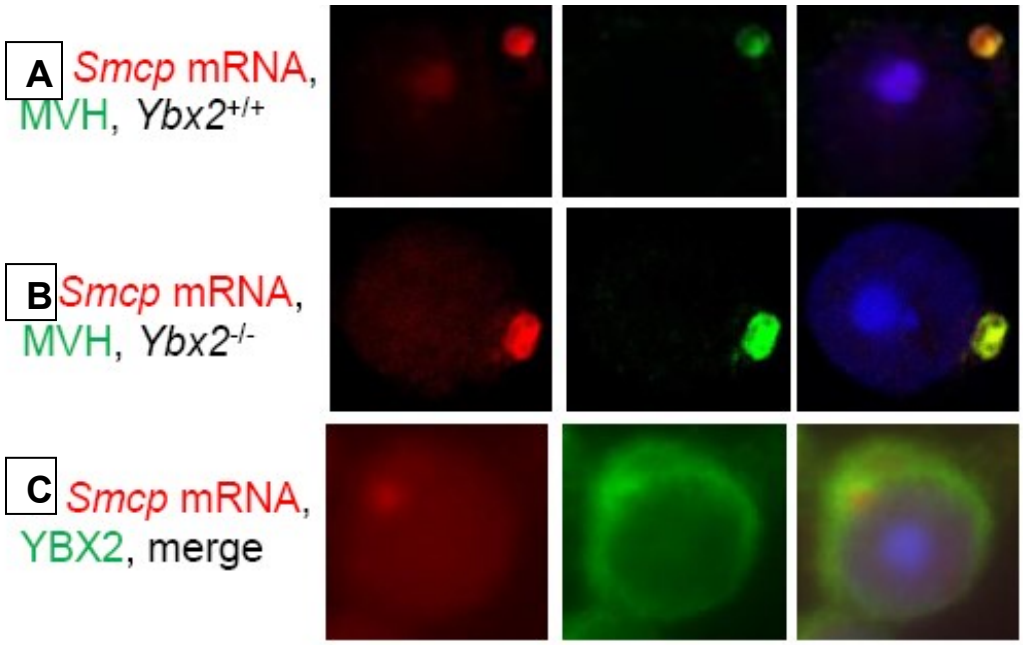


Figure 3.5 Localization of the *Smcp* mRNA in *Ybx2*$^{+/+}$ and *Ybx2*$^{-/-}$ mice. RNA-FISH analysis of the localization of *Smcp* mRNA in the chromatoid body in *YBX2*+/+ and *YBX2*-/- mice. Probe set for *Smcp* coding region were hybridized to dried-down cells from stages II-VI seminiferous tubules from adult testes. RNA-Fish was followed by immunocytochemistry with antibody to MVH/DDX4 or YBX2. The RNA-FISH hybridization was detected by confocal microscopy (Fig.3. 5A and 3.5B) or conventional fluorescence microscopy (3.5C) All images were counterstained with DAPI to display the nuclei of round spermatids. Adapted from (Cullinane et al., 2014).

3.5 Discussion

These observations show that YBX2 binds the 3' terminus of the *Smcp* 3'UTR (Chapter 2, Figure 2.4), thereby causing an effect on the regulation of translation of the transcript *in vitro and in vivo*. Sucrose gradient analysis clearly displayed an increase in polysomal loading with the *Smcp* and *Prm1* mRNAs in *Ybx2*$^{-/-}$, when compared to *Ybx2*$^{+/+}$ and *Ybx2*$^{+/-}$ mice at 25 dpp (Figure 3.4). The inference that YBX2 is a specific repressor of the *Smcp* mRNA agrees with reports in the literature that Y-box proteins are

sequence-specific effectors of translational repression (Giorgini et al., 2001; Matsumoto et al., 1996; Skabkin et al., 2006). Reports that YBX2 is the predominant RBP in free-mRNPs in testis tissue also supports the idea that YBX2 is a major translational regulator (Herbert et al., 1999). The critical experiments that need to be performed are identification of YRSs in the 3'terminin of the 3'UTR of various mRNAs. The *Smcp* 3T3U lacks elements that can be recognized in the degenerate YRS, [ACGU][AC]CA[UC]C[ACU] (Chowdhury et al., 2012; Giorgini et al., 2001).

YBX3 and YBX2 are both expressed in a stage specific manner. However *Ybx3*-null mice are fertile indicating that YBX2 is the major regulator in spermatogenesis (Davies et al., 2000; Giorgini et al., 2002). YBX1 was shown to be expressed at fairly even levels throughout all of spermatogenesis (Figure 3.2). As mentioned earlier it is not a surprising finding that YBX1 is uniformly expressed given all the reports stating all the multi-functions of the protein (Skabkin et al., 2006; Eliseeva et al., 2011). In contrast to YBX1, the levels of YBX2 are very high in round spermatids and gradually decrease in elongating and elongated spermatids (Figure 3.1) and this correlates with the repression and activation of many mRNAs (Oko et al., 1996).

Reports in the literature that YBX2 localized to the chromatoid body as well as the cytoplasm in round spermatids (Oko et al., 1996), led us to hypothesize that YBX2 might be the factor that causes repression in the chromatoid body. As mentioned above the function of this germ cell specific granule has remained an enigmatic over the years and the *Ybx2*$^{-/-}$ construct made it possible to attempt to assign function to the chromatoid, at least concerning the *Smcp* mRNA. (Figure 3.5B) clearly demonstrates *Smcp* mRNA

localization to the chromatoid body in the absence of YBX2. This granule was identified as the chromatoid body by colocalizing to MVH, a well documented marker for the chromatoid body. This infers that the *Smcp* mRNA is not repressed in the chromatoid body because preceding figures showed early activation of translation in the absence of YBX2. It is evident that localization in the chromatoid body is not sufficient for prolonged translational repression, and sucrose gradient analysis suggests that free-mRNPs as well as polysomes are present in the general cytoplasm (Kleene et al., 2011). There have been reports in the literature showing *in situ* hybridization of *Tnp2* and *Prm2* mRNAs in both the chromatoid body and cytoplasm in round spermatids (Saunders et al., 1992; Fukuda et al., 2013), indicating these mRNAs are not stored in the chromatoid body for prolonged periods of time. Also supporting these data; RNAseq studies that have purified active and repressed mRNAs from the chromatoid body (Meikar et al., 2014). Taken together this data indicates that the major role of the chromatoid body is not storing repressed messages and will be discussed in greater detail in the following chapter.

It is possible to speculate that the *Smcp* mRNA is transcribed and immediately moved into the chromatoid body where YBX2 first binds to the 3'UTR, then exported to the cytoplasm as a free-mRNP until proper activation of translation. The next chapter will discuss in detail developmental localization of the *Smcp* mRNA and provide a theoretical model.

CHAPTER 4

DEVELOPMENTAL LOCALIZATION OF THE SPERM MITOCHONDRIA-ASSOCIATED CYSTEINE-RICH PROTEIN mRNA

4.1 Abstract

The chromatoid body is a dynamic organelle that is thought to coordinate the cytoplasmic regulation of mRNA translation and degradation in mammalian spermatids. The chromatoid body is also postulated to function in repression of mRNA translation by sequestering dormant mRNAs where they are inaccessible to the translational apparatus. The goal of this study is to determine if RNA-FISH can detect regulated as well as non-regulated mRNAs in the chromatoid body to resolve once and for all the argument that the chromatoid body is solely a site for storage for repressed mRNAs.

We were able to accomplish this by designing probes complementary for two endogenous mRNAs in spermatogenesis, *Smcp* and *Ldhc*, as well as designing a *Gfp* probe for use on the testes of two well characterized transgenic mouse lines in our lab ($S^5G^CS^3$ and $G^5G^CS^3$-mut2). The intense RNA-FISH staining of the translationally active *Ldhc* and $G^5G^CS^3$-mut2 mRNAs and the repressed *Smcp and* $S^5G^CS^3$ mRNAs in the chromatoid body suggests that localization in the chromatoid body is independent of translational activity. Furthermore, all probes detected a dull cytoplasmic signal, indicating that these mRNAs are not stored in the chromatoid body for prolonged periods

of time. Unexpectedly, a probe designed for the *Smcp* intron also displayed an intense RNA-FISH signal coming from the chromatoid body, uncovering new insights into the function of this enigmatic germ cell granule.

4.2 Introduction

It is generally accepted that specific mRNAs are transcribed and this is temporally disconnected with protein synthesis in male germ cells (Kleene et al., 1996). Late-stage specific protein synthesis relies on the appropriate storage of translationally inactive mRNAs in spermatids (Nguyen-Chi et al., 2009). The chromatoid body has been proposed to be a site of storage or mRNA processing for repressed mRNAs, which has implications for control of mRNA translation. The chromatoid body is a male germ cell specific nuage, composed of thin filaments that are consolidated into dense strands of varying thickness that branch to form an irregular network (Parvinen et al., 2005). The fibrous portion of the chromatoid body is electron dense, is not surrounded by a membrane, and is frequently interspersed with small vesicles (Yokota et al., 2008). A less compact form of nuage consisting of many scattered pieces interspersed with mitochondria, which is known as the intermitochondrial cement, first appears in late pachytene spermatocytes and disperses during the first meiotic division. The small pieces of nuage condense to its final compact shape in post-meiotic round spermatids. The chromatoid body migrates towards the caudal pole of the nucleus of early elongating spermatids and forms a ring around the base of the flagellum then moves in front of the mitochondria and eventually disappears (Kotaja et al., 2007).

