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INVITED REVIEW

Sperm Biology

RNA binding proteins in spermatogenesis: an in depth focus on the Musashi family

Jessie M Sutherland[1], Nicole A Siddall[2], Gary R Hime[2], Eileen A McLaughlin[1]

Controlled gene regulation during gamete development is vital for maintaining reproductive potential. During the complex process of mammalian spermatogenesis, male germ cells experience extended periods of the inactive transcription despite heavy translational requirements for continued growth and differentiation. Hence, spermatogenesis is highly reliant on mechanisms of posttranscriptional regulation of gene expression, facilitated by RNA binding proteins (RBPs), which remain abundantly expressed throughout this process. One such group of proteins is the Musashi family, previously identified as critical regulators of testis germ cell development and meiosis in *Drosophila*, and also shown to be vital to sperm development and reproductive potential in the mouse. This review describes the role and function of RBPs within the scope of male germ cell development, focusing on our recent knowledge of the Musashi proteins in spermatogenesis. The functional mechanisms utilized by RBPs within the cell are outlined in depth, and the significance of sub-cellular localization and stage-specific expression in relation to the mode and impact of posttranscriptional regulation is also highlighted. We emphasize the historical role of the Musashi family of RBPs in stem cell function and cell fate determination, as originally characterized in *Drosophila* and *Xenopus*, and conclude with our current understanding of the differential roles and functions of the mammalian Musashi proteins, Musashi-1 and Musashi-2, with a primary focus on our findings in spermatogenesis. This review highlights both the essential contribution of RBPs to posttranscriptional regulation and the importance of the Musashi family as master regulators of male gamete development.

INTRODUCTION

Spermatogenesis

Spermatogenesis defines the maturation process of male gametes and is one of the most complex differentiation events that occur within developmental biology, necessitating the controlled regulation of gene expression (**Figure 1**). Within the male testis, spermatogenesis commences shortly after birth with the differentiation of prespermatogonial gonocytes to spermatogonia. These spermatogonia provide the pool of stem cells essential for the continual production of spermatozoa throughout postpubertal life. Spermatogonial stem cells undergo a series of mitotic amplifications to produce primary spermatocytes. These spermatocytes then go through two rounds of meiosis to form haploid round spermatids. Postmeiotic spermatid differentiation (spermiogenesis) defines the profound morphological changes that mark transition into elongating spermatids and the progressive development of immature spermatozoa.

During the process of spermatogenesis, two phases of the inactive transcription have been described. The first of these phases takes place during homologous recombination of spermatocytes entering early meiosis,[1] whereby the genome is damaged and repaired for crossing over and transcription is blocked.[2] The second phase marks the cessation of mRNA synthesis, and takes place in late elongating spermatids, at the time of chromatin condensation in spermiogenesis, denoted by the mass degradation of mRNAs and the gradual decline of translation.[3,4] These extended periods of transcriptional cessation that occur during the continued differentiation program of spermatogenesis necessitate extensive regulation of translation.[5] Consequently, the mechanisms of posttranscriptional regulation, controlled primarily by RNA binding proteins (RBPs), become of utmost importance. In instances where the mechanisms for posttranscriptional control function abnormally, spermatogenesis can fail resulting in the production of nonviable gametes.[6,7]

RNA binding proteins

RBPs are an extensive class of proteins defined by their ability to recognize and bind to specific sequences of RNA, and regulate the function utilizing an array of mechanisms. They contain at least one RNA recognition site responsible for identifying a particular motif within target sequences.

The sub-cellular level at which RBPs are expressed is important in determining the function. Consequently, RBPs can be divided into nuclear RBPs and cytoplasmic RBPs. Nuclear RBPs localize to the nucleus where they primarily regulate nascent mRNA (pre-mRNA) processing events, including capping, polyadenylation, and splicing.[7] Cytoplasmic RBPs bind mature mRNA sequences in the cytoplasm as

