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# TARBP2 -Mediated Post-Transcriptional Regulation of Gene Expression During Murine Embryonic Development and Spermatogenesis

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# TARBP2 -MEDIATED POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION DURING MURINE EMBRYONIC DEVELOPMENT AND SPERMATOGENESIS

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An Abstract of the Dissertation Presented
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Micro RNAs (miRNAs), which are ~22 nucleotide (nt) long RNA molecules along with several RNA binding proteins (RBPs), engage in an RNA dependent post-transcriptional gene silencing process known as RNA interference (RNAi). In the canonical miRNA biogenesis pathway, an enzyme known as DICER cleaves the ~70nt pre-miRNA to a ~22nt long miRNA that is loaded into the RNAi effector mechanism, the RNA induced silencing complex (RISC).

Several *in vitro* studies provide suggestive evidence that mammalian double stranded RNA binding proteins (dsRBPs), such as TARBP2, act as DICER cofactors in miRNA processing and RISC loading to promote RNAi activity. A screen attempting to identify translational regulators of the murine *Protamine1* gene identified TARBP2 as a potential translation regulator. At the time, I initiated my pre-doctoral studies, it was

unknown if TARBP2 has a role in miRNA biogenesis *in vivo,* or if the translational regulation of *Prm1* during murine spermatogenesis is dependent on TARBP2 mediated miRNA biogenesis.

To investigate the role of TARBP2 in miRNA biogenesis and TARBP2 mediated post-transcriptional gene regulation during spermatogenesis murine embryos with a constitutive null allele of *Tarbp2* and adult mice with a germ cell-specific loss of TARBP2 were generated. I show here that TARBP2 regulates the biogenesis of a sub-set of miRNAs during murine embryonic development and spermatogenesis and that TARBP2-dependent miRNAs regulate translation elongation.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 5'-3' exonuclease | XRNI |
| 60s ribosomal protein L22 | RPL22 |
| A-kinase anchoring protein | AKAP |
| Adenosine | A |
| Adenosine tri phosphate | ATP |
| Adenylate-Uridylate rich elements | ARE |
| Alkaline phosphatase | TNAP |
| Amino-terminus | N-terminal |
| Amyloid β-precursor protein | APP |
| Ankyrin repeat SAM and basic leucine zipper domain containing | GASZ/ASZ |
| Anti-Mullerian Hormone | AMH |
| Arginine | R |
| β-Galactosidase | lacZ |
| Brain derived neurotrophic factor | BDNF |

| | |
|---|---|
| Carboxyl-terminal | C-terminal |
| CCR4-NOT complex transcription factor | CAF1 |
| Chemokine (C-C) motif receptor 4 | CCR4 |
| Complementary DNA | cDNA |
| Cre-Recombinase | Cre |
| Cytosine | C |
| Dead box(Asp-Glu-Ala-Asp) polypeptide | DDX, MVH |
| Decapping protein | DCP |
| Deleted in azoospermia like | DAZL |
| Delta delta CT | ddCT |
| Deoxyribonucleic acid | DNA |
| DGCR8 microprocessor complex sub unit | DGCR8 |
| Dimethyl sulfoxide | DMSO |
| Double stranded | ds |

| | |
|---|---|
| Dystonia | DYT |
| Eukaryotic elongation factor | eEF |
| Eukaryotic initiation factor | eIF |
| Eukaryotic releasing factor | eRF |
| Eukaryotic translation initiation factor-2 alpha kinase 2 | PKR |
| Exon-junction complex | EJC |
| FK506 binding protein 6 | FKBP6 |
| Guanosine | G |
| Guanosine-5'-Triphosphate | GTP |
| Heart and Neural crest derivatives expressed 2 | HAND2 |
| Heat shock protein | HSP |
| Human Immunodeficiency Virus-Trans activating response | HIV |
| Interferon | IFN |
| Long interspersed nuclear elements | LINE |

| | |
|---|---|
| Lysine | K |
| Messenger Ribonucleic acid | mRNA |
| Messenger ribonucleoprotein particle | mRNP |
| Micro RNA | miRNA, miR |
| Microtomography | microCT |
| Mouse embryonic fibroblast | MEF |
| Mov10 RISC RNA helicase like 1 | MOV10L1 |
| Nanos C2HC-type zinc finger 2 | NANOS2 |
| Neurogenin | NGN |
| Non-coding | nc |
| Non-sense mediated Decay | NMD |
| Nuclear Receptor sub-family 2 group E member 1 | NR2E1 |
| Phosphoglycerate kinase | PGK |
| Piwi Argonaut and Zwille | PAZ |

| | |
|---|---|
| Piwi interacting RNA | piRNA |
| Poly A-specific Ribonuclease | PAN |
| Poly-A binding protein | PABP |
| Poly-A binding protein cytoplasmic | PABPC |
| Polymerase chain reaction | PCR |
| Polypyrimidine tract binding protein | PTBP |
| Pre-Initiation complex | PIC |
| Pre-termination codon | PTC |
| Primary | pri |
| Primordial germ cell | PGC |
| Protamine | PRM |
| Protein Kinase interferon inducible double stranded RNA dependent activator | PRKRA |
| Repeat associated small interfering | rasi |
| Replication protein A1 | RPA1 |

| | |
|---|---|
| Reverse transcription PCR | RT-PCR |
| Ribonuclease | RNAse |
| Ribonucleic acid | RNA |
| Ribonucleic acid binding protein | RBP |
| Ribosomal-RNA/DNA | rRNA/rDNA |
| Ribosome | Ribo |
| RNA binding domain | RBD |
| RNA helicase and ATPase | UPF1 |
| RNA induced silencing complex | RISC |
| RNA-interference | RNAi |
| Sex-determining region box-6 | SOX6 |
| Silencing RNA | siRNA |
| Single stranded | ss |
| Small nucleolar | Sno |

| | |
|---|---|
| Sperm mitochondria associated cysteine rich protein | SMCP |
| Spermatid Perinuclear RNA binding protein | SPNR |
| Spermatogenesis associated protein | SPATA |
| Stimulated by retinoic acid gene 8 | STRA8 |
| Synaptonemal complex protein | SYCP |
| Testis specific poly-A protein | TPAP |
| Trans-Activating Responsive Element Binding Protein | TARBP |
| Transfer ribonucleic acid | t-RNA |
| Transition protein | TNP |
| Trinucleotide repeat containing 6 | TNRC6/GW182 |
| Untranslated region | UTR |
| Uracil | U |
| Zinc finger protein 395 | ZNF395 |
| Zona pellucida sperm binding protein | ZP3 |

# CHAPTER 1. INTRODUCTION

" The Central Dogma. This states that once 'information' has passed into protein it cannot get out again. In more detail, the transfer of information from nucleic acid to nucleic acid, or from nucleic acid to protein may be possible, but transfer from protein to protein, or from protein to nucleic acid is impossible. Information means here the precise determination of sequence, either of bases in the nucleic acid or of amino acid residues in the protein."

-Francis H. Crick, 1958

Flow of genetic information from deoxyribonucleic acid (DNA) to messenger RNA (mRNA), and from mRNA to a protein is regulated continuously. Several key players, including transcription factors, RNA binding proteins, and small non-coding RNAs, are involved in this regulation. My research is aimed to understand the role of an RNA binding protein during this regulation, especially at the level of where the genetic information is translated from mRNA into protein.

## 1.1 Steps in post-transcriptional gene expression

Upon transcription of an encoding gene, its mRNA undergoes a series of steps including 5'-capping, excision of introns through splicing, followed by 3'-polyadenylation to generate mature mRNA (Singh, Pratt et al. 2015). Several RNA binding proteins (RBPs) bind to an mRNA generating an mRNP complex, which is then transported to the cytoplasm through the interaction of mRNP components with nuclear pore complexes (Natalizio and Wente 2013, Xing and Bassell 2013). Once inside the cytoplasm, mRNA is localized to cytoplasmic bodies found within specific regions of the cell and is ready to be translated. This is followed by degradation (Buxbaum, Haimovich et al. 2015). Among all these steps,

translation and mRNA degradation in the cytoplasm are key ones. The regulation of both of these steps allows for changes in protein concentrations during the maintenance of cell homeostasis (Garneau, Wilusz et al. 2007, Schoenberg and Maquat 2012).

## 1.2 Translation

Translation of mRNA is carried out in three steps: initiation, elongation, and termination. Translation initiation in eukaryotes occurs mainly in a 5'cap- dependent manner.

Several initiation factors including eIF4E (cap-binding protein), eIF4A (RNA helicase) and eIF4G (large scaffolding protein) are recruited onto the 5' end of the mRNA and form a translation initiation complex (eIF4F). This complex then binds to PABP on the 3' untranslated region (UTR) and facilitates the recruitment of a 43S ribosome to the mRNA resulting in the formation of the pre-initiation complex (PIC) (Wells, Hillner et al. 1998). A closed loop structure formed by the association between PABP and eIF4E renders stability to the translating complex. The pre-initiation complex then starts scanning along the 5'-UTR to detect the start codon (AUG) near the 5' end of the mRNA. The presence of the "Kozak consensus" sequence, 5'(A/G)CCAUGG 3' is considered as a favorable sequence for translation initiation (Kozak 1986). The presence of secondary structures (Kozak 1990) in the 5' UTR and the size of 5' UTR (Pestova and Kolupaeva 2002) can alter the efficiency of PIC to scan for the AUG start codon. Inefficient scanning by PIC for a favorable start codon results in a translation initiation block or protein isoform synthesis (Sedman, Gelembiuk et al. 1990). Once the favorable start codon is detected, a 60S ribosomal sub-unit joins the PIC, resulting in the formation of an 80S ribosome (Hinnebusch and Lorsch 2012).

### 1.2.1 Translation elongation and termination

The addition of each amino acid into a nascent peptide during translation elongation is characterized by moving of the 80S ribosome along the length of the mRNA at a distance of three nucleotides precisely. During the initial process of decoding, amino-acyl-t-RNAs form a complex with eEF1A bound to GTP to form a complex that is transferred to the A-site of a ribosome. When a match is found between the amino-acyl-t-RNA and a codon on the mRNA, a new amino acid is added to the nascent peptide. The addition of the amino acid to the nascent peptide results in a massive rearrangement of the ribosome in which each sub-unit rotates relative to each other. This causes the translocation of the ribosome along the mRNA, which is facilitated by the energy released from GTP-hydrolysis by eEF-2. With this movement, a new codon is exposed to the A-site while the P-site is still attached to the nascent peptide. This process continues until the A-site encounters a termination codon. Once a ribosome reaches a termination codon, releasing factors bind to the P-site and promote hydrolysis of peptidyl-tRNA. This results in the termination of translation coupled with the release of the poly-peptide chain from the translation complex (Lareau, Hite et al. 2014, Ali, Ur Rahman et al. 2017). This process is illustrated in Figure1.1

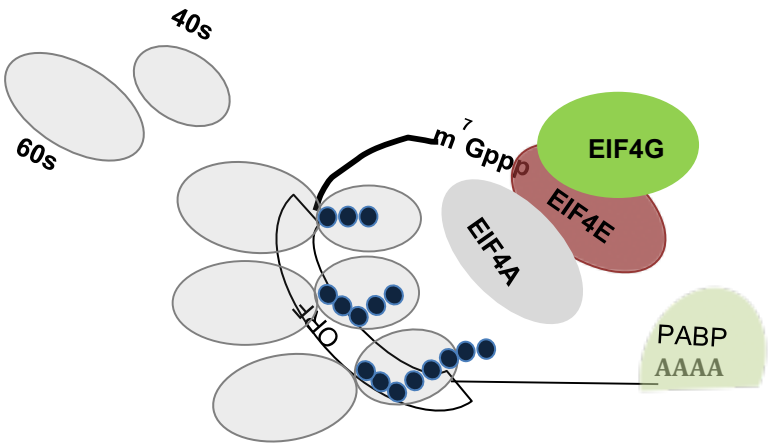



**Figure 1.1: Translation initiation and elongation**

## 1.3 mRNA degradation

Once an mRNA is exported in to the cytoplasm, it either undergoes translational repression, or gets translated and eventually undergoes degradation (Moore 2005). mRNA degradation is a common process by which to regulate the turnover of mRNA, thereby regulating protein expression. The most common form of mRNA degradation is de-adenylation of mRNA followed by exonuclease digestion. As soon as an mRNA enters the cytoplasmic region of a cell, de-adenylation is initiated. Usually, 5'-capping and 3' polyadenylation along with the binding proteins (PABP) causes an mRNA to become resistant to 5' and 3' exonucleases (Muhlrad, Decker et al. 1994, Mangus, Evans et al. 2003). Thus, mRNA degradation is characterized by removing of cap structures, de-adenylation on the 3'UTR, followed by exonuclease activity on the 5' and 3' ends of the mRNA. De-adenylation is initiated by the CCR4-CAF1 complex in eukaryotes leading to decapping of the 5' end by DCP1-DCP2 complex, and subsequent exo-nucleolytic degradation by XRN1 (Beelman and Parker 1995, Tucker, Valencia-Sanchez et al. 2001, Chen, Chiang et al. 2002, Coller and Parker 2004, Yamashita, Chang et al. 2005, Zheng, Ezzeddine et al. 2008). During the normal translation process, once the ribosome recognizes the stop codon at the end of the mRNA transcript, a UPF1-eRF1-eRF3 trimer complex interacts with PABP in the 3'UTR (Ivanov, Gehring et al. 2008, Shyu, Wilkinson et al. 2008). This interaction results in the recruitment of PAN3 which allows for the recruitment of PAN2 (Zheng, Ezzeddine et al. 2008). This PAN3-PAN2 complex also interacts with and recruits a CCR4/CAF1 deadenylase complex, resulting in the formation of a super-deadenylase complex (Boeck, Tarun et al. 1996, Brown, Tarun et al. 1996, Uchida, Hoshino et al. 2004).

During conditions in which a stop codon exists in the open-reading frame of an mRNA, deadenylation at the 3' end is triggered, leading to mRNA degradation. This process is known as non-sense mediated decay (NMD) (Nagy and Maquat 1998). During NMD, ribosomes are able to recognize and distinguish a pre-termination codon (PTC) from normal termination codon due to the presence of proteins in exons that are responsible for splicing that helps in the formation of exon junction complexes (EJCs) (Kashima, Yamashita et al. 2006, Rebbapragada and Lykke-Andersen 2009). Once the ribosome recognizes a PTC in conjunction with EJC's, this recognition amplifies the recruitment of PAN3 to PABP, and triggers de-adenylation followed by mRNA degradation. Figure 1.2, shows some of the main players involved in the deadenylation and degradation of mRNA.