There are several ideas for the function of the chromatoid body. One hypothesis on the function of the chromatoid body is that it is a site where repressed mRNAs are sequestered in the cytoplasm apart from ribosomes as a mechanism of translational repression. However, there are many new emerging theories as to what role the chromatoid body may be playing in spermatogenesis; it is proposed as a site for processing of reproductive cell-specific RNAs in male germ cells (Kotaja et al., 2007), or it is also possible that miRNA and RNA-decay pathways converge to the chromatoid body. This could mean that chromatoid bodies have an analogous function to P-bodies in somatic cells (Kotaja et al., 2007). Much of the evidence that the chromatoid body plays a role in storage and degradation of transcripts comes from immunofluorescence assays, many different RNA binding proteins (RBPs) and small non-coding RNAs have been shown to localize to the chromatoid, leaving the function up to broad speculation. In 2011, our lab published a review stating we believed the chromatoid body to be a remodeling center, where mRNAs enter the chromatoid body immediately after transcription, change RBPs or small non-coding RNAs, and exit the nuage for prolonged storage in the cytoplasm (Kleene et al., 2011). This function would be analogous to P-granules in *C. elegans* (Sheth et al., 2010). More recently (Meikar et al., 2014) have purified the chromatoid body and found the majority of transcripts in spermatogenesis can be detected in the chromatoid body, further supporting our hypothesis.

This study aims to claify the function of the chromatoid body by following localization patterns of different mRNAs throughout spermatogenesis. The sperm mitochondria-associated cysteine rich protein (*Smcp)* mRNA is transcribed in early

spermatids, stored as translationally inactive messenger ribonucleoprotein particles (free-mRNPs) for several days to a week before translation is activated in transcriptionally inactive late haploid cells, elongated spermatids (Kleene et al., 1989; 2003; 2013). Repression of mRNA translation in round spermatids is necessary for normal sperm development because premature activation of translation of many mRNAs in round spermatids in transgenic mice decreases male fertility (Lee et al., 1995; Tseden et al., 2007). As mentioned previously, the chromatoid body is believed to contain repressed mRNAs; therefore following *Smcp* localization throughout spermatogenesis could reveal if/or how long *Smcp* mRNA remains localized in the chromatoid body. Our lab also implements the use of well-characterized transgenes to determine if regulated as well as non-regulated mRNAs can be detected in the chromatoid body. This study revealed a few novel findings. To begin with, this is the first time multiple mRNAs have been detected in the chromatoid body using RNA-FISH. Second it is the first time non-regulated mRNAs have been detected in the chromatoid body, and lastly RNA-FISH detected the *Smcp* intron in the chromatoid body. This data corresponds to other studies detecting splicing proteins localized in the chromatoid body (Meikar et al., 2014), and yields exciting new leads for how the direction of future research concerning the chromatoid body.

4.3 Material and Methods

4.3.1 Construction of the $S^5G^CS^3$ and $G^5G^CS^3$-mut2 transgenes

The $S^5G^CS^3$ transgene was constructed from $G^5G^CS^3$ and $S^5G^3G^3$ transgenes constructed previously (Hawthorne et al., 2006). Briefly, plasmids containing the $G^5G^CS^3$

and $S^5G^3G^3$ transgenes were digested with *Bsrg* I and *Afl* II, and the large $S^5G^3G^3$ and small $G^5G^CS^3$ fragments were purified by agarose gel electrophoresis and a Gene Clean II kit (Bio101), and the small $G^5G^CS^3$ fragment was ligated into the large $S^5G^3G^3$ fragment.

The $G^5G^CS^3$-mut2 transgene was constructed from the $G^5G^CS^3$ and $G^5G^CG^3$ transgenes in several steps. A *Swa* I site was inserted overlapping the upstream *Smcp* poly(A) signal with overlap extension PCR in the $G^5G^CS^3$ transgene (Higuchi et al., 1988). The *Swa* I-*Afl* II fragment from the $G^5G^CG^3$ transgene was inserted into the Swa I-Afl II sites of the $G^5G^CS^3$. Finally, the Swa I site was reversed to that of the original *Smcp* 3'UTR with a second round of overlap extension PCR. The sequences of both transgenes, and the PCR primers used to construct the $G^5G^CS^3$-mut2 transgene are described in chapter 2 of this manuscript. All of the remaining techniques for the production and maintenance of transgenic mice have been described previously (Bagarova et al., 2010). The plasmids were electroporated into *E. coli* DH5α, plated on LB agar containing 50 μg/ml kanamycin, and the sequence of the transgene was verified by sequencing on both strands, the small *Xho* I and *Afl* II fragment containing the transgene is purified with agarose gel electrophoresis, extracted with a NucleoTrap kit (Clontech), filtered, and adjusted to 50 ng/μl in 0.1 mM EDTA, 5 mM Tris-HCl (pH 7.4). One-cell C57BL/6 X SJL F2 embryos are injected and implanted into pseudopregnant females at the University of Massachusetts Medical Center Transgenic Core facility and tail-biopsies were analyzed to determine which pups contain the transgene. After weaning, the founders are transferred to the UMass Boston Animal Care Facility. Transgenic founders are bred to C57BL/6 X SJL mice of the opposite sex to produce lines. To identify transgenic mice, 5 mm is excised from the end of the tail of 10-21 day old pups in accord

with NIH guidelines for genotyping transgenic mice, and the DNA is purified with a DNAeasy Blood and Tissue kit (Qiagen). The presence of transgenes is assayed by PCR using *Gfp*-specific primers (Hawthorne et al., 2006).

4.3.2 Sucrose and Nycodenz Gradient Analysis

Cytoplasmic extracts of 21/25 dpp and adult testes were prepared by dissecting testes (1 testis for adult mouse and 2 testes for 21/25 day mice), removing the *tunica albuginea* and homogenizing the testes in 300 µl HNM buffer (20 mM HEPES, pH 7.4, 0.1 M NaCl, 3 mM $MgCl_2$) containing 0.5% Triton X100 and 1 unit/µl RNasin Plus (Promega Biotech). The nuclei were pelleted by centrifugation at 13,000 X g for 2 minutes, and 250 µl of the supernatant was layered on either a 3.8 ml linear 15-40% sucrose gradient in HNM buffer (w/w) or a 3.8 ml 20-60% (w/v) Nycodenz gradient prepared by layering 760 ml of 60, 50, 40, 30, and 20% Nycodenz (Accurate Scientific Chemical Corporation, Westbury, NY, USA) in HNM (w/v) in polyallomer centrifuge tubes for the Beckman SW60 rotor. Sucrose gradients were centrifuged for 80 min at 35,000 rpm at 4°C, and ~0.4 ml fractions were collected onto 0.3 g guanine thiocyanate, and RNA was extracted as described previously (Kleene et al., 2010). Nycodenz gradients were centrifuged for 24 hr at 37,000 rpm at 4°C, and 0.2 ml fractions were collected, and RNA was extracted as for sucrose gradients with adjustments for the smaller volume of fractions. RNA was extracted from each fraction of sucrose or Nycodenz gradients with techniques that recover of equal amounts of RNA from each fraction (Kleene et al., 2010). Quantitative Reverse Transcriptase real-time PCR (RT-qPCR) was carried out as described by (Bagarova et al., 2006).

4.3.3 Immunocytochemistry and RNA-FISH

Stage 2-6 seminiferous tubule segments were identified with transillumination and dissected in DEPC-treated PBS, mechanically dispersed in DEPC-treated 100mM sucrose and fixed and spread as dried down preparations on slides dipped in 0.05% Triton-X-100 and freshly prepared 4% formaldehyde (EM Sciences, Hatfield, PA, USA) (Kotaja et al., 2004). For RNA-FISH, 20nt oligo probe sets for *Smcp* coding region were selected with the Stellaris Probe Designer at the Biosearch Technologies (Petaluma, CA) website. The probe set consists of 24-48 oligos, were tagged with Quasar 570. Cells were permeabilized with 70% ethanol at room temperature (RT) for 1 hour, rinsed twice in wash buffer (2X SSC, 10% deionized formamide (Ambion AM 9342, Austin TX, USA) 5min at 37°C with a 1:50 dilution in hybridization buffer (10% dextran sulfate (Sigma-Aldrich D8906) in wash buffer). After hybridization slides were washed 3X for 30min at 37 degrees Celsius in wash buffer, rinsed with DEPC-treated PBS and mounted in Prolong Gold Antifade reagent with DAPI (Life Technologies P36931, Bedford, MA).

For immunocytochemistry , dried down preparations were rehydrated in PBS, blocked with 10% normal goat serum (S-1000, Vector Laboratories, Youngtown, OH) for 20min at RT, washed with DEPC PBS, and incubated with rabbit polyclonal antibody to mouse vasa homologue MVH (Abcam, Eugene, OR, ab13840, 1:200, overnight at 4 degrees Celsius), washed with PBS, reacted with goat anti-rabbit secondary antibody (Alexa Fluor 488, A11008) or 594 (A11037) (1:500, 30min, RT), washed in PBS, and mounted and counterstained with DAPI as described above. Cells were photographed with an Olympus BX51 microscope 100X panfluorite objective equipped with Olympus filters, U-N31000 (excitation 360 emission 460) and U-N31004 (excitation 560 emission

630), or scanned at 0.8µm Zeiss LSM 510 confocal microscope equipped with Zeiss PlanApo 63x NA oil objective. The wavelengths (nm) used for the confocal excitation and emissions of the fluors in this study follow: DAPI, 405 and 460; Quasar 570, 547 and 570; Quasar 670, 644 and 670; Alexa Fluor488, 488 and 525; Alexa Fluor 594, 594 and 617.

4.4 Results

4.4.1 Poly(A)-containing mRNAs and *Smcp* localize to the chromatoid body

To confirm reports in the literature that poly(A) containing mRNAs are located in the chromatoid body (Kotaja et al., 2006), we designed a probe for oligo(dT) and digoxigenin labeled using terminal transferase, and visualized by a secondary anti-dig antibody labeled with FITC. *In situ* hybridization was performed on dried down preparations (Kotaja et al., 2004) and analyzed using fluorescent microscopy. Intense signal could be seen coming from a small round granule at the periphery of round spermatids as well as a less intense signal located in the cytoplasm (Figure 4.1A). These images verified what was previously seen in the literature (Kotaja et al., 2006), therefore indicating that our probe was hybridizing to the chromatoid body. The same *in situ* hybridization technique was used to visualize the *Smcp* mRNA, however due to the much lower abundance of the *Smcp* mRNA no hybridization was detected in the chromatoid body.