[1]School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308 Australia; [2]Anatomy and Neuroscience, University of Melbourne, Parkville, VIC 3010 Australia.
Correspondence: Prof. Eileen A McLaughlin (eileen.mclaughlin@newcastle.edu.au)
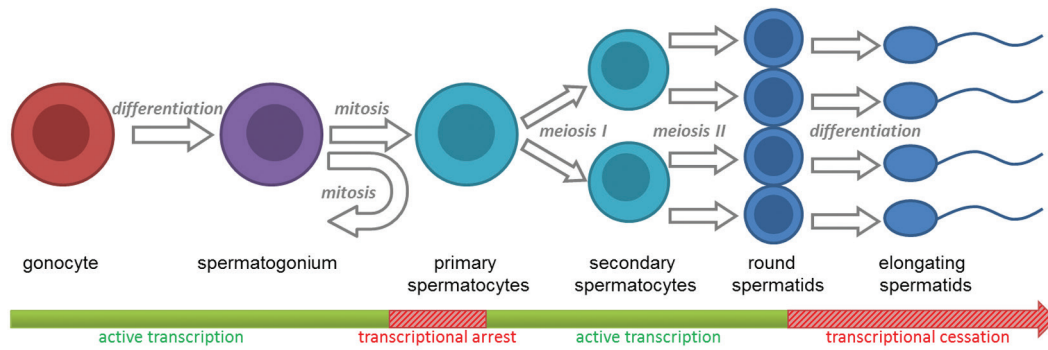
Figure 1: Important stages of spermatogenesis relative to the transcription. Here, we commence with the differentiation of prespermatogonial gonocytes in spermatogonia. Spermatogonial stem cells then undergo mitotic self-maintenance and mitotic amplifications to produce primary spermatocytes. These spermatocytes are transcriptionally arrested during homologous recombination and then continue through two rounds of meiosis to form haploid round spermatids. Postmeiotic spermatid differentiation (spermiogenesis) defines the profound morphological changes that mark transition into elongating spermatids and the progressive development of immature spermatozoa which occurs independently of RNA synthesis. The bar describes the transcriptional status relative to the stage of germ cell development.

they are released from the nucleus. Cytoplasmic RBPs operate more directly in the translation via: directing mRNA transport, competitive or co-operative interactions with translation machinery, and regulating mRNA stability. A number of RBPs are both nuclear and cytoplasmic and can, therefore, be involved in a combination of the previously mentioned processes, as well as uniquely in mRNA nuclear export.

Importantly RBPs are highly expressed throughout spermatogenesis and have been well documented as being essential to posttranscriptional control during all stages of germ cell development. Characterization studies using transgenic models of testis-expressed RBP disruption commonly identify an irregular spermatogenesis phenotype and often exhibit various stages of spermatogenic arrest and resultant sterility.[7] Piecing together the mechanisms that these RBPs utilize to make them fundamental during testis germ cell development is vital to our overall understanding of spermatogenesis and male factor infertility.

FUNCTIONAL MECHANISMS OF RNA BINDING PROTEINS
Within the cell, RBPs function at all levels of RNA metabolism. Assembling on nascent and processed mRNAs, RBPs have the ability to govern gene regulation at the posttranscriptional level in both health and disease. Here, we divide the mechanisms utilized by RBPs into six sub-categories in terms of posttranscriptional regulatory processes (**Figure 2**).

Capping
Messenger RNA production requires synthesis of a pre-mRNA and processing of the nascent precursor by 5' capping, splicing of introns, and 3' cleavage/polyadenylation to make mature mRNA. Although these processes are interconnected: the 5' cap can enhance splicing of the first intron and 3' processing,[8] while 3' processing depends on splicing of the last intron,[9] it is important to assess each step individually. Capping describes the addition of a 7-methylguanosine linked via a triphosphate bridge, to the first nucleotide of the 5' end of pre-mRNA and occurs co-transcriptionally. Capping is essential for the growth of eukaryotic cells: the guanylate protects the mRNA from untimely degradation, while the methyl group facilitates translation.[10]

Capping of transcripts confers stability. One main function of the 5' cap is to prevent Xrn1 and Xrn2 exoribonuclease-mediated digestion of mRNAs.[11] In the cytoplasm, the cap is also used as a signal to direct translational machinery to the 5' end of protein-encoding RNAs. The eukaryotic translation initiation factor eIF4E recognizes the cap-structure and recruits the 40S ribosome subunit to the 5' end of the mRNA to signal translation initiation.[12]

In mammalian cells, the caps of pre-mRNAs and newly synthesized mRNAs are bound by the nuclear cap binding complex (CBC) heterodimer of RBP cap binding protein 20 (CBP20) and auxiliary protein CBP80. This complex promotes nuclear cap function during pre-mRNA processing, but also functions in the cytoplasm in the pioneer round of translation, responsible for monitoring mRNA quality via the nonsense-mediated mRNA decay pathway.[13]

It is important to note that within the CBC, CBP20 is responsible for cap binding through its ribonucleoprotein domain while CBP80 causes a conformational change in CBP20 and is required for CBC to bind with high affinity. In mammalian cells, siRNA-mediated depletion of CBP80 results in deregulation of ~400 genes and a significant reduction in the cell proliferation rate.[14] Deletion of the CBC *in vitro* prevents spliceosome assembly at an early step in complex formation.[15]