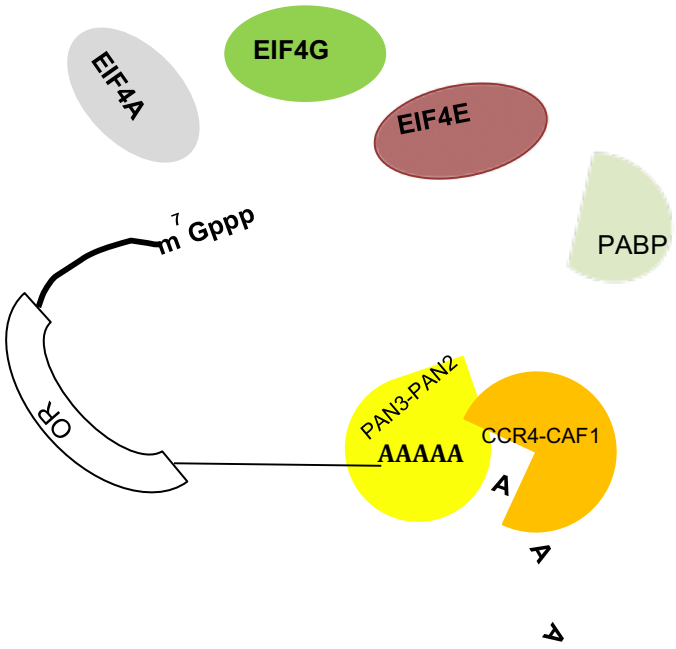



**Figure 1.2: mRNA degradation by deadenylation**

## 1.4 Post-transcriptional regulation of gene expression

The regulation exerted during mRNA translation or degradation is termed post-transcriptional regulation of gene expression. The relationship of this process to transcriptional and translation regulation is shown in Figure 1.3. Each step during post-transcriptional gene expression is tightly regulated, and this regulation is essential for physiological responses of cells, especially in mammals. Sequence or structure-specific, single stranded (ss)-RBPs or double stranded (ds)-RBPs and non-coding (nc)-RNAs including micro-RNAs (miRNAs), are key factors that regulate both translation and mRNA degradation. In this section, I will discuss miRNA-mediated post-transcriptional regulation of gene expression.

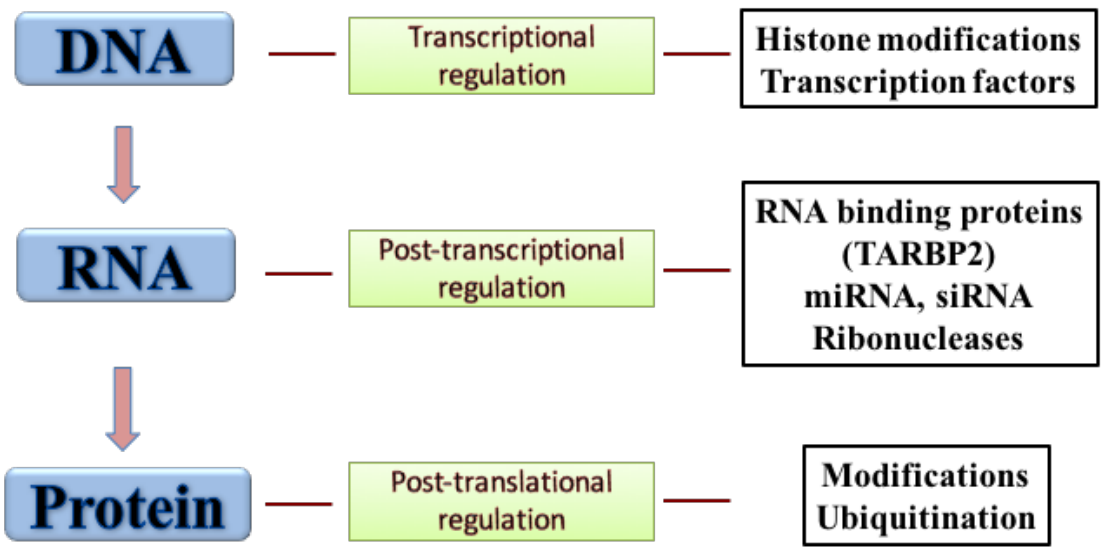



**Figure 1.3: Gene expression stages and regulation**

### 1.4.1 miRNA mediated post-transcriptional regulation of gene expression

miRNAs are a large family of endogenous ~22 nucleotide (nt) nc-RNAs that regulate the translational efficiency and decay of mRNAs (Krol, Loedige et al. 2010). The mouse genome is estimated to encode about 722 miRNAs, and these are predicted to control the activity of ~50% of all protein-coding genes (Baek, Villen et al. 2008, Griffiths-Jones, Saini et al. 2008, Selbach, Schwanhausser et al. 2008, Friedman, Farh et al. 2009). Although a miRNA is ~22nt in length, a seed sequence of ~2-8nt long is essential for an interaction of a miRNA with its mRNA target. miRNA interaction with a target mRNA results in lower mRNA levels. This miRNA-mediated lowering of mRNA levels has been shown to account for ~75-85% of the resultant protein level change, suggesting the impact of miRNAs on gene expression occurs predominantly during the post-transcriptional stage (Hendrickson, Hogan et al. 2009, Guo, Ingolia et al. 2010).

The mechanisms by which miRNAs regulate the process of translation and mRNA degradation have been well characterized. Extensive base pairing of the miRNA "seed" sequence to the target mRNA inhibits protein synthesis (Bartel 2009). Translational repression, or an Argonaut (AGO)-catalyzed cleavage of the target mRNA, ensues upon binding (Fabian, Sonenberg et al. 2010, Djuranovic, Nahvi et al. 2011). When a seed sequence is one hundred percent complementary to the target mRNA, a trinucleotide repeat containing protein, TNRC6/GW182 is recruited onto the 3'UTR region of the mRNA by AGO. Binding of the TNRC6/GW182 then recruits PAN2-PAN3 followed by the CCR4-NOT exonuclease, resulting in deadenylation and mRNA degradation respectively (Fabian, Sonenberg et al. 2010, Jonas and Izaurralde 2015).

The actual role of miRNAs in translational regulation is still inconclusive, as some studies report that miRNAs promote translation activation or elongation instead of repression (Vasudevan, Tong et al. 2007, Zhang, Tang et al. 2017). The role of miRNAs in regulating translation is likely tissue specific and would vary depending on the features (e.g. presence of AU-Rich elements (ARE) or short 3'UTR sequence) and the fate of the mRNA (storage or translation). Although the exact mechanism by which miRNAs promote translation needs further understanding, the mechanism by which miRNA dependent translational repression occurs has been well characterized. Binding of both a miRNA that contains a mismatched seed sequence, along with AGO, in the 3'UTR of an mRNA leads to the recruitment of a TNRC6/GW182 complex, which dissociates PABP (Huntzinger, Braun et al. 2010, Moretti, Kaiser et al. 2012, Zekri, Kuzuoglu-Ozturk et al. 2013). This leads to the release of a closed loop structure that would otherwise stabilize the PIC. This is followed by recruitment of a CCR4-NOT complex for deadenylation along with the decapping enzyme, DDX6 (Chen, Boland et al. 2014, Mathys, Basquin et al. 2014). Figure 1.4 illustrates some of the players that contribute to miRNA mediated mRNA degradation and translational inhibition.

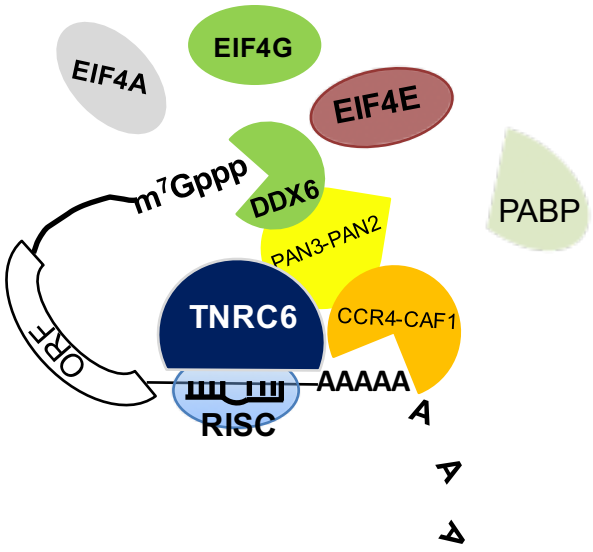



**Figure 1.4: miRNA mediated translation inhibition and mRNA degradation**

The expression and conservation of miRNAs along with their associated proteins in plants, flies, nematodes and vertebrates suggests that miRNA-mediated regulation of gene expression is conserved and is important for regulating cell fates in multiple forms of life. miRNA mediated post-transcriptional regulation was first identified *in Caenorhabditis.elegans*. During the first larval phase of *C.elegans,* mRNA encoding the transcription factor LIN-14 is degraded by binding of a small RNA, *lin-4,* to its 3'UTR region (Lee, Feinbaum et al. 1993, Wightman, Ha et al. 1993). During embryonic development in zebrafish, maternal oocyte mRNAs are translationally inhibited and then degraded by the expression of miR-430, which targets hundreds of mRNAs in the 3'UTR region (Bazzini, Lee et al. 2012).

In mice, many miRNAs exhibit strict developmental stage and tissue-specific expression patterns (Landgraf, Rusu et al. 2007). Several studies have shown that miRNAs are essential in the development of organs, including heart, brain, skin and skeletal muscle,

the development of male and female germline, and in blood cell differentiation. A failure in miRNA-mediated post-transcriptional regulation leads to several developmental disorders or diseases in adult systems (Coffre and Koralov 2017, Horsburgh, Fullard et al. 2017, Li, Fu et al. 2017, Rajman and Schratt 2017, Weiss and Ito 2017, Wojciechowska, Braniewska et al. 2017, Tesfaye, Gebremedhn et al. 2018).

During the early stages of heart muscle development in mice, miR-133a and miR-1a suppress smooth muscle generation and enhance cardiomyocyte proliferation by targeting the 3'UTR regions of *CyclinD2* and *Hand2*, respectively (Liu, Bezprozvannaya et al. 2008, Heidersbach, Saxby et al. 2013). Elevated levels of *Sox6* expression, creating an imbalance between the differentiation of fast and slow twitch muscles, was observed with the loss of a heart-specific miR-203a during heart muscle differentiation in mouse (Ding, Chen et al. 2015). miRNAs are also needed for proper development of the nervous system. For example, miRNAs, miR-9 and let-7b directly target the 3'UTR and suppress NR2E1 receptor expression and translation of *CyclinD1,* thereby regulating neuronal stem cell self-renewal and differentiation during early neuronal development in mice. miR-9 also regulates neuronal differentiation in the developing mouse retina (Qu, Sun et al. 2010, Ding, Chen et al. 2015). Anti-proliferative and pro-differentiative *Tis21* expression is regulated by miR-92 interaction with the 3'UTR region of its mRNA. Loss of this regulation results in microcephaly in mouse (Fei, Haffner et al. 2014).

During germ line development of mice, several miRNAs are differentially expressed, among which two major classes-miR17-92 and miR290-295 clusters have been shown to be critical (Hayashi, Chuva de Sousa Lopes et al. 2008, Ventura, Young et al. 2008). miR-17-92 micro RNAs are highly expressed in early primordial germ cell stages and then become

downregulated in female primordial germ cells (PGCs) (Tang, Kaneda et al. 2007). Whole body knock out of the miR290-295 cluster results in female mice that can survive up to adulthood, but are sterile, suggesting the importance of this cluster in successful female gamete development (Blakaj and Lin 2008). The role of miRNAs in male gamete development will be discussed in detail in section 1.5.

### 1.4.2 miRNA biogenesis

miRNAs are transcribed by RNA polymerase II as long pri-miRNAs that are capped, polyadenylated and frequently spliced, while a minor group of miRNAs are transcribed from the introns of protein-coding genes. The formation of a stem-loop structure in the primary (pri)-miRNA acts as a substrate for two members of the RNase III family of enzymes, DROSHA and DICER (Bernstein, Caudy et al. 2001, Grishok, Pasquinelli et al. 2001, Hutvagner, McLachlan et al. 2001, Ketting, Fischer et al. 2001, Knight and Bass 2001).

pri-miRNAs are characterized by the presence of a UGU motif in the apical loop, a GHG motif in the stem, and a UG and CNNC motif in the basal region (Auyeung, Ulitsky et al. 2013). DGCR8 first recognizes and binds to the apical UGU motif recruiting DROSHA through its C-terminal tail. DROSHA is then placed at the UG motif close to a hairpin loop, cleaving the pri-miRNA to an ~70 nucleotide stem-loop containing, pre-miRNA (Denli, Tops et al. 2004, Gregory, Yan et al. 2004, Han, Lee et al. 2004, Landthaler, Yalcin et al. 2004, Nguyen, Jo et al. 2015). The pre-miRNA is actively transported to the cytosol via Exportin-5 and incorporated into a multiprotein complex containing the second RNase III enzyme, DICER (Yi, Qin et al. 2003, Lund, Guttinger et al. 2004).

DICER contains an ATPase/RNA helicase domain, a PAZ domain, two RNAseIII domains and a C-terminal RNA binding domain (Yan, Yan et al. 2003, Zhang, Kolb et al.

2004, Lau, Guiley et al. 2012). DICER binds to pre-miRNA upon recognition of a 5'end free phosphate group by the PAZ domain (Park, Heo et al. 2011). The distance between the PAZ and RNAse domains acts as ruler for DICER cleavage, leading to cleavage of a hairpin loop structure (Macrae, Zhou et al. 2006). This cleavage results in the formation of a mature mi-RNA duplex, among which, one strand acts as a lead strand that targets mRNA while the other strand gets degraded. While DICER alone can process miRNA, the presence of dsRBPs, such as TARBP2 or PRKRA, helps DICER to recognize miRNAs from other types of small RNAs in an RNA crowded environment (Lee, Zhou et al. 2013, Wilson, Tambe et al. 2015). Mature miRNA, along with TARBP2 and DICER, recruits AGO proteins, resulting in the formation of RNA induced silencing complex (RISC) and the occurrence of RNA interference (RNAi) as illustrated in Figure 1.5 (Chendrimada, Gregory et al. 2005, Haase, Jaskiewicz et al. 2005, Chakravarthy, Sternberg et al. 2010, Kawamata and Tomari 2010, Czech and Hannon 2011). The role of some of these biogenesis factors in regulating key physiological processes will be described in detail in the following sections.