In order to analyze *Smcp* localization we used a recently developed *in situ* hybridization protocol in which 28-48 20-base oligonucleotides specific for individual mRNA species are 5' end labeled with fluorochromes (Raj et al., 2008). These 20 nt

probe sets are tiled along the coding region of interest and provide high sensitivity by combining excellent probe penetration and high ratio of fluorochromes to bases.

Confocal microscopy with probe sets for the *Smcp* coding region detected intense *in situ* hybridization signals in a ~1 μM diameter irregular perinuclear spot in dried down preparations from stage II-VI seminiferous tubules (Figure 4.1B). The *Smcp* RNA-FISH co-localizes with immunocytochemical staining of mouse vasa homolog, MVH/DDX4, (Figure 4.1C-D) a well known marker in the literature for the chromatoid body (Parvinen et al., 2006; Yokota et al., 2012; Meikar et al., 2014). Note the lack of *Smcp* mRNA signal in spermatocytes (Figure 4.1D); this finding is consistent with reports that *Smcp* mRNA is first detected with ^{3}H-labeled RNA probes in step 3 spermatids (Shih et al., 1992).

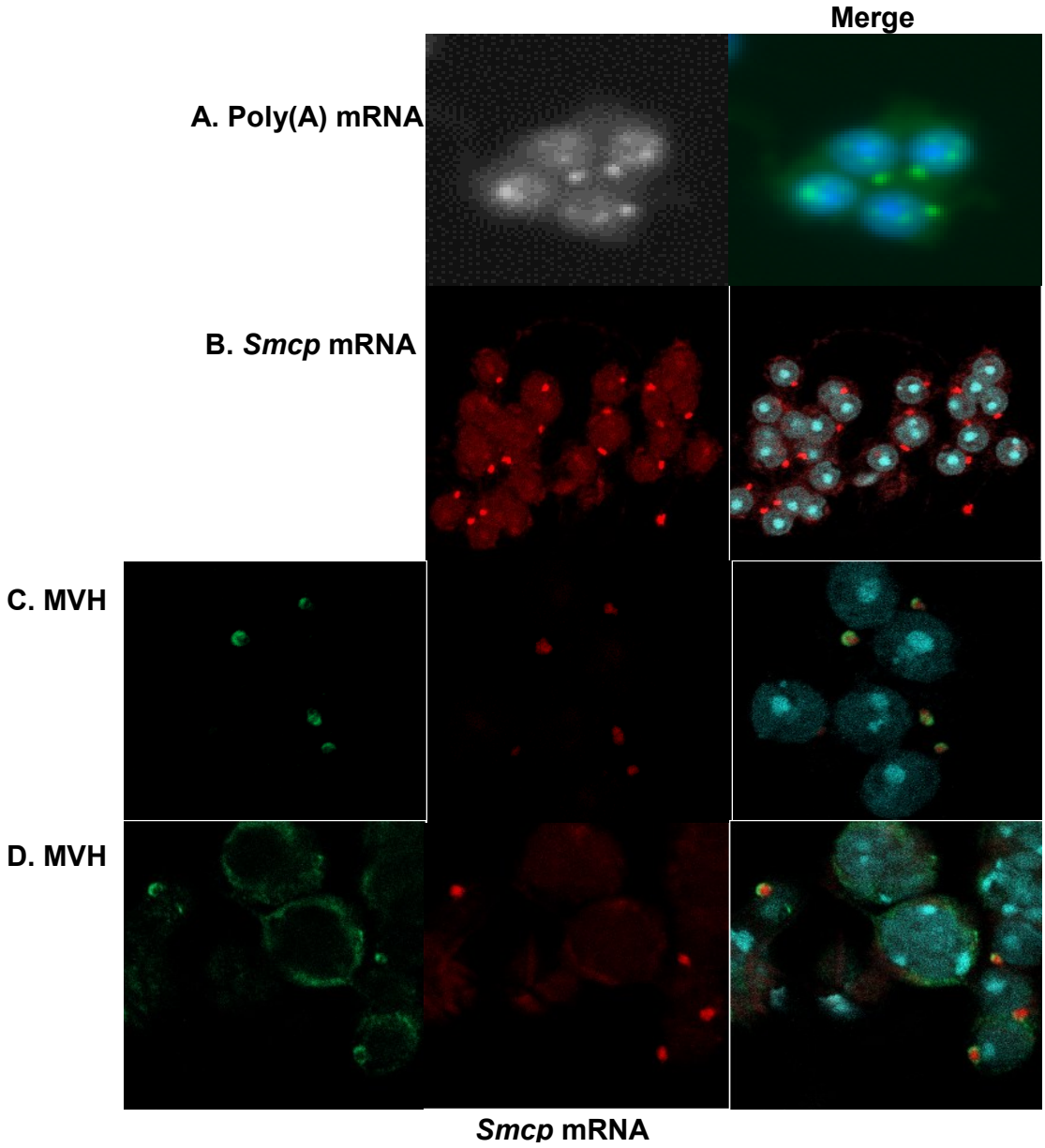


Figure 4.1 Poly(A) and *Smcp* mRNAs localize to the chromatoid body. RNA-FISH analysis of the localization of poly(A)-containing and *Smcp* mRNAs in the chromatoid body in CD-1 mice. Probe sets for oligo(dt) and *Smcp* coding region were hybridized to dried-down cells from stages 2-6 seminiferous tubules from adult testes. RNA-Fish was followed by immunocytochemistry with antibody to MVH/DDX4. The RNA-FISH hybridization was detected by confocal microscopy or fluorescence microscopy (4.1A.) All images were counterstained with DAPI in order to visualize the nuclei of round spermatids.

4.4.2 *Smcp* probe set is specific for the *Smcp* mRNA coding region

To test the specificity of the *Smcp* 27 oligo probe set, the oligos were split at random into two oligo probe sets, one containing 14 probes and the other containing 13 probes, in order to determine if they overlap. The specificity is supported by finding that subsets consisting of 14 odd and 13 even numbered oligos exhibit identical patterns of hybridization (Figure 4.2 A. Ortajo). In addition, RNAseq shows that the *Smcp* mRNA is the 16th most abundant mRNA in purified chromatoid bodies (Meikar et al., 2014). These data combined allow us to reliable confirm the intense signal coming from the chromatoid body is indeed *Smcp* mRNA.

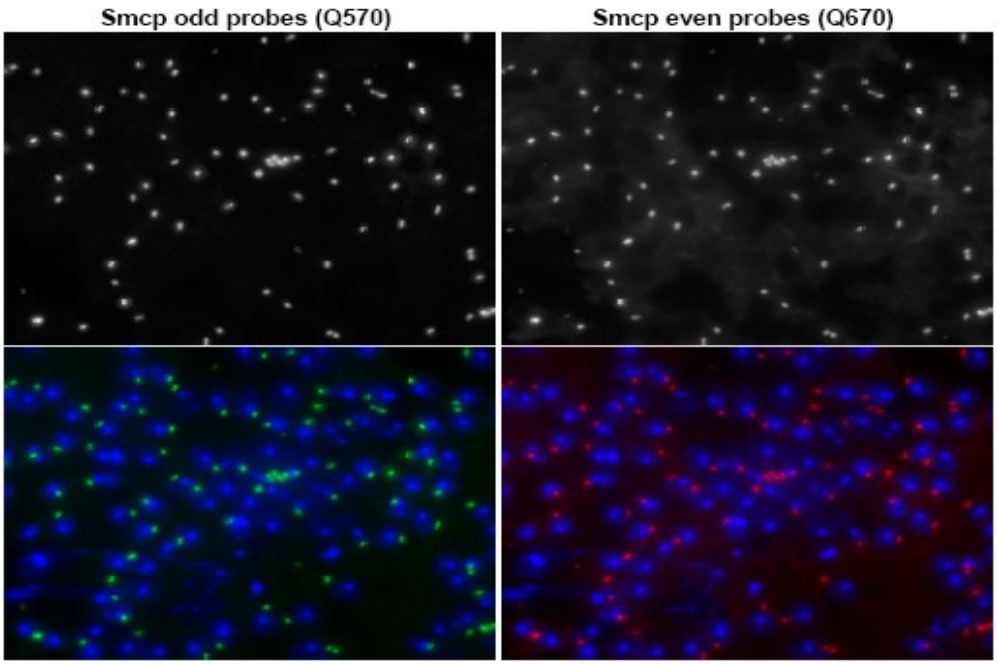


Figure 4.2 Testing the specificity of the Smcp probe set. Odd and even *Smcp* probe sets exhibit hybrideize to the chromatioid body. This experiment was performed by A. Ortajo and through personal communication he follows the (Raj et al., 2008) protocol and analyzed using a Nikon Eclipse Ti-E Inverted Microscope. The dried down preparations were sent to him by our lab and followed the (Kotaja et al., 2004) protocol. Adapted from (A. Ortajo).

4.4.3 Developmental localization of the *Smcp* mRNA

Little is known about the trafficking of spermatogenic messages after transcription, therefore we wanted determine where *Smcp* mRNA localized throughout different stages in spermatogenesis. A study performed in 1991 by Morales et al. observed that protamine 1 (*Prm1*) and transition protein 1 (*Tnp1*) mRNA were not

localized to the chromatoid body. Instead these studies revealed *Prm1* and *Tnp1* mRNAs distributed throughout the cytoplasm of step 7 to 9 spermatids (Morales et al., 1991). Fukuda et al. reported that the *Prm2* mRNA transits through chromatoid bodies of round spermatids and localizes to cytosol of elongating spermatids for repression and translation. We knew that the *Smcp* mRNA localized to the chromatoid body (Figure 4.1), but the question remained does the *Smcp* mRNA stay in the chromatoid body until activation of translation or does it move out into the cytoplasm for prolonged storage?