Pre-mRNA splicing
Splicing describes the precise removal of noncoding intervening sequences (introns) from pre-mRNAs and is essential for proper protein expression. This process of intron removal is efficient and precise, constituting the majority of constitutive splicing events in the cell. However, most transcripts in higher eukaryotic cells also contain regions that are subjected to selection of alternative exons, resulting in the production of different mRNA isoforms. This process, known as alternative splicing, has the capacity to alter gene coding and affect RNA stability and has been recognized as a mechanism for increasing the functional diversity of the proteome, altering the expression of a protein 3- to 4-fold.[16,17] Both constitutive and alternative splicing can occur co- and post-transcriptionally and is catalyzed by the spliceosome, a macromolecular complex comprised of the U1-U6 family of small nuclear ribonucleic particles (RNPs) in conjunction with >100 different additional proteins.[18] Unsurprisingly, mutations in spliceosome components and subsequent splicing errors underlie a large number of human diseases.[19]

Spliceosome proteins are a well-studied class of RBPs. There are also a number of RBPs involved in alternative splicing that bind pre-mRNA either to encourage or block specific splicing events. DExD/H-type RNA-dependent ATPases/helicases have long been implicated in rearrangements within the spliceosome and act at discrete
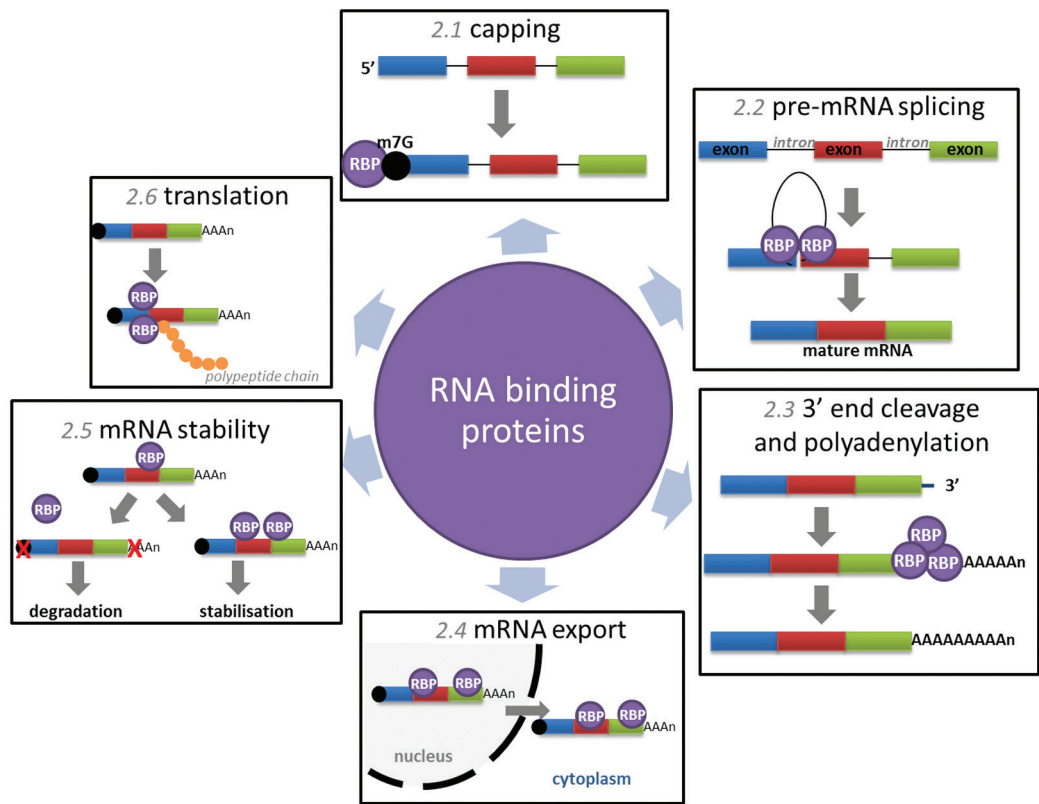
Figure 2: Mechanisms of posttranscriptional control regulated by RNA binding proteins. Capping (**section in this article**) describes the addition of a 7-methylguanosine to the 5' end of nascent mRNA, RBPs bind to the cap and promote mRNA stability. Pre-mRNA splicing (**section in this article**) describes the excision of noncoding introns from nascent mRNA regulated by numerous RBPs within the macromolecular spliceosome. 3'-end cleavage and polyadenylation (**section in this article**) involves cleavage at a defined site and the 3'-end of fully transcribed pre-mRNA followed by the addition of 150–200 adenosine residues, facilitated by a complex of RBPs. mRNA export (**section in this article**) refers to the shuttling of mature mRNAs through the nuclear pore complex to the cytoplasm, mediated by the association of RBPs with specific transcripts. MRNA stability (**section in this article**) can be modulated by transcript associations with specific RBPs, poly(A) tail alterations and decapping often precede rapid degradation. Translation (**section in this article**) is orchestrated by a complex of RBPs, known as polysomes, RBPs can also modulate translation via exonuclease degradation or sequestering of transcripts in protective cytoplasmic compartments. RBP: RNA binding proteins.