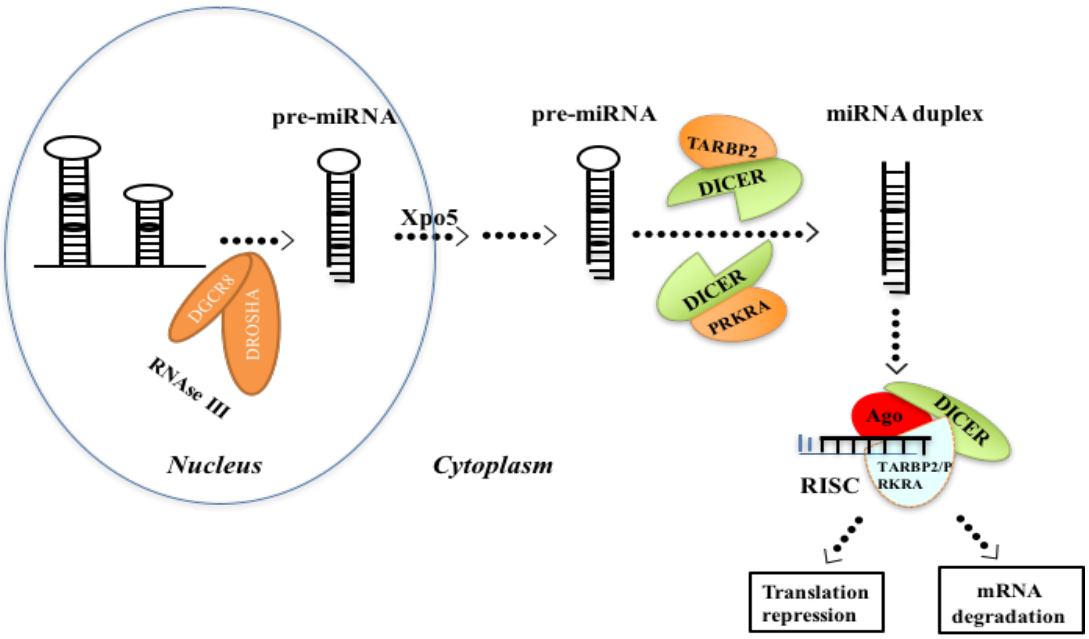



**Figure 1.5: Canonical miRNA biogenesis**

### 1.4.3 DICER

The RNAseIII enzyme DICER has been extensively studied as an endonuclease that cleaves dsRNA into small non-coding RNA including miRNAs and silencing (si) RNAs. DICER expression is conserved in many eukaryotes exhibiting RNAi mechanisms. RNAi mediated through siRNA and miRNA relies on expression of a single DICER protein in many vertebrates including mammals (Gao, Wang et al. 2014, Svobodova, Kubikova et al. 2016). In contrast to mammals, which express one *Dicer* gene, some animals, including *Drosophila melanogaster*, the shrimp *Litopenaeus vannamei* and the planarian *Schmidtea mediterranea*, two *Dicer* genes, *Dicer1* and *Dicer2,* are expressed. *Dicer1* is required for miRNA biogenesis, and ATP-dependent *Dicer2* is required for siRNA biogenesis (Lee, Nakahara et al. 2004).

Recent studies have shown the ability of DICER to process small nc-RNAs other than miRNAs or siRNAs. These include tRNA fragments (tRFs). Among several groups of tRFs,

the major groups have been classified as 3' U tRFs, 5' U trFs and 3' CCA tRFs (Lee, Shibata et al. 2009). Among these groups, 5' U trFs and 3' CCA tRFs are characterized by the presence of 5' phosphate and 3' hydroxyl groups, which can be recognized and cleaved by DICER (Babiarz, Ruby et al. 2008). *In vitro* and *in vivo* experiments have shown that mammalian DICER generates CU1276, which is a ~22nt long tRNA (Gly)-derived small RNA that inhibits the translation of *Rpa1* mRNA by binding to its 3'UTR region (Cole, Sobala et al. 2009, Langenberger, Cakir et al. 2013, Maute, Schneider et al. 2013). DICER has also been shown to be required for production of miRNAs from snoRNAs and rasiRNAs from double stranded structures of LINE-1elements (Saraiya and Wang 2008, Faulkner 2013). In mice, the depletion of DICER is associated with ectopic expression of Alu-repeat RNAs. This causes Geographic Atrophy in the eye, suggesting DICERs ability to cleave Alu repeats (Kaneko, Dridi et al. 2011).

DICER localization within the cell is different for humans and mice. Human DICER is localized inside the cell cytoplasm and nucleus, where it mediates multiple functions, including regulating the synthesis of siRNA, the synthesis of ribosomal (r) RNA by binding to rDNA, and formation of heterochromatin structure (Fukagawa, Nogami et al. 2004, Sinkkonen, Hugenschmidt et al. 2010, Kalantari, Chiang et al. 2016). However, in mouse using a reporter system *in vivo*, DICER was shown to be exclusively cytoplasmic in cells from several tissues, suggesting its role in post-transcriptional regulation is localized to the cytoplasm (Much, Auchynnikava et al. 2016).

DICER in mice has been shown to be essential for the development and normal physiological function of almost every organ. During early embryonic development in mice, maternal DICER and DICER-dependent miRNAs are expressed until the 8-cell stage and

then taken over by endogenous DICER (Murchison, Stein et al. 2007, Tang, Kaneda et al. 2007). Complete loss of DICER expression in the developing mouse embryo results in embryonic lethality by embryonic day7.5 (E7.5), just before gastrulation (Bernstein, Kim et al. 2003).

Cell-specific knock out studies in male and female reproductive organs of mice suggest a role of DICER in successful gamete development (Hong, Luense et al. 2008). In mice, DICER is expressed in both somatic and germ cells of the testis (Comazzetto, Di Giacomo et al. 2014). *Dicer* ablation in PGCs as early as E10 using *Tnap-cre* causes defects in proliferation of germ cells, and in post-natal spermatogenesis (Hayashi, Chuva de Sousa Lopes et al. 2008, Maatouk, Loveland et al. 2008). *Ddx4-cre* mediated deletion of *Dicer* expression in spermatogonia at E18 results in defective meiotic progression characterized by a delay in transition from leptotene to zygotene or pachytene, and apoptosis of pachytene spermatocytes during germ cell development (Romero, Meikar et al. 2011). Sertoli cell specific deletion using *Amh-cre* results in the absence of Sertoli cell maturation followed by germ cell loss and degeneration of seminiferous tubules (Papaioannou, Pitetti et al. 2009, Papaioannou, Lagarrigue et al. 2011). Post-natal ablation of DICER expression in spermatogonia of a mouse testis using *Stra8-icre* or *Ngn3-Cre* results in defective spermiogenesis (Korhonen, Meikar et al. 2011, Greenlee, Shiao et al. 2012). However, *Stra8-icre* mediated deletion also causes a delay in meiotic cell stage progression, whereas *Ngn3-cre* mediated deletion leads to a defect in cell-cell junction formation between haploid spermatids and Sertoli cells (Korhonen, Yadav et al. 2015). *Prm1-Cre* mediated deletion of *Dicer* in haploid spermatids results in improper chromatin condensation, abnormalities in

sperm shape and defective translation regulation due to loss of miRNA expression (Chang, Lee-Chang et al. 2012).

Of interest, the phenotype of *Dgcr8*$^{-/-}$ mice is less severe than that of *Dicer*$^{-/-}$ (Wu, Song et al. 2012, Zimmermann, Romero et al. 2014). As described in section 1.4.2, DGCR8 has an important role in miRNA biogenesis. Together, these results, along with the role of DICER in regulating multiple cellular processes, suggests that the severe phenotype observed in many organs due to loss of DICER expression cannot be due to improper miRNA biogenesis alone.

### 1.4.4 TARBP2

Protein-RNA binding screens using HIV-TAR RNA helped lead to the identification of human TRBP1 and TRBP2 and cloning of the encoding gene from Hela cells (Gatignol, Buckler-White et al. 1991). These two proteins are identical to each other except for the presence of an additional 21amino acids towards the N-terminus of TRBP2, due to translation of an alternative first exon (Bannwarth, Talakoub et al. 2001). The gene encoding TRBP is present on human chromosome 12 and in mouse, *Tarbp2* gene is located on chromosome 15. Usage of alternative promoters adjacent to each other during transcription results in the inclusion of alternative exons in its mRNA. The murine homolog of human TRBP, termed PRBP or TARBP2, was identified and characterized several years after the cloning of the human gene. It was identified by screening a cDNA library for RNA binding proteins from mouse testes (Lee, Fajardo et al. 1996). TARBP2 is expressed at high levels in both human and murine testis compared to any other organs (Lee, Fajardo et al. 1996, Siffroi, Pawlak et al. 2001). Homologs of TRBP were identified in other animals including *Xenopus* (homolog is termed *Xlrbpa*) (Eckmann and Jantsch 1997), *Drosophila* (homolog is named

Loquacious or LOQ) (Forstemann, Tomari et al. 2005) and *C. elegans* (termed RDE-4) (Blanchard, Parameswaran et al. 2011).

Murine PRBP or TARBP2 is 93% identical to human TRBP with three structural domains: dsRBD1, dsRBD2 and dsRBD3. dsRBD1 and dsRBD2 together are essential for binding of these proteins to double stranded RNA. Among these domains, the second domain has the strongest affinity for RNA due to the presence of a KR-helix motif in its structure (Daviet, Erard et al. 2000, Benoit, Imbert et al. 2013). Previously, it was shown that TARBP2 binds to asymmetric structures formed by perfectly matched, GC rich regions of double stranded RNA (Lee, Fajardo et al. 1996, Parker, Maity et al. 2008, Kini and Walton 2009, Gredell, Dittmer et al. 2010). However, it is still unclear if sequence or structure determines the binding of TARBP2 to mRNA as recent studies indicate that TARBP2 binds to bulge structures formed by unpaired regions of miRNA stem (Zhu, Kandasamy et al. 2018). The role of dsRBD3 in binding to RNA is unknown even though it exhibits structural homology with the other two domains. However, this domain plays a role in the binding of TARBP2 with other proteins, including DICER, PRKRA, MERLIN. Hence, this domain is also known as the MeDiPal domain (Cosentino, Venkatesan et al. 1995, Lee, Kim et al. 2004, Laraki, Clerzius et al. 2008, Daniels, Melendez-Pena et al. 2009, Chukwurah, Willingham et al. 2018).

Although TARBP2 was first identified as a protein that binds to HIV-TAR RNA and enhances its replication in human cells, it has been extensively studied as a protein that regulates RNAi (Chendrimada, Gregory et al. 2005, Forstemann, Tomari et al. 2005, Haase, Jaskiewicz et al. 2005). Inside a cell containing several types of RNAs, TARBP2 binds to the 3' ends of siRNA (Gredell, Dittmer et al. 2010) or the stem structure of miRNA (Zhu,

Kandasamy et al. 2018) in an ATP-independent manner, and recruits DICER to the opposite end near the stem loop structure of the pre-miRNA (Koh, Kidwell et al. 2013, Wilson, Tambe et al. 2015). Using high throughput sequencing (HTS), Morin and co-workers established that each pre-miRNA can be processed to produce mature miRNAs that deviate from the annotated reference miRNAs (Morin, O'Connor et al. 2008). These "isomiRs" are loaded into the RISC and mediate post-transcriptional gene regulation (Cloonan, Wani et al. 2011). IsomiRs can be generated by changing the DROSHA or DICER cleavage sites in the pri-miRNA and pre-miRNA, respectively. Regarding the roles of TARBP2, several independent groups have shown the importance of this protein in regulating the isomiR production using different model systems (Fukunaga, Han et al. 2012, Lee and Doudna 2012, Lee, Zhou et al. 2013). For example, Loquacious (Loqs), the Drosophila ortholog of TARBP2, can change the DICER dependent cleavage site up to 2nt positions (Fukunaga, Han et al. 2012). With the loss of LOQS expression, miRNAs of different lengths with altered seed sequences are generated, even though they are derived from the same pre-miRNA. Such a sequence change to the miRNA seed sequence could dramatically influence the repertoire of targeted mRNAs. Loss of TARBP2 expression in human cells results in deregulation of mature miRNA expression and accumulation of isomirs (Kim, Yeo et al. 2014). In summary, the above studies have shown roles of TARBP2 in miRNA biogenesis and with respect to this, being able to regulate miRNA diversity and downstream mRNA targets by controlling DICER cleavage sites.

Based on its above described roles, it might be hypothesized that TARBP2 would be involved in mediating normal development, and the findings from number of studies support this. For example, results from cell specific ablation of TARBP2 expression in cardiac cells

suggest a role for TARBP2 in regulating the switch between fast and slow twitching muscles through RNAi mediated translation regulation (Ding, Chen et al. 2015). Recent studies have shown that TARBP2 regulates localized translation of BDNF through an RNAi dependent mechanism in neuronal cells, thereby regulating BDNF mediated dendritogenesis (Antoniou, Khudayberdiev et al. 2018). Overall, all these studies suggest the role of TARBP2 in post-transcriptional regulation during development could be due to miRNA dependent translation regulation.

A constitutive null allele of *Tarbp2* on the C57BL/6J strain mouse was shown to be lethal. On a mixed genetic background (B6129S4) animals survive up to adulthood, although they are smaller than littermate controls and have a shorter life span (Zhong, Peters et al. 1999). Mice that survive and live up to reproductive age are sterile with accompanying defects in translational activation of *Protamine 1* (*Prm1)* mRNA, which is under temporal translational regulation during spermiogenesis (Lee, Fajardo et al. 1996, Zhong, Peters et al. 1999). It is unknown if the defective translational activation of the *Prm1* mRNA is mediated through the action of TARBP2 as a cofactor of DICER and whether the effect is dependent on miRNAs.