We were able to determine that the *Smcp* mRNA is transported from the chromatoid body to the cytoplasm long before activation of translation (Figure 4.3). *Smcp* mRNA is first detected in early haploid spermatids, ~steps 3-5, an intense signal can be detected in the chromatoid body at this stage; however a duller cytoplasmic signal can also be detected (Figure 4.3A). Presumably, as spermatogenesis progresses (~steps 6-8), the *Smcp* mRNA transits out of the chromatoid body and into the cytoplasm (Figure 4.3B), and by step 9 and 10 spermatids the message is completely localized in the cytoplasm (Figure 4.3C).

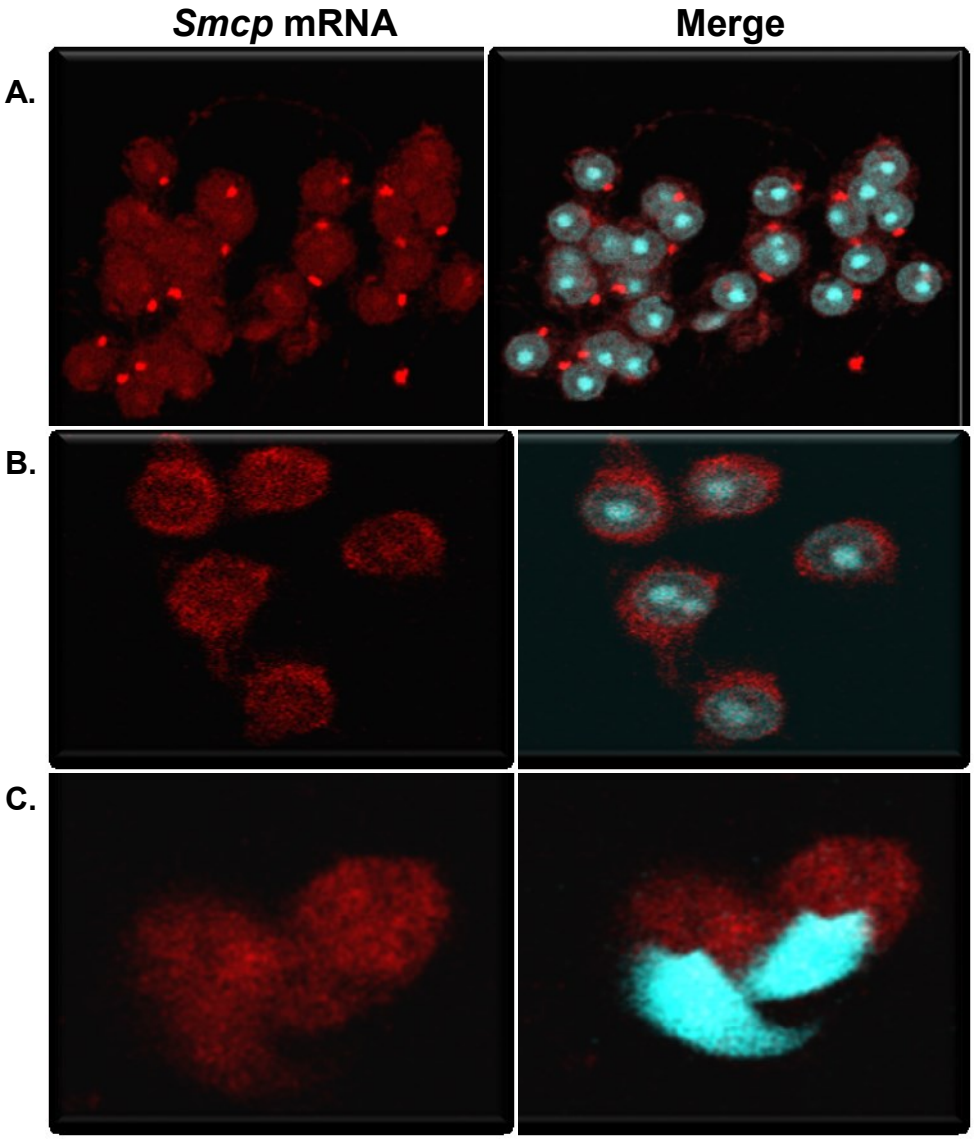


Figure 4.3 Localization of the *Smcp* mRNA throughout spermatogenesis. RNA-FISH analysis of the localization of the *Smcp* mRNA in the chromatoid body and the cytosol of CD-1 mice. 3A contains early round spermatids and observes an intense signal coming from the chromatoid body as well as a duller cytoplasmic signal. 3B contains later round spermatids with a stronger cytoplasmic signal. 3C contains elongating spermatids with a cytoplasmic signal. Probe sets for *Smcp* coding region were hybridized to dried-down cells from stages III-VIII seminiferous tubules from adult testes. The RNA-FISH hybridization was detected by confocal microscopy. All images were counterstained with DAPI.

However, it was extremely difficult to determine the precise step spermatid when *Smcp* is transported to the cytoplasm because the exact morphology of the round spermatid cannot be determined through fluorescence microscopy. This creates difficulties in distinguishing between the different steps in spermiogenesis.(Figure 4.3A) shows the *Smcp* mRNA in step 2-6 round spermatids, while (Figure 4.3B) shows round step 7-8 spermatids, and(Figure 4.3C) shows step 10-11 elongating spermatids. These data indicate that the *Smcp* mRNA may be continuously transcribed throughout early stages in spermatogenesis, and there is a constant flow of the mRNA from the nucleus, to the chromatoid body, followed by transport of the *Smcp* mRNA to the cytoplasm. Currently, it is not known when transcription of the *Smcp* gene is turned off.

4.4.4 Repressed and Active mRNAs localize to the chromatoid body

We previously stated there was no convincing evidence that dormant mRNAs are localized exclusively in the chromatoid body (Kleene et al., 2011). It is feasible that these discrepancies of whether or not repressed mRNA is stored in the chromatoid body, can be explained by a variety of possibilities. Experimental artifacts, possibly related to peculiarities of the structure and function of the chromatoid body, might prevent obtaining an accurate indication of mRNA localization. It is also possible that mRNA is not stored in the chromatoid body, because, like perinuclear P granules in *Caenorhabditis elegans*, the chromatoid body functions as a center for mRNP remodeling and export to other cytoplasmic sites (Kleene et al., 2011).

The dramatic differences in translational activity of the $S^5G^CS^3$, $G^5G^CS^3$-mut2, *Smcp* and *Ldhc* mRNAs reported previously in this manuscript provided an opportunity to analyze localization of regulated and non-regulated mRNAs in the chromatoid body. Two transgenes were studied to unveil the functions of the *Smcp* UTRs in regulating translational control during spermatogenesis. Both transgenes contain 511 nt of *Smcp* 5' flanking region, which directs transcription of the *Smcp-Gfp* transgenic mRNAs in round spermatids at the same start site as the natural *Smcp* mRNA (Hawthorne et al., 2006). The first transgene $S^5G^CS^3$, contains the complete Smcp 5' and 3'UTRs, was designed to show the importance of 5' and 3'UTRs interaction for mimicking wildtype *Smcp* translational regulation, and displayed ~4% polysomal loading in 21dpp mice (Cullinane et al., 2014). The second transgene $G^5G^CS^3$-mut2, contained the pEGFP 5'UTR and the *Smcp* 3'UTR with the 16 nt downstream the first poly(A) signal replaced with 17nt downstream of the poly(A) signal in the pEGFP 3'UTR, and displayed ~35% polysomal loading in 21dpp mice (Cullinane et al., 2014). In wildtype prepubertal mice, there is only ~4% polysomal loading. Therefore replacing the sequence downstream of the first poly(A) signal in the 3'UTR in the $G^5G^CS^3$-mut2 transgene abolished repression in round spermatids. A probe set complementary for the *Ldhc* mRNA coding region was designed in order to analyze if an endogenous non-regulated mRNA localizes to the chromatoid body. The *Ldhc* mRNA exhibits high and constant polysome loading in pachytene spermatocytes, round spermatids, prepubertal and adult testes (Bagarova et al., 2010; Kleene et al., 2010).

Probe sets designed for the *Ldhc* coding region and *Gfp* coding region exhibited intense *in situ* hybridization signals in a small irregular perinuclear spot in dried down

preparations from stage IV-VI, in $S^5G^CS^3$, $G^5G^CS^3$-mut2 seminiferous tubules (Figure 4.4B, C and D). The general cytoplasm exhibits a much weaker RNA-FISH signal. The sharp boundaries of the RNA-FISH images at the edges of the chromatoid body demonstrate that mRNAs in the general cytoplasm do not originate by diffusion out of the chromatoid body during fixation of the dried-down preparations. The absence of RNA-FISH signals in the general cytoplasm and chromatoid bodies in wild type mice demonstrate that the RNA-FISH signals for the *Gfp*-probe sets in both compartments are specific for *Smcp-Gfp* transgenic mRNAs (Figure 4.4E). The intense RNA-FISH staining of the translationally active *Ldhc* and $G^5G^CS^3$-mut2 mRNAs, and the repressed *Smcp and* $S^5G^CS^3$ mRNAs in the chromatoid body (Figure 4.4) suggests that localization in the chromatoid body is independent of translational activity.