stages of splicing including single-strand RNA translocation, strand annealing, and protein displacement.[20]

Despite being composed of a large number of RBPs, very few mutations in core spliceosome components have been found, suggesting that such mutations are nonviable either at the cellular level or in early development.[21] A diverse set of diseases is also associated with even moderate changes in expression of any number of RBPs involved in splicing and splicing regulation.[22,23] Recently, an unbiased genetic screen for essential male fertility genes in the mouse identified the RBP, RBM5, as an essential regulator of haploid male germ cell pre-mRNA splicing and fertility. Mice carrying a missense mutation in the second RNA recognition motif of RBM5, affecting pre-mRNA splicing of putative targets, exhibited spermatid differentiation arrest, which led to azoospermia and male sterility.[24]

Cleavage and polyadenylation
Endonucleolytic cleavage marks the final stage of transcription and is followed by addition of a poly(A) tail at the 3'-end. Similar to the 5' cap, the poly(A) tail is important for the stability and translational efficiency of the mRNA transcript.[25] And like splicing, transcripts can be alternatively polyadenylated, altering stability, localization, and transport. More than half of the genes in the human genome are estimated to be subject to alternative 3'-end processing, generating isoforms that differ in 3' UTR length or encoding different proteins.[26]

With the exception of replication-dependent histone genes, all protein encoding mRNAs contain a uniform 3'-end consisting of around 200 adenosine residues. The formation of this poly(A) tail is directed by sequences present on the pre-mRNA and the polyadenylation machinery. In mammals this consists of six multimeric proteins which come together to firstly mediate cleavage of the nascent mRNA 3'-end, and secondly facilitate coupled polyadenylation, namely: cleavage and polyadenylation specificity factor, cleavage stimulation factor (CstF), cleavage factors I and II (CFI and CFII), poly(A) polymerase, and poly(A)-binding protein II.[27]

A number of reports suggest that there are testis-specific mechanisms which support nuclear polyadenylation in male germ cells. One example of this is the testis-specific CstF paralog, τCstF-64 (gene name: *Cstf2t*), considered necessary for germ-cell polyadenylation and gene expression during spermatogenesis.[28] Targeted deletion of *Cstf2t* resulted in male infertility due to aberrant meiotic and postmeiotic development.[29]

mRNA export
Transport through the nuclear pore complex (NPC) represents the link between the nucleus and cytoplasm. With nuclear export of mature mRNAs likely to involve distinct docking, translocation and release steps from the NPC.[30] Export is mediated by protein factors associated with the mRNA, and mRNAs without the necessary adaptor and export

factors remain held in the nucleus. The mRNA export machinery includes numerous RBPs, ATPase/RNA helicases, and NPC-associated proteins. Most of these are essential, with conditional mutations for many these genes in yeast showing rapid and strong defects in mRNA export.[31]

Following mRNA export from the nucleus, interacting RBPs either remain in the nucleus or accompany the transcript into the cytoplasm, where the transcript either remains bound to the same RBPs or is recruited by others. This ultimately determines the cytoplasmic compartment localisation.[7]

mRNA stability

Maintenance of mRNA stability is essential for the translation of essential proteins and also the proteosomal-mediated degradation of unwanted transcripts. Stability of mRNAs can be rapidly modulated to alter the expression of specific genes, providing flexibility in affecting changes in patterns of protein synthesis. Degradation of transcripts is highly variable and thought to be controlled mainly by RBPs. An important step commonly observed in the regulation of stability is an alteration in the length of the poly(A) tail, which often precedes decapping, and 5ingth of the poly(A).[32] It is often the case that RBPs that accompany the transcript from the nucleus to the cytoplasm, with the assistance of newly assembled translation initiation factors, aid in the recruitment of the translation machinery.[33] In contrast, mRNA binding of the miRNA-induced silencing complex brings about decapping, deadenylation, and translational repression of the mRNA.[34]

Translation

Perhaps the best-described and also most important event that RBPs control is translation. Regulation of translation is controlled through three main mechanisms: poly(A) tail modification, association with RNPs, and competition with translation machinery. Lengthening the poly(A) tail of a transcript can significantly increase translation rates. This is achieved through the regulation of binding of the RBP poly(A)-binding protein (PABP), which recruits factors essential for translation initiation.[35] The most significant mechanism involves directing the transcripts to either cytoplasmic compartments - correlated with translational inhibition, or polyribosomes - known sites of translation.[36] Cytoplasmic compartmentalization of transcripts in RNPs represents a targeted location for processing, sorting, storage, or degradation. Generally associated with translational arrest (stress granules), some RNPs are associated with exonucleases (exosome complexes) and are thus sites for mRNA degradation, while others represent locations where transcripts can be protected and remain translationally silent until they are needed (processing bodies, intermitochondrial cement/nuage, and chromatoid bodies).[7] Polyribosomes or polysomes are groups of ribosomes clustered around a single mRNA transcript, allowing for simultaneous translation of the transcript, resulting in rapid protein production.[7] Polysome aggregates represent the main sites of translation, controlled to correspond with cell needs.