Recently, some studies have elucidated the role of TARBP2 in RNAi independent mechanisms. In metastatic breast cancer cells, TARBP2 was shown to bind directly to secondary structure elements in 3'UTR regions of *App* and *Znf395* transcripts. This binding lead to the destabilization of these transcripts, thereby promoting the invasion of metastatic cells (Goodarzi, Zhang et al. 2014). Under stress conditions, especially during viral infections or during the M1-G transition in normal cell cycle progression, TARBP2 inhibits eIF2 phosphorylation, thus promoting global translation in Hela cells (Park, Davies et al. 1994,

Kim, Yeo et al. 2014). While studies such as these suggest a role for TARBP2 in RNAi independent mechanism, further studies are required to provide more evidence in this regard.

Even though the role of TARBP2 in siRNA and miRNA biogenesis in mammalian systems is evident based on biochemical and *in-vitro* analyses, there is a need to investigate if TARBP2 has a role in miRNA biogenesis *in vivo* and if the translation regulation observed during murine spermatogenesis is dependent on miRNAs. My research is focused on providing some insights in this regard and I will discuss my findings in the next two chapters.

### 1.4.5 PRKRA

PRKRA was identified as a protein that binds to Protein kinase regulated by RNA (PKR) under stress conditions resulting in enhanced phosphorylation of eIF2α thereby shutting down global translation (Patel and Sen 1998, Patel, Handy et al. 2000). Through this mechanism, PRKRA acts as a key modulator of the anti-viral and anti-proliferative effects of IFN. Although several studies suggest the role of PRKRA is to activate PKR, one study suggests the role of PRKRA is to negatively regulate PKR activity during anterior pituitary development (Dickerman, White et al. 2015). Together, these studies suggest that the actual mechanism of PRKRA-mediated translation regulation varies during normal versus stress conditions.

In one study using human cell lines, it was shown that under normal cell homeostasis conditions, TARBP2 directly binds to PRKRA, inhibiting PRKRA-mediated activation of PKR and promoting translation. However, this TARBP2-PRKRA interaction was absent under stress conditions, leading to global translation shutdown (Daher, Laraki et al. 2009). This study suggests the antagonistic roles of TARBP2 and PRKRA in regulating global translation.

*Prkra* is the only mammalian *Tarbp2* paralog and encodes a protein with high sequence and structural similarity to TARBP2. Like TARBP2, PRKRA contains two dsRBDs and a C-terminal "Medipal" domain that interacts with DICER (Laraki, Clerzius et al. 2008). Studies on the role of PRKRA in small RNA biogenesis have been conflicting. Several biochemical studies have suggested a role of *Prkra* in small RNA biogenesis as a cofactor of DICER. However, loss of *Prkra* in HeLa cells has no effect on mature miRNA levels suggesting PRKRA has no role in miRNA biogenesis in mammalian systems. (Kim, Yeo et al. 2014).

Like TARBP2, PRKRA plays an important role during murine development. *Prkra*$^{-/-}$ mice exhibit developmental defects with reduced body size. These mice have severely impaired hearing due to abnormalities in the outer ear and inner ear, exhibiting microtia in the outer ear (Rowe, Rizzi et al. 2006). Some mutant mice exhibit pituitary anterior lobe hyperplasia, and these mice are sterile due to defects in pituitary function (Peters, Seachrist et al. 2009).

In human patients with Dystonia, using high-density genome wide SNP genotyping, a mutation in the *Prkra* gene was identified in a novel DYT16 genome locus suggesting its role in causing Dystonia among humans. These patients were resistant to translation-based levodopa therapy, suggesting the mechanism of disease phenotype is related to PRKRA interplay in translation regulation (Camargos, Scholz et al. 2008).

Although there is enough evidence to suggest an interaction between PRKRA and PKR under stress conditions, there is a need to explore the actual mechanism by which PRKRA regulates translation during normal cell homeostasis. Furthermore, none of these studies have characterized the role of PRKRA in small RNA biogenesis *in vivo,* which

introduces an important gap, considering the structural similarity with TARBP2 and binding properties with DICER. In Chapter.2, I present data addressing the role of PRKRA in miRNA biogenesis during murine embryogenesis.

## 1.5 Spermatogenesis and post-transcriptional regulation of gene expression

Spermatogenesis is the process during which spermatozoa are produced from diploid spermatogonia after mitotic proliferation, meiosis and haploid spermatid differentiation (referred to as spermiogenesis). New transcription, and presumably, regulated RNA degradation, effect the major changes in mRNA populations during the cell state transitions that occur during spermatogenesis. As transcription ceases by the end of meiosis, and translation continues throughout spermatogenesis, translational regulation is required for correct temporal protein synthesis of many proteins involved in spermatid differentiation, including the highly basic transition proteins (TNP1, TNP2) and protamines (PRM1, PRM2) that are required for chromatin condensation in mature sperm (Monesi 1964, Monesi, Geremia et al. 1978, Braun 1998, Brewer, Corzett et al. 2002). RNA degradation, RNA stabilization and temporal translation during spermatogenesis rely on RNA-binding proteins, small non-coding RNAs, and nucleases.

As mentioned earlier, transcript stability in cells, including germ cells, is dependent on adenylation of mRNA at the 3' end. A number of proteins have been characterized that regulate this adenylation. Testis specific poly-adenylation protein, TPAP was identified as an important regulator of murine spermatogenesis (Kashiwabara, Zhuang et al. 2000, Kashiwabara, Noguchi et al. 2002). Germ cell specific loss of TPAP results in improper spermatid differentiation due to defective polyadenylation of mRNA (Kashiwabara, Tsuruta et al. 2016). Several poly-A binding proteins including, PABPC1, testis-specific PABPC2 and

EPAB bind to mRNAs and protect them from enzyme-mediated deadenylation during murine spermatogenesis (Kimura, Ishida et al. 2009, Ozturk, Guzeloglu-Kayisli et al. 2012, Ozturk, Guzeloglu-Kayisli et al. 2014, Kashiwabara, Tsuruta et al. 2016).

The regulation of mRNAs expressed during germ cell development is also mediated at the level of storage and control of translation. Several sequence-specific or structure-specific RBPs bind to germ cell specific mRNAs to promote their storage or translation. The sequence specific RNA binding proteins include the Y-box proteins (YBX) including, YBX-1 (MSY-1), YBX-2 (MSY-2) and YBX-3 (MSY-4), which are expressed in murine testis and are shown to be required for successful male gamete development (Gu, Tekur et al. 1998, Davies, Giorgini et al. 2000, Mastrangelo and Kleene 2000, Snyder, Soundararajan et al. 2015). Indicative of their role in regulating mRNA translation, these proteins bind to Y-box recognition sequences of mRNA (Chowdhury and Kleene 2012) and stabilize mRNA on the mRNP complex (Yang, Morales et al. 2007, Xu and Hecht 2008). Studies indicate that these proteins are redundant for each other in stabilizing and regulating the translation of mRNAs including those encoded by *Tnp1*, *Tnp2*, *Prm1*, *Prm2*, *Akap4*, *Akap3*, *Smcp* and *Spata18* during murine male gamete development (Giorgini, Davies et al. 2001, Chowdhury and Kleene 2012, Snyder, Soundararajan et al. 2015). A sequence-independent RBP, PTBP2, binds to 3'UTR region of *Pgk2* mRNA*,* thereby stabilizing this mRNA after its transcription in spermatocytes until its translation in haploid spermatids (Xu and Hecht 2007, Xu and Hecht 2008).

*Prm1* mRNA is under tight translational regulation during haploid spermatid differentiation, and its precocious translation leads to sterility (Lee, Haugen et al. 1995). RNA binding proteins including SPNR (Schumacher, Lee et al. 1995, Schumacher, Artzt et

al. 1998), and TARBP2 (also known as PRBP, see section 1.4.4) (Lee, Fajardo et al. 1996, Zhong, Peters et al. 1999) bind to specific sites on the 3'UTR-region of *Prm1* and help regulate its proper translation. However, the exact mechanism by which these proteins regulate *Prm1* translation remains to be elucidated. Overall, these findings suggest that mammalian germ cells express sequence-specific or sequence-independent proteins binding specific or non-specific mRNAs to ensure proper storage and translation.

Small noncoding (snc) RNAs including siRNAs, miRNAs and piRNAs, along with proteins involved in their biogenesis or RISC formation, are also expressed in germ cells (Saxe and Lin 2011). piRNAs are a class of germ cell specific sncRNAs that interact with PIWI proteins including MILI and MIWI2, and lead to epigenetic silencing of transposon encoding regions of germ cells (Aravin, Gaidatzis et al. 2006, Girard, Sachidanandam et al. 2006). piRNAs are expressed in the pre-meiotic and meiotic cell populations, with highest levels in pachytene spermatocytes (Li, Roy et al. 2013). Several proteins, including MVH (Kuramochi-Miyagawa, Watanabe et al. 2010), Protein maelstrom homolog (Soper, van der Heijden et al. 2008), MOV10L1 (Zheng, Xiol et al. 2010), GASZ/ASZ1 (Ma, Buchold et al. 2009), MITOPLD (Watanabe, Chuma et al. 2011) and FKBP6 (Xiol, Cora et al. 2012), which contribute to successful male gamete development in mice, have been identified to play a role in piRNA biogenesis pathways. Although there is an indication of piRNAs involvement in translation regulation, the exact mechanism by which piRNAs regulate translation during murine spermatogenesis still needs to be explored.

The other class of sncRNAs that are extensively studied and reported in regulating transcript stability and translation are miRNAs, which were introduced earlier in this chapter. Phenotypes that result from the loss of the enzymes DROSHA and DICER, which, as

described earlier are involved in miRNA synthesis in murine testis, suggest the importance of miRNA expression during male germ cell development. The level of miRNA expression changes during cell state transitions of murine male gametogenesis, with highest levels reported in spermatocytes and haploid cells (Ro, Park et al. 2007). Consistent with their expression patterns, miRNAs are required for successful germ cell stage progression during murine male gamete development (Hayashi, Chuva de Sousa Lopes et al. 2008).

In mice, specific miRNAs like miR-34b/c and miR-449 are essential for post-meiotic gene expression regulation (Yu, Raabe et al. 2005, Comazzetto, Di Giacomo et al. 2014). The key spermatid differentiation factor gene *Tnp2,* which marks the initiation of the spermiogenesis process, is regulated at the transcript level and translation level by miR-122a and miR-469, respectively (Yu, Raabe et al. 2005, Dai, Tsai-Morris et al. 2011). miR-469 also regulates translation of *Prm2,* expression of which is need for spermiogenesis (Dai, Tsai-Morris et al. 2011). It is still unclear if the binding of miRNAs to target mRNAs in germ cells inhibit or promote their translation, so this needs to be investigated.

As discussed earlier, miRNA-dependent or independent mRNA degradation is characterized by deadenylation and decapping, and several genes regulate these processes during germ cell development. The deadenylase enzyme CCR4-NOT is expressed in murine germ cells and is recruited onto 3'UTR regions of several mRNAs, including *Nanos*, *Sycp3* and *Dazl* by a RBP DND to promote deadenylation followed by degradation of transcripts (Cook, Munger et al. 2011, Suzuki, Niimi et al. 2016, Yamaji, Jishage et al. 2017). The expression of testes specific decapping enzymes, including DDX4 and DDX25 are essential for germ cell proliferation, meiotic progression and haploid sperm differentiation (Tanaka, Toyooka et al. 2000, Gutti, Tsai-Morris et al. 2008).

Overall spermatogenesis is a complex process during which, post-transcriptional regulation of gene expression involves an interplay of several RBPs and sncRNAs. The exact molecular mechanisms through which these factors coordinate to regulate germ cell stage progression needs to be explored in detail to obtain a better understanding of gene expression regulation during mammalian spermatogenesis.

The current state of knowledge regarding miRNAs and a key miRNA biogenesis factor, DICER, in post-transcriptional regulation of gene expression *in vivo* is substantial. However, the roles of the DICER co-factors, TARBP2 and PRKRA, in post-transcriptional regulation of gene expression *in vivo* remain relatively unknown. The following chapters will describe a series of experimental analyses aimed at understanding the role of TARBP2 and PRKRA in miRNA biogenesis *in vivo*. In an effort to provide information that will increase understanding of the role of TARBP2 during post-transcriptional regulation of gene expression, future chapters will also describe a possible role of miRNAs in regulating translation elongation during spermatogenesis.

# CHAPTER 2. ROLE OF TARBP2 DURING MURINE EMBRYONIC DEVELOPMENT

## 2.1 Introduction

The following chapter will outline a series of experiments and analyses that have led to the conclusion that TARBP2 has a role in miRNA biogenesis during embryonic development. This is consistent with *in vitro* validation of the role of TARBP2 as a co-factor of DICER. Additionally, the following analyses present novel evidence for possible redundancy between TARBP2 and PRKRA, independent of miRNA biogenesis.

Conditional ablation of *Dicer* in oocytes using *Zp3Cre* results arrest in meiosis I as a consequence of spindle dysfunction and defects in chromosome congression (Murchison, Stein et al. 2007, Tang, Kaneda et al. 2007). Furthermore, conditional gene targeting has revealed DICER functions in the development or homeostasis of embryonic and fetal organs including the cardiovascular, genitourinary, musculoskeletal and nervous systems (Bernstein, Kim et al. 2003, O'Rourke, Georges et al. 2007, Saal and Harvey 2009, Zehir, Hua et al. 2010, Small and Olson 2011). These data are consistent with a model where miRNAs function in the proper development of numerous mammalian organs and that disruptions in this small RNA biogenesis pathway can result in congenital birth defects and in extreme cases fetal death. The mouse *Tarbp2* gene encodes a 365-amino acid protein that is localized predominantly to the cytoplasm and *Tarbp2* $^{-/-}$ mice on a hybrid background are viable but have reduced body size and are male sterile (Zhong, Peters et al. 1999). Previously characterized *Prkra* mutant mice are homozygous viable with cranial-facial defects (Rowe, Rizzi et al. 2006, Dickerman, White et al. 2015) and like *Tarbp2* mutants exhibit postnatal

growth retardation on several genetic backgrounds, including C57BL/6J. The developmental defect phenotype in different tissues of *Tarbp2* $^{-/-}$ and *Prkra* $^{-/-}$ mutants led us to investigate their role as DICER cofactors in vivo.

## 2.2 Experimental Methods

### 2.2.1 Mouse mutants

*Tarbp2* $^{tm1reb}$ (Zhong, Peters et al. 1999) mice were genotyped as described previously. Genotyping for the *Prkra* $^{lear1J}$ was carried out as recommend by the Jackson Laboratory mutant repository.