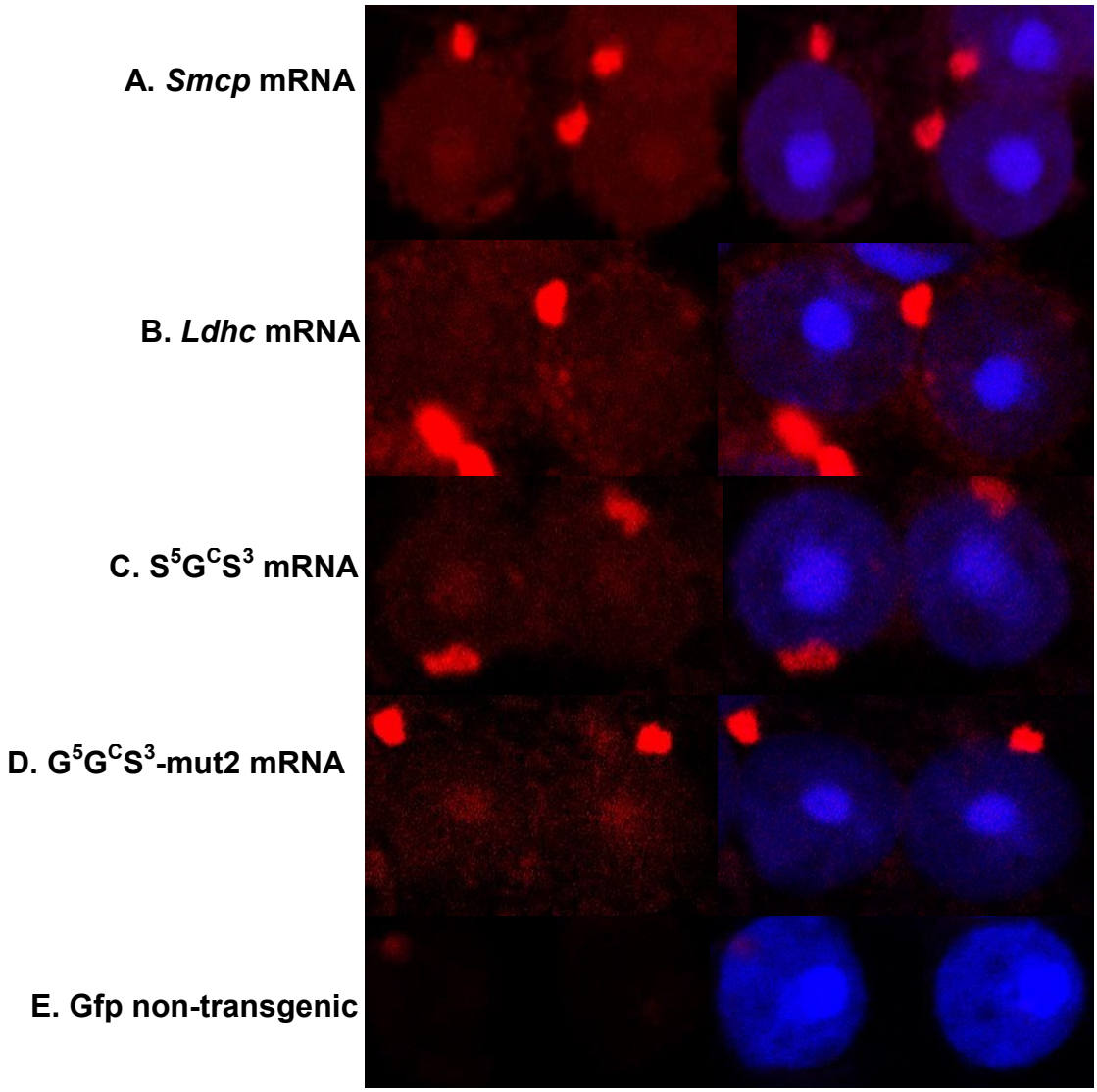


Figure 4.4 Repressed as well as Active mRNAs localizes to the chromatoid body. RNA-FISH analysis of the localization of various mRNAs in the chromatoid body. Probe sets for *Smcp* coding region, *Ldhc* coding region, and *Gfp* coding region were hybridized *in situ* to dried-down cells from stage 2-6 seminiferous tubules from adult testes. The RNA-FISH staining was detected with laser scanning confocal microscopy. The nuclei of DAPI stained round spermatids were identified by their round shape and bright chromocenter. With the exception of the negative control all probe sets exhibited strong staining to the chromatoid body, an irregular ~1 μm diameter organelle that is located adjacent to nuclei on round spermatids. Adapted from (Cullinane et al., 2014).

4.4.5 Intron *In Situ* Hybridization

As noted above, the evidence that regulated and non-regulated mRNAs are both localized in the chromatoid body is inconsistent with the idea that repressed mRNAs are stored in the chromatoid body. This prompted a new hypothesis: mRNAs that are transcribed in the nucleus are transferred to the chromatoid body and spend a fairly short period of time in that organelle before they are transferred to the cytoplasm.

To test this hypothesis, we developed probe sets for the *Smcp* and *Ldhc* introns with the goal of detecting pre-mRNA transcription in round spermatids in the absence of the intense chromatoid body RNA-FISH signal (Raj et al., 2006). Unexpectedly, the *Smcp* intron probe set strongly stained the chromatoid body (Figure 4.5B. and C) , while the *Ldhc* intron probe set did not (Figure 4.5A). We were able to detect discrete spots of *in situ* hybridization in some nuclei and not others for both the *Smcp* and *Ldhc* in spermatocytes. These spots are consistent with low levels of pulsatile transcription. Interestingly, no cytoplasmic signal could be detected in round or elongating spermatids with either the *Smcp* or *Ldhc* intron. As noted earlier both the *Smcp* and *Ldhc* mRNA probes were able to detect a dull cytoplasmic signal in round spermatids. This observation leads to speculation as to why *Smcp* intron localizes to the chromatoid body and *Ldhc* intron does not, this topic will be re-visited in the Discussion.

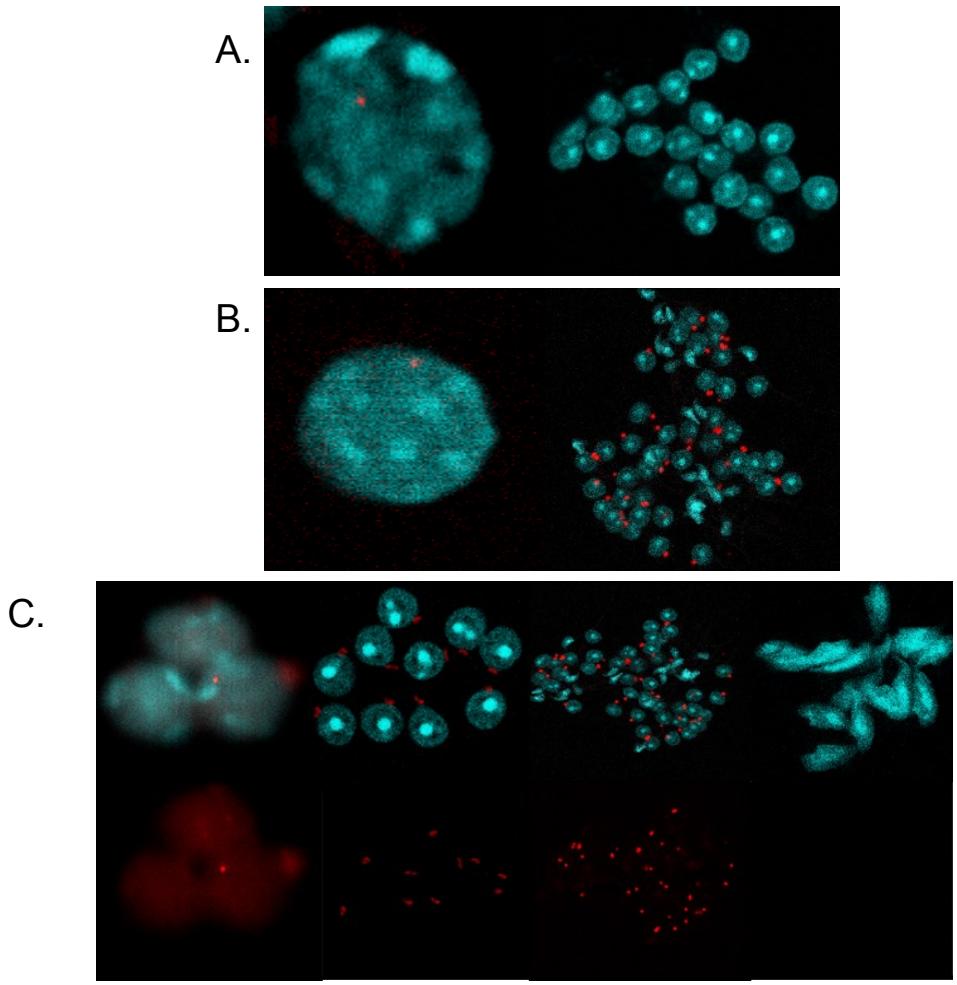


Figure 4.5 *In situ* hybridization using probe sets for *Smcp* and *Ldhc* introns. *Smcp* intron, but not *Ldhc* intron localizes to the chromatoid body. *In situ* hybridization using probes sets for *Smcp* and *Ldhc* introns on dried down preparations from adult CD-1 mice. The RNA-FISH staining was detected with laser scanning confocal microscopy. The nuclei of DAPI (blue) stained round spermatids were identified by their round shape and bright chromocenter. 4.5A displays a spermatocyte with an *Ldhc* transcription site (red) (left) and lack of hybridization in round spermatids (right). 4.5B displays *Smcp* transcription site in a spermatocyte (red) (left) and intense signal coming from the chromatoid body in round spermatids (right). 4.5C. displays a panel following *Smcp* intron (red) localization throughout spermatogenesis. Starting from the left with a transcription site in a spermatocyte, moving to the IMC, transported to the chromatoid body in round spermatids and disappears in elongating spermatids.