STAGE-SPECIFIC EXPRESSION OF RNA BINDING PROTEINS DURING SPERMATOGENESIS

The stage at which RBPs are expressed during spermatogenesis is also essential in determining their role. Given the sequential process of germ cell differentiation, defining the expression patterns of testis-expressed RBPs in terms of the differentiation status is relatively straightforward. We can sub-divide these RBPs into three major germ cell categories: mitotic, meiotic, and postmeiotic and in the following section explore all three groups in depth, providing examples of each (**Table 1**). It is important to note that some RBPs are expressed throughout all stages of spermatogenesis, quite often serving a variety of functions.

Mitotic RNA binding proteins

Mitotic RBPs consist of those expressed during early stage spermatogenesis, primarily in gonocytes and spermatogonia. Transcription remains active during this period and, as a result, the cell is not as heavily reliant upon posttranscriptional gene regulation. Fewer RBPs have been identified during this stage; however those that are expressed appear to be essential.

Two well-characterized RBPs expressed during mitosis are *Nanos2* and *Nanos3*. *Nanos2* is predominantly expressed in early male germ cells, and the elimination of this gene results in a complete loss of spermatogonia.[37] *Nanos3* is expressed slightly earlier, in migrating gonocytes,[38] with deletion of this factor resulting in complete germ cell loss.[39]

Similarly, the RBP DAZ1, present only in higher primates, is known to be expressed in gonocytes, with increasingly high levels in observed spermatogonial stem cells plateauing at the first wave of meiosis.[40] This unique pattern of expression indicates that DAZ1 participates in differentiation, proliferation, or maintenance of germ cells during early spermatogenesis. Deletion of DAZ1 is associated with azoospermia, strongly suggesting that DAZ1 plays a critical role in normal spermatogenesis,[41] with a predicted function in translational activation.[42]

HnRNP proteins are a class of nuclear pre-mRNA binding proteins with important roles in the biogenesis of mRNA. In the testis, hnRNP proteins are abundant, yet their expression remains tightly regulated. Specifically, hnRNPA1 is highly expressed only in early spermatogonia and absent in later stages.[43] Although no hnRNPA1 mutations have been described, it is predicted based on its role in splicing regulation in other cell systems,[44,45] that this protein would serve a similar essential function during spermatogenesis.

Meiotic RNA binding proteins

These RBPs are present in cells undergoing either of the two meiotic divisions that occur during spermatogenesis. These cells, spermatocytes, initially undergo homologous recombination to differentiate their genome from the parental ones. During this period, the genome is damaged and repaired for crossing over and transcription is blocked.[2] Following this, pachytene spermatocytes begin to synthesize large amounts of stored mRNAs into proteins that will allow them to sustain two consecutive rounds of cell divisions without a real interphase.[4] Round spermatids are the resultant germ cells. Given the importance of posttranscriptional regulation during this period, it is not surprising that there is an abundance of RBPs expressed in meiotic spermatocytes.

TRBP2 (formerly PRBP) is a protamine-1 RBP present in the cytoplasmic compartment of late-stage meiotic cells and haploid round spermatids. Recombinant TRBP2 protein inhibits the translation of multiple mRNAs, suggesting that TRBP2 acts as a translational repressor during spermatogenesis.[46] Mice that carry a targeted disruption of *Tarbp2* are sterile and severely oligospermic due to failure of late-stage spermiogenesis.[47]

Khdrbs1 (formerly SAM68) belongs to the STAR family of RBPs, which regulate a range of processes, including RNA stability, export, splicing, and mRNA translation.[48] The expression of *Khdrbs1* protein during spermatogenesis peaks in spermatocytes as the meiotic cells prepare for division. *Khdrbs1* is a nuclear protein during most of spermatogenesis but is found within the cytoplasm in meiosis.[49] Analysis of the reproductive phenotype of *Khdrbs1* knockout mice