### 2.2.2 High throughput sequencing of small RNAs

E15.5 C57BL/6J-*Tarbp2* $^{tm1reb}$ and C57BL/6J-*Prkra* $^{lear1J}$ embryos were generated through timed mating intercrosses of heterozygous male and female mutant mice. Embryos were dissected at 15.5 days post coitum and the associated yolk sac was used for genotyping. The developmental stage was confirmed using Theiler staging criteria for mouse embryo development. Embryos were decapitated and both body and head were immediately immersed in RNAlater (ThermoFisher). Whole fetuses (head and body) were homogenized in Trizol followed by purification of total RNA using the QIAGEN miRNeasy kit. High-throughput sequencing libraries containing small non-coding RNAs (e.g. miRNA or piRNAs) were generated using 1ug of total RNA and the Illumina TruSeq small RNA library preparation kit (Illumina Inc., USA). Individually barcoded fetal libraries were pooled and sequenced (100 base pair-end reads) on a single HiSeq 2000 flow cell lane running version 3 chemistry. Illumina CASAVA software was used to carry out BCL to Fastq conversion. All the samples were passed through quality control and sequence with poor base qualities were

removed. The adaptors sequences were removed using clip_adaptors.pl module of mirdeep (v2.0.0.5) (Friedlander, Mackowiak et al. 2012). Raw reads were collapsed based on sequence identity using collapse_reads_md.pl module of mirdeep2. Collapsed reads were further annotated for miRNA by mapping to miRBase version 21 (Griffiths-Jones, Saini et al. 2008, Kozomara and Griffiths-Jones 2011, Friedlander, Mackowiak et al. 2012) using the miraligner module of seqbuster (Pantano, Estivill et al. 2010). IsomiR's were identified using R isomiRs (v1.3.0) package of seqbuster package. Pairwise differential expression was performed among different groups using DESEQ2 (v1.12.4) (Love, Huber et al. 2014).

### 2.2.3 Quantitative RT-PCR and TaqMan assays

Adult tissues were used for total RNA extraction (TRIzol-Invitrogen), followed by the production of random primed cDNA (Invitrogen-18080051). Real time PCR using SYBR green (ABI 7500, ThermoFisher) and the ddCT method for calculating relative gene expression were used to determine *Prkra* and *Tarbp2* transcript levels. *b-actin* amplification was used as an endogenous control. For miRNA taqman assays, total RNA is extracted (TRIzol extraction-Invitrogen) from transformed MEFs, followed by production of cDNA specific to each miRNA (Thermo Fischer- 4427975) using TaqMan miRNA reverse transcription kit (Thermo Fischer- 4366596). TaqMan PCR using TaqMan Universal Master Mix II (Thermo Fischer- 4440042) and the ddCT method for calculating relative transcript expression were used to determine mature miRNA levels. U6 amplification was used as an endogenous control.

### 2.2.4 Western blotting

PRKRA and TARBP2 protein levels were assayed from the tissues and MEFs using standard western blotting protocols. Rabbit monoclonal antibody raised against a synthetic peptide corresponding to the C-terminal end of human PRKRA (AbCam #ab75749) and TARBP2 antibody from previous study (Zhong, Peters et al. 1999) was used to quantify the protein levels. GAPDH was used as a loading control.

### 2.2.5 Cell culture, transformation and cell number calculation

Mouse embryonic fibroblast cells (MEFs) were isolated at E11.5 and were transformed by infecting with viral supernatant from SV40 T antigen (pBABE Sv40 T antigen from Addgene #13970) packaged cells (Plat-E ecotropic packaging). To obtain stably transfected clones, cells were selected with 2μg/ml Puromycin and single clones were isolated using clonal rings. Transformed MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM containing high glucose level; GIBCO) supplemented with 10% fetal bovine serum (Invitrogen). For cell number quantification transformed MEFs were seeded at an initial density of $1 \times 10^5$ cells/well on the day prior to treatment with 40mg/ml DMSO, enoxacin and incubated at 37 °C in 5% $CO_2$. The cell numbers were calculated on Day0, Day3, Day5 using Trypan blue staining and Countess cell counter.

### 2.2.6 Embryo dissesction and β-gal staining

Time matings were set up to collect the embryos varying from embryonic day E8.5 to E13.5. Embryo genotypes were determined by PCR from yolk sac DNA. Embryos were fixed in 4% paraformaldehyde and stained overnight in X-Gal containing solution to visualize β-galactosidase activity derived from the *lacZ* gene tag in *Tarbp2* targeted mutation. For tissue

sections, embryos were frozen in OCT and cryostat sectioned. Sections were fixed (0.2% Gluteraldehyde) and stained as above. Sections were post-fixed, counterstained with Nuclear Fast Red, mounted and imaged under the Olympus Nanozoomer with the desired magnification.

### 2.2.7 Skeletal staining and histology

For ossified bone and cartilage staining, E15.5 embryos were processed using an alizarin red/alcian blue standard staining protocol (Hogan, Beddington et al. 1994). Embryos collected at E18.5 were fixed for histology in Bouin's fixative, then paraffin embedded. 10um serial sections were processed using hematoxylin and eosin (H&E) staining.

### 2.2.8 Data and reagent availability

miRNA sequencing data has been uploaded to BioprojectID: PRJNA423238: Mus musculus musculus. Raw sequence reads (TaxId: 39442) SRA ID: SRP127346.

## 2.3 Results

### 2.3.1 *Tarbp2* is broadly expressed during embryonic development

To determine where *Tarbp2* is expressed during embryonic development, we analyzed β-galactosidase expression in heterozygous *Tarbp2* $^{tm1reb/+}$ embryos (herein referred to as *Tarb2* $^{-/+}$) containing a LacZ insertion that transcriptionally tags the *Tarbp2* gene (Figure 2.1.A). In whole-mount staining of *Tarbp2* $^{+/-}$ embryos, we observed high levels of β-galactosidase activity throughout all stages of development, as early as E8.5 when *Dicer* is expressed (Figure 2.1.B). In sections of E13.5 organs, we detected expression in some, but not all, cells in the heart, hindbrain and liver (Figure 2.1.C). This organ expression was confirmed by RT-PCR analysis of *Tarbp2* in wild-type E13.5 extracts (Figure 2.1.C). *Tarbp2*

is expressed in all adult mouse tissues, with abundant transcript levels in the testis, although expression is restricted by cell type (Zhong, Peters et al. 1999). Thus, mouse *Tarbp2* is broadly expressed from early embryonic stages through to adulthood with likely cell-type specific expression in many, if not all, tissues.

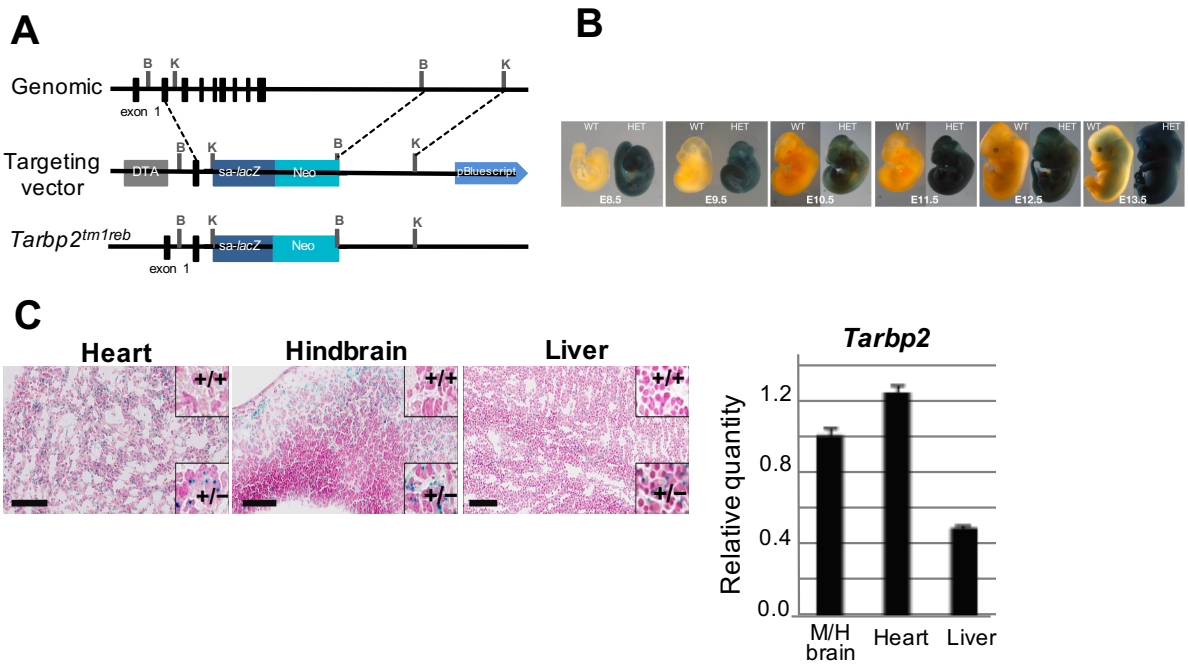



**Figure 2.1. TARBP2 is expressed in murine embryos**

(A) Schematic representation of the *Tarbp2*$^{tm1REB}$ allele. A cassette containing negative (DTA) selection, positive selection (Neomycin, Neo) and lacZ was inserted into exon2 with a 5' splice acceptor (sa) sequence. B=BamHI, K=KpnI. (B) β-galactosidase activity in Tarbp2 tm1Reb/+ embryos from E8.5 to E13.5 stage of development. (C) β-galactosidase activity in sections of E13.5 organs of *Tarbp2*$^{tm1REB/+}$ transcriptional reporter mice (blue= β-gal, red=nuclear fast red). Top and bottom insets show higher magnification images of indicated genotypes. Scale bar, 200um. RT-PCR in wild-type E13.5 organs total RNA extracts for *Tarbp2* (M/H Brain, mid/hind brain).

### 2.3.2 *Tarbp2* null animals are smaller but exhibit no gross anatomical defects

Previously, we reported a smaller body size, early postnatal lethality and male sterility in *Tarbp2* $^{-/-}$ mice on a mixed genetic background (Zhong, Peters et al. 1999). To determine if early defects in ossification could explain the size difference in mutant animals, we stained E15.5 embryos with alizaran red/alcian blue to mark bone and cartilage, respectively. Stained embryos were smaller than control littermates (Figure 2.2.A) with delayed ossification of tympanic and spinal column. To compare the size of organs and soft tissues in *Tarbp2* mutants, we performed microCT on E15.5 embryos. We observed a 20% decrease in total volume in *Tarbp2* mutants compared to controls (red outline, Figure 2.2.B) but no significant relative difference in the size of any single organ or tissue. We conclude growth retardation defects begin in utero, as early as E15.5.

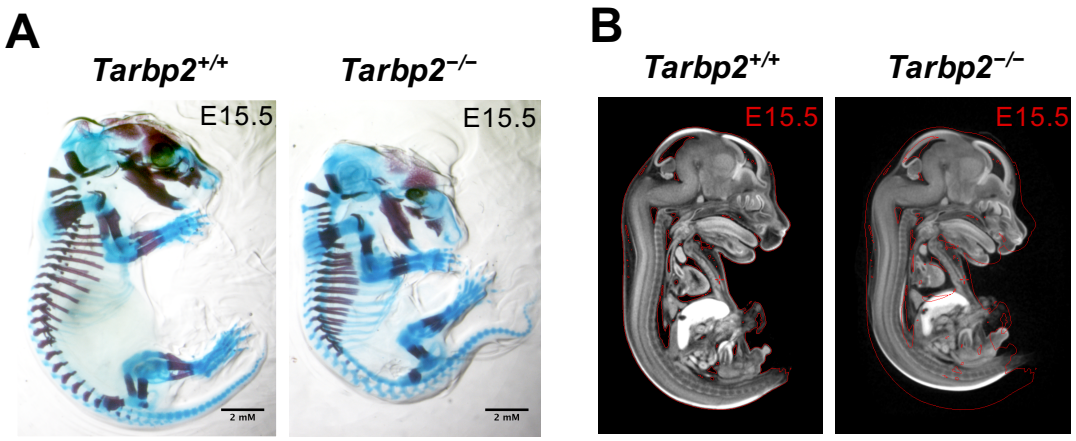



**Figure 2.2 Anatomical defects in *Tarbp2* null animals**

(A) Alizarin red (bond) and alcian blue (cartilage) staining of E15.5 wild type and TARBP2 $^{-/-}$ B6 embryos showing defects in developmental patterning. (B) microCT scans of E15.5 wild-type and TARBP2 $^{-/-}$ B6 embryos, red outline shows smaller volume in mutant.

### 2.3.3 *Tarbp2* null allele is embryonic lethal on the C57BL/6J mouse strain

*Tarbp2* $^{-/-}$ mice have reduced body mass and perinatal lethality on mixed strain background (Zhong, Peters et al. 1999). Because genetic background can impact phenotypic variation, we tested whether strain background had an effect on the phenotypes observed in *Tarbp2* mutants, and therefore compared the frequency of observed homozygotes on the 129S4 (129) background to C57BL/6J (B6) background. In heterozygous (het) x het breeding crosses in the 129 background, we observed a significant reduction in the number of *Tarbp2* $^{-/-}$ homozygotes at P21, suggesting reduced viability on the 129 background (Table2.1). *Tarbp2* $^{-/-}$ mutants on the B6 genetic background exhibited an even more extreme phenotype. In het x het crosses, we were unable to recover any homozygous animals at P14. Furthermore, at E17.5, we observed only 13% of the expected 25% homozygous animals. In crosses between 129 *Tarbp2* $^{+/-}$ females and B6 *Tarbp2* $^{+/-}$ males, we observed the expected Mendelian ratios at P21, suggesting that the 129 genetic background has one or more modifiers that can suppress the lethality observed on the B6 background.

| Strain | Age | +/+ | +/− | −/− |
|---|---|---|---|---|
| 129S4 (129) | P21 | 29 (26%) | 66 (58%) | 18 (16%) |
| C57BL/6J (B6) | E17.5 | 11 (21%) | 35 (66%) | 7 (13%) |
| | P14 | 23 (31%) | 53 (69%) | 0 (0%) |
| 129 x B6 F1 | P21 | 22 (23%) | 51 (53%) | 23 (24%) |

Table 2.1. Background strain dependence of *Tarbp2*

### 2.3.4 *Prkra*$^{1J}$ mutants are viable with anatomical defects

Like TARBP2*,* PRKRA binds DICER and has been shown to influence miRNA biogenesis *in vitro*. To compare the requirements for *Tarbp2* and *Prkra* during development, we next analyzed *Prkra* mutant mice. Three spontaneous mutant *Prkra* alleles (*lear*, *little*

*ear)* have arisen in The Jackson Laboratory repository colony, displaying small body size and reduced ear size. These phenotypes are similar to the previously characterized *Prkra* targeted mutant (Rowe, Rizzi et al. 2006) and chemically induced mutants (Dickerman, White et al. 2011) that produce a complete PRKRA protein deficiency or a protein isoform deficient in dsRNA binding, respectively. The *lear1J* allele (referred to herein as *Prkra* $^{-}$) contains a splice donor mutation in intron 5 (Figure 2.3.A). Using RT-PCR on RNA from E13.5 *Prkra* $^{-/-}$ mutant organ extracts, we found a significant decrease in *Prkra* expression in heart and kidney (Figure 2.3.B). Furthermore, immunoblots for PRKRA protein showed dramatically decreased levels in liver, lung, kidney and spleen, with complete loss of protein in the heart (Figure 2.3.C). Thus, *Prkra*$^{1J}$ is a strong hypomorphic or null allele that phenocopies previously characterized spontaneous and targeted alleles.