4.4.6 Following *Smcp* mRNA developmental localization throughout spermatogenesis

We were able to track *Smcp* throughout all stages of spermatogenesis. The earliest detection of the *Smcp* intron was in spermatocytes as pulsatile transcription sites (Figure 4.6). This was unexpected because previous *in sit*u hybridizations with [^{3}H]-riboprobes and autoradiography demonstrate that the *Smcp* mRNA is first detected in step 3 spermatids (Shih et al., 1992). In addition, grossly overexposed northern blots fail to detect the *Smcp* in pachytene spermatocytes from 18 dpp testes (Shih et al., 1992) However, RT-qPCR analysis on testes of a 16 dpp mouse detected low levels of *Smcp* mRNA (data not shown). It is therefore possible that small amounts of *Smcp* mRNA are transcribed earlier than previously believed and the present RNA-FISH technology has allowed for more sensitive detection. Interestingly, no intron cytoplasmic signal can be detected in round spermatids, indicating that the *Smcp* mRNA is degraded in cytoplasm and is not exported to the cytoplasm. Supporting this data is the observation that *Smcp* mRNA was shown to be detected in the chromatoid body of round spermatids (Figure 4.6B and 4.6C), and a duller cytoplasmic signal coming from the cytoplasm in round spermatids, elongating spermatids, and elongated spermatids. The *Smcp* intron and *Smcp* mRNA probes colocalize in the chromatoid body (Figure 4.6C), suggesting that splicing may occur in the chromatoid body since the *Smcp* intron probe is not detectable after this specific stage in spermatogenesis. These findings consitutue provocative insights into the function of the chromatoid body; further work will need to be performed to show whether *Smcp* pre-mRNA is spliced within this granule.

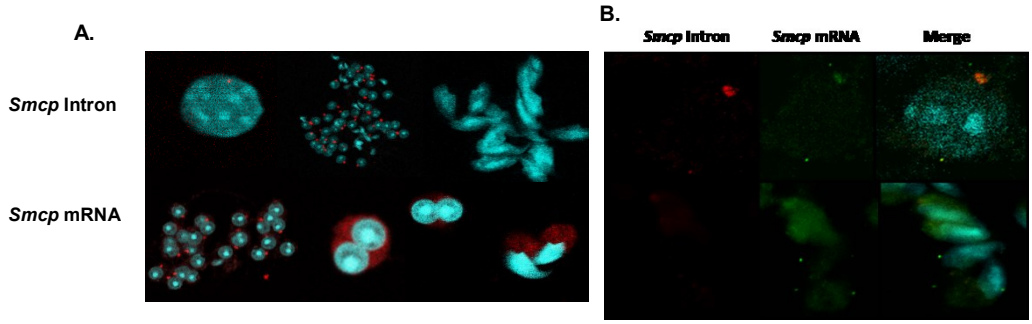


Figure 4.6 Developmental localization of *Smcp* intron and *Smcp* mRNA. 6A. Top panel (starting from left to right) displays transcription sites in the nucleus of spermatocytes from adult CD-1 mouse testis, next moves to chromatoid body in round spermatids and no detection of signal in elongating spermatids. Bottom panel (starting left to right), first detection of *Smcp* mRNA is in the chromatoid body and cytoplasm of round spermatids, *Smcp* mRNA can also be detected in the cytoplasm of elongating spermatids. 6B. Colocalization of the *Smcp* intron and *Smcp* mRNA at the chromatoid body, and a cytoplasmic signal from the *Smcp* mRNA in elongating spermatids. The RNA-FISH staining was detected with laser scanning confocal microscopy. The nuclei of DAPI stained round spermatids were identified by their round shape and bright chromocenter.

4.5 Discussion

Our lab has directed efforts towards developing *in situ* hybridization techniques that can reliably detect mRNA-localization in the chromatoid body using the *Smcp* mRNA as a test. Reproducing observations in previous studies that the 3' poly(A) tail is concentrated in the chromatoid body was straightforward (Kotaja et al., 2006; Tanaka et al., 2011) (Figure 4.1A.). However, detecting individual messages turned out to be much more challenging. Protocols using digoxigenin labeled oligos and a single locked-nucleotide fluorescently labeled oligonucleotide were extolled in the literature as being highly sensitive, but failed to detect the *Smcp* mRNA in the chromatoid body. These difficulties with *in situ* hybridization techniques were solved with the probe set

containing 27 small antisense oligos to the *Smcp* coding region, each individually labeled with Quasar 570, enhanced the signal of our transcript of interest. This allowed a complete analysis of the localization patterns throughout spermatogenesis (Figures 4.1B and 4.3). To ensure the signal was localized in the chromatoid body, colocalization assays were performed with mouse vasa homolog (MVH), a well-documented chromatoid body marker (Kotaja et al., 2006; Onohara et al., 2010). Perfect colocalization was observed between MVH and *Smcp* mRNA in the chromatoid body (Figure 4.1C and4.1D).This data is a clear indication that mRNA is indeed localized in the chromatoid body and the first proof of *Smcp* mRNA localization to the chromatoid body.

In order to ensure the *Smcp* probe was specific, the probe set was split in half; odd numbered probes were directly labeled with Quasar 570 and even numbered probes were labeled with Quasar 670. This experiment was done to test whether any of the ~27 probes were non-specifically binding, as they would not show the same localization pattern. We did indeed observe the exact same localization pattern between the two probes, suggesting they bind specifically (Figure 4.2).

We show here that the *Smcp* and $S^5G^CS^3$ mRNAs, which are repressed in round spermatids, and *Ldhc* and $G^SG^CS^3$-mut2 mRNAs, which are translationally active in round spermatids, are both present in the chromatoid body. The presence of the *Smcp* and *Ldhc* mRNAs is supported by high levels of both mRNAs in the chromatoid body (Miekar et al., 2014) and the presence of $G^SG^CS^3$-mut2 and $S^5G^CS^3$ mRNAs is supported by enormous reduction in RNA-FISH signal non-transgenic round spermatids. The finding that translationally active and repressed mRNAs are present in the chromatoid

body argues that this granule does not function solely as a site for storage of repressed transcripts. In addition, the findings here that *Smcp* mRNAs are initially present in both the chromatoid body and the cytoplasm agree with non-isotopic *in situ* hybridization studies of the *Tnp2* and *Prm2* mRNAs (Saunders et al., 1992; Fukuda et al., 2013), although those studies did not explicitly note the cytoplasmic mRNA in round spermatids. All of these *in situ* hybridization reports are generally consistent with RNAseq findings that translationally repressed mRNAs in round spermatids are abundant in purified chromatoid bodies (Meikar et al., 2014).

We did observe that MVH and the *Smcp* mRNA do not co-localize within the chromatoid body in accord with previous reports that DDX4/MVH and other RBPs are not uniformly distributed in the electron dense, fibrous stroma of the chromatoid body (Nguyen Chi et al., 2009; Onohara et al., 2010; Onohara et al., 2012). Clearly, the amorphous chromatoid body is compartmentalized, but it is unknown whether the localization of protein constituents corresponds to specific processes in RNA biology.

The striking concentration of the *Smcp* intron in the chromatoid body was unexpected (Figure 4.5B and4.5C). However, non-canonical splicing in the cytoplasm has been well documented in the literature, and the idea that pre-mRNA is spliced in the chromatoid body agrees with immunohistochemical, RNAseq and proteomics evidence that introns and proteins with functions in splicing (snRNPs, hnRNP proteins, exon-junction complex proteins) are enriched in the chromatoid body (Biggiogera, et al., 1990; Meikar et al., 2014; Moussa et al., 1994). There was no cytoplasmic signal detected with either *Smcp* or *Ldhc* intron probe sets in round spermatids.

Interestingly, the *Ldhc* intron was not localized in the chromatoid body (Figure 4.5A). In explaining this observation, it is relevant to note that the *Ldhc* gene is transcriptionally active in pachytene spermatocytes and transcriptionally inert in round spermatids (Tang et al., 2008). This implies that the *Ldhc* mRNA in the chromatoid body in round spermatids could either represent extended storage in the RNP granule in pachytene spermatocytes known as the intermitochondrial cement and the chromatoid body in round spermatids, or import of the *Ldh*c mRNA from the cytoplasm into the chromatoid body in round spermatids. Alternatively, it is possible that splicing within the chromatoid body serves as a signal for the repression of some mRNAs, since the *Ldhc* mRNA, in contrast to the *Smcp* mRNA and *Smcp* transgenic mRNAs, is considered to be an actively translated message. Translational regulation in spermatids is extremely mRNA specific, as demonstrated by reports that mRNAs that are repressed in round spermatids are first detected at different stages. For example the *Pgk2*mRNA is first detected in leptotene/zygotene spermatocytes, the *Smcp* mRNA is first detected in step 3 spermatids, and the *Prm1*mRNA is first detected in step 7 spermatids (Chapter 1, Table 1.2). In addition, translation is activated at different stages: the ACEV2, PRM1, SMCP, PRM2 and ODF1 proteins are first detected respectively steps 9, 10, 11, 13 and 16, respectively (Table 1.2) and the proteins corresponding to the *Acr* and *Acrv2* mRNAs are first detected in round spermatids (Kleene et al., 2013).

RNA-FISH *in situ* hybridization studies of mRNA levels and intracellular localization in individual cells provide insights into the mechanisms of regulation of mRNA expression in individual cells that cannot be achieved by biochemical techniques.

The interpretation of RNA-FISH studies of the distribution of mRNA between chromatoid body and general cytoplasm should begin by recognizing that the chromatoid body represents only ~0.4% of the volume of the cytoplasm in round spermatids (Kleene et al., 2011). This implies that if 5% of the *Smcp* mRNA was in the chromatoid body and 95% was in the general cytoplasm, the RNA-FISH signal in the chromatoid body would be 13-fold stronger than that in the general cytoplasm. The difference in mRNA concentration in the chromatoid body and cytoplasm is further exaggerated by thin optical sections of confocal microscopy which detect many mRNAs in a small object, the chromatoid body, and fewer mRNAs in a thick object, the cytoplasm. Notably, the presence of the *Smcp* mRNA in the thin layer of cytoplasm surrounding the nuclei in dried down preparations can be detected with conventional fluorescence microscopy which co-localizes with YBX2, a marker for cytoplasmic free-mRNPs, (Chapter 3, Figure 3.5C) (Oko et al., 1996; Yang et al., 2007).