Table 1: RBP expressed during mammalian spermatogenesis, function, and phenotype

RBP	Expression	Function	Phenotype	Reference
RNA binding motif 5 (RBM5)	Nucleus and cytoplasm from spermatogonia to round spermatids	Pre-mRNA splicing	Sterile: spermatid arrest	24
Cleavage stimulation factor 64 testis-specific (τCstF-64)	Nuclei of pachytene spermatocytes through to early spermatids	Nuclear polyadenylation (testis-specific)	Sterile: disrupted meiotic and post-meiotic development	28 and 29
Nanos homolog 2 (NANOS2)	Cytoplasmic p-bodies: gonocytes and spermatogonia	mRNA degradation	Sterile: complete loss of spermatogonia	37 and 39
Nanos homolog 3 (NANOS3)	Stress granules and p-bodies: primordial germ cells to spermatogonia	Translational repression	Sterile: total germ cell loss	38 and 39
Deleted in azoospermia 1(DAZ1)*	Predominately cytoplasmic: gonocytes, spermatogonia, and early spermatocytes	Translational activation	Deletion associated with idiopathic oligozoospermia	40, 41, and 42
Heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1)	Spermatogonia nuclei	pre-mRNA splicing	Not described	43, 44, and 45
TAR (HIV) RNA binding protein 2 (TARBP2)	Cytoplasm of late-stage meiotic cells and round spermatids	Translational repression	Sterile: late stage failure of spermiogenesis	46 and 47
KH domain containing, RNA binding, signal transduction associated 1 (KHDRBS1)	Cytoplasm and nucleus of spermatocytes, decreased levels in round spermatid nuclei	Alternative splicing and translational activation	Sterile: reduced post-meiotic cells and defects in spermiogenesis	49 and 50
Adenosine deaminase domain containing 1 testis-specific (ADAD1)	Nucleus of round and early elongating spermatid cells	pre-mRNA processing	Sterile: defective spermatid morphogenesis	51 and 52
Polyadenylate-binding protein-interacting protein 2 (PAIP2)	Cytoplasm of late elongating spermatids	Major translational regulator	Sterile: multiple defects in late spermiogenesis	53 and 54
Y-box-binding protein 2 (YBX2)	Cytoplasm of post-meiotic round spermatids	mRNA stability	Sterile: late stage spermatid failure	56
Musashi RNA-binding protein 1 (MSI1)	Cytoplasm of spermatogonia, XY body of pachytene spermatocytes	Translational regulation	Not described	100
Musashi RNA-binding protein 2 (MSI2)	Nucleus of spermatocytes and spermatids	Pre-mRNA processing	Sterile: multiple defects in late spermiogenesis**	100

*Description of human RBP; **Testis-specific over-expression. Each RBP described is listed by full name and protein symbol, expression describes the sub-cellular localization of the RBP during mammalian spermatogenesis, function related to method of posttranscriptional regulation, and phenotype describes the spermatogenesis related effects from targeted mutation/knockout of RBP in mice, with related references. RBP: RNA binding proteins

revealed that males are completely infertile due to azoospermia,[50] indicating the requirement of *Khdrbs1* expression in fertility.

Postmeiotic RNA binding proteins

Postmeiotic RBPs describe those expressed in both round and elongating spermatids during the differentiation process known as spermiogenesis. The cessation of mRNA synthesis during spermatid elongation necessitates extensive posttranscriptional regulation as a number of new proteins appear during the later stages of this process.[5]

Adad1 (formerly TENR) is a testis-specific nuclear RBP, detected in postmeiotic cells, primarily in round and early elongating spermatids, and in association with the nuclear scaffold.[51] *Adad1* is predicted to function in either in pre-mRNA editing or transport. Targeted mutation of the *Adad1* gene causes male sterility, through a combination of reduced sperm number, decreased motility, and increased malformed heads in remaining spermatozoa, indicating an essential function in spermatid morphogenesis.[52]

The RBP *Paip2* is a PABP binding partner expressed late elongated spermatids and acts in the translational repression of poly(A)-containing mRNAs.[53] *Paip2*-KO mice exhibit male infertility via translational inhibition of essential development proteins, resulting in defective elongated spermatids.[54]

Ybx2 (also MSY2) is a germ cell-specific member of the Y-box family of proteins with broad DNA and mRNA binding capacity. Largely localized in the cytoplasm, *Ybx2* exhibits maximal expression in postmeiotic round spermatids and is predicted to function in mRNA storage and translational delay.[55] The inactivation of *Ybx2* results in spermatogenic arrest and infertility, with incomplete nuclear condensation prominent in later-stage spermatids at the time of massive spermatid loss.[56] This occurs due to increased mRNA instability, as indicated by polysomal redistribution resulting in decreases to the abundance of numerous mRNAs normally expressed in these germ cells.[56]

MUSASHI FAMILY OF RNA BINDING PROTEINS

Historically, the Musashi family of RBPs has well-established roles in stem cell function and cell fate determination. All Musashi family members contain two tandem RNA recognition motifs located at the N-terminal of the protein, each composed of two highly conserved motifs; RNP-1 and RNP-2, that bind to target mRNAs through a Musashi binding element (MBE, (G/A) U1–3AGU) in the mRNA 3' untranslated region.[57–60] Originally described in *Drosophila*, Musashi has also been well-characterized in *Xenopus*. Both of these model species have provided keen insights into elucidating the pivotal functions of Musashi in the mammalian system, specifically in stem cell maintenance, nervous system development, and tumorigenesis.