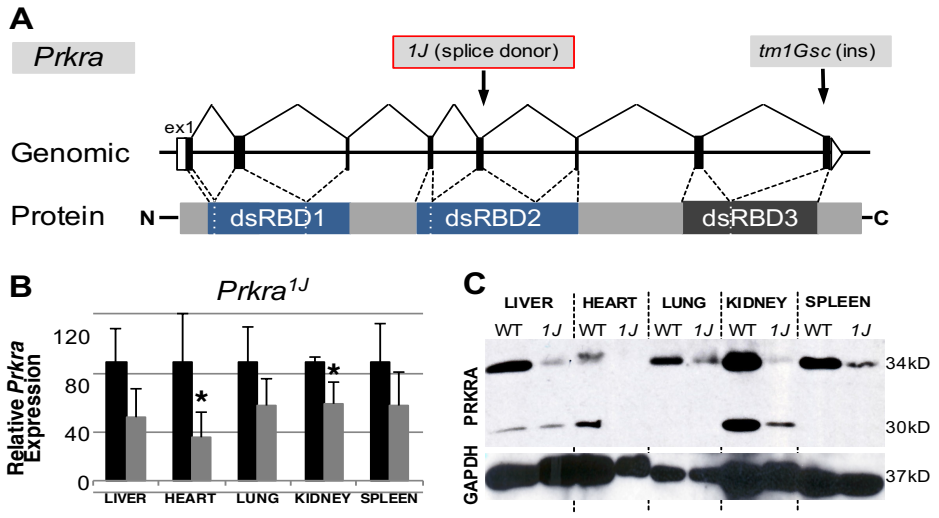



**Figure2.3 *Prkra*$^{lear1j}$ mutant characterization**

(A) Summary of genomic/protein locations of the spontaneous *learJ* (little ears, The Jackson Laboratory) *Prkra* allele (1j), and an engineered insertion in exon (ex) 8 (tm1Gsc). (B) RT-PCR of *Prkra* transcripts in E13.5 organ extracts show significant decreases (*p-value<0.05) in heart and kidney. (C) Immunoblot for PRKRA shows decreased protein levels in all organs analyzed.

### 2.3.5 *Tarbp2* $^{-/-}$; *Prkra* $^{-/-}$ double-mutants die by mid-gestation

Because *Dicer* mutants die by E7.5 (Figure 2.4.A), while *Tarbp2* $^{-/-}$ mutants die peri-natally, and *Prkra* mutants are viable but have reduced body size and ear deformities, we asked whether there might be functional redundancy between *Tarbp2* and *Prkra* during early embryogenesis. To test this, we crossed *Tarbp2* $^{+/-}$; *Prkra* $^{+/-}$ double-heterozygotes and genotyped animals at birth. We failed to obtain any *Tarbp2* $^{-/-}$*; Prkra* $^{-/-}$ (B6) animals. We therefore looked at earlier embryonic time points and recovered one E18.5 double-mutant that was half-the-size of control littermates and exhibited severe shortening of the snout (Figure 2.4.C). Additionally, the heart was smaller and there were open cranial sutures (not shown). This animal was able to survive quite late considering we were only able to recover one double-mutant out of an expected eight, at E12.5 (Figure 2.4.B, chi-square $p<0.05$). To determine if open cranial features were a hallmark of *Prkra* mutants, and if they are affected by the *Tarbp2* locus, we analyzed cranial morphology using microCT in 8-week-old animals. We consistently observed open cranial sutures in *Prkra* $^{-/-}$ mutants, and reduction in *Tarbp2* gene dosage further exacerbated this phenotype, as exemplified in *Tarbp2* $^{-/+}$; *Prkra* $^{-/-}$ mutants (Figure 2.4.D). These data indicate the structurally similar dsRBPs, TARBP2 and PRKRA, genetically interact.

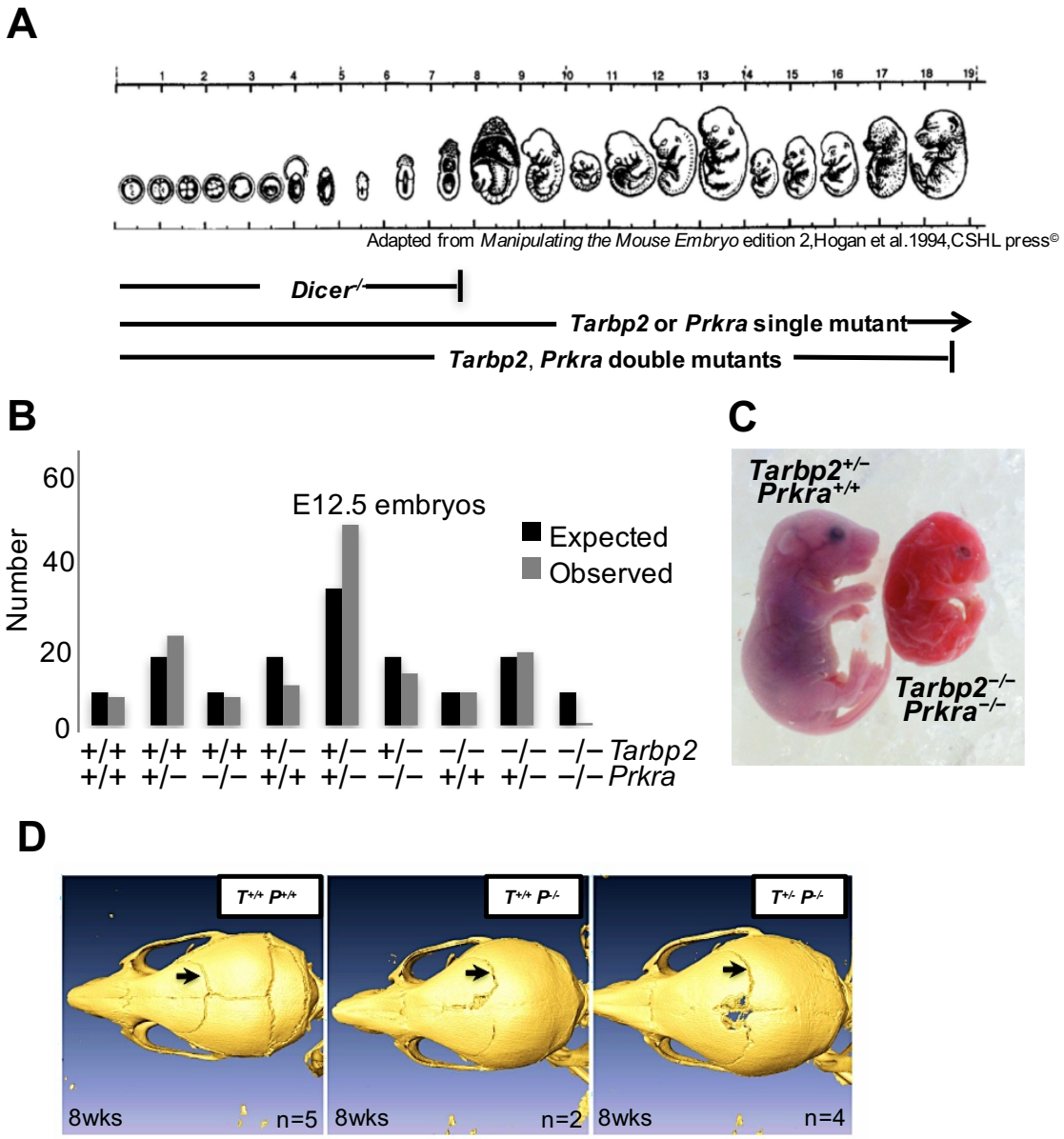



**Figure 2.4. *Tarbp2* $^{-/-}$; *Prkra* $^{-/-}$ mutants die at midgestation**

(A) Summary of developmental stages of survival or embryonic lethality of *Dicer* $^{-/-}$, *Tarbp2* $^{-/-}$ or *Prkra* $^{-/-}$ single-mutants and *Tarbp2* $^{-/-}$; *Prkra* $^{-/-}$ double-mutants. (B) Number of E12.5 embryos harvested from crossing *Tarbp2* $^{-/+}$; *Prkra* $^{-/+}$ double-heterozygotes. $X^2$=19.283 (8 degrees of freedom), two-tailed p-value=0.0134. (C) An E18.5 *Tarbp2* $^{-/-}$;*Prkra* $^{-/-}$ mutant that is significantly smaller than a *Tarbp2* $^{+/-}$;*Prkra* $^{+/+}$ sibling, and has a shorter snout. (D) MicroCT images of skull showing immature fusion and opening of cranial sutures. RTPCR in wild-type E13.5 organs total RNA extracts for *Prkra* (M/H Brain, mid/hind brain).

### 2.3.6 miRNA processing is altered in *Tarbp2* $^{-/-}$ but not *Prkra* $^{-/-}$ mutants

The difference in phenotypes between *Dicer*, *Tarbp2* and *Prkra* single and double-mutants led us to ask whether TARBP2 and PRKRA influenced DICER-mediated miRNA biogenesis in vivo. We collected total RNA from E15.5 B6 *Tarbp2* $^{-/-}$ and *Prkra* $^{-/-}$ embryos and generated high-throughput sequencing libraries of small non-coding RNAs for RNA-sequencing (RNA-seq). Using an FDR pass of <0.05, we found that the expression levels of 74 mature miRNAs were significantly changed in *Tarbp2* $^{-/-}$ mutants (Figure 2.5.A). Interestingly, these transcripts were both decreased (n=46, 62%) and increased (n=28, 38%) suggesting that TARBP2 is required for a sub-population of miRNAs during this stage of development. Conversely, there was no change in the expression of miRNAs in *Prkra* $^{-/-}$ embryos (Figure 2.5.B), suggesting that PRKRA has no discernable role in miRNA biogenesis during this stage of development. Biochemical data suggests the dsRBPs can influence strand selection of the processed pre-microRNA. As TARBP2 determines the site of cleavage by DICER, and addition or deletion of a nucleotide can alter the stability of processed miRNA arms, we compared the overall expression level changes of 3p-miRNAs and 5p-miRNAs. Significant expression level changes were observed in both populations, however there were more changes to 3p-miRNAs (44 out of 74) than 5p-miRNAs (30 out of 74) in *Tarbp2* $^{-/-}$ embryos compared to wild-type embryos, suggesting a preferential defect in processing of 3p-miRNAs in the absence of TARBP2 (Figure 2.5.C). Also, in *Tarbp2* $^{-/-}$ embryos we observed that there was an increase in the number of reads mapped to 3p-miRNAs with an addition ($A_n$) or deletion ($D_n$) of nucleotides at the 5' end compared to wild-type embryos (Figure 2.5.D) suggesting an improper cleavage site selection by DICER in the absence of TARBP2.