This study shows that the chromatoid body functions as more than a site for storage for repressed mRNAs by observing that both repressed and active messages localize to the chromatoid body. Furthermore, it has shed insight as to why *Smcp* mRNA is passing through the chromatoid body, leading to speculation that all strongly repressed mRNAs in round spermatids might be spliced within the chromatoid body. However, more experiments are necessary to validate this hypothesis. For instance it would be possible to determine whether translationally repressed and translationally active mRNAs transit at different rates through the chromatoid body in murine round spermatids by coexpressing an mRNA containing a 3′-UTR-binding site for the bacteriophage MS2 coat protein and an mRNA encoding a GFP–MS2 coat protein fusion in prepubertal transgenic

mice (Sheth et al., 2003; Kedersha et al., 2005; Lionnet, et al., 201; Wu et al., 2014). This tethering methodology provides a technique for monitoring the movements of specific mRNAs through the nuclei, chromatoid body and cytoplasm. It would also be interesting to analyze if there is a difference in the type of introns that localize to the chromatoid body. Probe sets could be designed for introns contained within regulated and non-regulated mRNAs and one could observe if there is a difference in localization patterns between the probe sets.

CHAPTER 5

SUMMARY AND PERSPECTIVES

I studied a mutation in the *Smcp* 3'UTR, $G^5G^CS^3$-mut2 that replaced the 16 nt segment downstream of the first *Smcp* poly(A) signal with 17 nt downstream of the pEGFP 3' UTR AAUAAA polyadenylation signal. This mutation resulted in complete loss of translational repression in round spermatids: GFP fluorescence was first detected in step 3 spermatids, and ~35% of the $G^5G^CS^3$-mut2 mRNA is associated with polysomes in 21 dpp testis sucrose and Nycodenz gradients (Chapter 2). The phenotype of the $G^5G^CS^3$-mut2 mutation concurs with evidence that the elements that repress mRNA translation in the *Prm1* 3'UTR must be located at the 3' terminus of the 3'UTR, in close proximity to the poly(A) signal (Fajardo et al., 1997; Giorgini et al., 2001; Zhong et al., 2001; Soundajaram et al., 2010). Since the $G^5G^CS^3$-mut2 mutation binds the La protein, YBX2 and YBX3L, we do not know whether the release of translational repression is caused by a failure to assemble a repressive complex or the stimulation of mRNA translation by another pathway.

I also demonstrated that depletion of YBX2 with the *Ybx2*-null mutation resulted in complete release of translational repression of the *Prm1* and *Smcp* mRNAs in 25 dpp mice. The implication of these findings that YBX2 is an important translational repressor

of two mRNAs likely extends to other mRNAs because YBX2 is the most abundant protein in translationally repressed free-mRNPs (Herbert et al.,1999). In addition, YBX2 YRSs are present in the 3'UTRs 1-4 nt upstream of the poly(A) signals in the 3' UTRs of several mRNAs that are strongly repressed in round spermatids, the *Tnp1*, *Dazap1* and *Prm2* mRNAs (Kleene, unpublished). Interestingly, the *Ybx2* 3'UTR has a YRS 8 nt upstream of its poly(A) signal, suggesting that translation of the *Ybx2* mRNA may be autoregulated by YBX2. As previously mentioned, *Prm1* mRNA is transcribed and stored for 3 days until proper activation of translation; loss of PRM1 results in inability of the chromatin to be able to condense. Studies performed by (Giorgini et al., 2001; Zhong et al., 2001) reported that the position of the YRS at the distal end of the *Prm1* 3'UTR is crucial for delay of translation. It has been shown that YRSs 33 nt upstream of the *Smcp* poly(A) signal, and 16 nt upstream of the *Prm1* poly(A) signal result in negligible and partial repression in transgenic mice, respectively (Bagarova et al., 2010; Fajardo et al., 1997). In addition high affinity YRSs in the *Prm1* and *Smcp* 5' UTRs result in negligible repression (Bagarova et al., 2010; Braun et al., 1989). Furthermore, the *Prm1* translational control element (TCE) strongly represses translation in its natural position 3 nt upstream of the *Prm1* poly (A) signal, and no repression in the *Prm1* 5' UTR and 110 nt upstream of the *Prm1* poly(A) signal (Soundajaram et al., 2010).

There are many unusual features of translational control in spermatids; therefore it is important to validate the functions of YRSs by analyzing point mutations that abolish the binding of YBX2 to the YRS in transgenic mice (Kleene et al., 2013). A systematic search for YRSs in 3'terminus of the *Smcp* 3'UTR needs to be performed using

recombinant YBX2. It would then be possible to identify mutations that eliminate YBX2 binding, and analyze effects of mutations on translational activity in transgenic mice. Unfortunately, because of studies of mutations in transgenic mice are considered to be excessively risky, costly and laborious, this very important approach is seldom used. I believe that quantification is necessary to establish whether the mutated-YRS results in partial or complete release of repression, thereby indicating whether repression requires additional *cis*-elements, such as YRSs or binding sites of other RBPs and miRNAs (Bagarova et al., 2010).

The role of YBX2 in translational repression in round spermatids would also be clarified with HITS-CLIP, a procedure in which proteins that contact RNA bases are crosslinked with ultraviolet light in living cells (Zhang et al., 2011). After lysis of the cells, the lysates are digested with RNase to produce short fragments of RNA surrounding the crosslinked protein, complexes are immunoprecipitated with antibody to YBX2, complexes are separated by SDS-PAGE, reverse transcribed and millions of cDNAs are sequenced with next generation sequencing. Importantly, amino acids that were crosslinked to bases *in vivo* produce mutations that can be mapped precisely.

A HITS-CLIP analysis of YBX2 binding in testis would produce important insights into the scope and mechanisms of translational repression by YBX2. First, YBX2 binding sites identified with HITS-CLIP would define the sequences to which YBX2 binds *in vivo*. The binding specificity of RNA-binding proteins determined *in vitro* does not necessarily agree with those determined *in vivo*, since the binding of proteins to

mRNAs is likely quantitatively and qualitatively influenced by interactions with RNA binding proteins in the nucleus, chromatoid body and cytoplasm (Ascano et al., 2012).

Second, YBX2 is postulated to partially repress translation of all mRNA and a strong repressor of a subset of mRNAs in round spermatids. The YBX2 binding sites identified with HITS-CLIP may correlate with strong translational repression, which based on very limited studies of mutations in transgenic mice, appear to be in close proximity to the poly(A) tail and poly(A) signal. Recent studies document the revolutionary idea that the interactions of two RNA binding proteins can dramatically alter the binding specificity and affinity of RNA binding proteins (Campbell et al., 2012). Of course, any potential insights into the configurations of cis-elements that are necessary for strong translational repression will need to be validated by analyzing the effects of mutations on translational activity in transgenic mice.

When I started this project the function of the chromatoid body in storage of translationally repressed mRNA was a popular and controversial idea (reviewed in (Kleene et al., 2011). The translationally regulated transition protein 2 *(Tnp2*) had been shown to localize to the chromatoid body (Saunders et al., 1992), but two other translationally regulated mRNAs, *Prm1* and *Tnp1* were reported not to localize to the chromatoid body (Morales et al., 1991).

All of the mRNA species I studied were intensely localized to the chromatoid body in round spermatids, including the *Smcp* and $S^5G^CS^3$ mRNAs, which are strongly repressed, the *Ldhc* and $G^5G^CS^3$-mut2 mRNAs, which are translationally active, as well as the prematurely translated *Smcp* mRNA in the *Ybx2*-null mice (Chapters 3 and 4). All

of these data suggest that prolonged sequestration of mRNAs in the chromatoid body is not sufficient for prolonged translational repression. My studies are the first to demonstrate using RNA-FISH that translationally active mRNAs to localize in the chromatoid body, and agree with recent RNAseq studies that both repressed and active mRNAs are present in the chromatoid body (Meikar et al., 2014). These studies suggest that all mRNAs transit from nuclei through nuclear pores into the chromatoid body and then transit from the chromatoid body into the general cytoplasm, where the mRNAs are translationally active on polysomes or stored as translationally repressed free-mRNPs.

The idea that high levels of mRNA are not stored in the chromatoid body is consistent with work on *Caenorhabditis elegans* demonstrating that there are multiple types of P granules (Schisa et al., 2001; Sheth et al., 2010). One type of granule is associated with nuclear pore complexes, as are chromatoid bodies in mammalian round spermatids. These perinuclear P-granules are proposed to function as mRNP remodeling and sorting centers: newly synthesized mRNAs pass through the P granules and are exported to the cytoplasm or other classes of cytoplasmic P granules (Kleene et al., 2011) Adding to this, a number of constituents of the chromatoid body are also localized in the cytoplasm, such as DICER, AGO3, DCP1A, and MIWI, indicating that these proteins may play a role in both the chromatoid body and the general cytoplasm (Kotaja et al., 2006). Also DEAD-box helicases such as MVH and GRTH are major constituents of the chromatoid body, and these helicases often function in melting RNA secondary structure thereby promoting the formation of protein-mRNA complexes (Arkov et al., 2010). It is known that GRTH and MVH localize to both the chromatoid body and the cytoplasm

where they may help to remodel mRNAs in preparation for active translation or degradation in the cytoplasm (Meikar et al., 2014). Differences in the mechanisms of post-transcriptional regulation of different mRNAs may be reflected in differences in the periods of time for specific mRNAs to transit through the chromatoid body.

The prediction that translationally repressed and translationally active mRNAs transit rapidly through the chromatoid body in murine round spermatids could be analyzed by coexpressing an mRNA containing a 3′-UTR-binding site for the bacteriophage MS2 coat protein and an mRNA encoding a GFP–MS2 coat protein fusion in prepubertal transgenic mice (Sheth et al., 2003; Kedersha et al., 2005; Lionnet et al., 2011; Wu et al., 2014). This experimental approach, which is known as tethering, provides a technique for monitoring the movements of specific mRNAs through the nuclei, chromatoid body and cytoplasm.