Musashi in Drosophila

The original *Drosophila* Musashi (*d*Msi), was discovered to function in the nucleus of adult external sensory organs. Loss-of-function *d*Msi mutants exhibit a phenotype of extra outer support cells.[61] Consequently, *d*Msi was shown to play an essential role in the asymmetric division of the external sensory organs progenitor cells; the sensory organ precursor cells. This was later found to be achieved through *d*Msi-mediated mRNA binding and subsequent translational repression of the neural differentiation inhibitory factor Tramtrack69 (*ttk69*).[62] Similarly, *d*Msi is required to negatively regulate *ttk69* expression in the photoreceptor cells of the developing eye.[63] *d*Msi is also expressed in the central nervous system (CNS) in proliferating neural stem/progenitor cells within the *Drosophila* larvae brain with overexpression resulting in the proliferation of undifferentiated cells.[64]

More recently, a second *d*Msi protein has been identified. Termed RNA-binding protein 6 (*Rbp6*), it is considered more closely related to the vertebrate Musashi proteins due to sequence homology.[65] *Rbp6* is expressed in multiple tissues throughout development, but interestingly, *Rbp6* mutants are viable and fertile, exhibiting only a delay in the timing of larval development.[65] Furthermore, this work showed no overlap in function between *Rbp6* and *d*Msi.

Musashi in Xenopus

Studies in *Xenopus laevis* have uncovered evidence for the additional roles of vertebrate Musashi in posttranscriptional regulation, both in retinal development and oocyte maturation. The *Xenopus* Musashi-1 homolog (*xMsi1*) is a heterogeneous nuclear RBP specifically expressed in the nervous system.[66] During *Xenopus* retina development *xMsi1* is expressed in retinal stem cells, mitotically active neural precursors, postmitotic photoreceptors, and retinal pigment epithelium.[67] In *Xenopus*, Musashi function is also considered essential to establishing the temporal order of maternal mRNA translation during the meiotic cell cycle in developing oocytes.[68] Here, *xMsi1* directs the activation of the mRNAs required for MAP kinase- and CDK-mediated promotion of cell cycle progression.[69] In to oocyte, it has been demonstrated that *Xenopus* Musashi proteins auto-regulate *xMsi1* translation,[70] while Musashi-mediated translational activation of the proto-oncogene *Mos* is considered a necessary event for meiotic cell cycle progression.[68]

Musashi-1

Mammalian Musashi-1 (*Msi1*) is strongly expressed in fetal and adult CNS and brain, with functional roles in the maintenance of stem-cell state, differentiation, and tumorigenesis.[64,71] *Msi1* null mice develop obstructive hydrocephalus and suffer early postnatal lethality.[72] This indicates a vital role for *Msi1* in the normal development of ependymal stem cells and highlights the importance of *Msi1* expression in the proliferation and maintenance of CNS stem cell population.

In mouse neural stem cells, *Msi1* inhibits translation by binding to consensus sequences of transcripts encoding *Numb*,[73] the cell cycle regulator *Cdkn1a*,[73,74] and *Dcx*.[75] The mechanisms described for *Msi1* translational repression competition with eIF4G for PABP binding demonstrates an essential mechanism for preventing the formation of the 80S ribosome.[76] Conversely, in the hindbrain, *Msi1* has been shown to facilitate the translational activation of *Robo3*, required for axonal midline crossing of precerebellar neurons.[77]

Interestingly, *Msi1* is often up-regulated in tumor cells, specifically in malignant gliomas and astrocytomas where *Msi1* is highly enriched when compared with nonneoplastic brain tissue. Furthermore, the level of increased expression was positively correlated with the aggressiveness of the tumour.[78] Considered a putative marker of intestinal stem cells,[79] *Msi1* expression is frequently detected at elevated levels in both premalignant gastric lesions and invasive gastric cancer.[80,81] Up-regulation of *Msi1* has also been linked with endometrial carcinoma[82] and of lymph node metastases.[83,84]

Musashi-2

Musashi-2 (*Msi2*) shares high sequence homology to mammalian *Msi1*, appearing to have arisen following gene duplication.[85] *Msi2*-deficient mice demonstrate 50% embryonic lethality, and subfertility when crossed among themselves.[86]