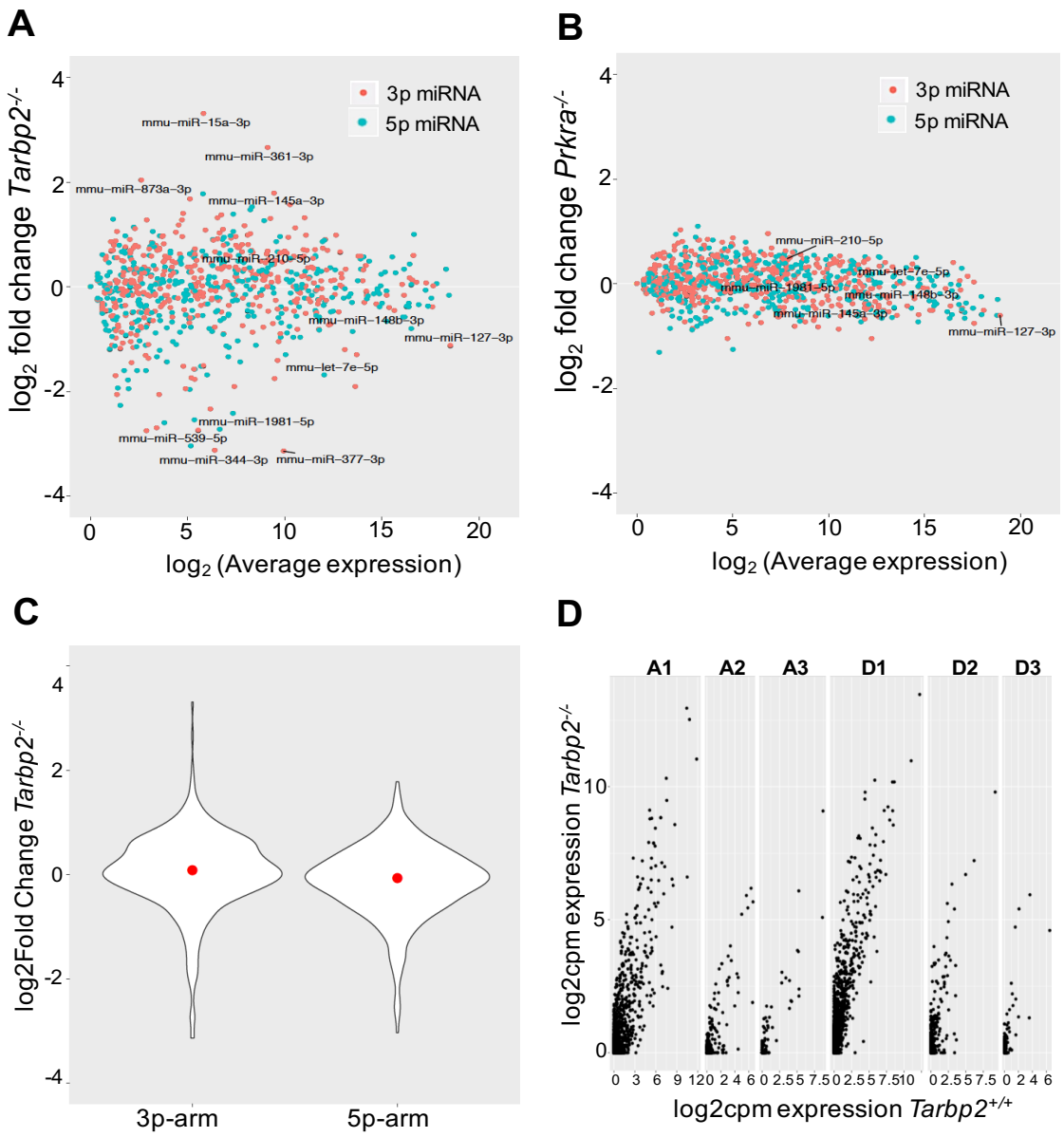
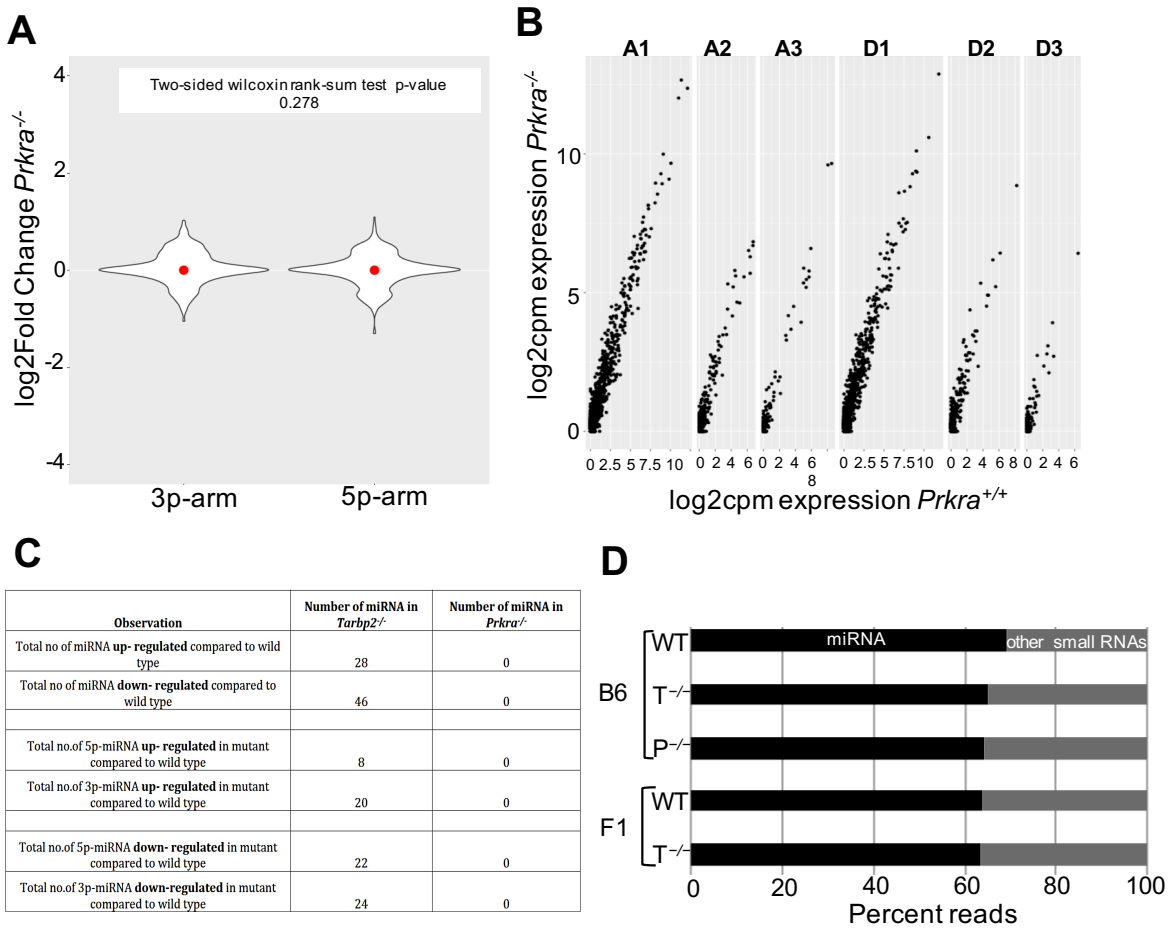



**Figure 2.5 *Tarbp2* regulates processing of a subclass of pre-miRNAs.**

(A) Plot of miRNA transcripts in *Tarbp2* $^{-/-}$ whole embryos at E15.5 and their fold-changes by average expression using RNA-seq. (B) Plot of miRNA transcripts in *Prkra* $^{-/-}$ whole embryos at E15.5 and their fold-changes by average expression using RNA-seq. (C) Plot of overall expression fold change of 3p and 5p miRNA transcripts in C57BL/6J *Tarbp2* $^{-/-}$ whole embryos at E15.5 compared to wild-type embryos. (D) Plot of 3p arm miRNA transcripts with one, two or three nucleotide (n=1,2,3) addition (An) or deletion (Dn) at 5' end in *Tarbp2* $^{-/-}$ whole embryos at E15.5 and their fold-changes by counts per million (cpm) using RNA-seq.

In *Prkra* $^{-/-}$ mutant embryos the level of expression of 3p-miRNAs and 5p-miRNAs remained the same as in wild-type embryos (Figure 2.6.A), as did the length of the 3p-miRNAs (Figure 2.6.B). A comparison of the observed changes in miRNA populations in *Tarbp2* $^{-/-}$ and *Prkra* $^{-/-}$ mutants is shown in Figure 2.6.C. Because an equal number of reads were mapped to miRNAs and other small non-coding RNAs independent of the genotype (Figure 2.6.D), we conclude that TARBP2 but not PRKRA is required for the processing of a subclass of miRNAs at E15.5.




| Observation | Number of miRNA in *Tarbp2*$^{-/-}$ | Number of miRNA in *Prkra*$^{-/-}$ |
|---|---|---|
| Total no of miRNA **up- regulated** compared to wild type | 28 | 0 |
| Total no of miRNA **down- regulated** compared to wild type | 46 | 0 |
| | | |
| Total no.of 5p-miRNA **up- regulated** in mutant compared to wild type | 8 | 0 |
| Total no.of 3p-miRNA **up- regulated** in mutant compared to wild type | 20 | 0 |
| | | |
| Total no.of 5p-miRNA **down- regulated** in mutant compared to wild type | 22 | 0 |
| Total no.of 3p-miRNA **down-regulated** in mutant compared to wild type | 24 | 0 |

**Figure 2.6 *Prkra* is not required for canonical miRNA biogenesis at E15.5**

(A) Fold-change of over-all expression of 3p and 5p miRNA transcripts in C57BL/6J *Prkra* $^{-/-}$ whole embryos at E15.5 compared to wild type embryos. (B) Plot of 3p arm miRNA transcripts with one, two or three nucleotide (n=1,2,3) addition (An) or deletion (Dn) at 5' end in *Prkra* $^{-/-}$ whole embryos at E15.5 and their fold-changes by counts per million (cpm) using RNA-seq. (C) A table chart summarizing the type of deregulation and number of miRNAs in *Tarbp2* $^{-/-}$ and *Prkra* $^{-/-}$ compared to wild-type (D) Chart of percent reads of miRNAs versus other small non-coding RNAs in the RNA-seq libraries, per genotype and background.

### 2.3.7 Pharmacological assessment of the role of TARBP2 and PRKRA on miRNA processing

In a screen for small molecules that modify RNAi activity, the synthetic antibacterial compound enoxacin was identified as an enhancer of RNAi (Shan, Li et al. 2008). Several studies have reported that enoxacin binds human TARBP2, increases TARBP2 affinity for pre-miRNAs and enhances the processing of pre-miRNAs to mature miRNAs (Melo, Villanueva et al. 2011, Cornaz-buros, Riggi et al. 2014). To confirm our findings that TARBP2, but not PRKRA, is involved in miRNA processing during embryogenesis, we examined the effect of enoxacin on growth of mouse embryonic fibroblasts (MEFs) derived from *Tarpb2* $^{-/-}$ and *Prkra* $^{-/-}$ mutant embryos. Western blot analysis showed that PRKRA and TARBP2 were detected in MEFs derived from wild-type embryos but not *Prkra* $^{-/-}$ or *Tarbp2* $^{-/-}$ mutants, respectively (Figure 2.7.A). To assess whether enoxacin differentially affected *Prkra* $^{-/-}$ but not *Tarbp2* $^{-/-}$ mutant MEFs, we treated the cells with DMSO and enoxacin as described previously (Melo, Villanueva et al. 2011). When cell numbers from Day 0, Day 3 and Day 5 were plotted comparing DMSO treated cells with enoxacin, we observed that *Tarbp2* $^{-/-}$ mutants were more resistant to enoxacin than wild-type and *Prkra* $^{-/-}$ mutant MEFs at Day 3, although by Day 5 all genotypes were similarly affected (Figure 2.7.B). To directly test the effect of enoxacin on miRNA levels, we measured the effect of enoxacin on the abundance of 10 miRNAs whose levels were altered in *Tarbp2* $^{-/-}$ E15.5 embryos. Treatment of wild-type MEFs with enoxacin significantly decreased the relative levels of 3/8 miRNAs (Figure 2.7.C top), although none of the levels of miRNAs were elevated as we had predicted. The levels of two of the miRNAs, miR-127 and miR145, remained significantly depressed in *Prkra* $^{-/-}$ MEFs, while the levels of one miRNA, miR-120, were rescued,

although not to wild-type levels (Figure 2.7.B middle). On the other hand, mutation of *Tarbp2* rescued levels of miR-127 and miR145 to wild-type levels, but had no effect on miR-120 and actually resulted in an increase in two miRNAs, let-7e and miR-484, that had been unaffected in wild-type MEFs. From these observations, we conclude that enoxacin does act solely through TARBP2 and there is no direct correlation with the increase in miRNAs and the effect of enoxacin on cell growth of transformed MEFs.

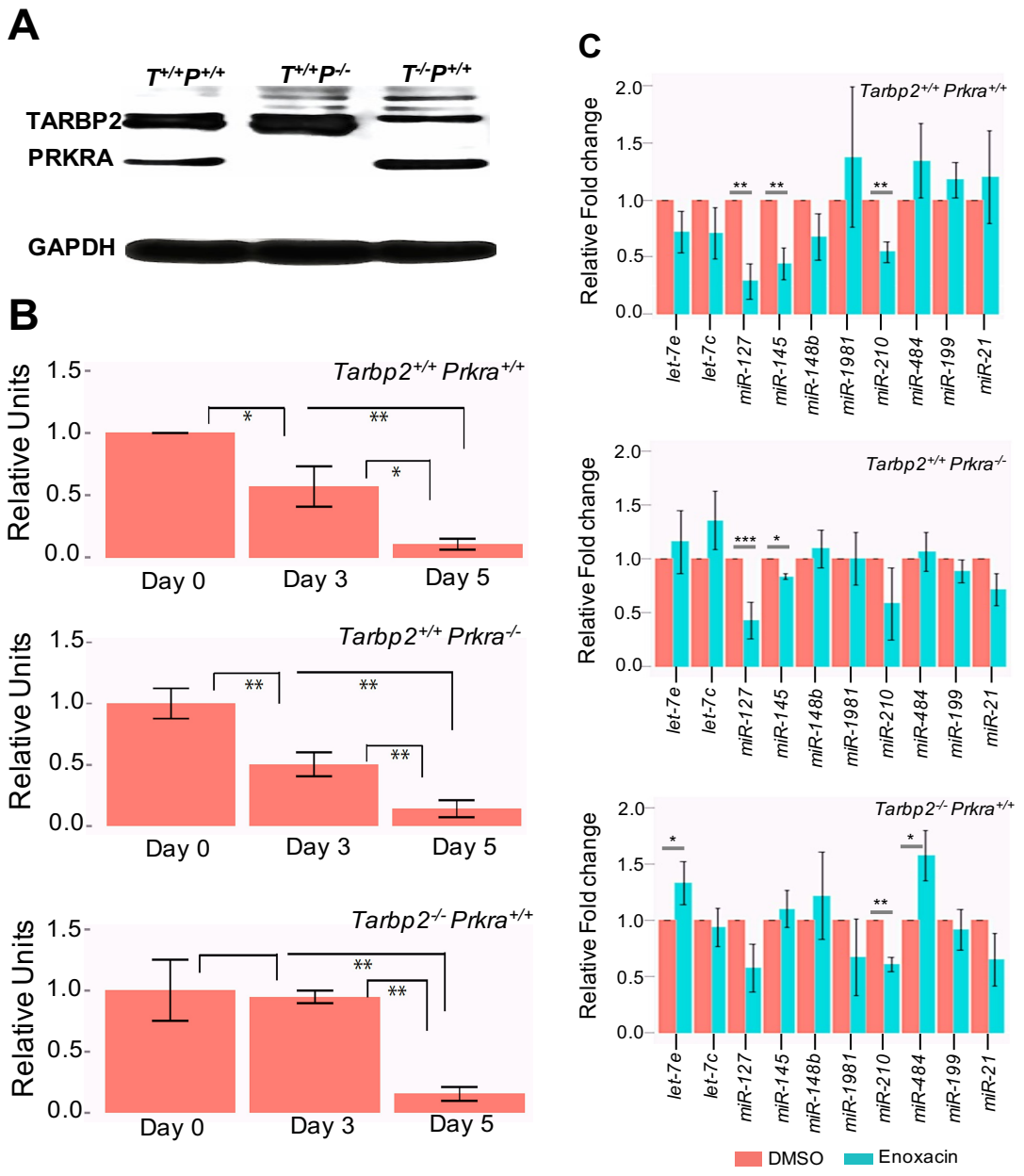



**Figure 2.7 Enoxacin has its effect on transformed MEFs independent of *Tarbp2* or *Prkra***

(A) Immunoblot for TARBP2 and PRKRA shows the expression or absence of these proteins based on their genotype in transformed MEFs. (B) Graphical representation of cell numbers for the indicated genotype after enoxacin treatment from Day 0 to Day 5. Relative units are obtained after normalizing the cell numbers from enoxacin treatment to the cell numbers with DMSO treatment. (*p-value<0.05, **p-value<0.005) (B) Expression fold-change of 10 quantified mature miRNAs in transformed MEF cell lines with the indicated genotype upon enoxacin treatment (*p-value<0.05, **p-value<0.005).