However, the question remains how much of these mRNA species are in the chromatoid body versus the general cytoplasm? The relative amounts of mRNAs in the cytoplasm and chromatoid body is difficult to quantify using RNAseq because the amount of RNA in the chromatoid body compared to that in round spermatids, ~2 pg, is unknown (Kleene et al., 1983). My own measurements of the *Smcp* mRNA has been very difficult to quantify as well because the chromatoid body represents only ~0.4% of the volume of the cytoplasm in round spermatids (Kleene et al., 2011). This implies that if 5% of the *Smcp* mRNA was in the chromatoid body and 95% was in the general cytoplasm, the RNA-FISH signal in the chromatoid body would be 13-fold stronger than that in the general cytoplasm. Simply quantifying this difference by intensity

measurements could possibly yield skewed results, indicating much more mRNA in the chromatoid body than in there is in reality. The difference in mRNA concentration in the chromatoid body and cytoplasm is further exaggerated by thin optical sections of confocal microscopy which detect many mRNAs in a small object, the chromatoid body, and fewer mRNAs in a thick object, the cytoplasm.

The striking concentration of the *Smcp* intron in the chromatoid body was unexpected; however recent studies using proteomics and RNAseq finds that introns as well as proteins known to have function in splicing have been detected in the chromatoid body (Meikar et al., 2014). In addition previous studies using immunocytochemistry and mass spec sequencing reveal that snRNPs, hnRNP proteins, and exon-junction complex proteins are enriched in the chromatoid body (Biggiogera et al., 1990; Meikar et al., 2014; Moussa et al., 1994).

The observation of *Smcp* intron in the chromatoid body leads to speculation of a possible model for *Smcp* regulation (Figure 5.1). It is reasonable to hypothesize that *Smcp* pre-mRNA is transcribed spermatids and immediately transported to the chromatoid body, as it moves dynamically around the nucleus making contact with nuclear pores. Once inside the chromatoid body the *Smcp* is spliced and YBX2 first binds the 3'UTR. The hypothesis that YBX2 binds the *Smcp* mRNA in the chromatoid body is consistent with evidence that the levels of YBX2 are highest in the chromatoid body and undetectable in nuclei (Oko et al., 1996). The *Smcp* mRNA is exported to the cytoplasm with bound YBX2 where it is stored for about a week as a free-mRNP until proper activation of translation. It should be noted that other factors likely affect these

pathways. These co-factors potentially include protein kinases and helicases and other proteins that influence the binding of YBX2 to mRNA (Matsumoto et al., 2005; Herbert et al., 1999; Tsai-Morris et al., 2004). Another set of co-factors may interact with other elements at the 3'terminus of the 3' UTR and YBX2 to strengthen repression and block initiation at the 5' end of the mRNA. Establishing the identity and importance of these co-factors will be another major area of future investigations.

If *Smcp* mRNA is spliced in the chromatoid body; it is possible that capping of the 5'end and polyadenylation may also occur there as well. Bentley (2014) suggests that transcription, splicing, polyadenylation, and capping are coupled. Maybe these are partially uncoupled because an extraordinarily high proportion of the genome is transcribed in round spermatids, even more than in brain (Soumillon et al., 2013). Too many RNAs floating around in nuclei might overwhelm the specificity of splicing in the nucleus.

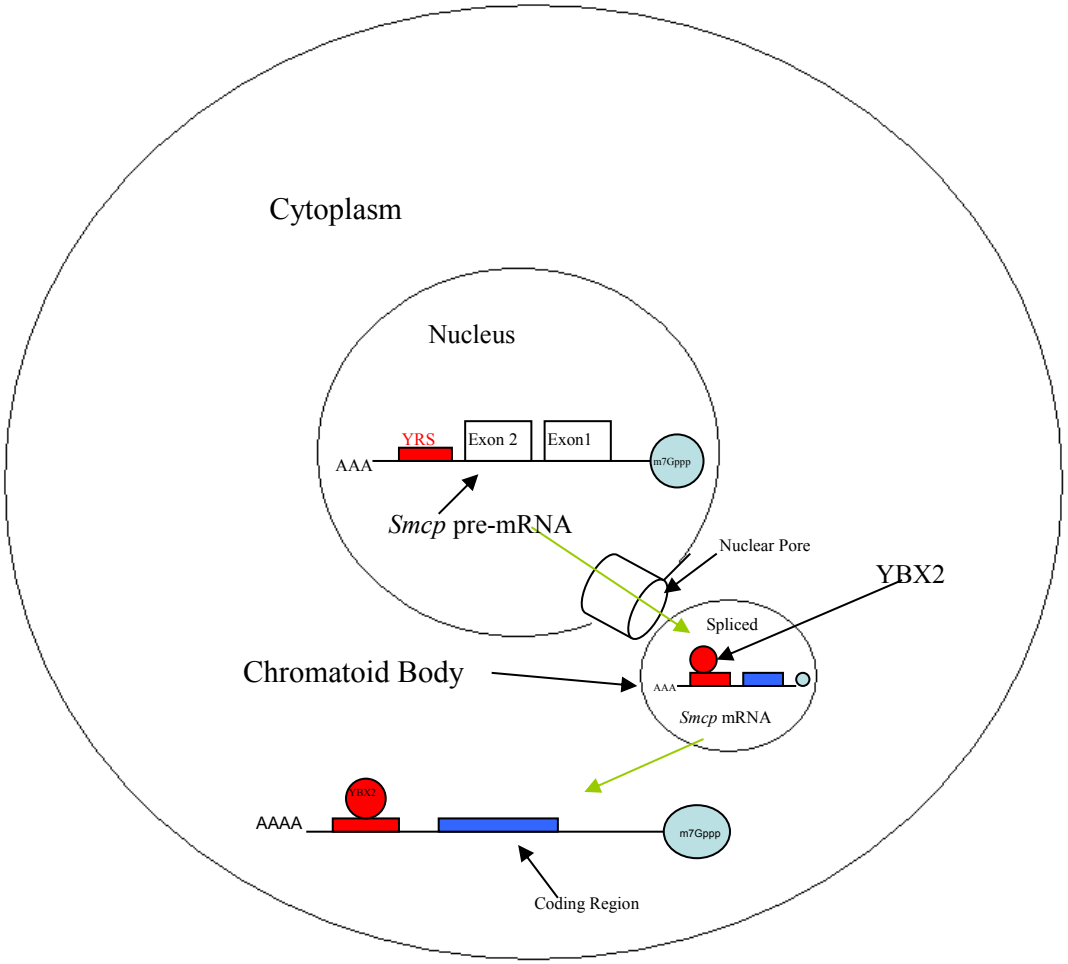


Figure 5.1 Speculative model for repression of the *Smcp* mRNA in round spermatids. *Smcp* mRNA is transcribed in the nucleus of round spermatids and is exported to the chromatoid body through nuclear pores as a pre-mRNA. Once in the chromatoid body *Smcp* is spliced and YBX2 binds the YRS in the 3'UTR. Smcp mRNA is then transported to the cytoplasm where it stored for about 8 days as a free-mRNP with YBX2 remaining bound until proper activation of translation.

Interestingly, *Ldhc* intron did not localize the chromatoid body in round spermatids (Chapter 4). Although the exact reason for this remains unknown, it is reasonable to believe when and where splicing occurs is mRNA specific. It is well documented in the literature that regulation of spermatogenic messages is mRNA specific due to the fact that many mRNAs are transcribed and translated at a variety of different times. Splicing in the chromatoid may serve as a marker for prolonged storage in the cytoplasm. Therefore, it is reasonable that *Ldhc* mRNA is not spliced in the chromatoid body because it is one of the few spermatogenic messages that are considered actively recruited onto polysomes and translated in spermatocytes and spermatids. The variety of different proteins that localize to the chromatoid body support this hypothesis; leading to speculation that the majority of mRNAs in spermatogenesis pass through the chromatoid body for a variety of reasons. Some may pass through the chromatoid body binding RBPs that signal for activation of translation, such as *Ldhc* and others may bind RBPs that signal for repression in the cytoplasm such as *Smcp*, both types of RBPs have been shown to localize to the chromatoid body (Meikar et al., 2014). Another hypothesis is that mRNA may transit from the cytoplasm into the chromatoid body, there is no data specifying that mRNA transition into the chromatoid body is unidirectional.

To verify that *Smcp* intron does indeed localize to the chromatoid body, long range RT-PCR would have to be performed on *Smcp* mRNA purified from the chromatoid body. For instance the *Smcp* pre-mRNA contains 2 exons for which complementary forward and reverse primers could be designed. After PCR amplification, the cDNA could be run out on a gel, theoretically if the intron did not exist within the

sequence there would be no amplification and no observation of a band on the gel, or a larger than expected band due to the presecence of the exons. If the intron was still intact one would observe of the band of designed length because amplification was able to occur.

Conclusion

My project has detailed information on the regulation of the *Smcp* mRNA, as well as insight into the function of YBX2 and the chromatoid body. I have identified a sequence 16 nt downstream the first poly(A) signal in the 3'UTR that may be necessary for regulation and have shown that the trans-factor YBX2, binds the 3T3U in the $G^5G^CS^3$-mut2 mice in much lower levels indicating the reason behind the loss of regulation.

I have also showed *in vivo* that loss of YBX2 has an effect on regulation of natural *Smcp* mRNA. *Smcp* displayed much higher amounts of polysomal loading in *Ybx2*-null prepubertal mice when compared to prepubertal wildtype mice. Also, the *Smcp* mRNA still localizes to the chromatoid body in the absence of YBX2. This indicates that YBX2 is not required for transport concentration of the *Smcp* mRNA in the chromatoid body to the cytoplasm and the chromatoid body is not a site for prolonged storage of repressed mRNAs.

Lastly, I have shown for the first time utilizing RNA-FISH that regulated as well as non-regulated mRNAs and *Smcp* intron localize to the chromatoid body. Thes findings

are significant because it puts to rest the debate of whether or not the chromatoid body is solely a site for storage for repressed messages, and sheds insight into the function of this enigmatic germ cell granule.

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