In neural precursor cells, *Msi2* and *Msi1* are strongly co-expressed and share similar RNA-binding target specificity, as well as being predicted to be co-operatively involved in the proliferation and maintenance of CNS stem cell population.[72] Indeed, extensive studies

of Musashi proteins in the CNS unequivocally favor a redundant and compensatory role for *Msi2* that is indistinct from *Msi1*.[72,87–89] In instances involving primary regulation via *Msi2*, it is common for *Msi1* expression is effectively undetectable.[90,91] However, a recent study in pancreatic islet (endocrine) cells did identify distinctive roles for *Msi1* and *Msi2*.[92]

Like *Msi1*, *Msi2* has also been linked with tumorigenesis. Specifically, up-regulation of *Msi2* has been implicated in brain tumor growth,[93] leukemia progression,[94,95] and the differentiation of hematopoietic stem cells (HSCs).[96] Further studies in HSC utilizing a *Msi2* conditional ablation model revealed failure of HSC maintenance and engraftment resulting from a loss of quiescence and increased commitment divisions.[97]

MUSASHI IN SPERMATOGENESIS

Genetic screening of *Drosophila* genes involved in germ cell biology identified Musashi as a critical regulator of testis stem cell maintenance and meiosis.[98] Using the fly testis as a model system, it has been demonstrated that loss of Musashi function disrupts the balance between germ-line stem cell renewal and later-stage differentiation, resulting in the premature differentiation of germ-line stem cells and meiotic defects.[98] Studies in the mouse have identified differential expression of the two primary mammalian orthologs: *Msi1* and *Msi2*, both at a developmental stage and sub-cellular level.[98,99]

Recent work has by our group detailed the distinctive expression of mammalian *Msi1* and *Msi2* and explored the outcomes of aberrant expression of both RBPs during the process of male gamete development.[100] The unique spatial and temporal expression patterns of the Musashi proteins throughout spermatogenesis indicate individual roles of both RBPs throughout during gamete development in the mammalian testis (**Figure 3**). Specifically, *Msi1* predominately localizes to the cytoplasmic compartments of mitotic gonocytes and spermatogonia, while *Msi2* shows nuclear expression in meiotic spermatocytes and differentiating spermatids. The two novel transgenic mouse models utilized, with germ cell-specific overexpression of full-length isoforms of *Msi1* or *Msi2*, demonstrated that aberrant
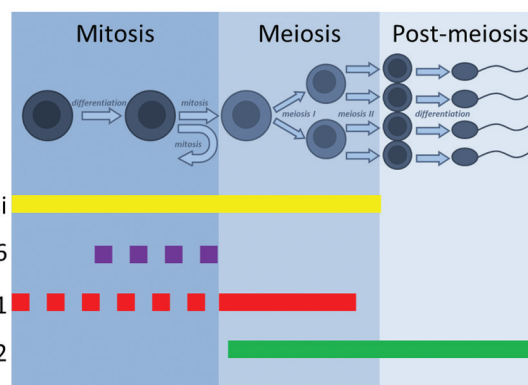

Figure 3: Musashi RBP expression during spermatogenesis. *d*Msi paralogs *d*Msi and *Rbp6* are differentially expressed in the fly testis: *d*Msi is nuclear and expressed in early germ cells and spermatocytes while *Rbp6* is cytoplasmic and localized to spermatogonial cyst cells. Mammalian *Msi1* is expressed in the cytoplasm of early mitotic germ cells before translocating to the nucleus upon transition to meiosis. Mammalian *Msi2* is entirely nuclear, expressed throughout meiosis and during spermatid differentiation. The dotted line refers to cytoplasmic localization while the solid line refers to nuclear-specific expression. RBP: RNA binding proteins; *Msi1*: Musashi-1; *Msi2*: Musashi-2; *d*Msi: *Drosophila* Musashi; *Rbp6*: RNA-binding protein 6.

expression of either gene was deleterious to normal spermatogenesis and detrimental to cell health. In addition to this, preliminary studies performed on human testicular seminoma tumors have provided further insights into the relevance of *Msi1* and *Msi2* over-expression as diagnostic markers to human stem cell cancers.[100]

SUMMARY

Herein, we have highlighted various mechanisms utilized by RBPs within the cell and emphasized the importance of sub-cellular localization and stage-specific expression on the function of these master regulators of posttranscriptional control. Through the detailed description of the Musashi family of RBPs, we acknowledge their fundamental role in stem cell maintenance, nervous system development, and tumorigenesis, and observe their major contributions to our general knowledge of RBPs. Finally, by focusing on the expression and function of Musashi in spermatogenesis, we have provided new evidence for the novel and unique roles of both *Msi1* and *Msi2* during male germ cell development.

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