## 2.4 Discussion

Previous *in vitro* studies suggested that TARBP2 and PRKRA act as co-factors of DICER for the processing of pre-miRNA to mature-miRNAs. The discordance between the phenotypes of *Dicer1* $^{-/-}$, *Tarbp2* $^{-/-}$, and *Prkra* $^{-/-}$ single mutants led us to test these findings and to determine if TARBP2 and PRKRA have redundant functions in miRNA biogenesis.

Our results strongly suggest that *Tarbp2* and *Prkra* genetically interact during embryonic development. Earlier studies have established the essentiality of *Dicer* during oogenesis and embryogenesis. Maternal Dicer mRNA, which is present throughout oocyte development (Murchison, Stein et al. 2007), is required for completion of meiosis 1 (Murchison, Stein et al. 2007, Tang, Kaneda et al. 2007), and elimination of zygotic *Dicer* expression results in an embryonic lethality phenotype by E7.5. We failed to recover the expected number of *Tarbp2* $^{-/-}$, *Prkra* $^{-/-}$ double-mutants at E12.5, and recovered only one double-mutant embryo, which was severely abnormal, at E18.5. It is possible that the missing embryos died prior to E7.5, similar to that of *Dicer*$^{-/-}$ embryos, and that the few embryos recovered at E12.5 and E18.5 could be due to incomplete penetrance of the double-mutant phenotype. Furthermore, we found that the allele status at *Tarbp2* affected the closure of cranial sutures observed in *Prkra* $^{-/-}$ mutants.

miRNA sequencing of wild-type and single mutants at E15.5 revealed that TARBP2, but not PRKRA, is essential for proper miRNA biogenesis of a set of miRNAs during embryonic development. We chose to analyze E15.5 embryos because of the lack of an observable phenotype in single mutants at that time point and because double-mutant embryos were absent, suggesting a requirement for both TARBP2 and PRKRA at that stage. We observed changes in abundance and length of both 3p and 5p miRNAs in *Tarbp2* $^{-/-}$

mutants, although the extent of aberrant processing of 3p-miRNAs was greater than for 5p-miRNAs. Our findings for TARBP2 are supported by a recently published study using knock-out cell lines (Kim, Yeo et al. 2014). We did not observe differences in abundance, sequence or length of miRNAs in *Prkra* $^{-/-}$ mutants, suggesting that PRKRA is not involved in miRNA biogenesis during murine embryogenesis. However, given that we were able to detect minor levels of PRKRA in some tissues (Figure 2.3.C), although not in MEFs (Figure 2.7.A), it remains an open possibility that PRKRA is involved in miRNA biogenesis.

Interestingly, the absence of TARBP2 affected some but not all miRNAs. Of the 74 miRNAs whose levels were affected, 28 were increased and 46 were decreased. We do not know the basis for the selectivity. In addition, because we used RNA isolated from whole embryos, we do not know if the affected miRNAs are expressed in the same cells or in different cells. These observations support the hypothesis that DICER acts independently of TARBP2 prior to E7.5, as not all miRNAs require TARBP2 for their biogenesis. We also observed a dependency for TARBP2 on isomiR biogenesis with a bias towards a stronger effect on 3p-miRNAs as previously reported (Wilson, Tambe et al. 2015). The effect of this change could alter the half-life or stability of improperly processed mature miRNAs and ultimately result in the deregulation of RISC complex formation. Improper targeting of mRNAs by isomiRs could result in the developmental defects observed in *Tarbp2* $^{-/-}$ animals. The viability of B6129F1 *Tarbp2* $^{-/-}$ hybrids may also be explained by the restricted effect of loss of TARBP2 on miRNA biogenesis. Heterozygosity at miRNA and target mRNA loci across the genome may alter the global miRNA/mRNA profile and suppress the relatively minor changes in miRNA levels and isomiR types observed in *Tarbp2* $^{-/-}$ mutants. In support of this, we attempted to identify quantitative trait loci in the 129 strain that suppress the

lethality and growth defects observed in B6. Assaying for both viability and body weight we mapped several loci with modest LOD scores across the genome that correlated with one or both phenotypes, indicating that there are multiple loci that contribute to enhanced survival and body weight in the F1 hybrid, N2 or mixed backgrounds (unpublished data).

In an attempt to pharmacologically strengthen our findings, we assessed the effect of enoxacin on MEFS derived from *Tarbp2* $^{-/-}$ and *Prkra* $^{-/-}$ mutant embryos. Enoxacin has been previously reported to inhibit growth of cancer cells by enhancing miRNA processing through a direct physical interaction with TARBP2 miRNA (Shan, Li et al. 2008, Melo, Villanueva et al. 2011, Cornaz-buros, Riggi et al. 2014). If TARBP2 and PRKRA are redundant, then cells expressing either of these proteins should have a similar response with enoxacin treatment. If enoxacin acts only through TARBP2, then *Tarbp2* $^{-/-}$, but not *Prkra* $^{-/-}$ mutant MEFS should be resistant to it effects. We initially found that the growth of transformed *Tarbp2* $^{-/-}$ MEFs was more sensitive to enoxacin than *Prkra* $^{-/-}$ MEFs, supporting the possibility that enoxacin may act through TARBP2. However, molecular analysis failed to reveal an increase in miRNA levels in any genotype, and the changes that were observed were opposite of what has been observed in cancer cells. Based on these observations, we conclude that the growth effects of enoxacin on transformed MEFs is independent of TARBP2 or PRKRA and that enoxacin may act through a different mechanism in transformed MEFs than in cancer cells.

The discordant phenotypes between *Dicer* and *Tarbp2* mutants, and the observation that only a subset of miRNAs are affected in *Tarbp2* mutants, suggest DICER does not require a co-factor for processing some pre-miRNAs, or that another as yet unidentified co-factor of DICER functions in early embryogenesis. It has recently been shown that DICER

interacts with a ADAR, and lack of this association in *Adar1* mutants impacts the biogenesis of mature miRNAs at E11.5 (Ota, Sakurai et al. 2013). These combined results suggest that DICER interacts with different dsRBPs in canonical miRNA biogenesis during embryonic development and this association is specific to developmental stage. DICER may also perform a function in early embryogenesis that is independent of miRNA biogenesis as has been previously suggested (Johanson, Lew et al. 2013).

The failure to detect defects in miRNA biogenesis in *Prkra* mutants at E15.5, and the discordant phenotypes between *Dicer* single mutants and *Tarbp2* $^{-/-}$*; Prkra* $^{-/-}$ double-mutants, suggests that double-mutant phenotype is not solely due to defects in miRNA biogenesis. The severity of the phenotype in *Tarbp2* $^{-/-}$*; Prkra* $^{-/-}$ double-mutant embryos could be due to the combination of defects in miRNA biogenesis, as a consequence of the absence of TARBP2, coupled with defects in endo-siRNA biogenesis as a consequence of the absence of PRKRA. That said, deletion of *Dicer* failed to affect the ability of siRNAs to repress gene expression in murine embryonic stem cells (Murchison, Partridge et al. 2005). Alternatively, TARBP2 and PRKRA could be functioning in a shared pathway that is independent of DICER-mediated pre-miRNA processing. TARBP2 has been shown to act independently of DICER to destabilize dsRNA (Goodarzi, Zhang et al. 2014), while PRKRA regulates translation by activating PKR (Daniels and Gatignol 2012), a global regulator of translation. The absence of both proteins could result in increased levels of dsRNA, hyper activation of PKR and loss of global control of translation leading to broad inhibition of development.

# CHAPTER 3. ROLE OF TARBP2 DURING MURINE SPERMATOGENESIS

## 3.1 Introduction

The following chapter will outline a series of experiments and analyses that have led to the conclusion that TARBP2 has a role in miRNA biogenesis during spermatogenesis. This is consistent with our observation based on *in vivo* studies in the previous chapter. Additionally, the following analyses present novel evidence for possible role of TARBP2 dependent miRNAs in regulating translation elongation during murine gametogenesis.

As discussed in Chapter 1.4.3, DICER is required at various stages of spermatogenesis to ensure the production of fully differentiated spermatozoa. It is also a component of chromatoid bodies, an intracellular focal domain that organizes and controls RNA processing in haploid spermatids (Comazzetto, Di Giacomo et al. 2014). Cell-specific and developmental stage-specific ablation of *Dicer1* in murine testes results in disruption of DICER1 mediated miRNA biogenesis leading to sterility (Maatouk, Loveland et al. 2008, Korhonen, Meikar et al. 2011, Romero, Meikar et al. 2011, Greenlee, Shiao et al. 2012). Together these studies highlight the importance of miRNA biogenesis pathways and miRNAs in murine male germ cell development. The role of specific miRNAs, like miR-34b/c and miR-449, miR-122a and miR-469 during post-transcriptional gene regulation of *Tnp1*, *Tnp2* and *Prm2* in post-meiotic germ cells was discussed in Chapter 1.5. (Yu, Raabe et al. 2005, Dai, Tsai-Morris et al. 2011, Comazzetto, Di Giacomo et al. 2014). Human patients with asthenozoospermia and oligoasthenozoospermia are characterized by downregulation of several miRNA expression levels in germ cells, suggesting a conserved role for miRNAs in

regulating cell state transitions during male gamete development in mammals (Liu, Cheng et al. 2012, Abu-Halima, Hammadeh et al. 2013).

Compared to somatic tissues, and to DICER expression in the testis, TARBP2 is highly expressed in the testis where it is predominantly expressed in germ cells (Lee, Fajardo et al. 1996). TARBP2 is first expressed at low levels in pachytene spermatocytes and then at high levels in round spermatids. Previously we generated a constitutive null allele of *Tarbp2* that is lethal on a C57BL/6J background. However, on a mixed genetic background some animals survive, although they are smaller than littermate controls and have a shorter life span (Zhong, Peters et al. 1999). Mice that survive and live to reproductive age are sterile with accompanying defects in translational activation of *Prm1* mRNA, which is under temporal translational regulation during spermiogenesis (Fajardo, Haugen et al. 1997, Zhong, Peters et al. 1999). Because these studies were completed before the discovery of miRNAs, it is unknown if the defective translational activation of the *Prm1* mRNA is mediated through the action of TARBP2 as a cofactor of DICER and whether the effect is dependent on miRNAs.

In the previous chapter, we reported that loss of TARBP2 during C57BL/6J murine embryonic development results in downregulation of a subset of mature miRNAs and increased isomir accumulation, demonstrating the essential role of TARBP2 in miRNA biogenesis *in vivo*. To overcome embryonic lethality, and to study the effect of loss of TARBP2 on a pure genetic background, we report here the consequences of conditional ablation of *Tarpb2* in spermatogenic cells.

## 3.2 Experimental Methods

### 3.2.1 Animals and tissue collection:

C57BL/6J mice bearing *Tarbp2*$^{fl/fl}$ (Ding, Chen et al. 2015) were generously provided by Dr. Da-Zhi Wang (Harvard University). All experimental animals were maintained on C57BL/6J background. *Tg(Stra8i-cre*$^{1Reb}$*)* was used to excise *Tarbp2* in the germ cells (Sadate-Ngatchou, Payne et al. 2008). To generate conditional *Tarbp2*$^{-/-}$ male mice, we first mated *Tarbp2*$^{fl/fl}$ males with wild type *Tarbp2* and *Tg (Stra8i-cre*$^{1Reb}$*)* females. *Tarbp2*$^{flox/WT}$; *Stra8*-icre$^{+}$ males were crossed with *Tarbp2*$^{fl/fl}$ females to generate *Tarbp2*$^{flox/\Delta}$*; Stra8*-icre+ males. These males have *Tarbp2*$^{-/-}$ germ cells and referred to as *Tarbp2*$^{\Delta/fl}$*; Stra8icre*$^{+}$ male mice. Excision of *Tarbp2* from germ cells was confirmed by measuring *Tarbp2* transcript and TARBP2 protein levels in *Tarbp2*$^{\Delta/fl}$ and *Tarbp2*$^{\Delta/fl}$*; Stra8icre*+ testes. For all procedures, mice were sacrificed by $CO_2$ exposure followed by cervical dislocation. The Institutional Animal Care and Use Committee at Jackson Laboratory (JL) approved all animal procedures (Permit Number: 07007).

### 3.2.2 Histology and immunofluorescence analysis:

Epididymes and testes were fixed overnight in Bouins fixative at 4°C and then washed in water prior to paraffin embedding by the Histology Core Facility. 5-µm sections were stained with hematoxylin and PAS for histological analyses. For immunofluorescence studies, slides containing 5-µm sections were deparaffinized in xylene (2 times for 5 min each), followed by a 5-min rinse in 100% ethanol. Slides were rehydrated by 5min incubations in 100%, 95%, 70% and 50% ethanol followed by citrate buffer antigen retrieval. Tissue sections were blocked in PBS containing 5% normal goat serum and then incubated

with primary antibodies overnight at 4°C. Primary antibodies were used at the following dilutions: TARBP2 (1:500) and PRM1 (1:200). Following 3x washes for 5 minutes in PBS, the sections were incubated at room temperature for an hour with goat anti-mouse conjugated to Alexa Fluor R568 (Life Technologies) (1:1000) in PBS containing 0.05% Tween-20. Following 3x washes in PBS-T in the dark, sections were mounted with Vectashield medium containing DAPI (Vector Laboratories). Fluorescence was imaged using a Nikon Eclipse E600 equipped with a digital camera and formatted using Photoshop software (Adobe Systems).

### 3.2.3 Sperm count and morphology

Epididymes were dissected from adult mice and diced in 1 ml of phosphate buffered saline (PBS). The diced tissue was incubated at 37°C for an hour to release sperm that were then diluted in PBS (1:10) and counted using a hemocytometer. Duplicate counts were evaluated for each mouse sample (N = 6) and expressed as mean ± S.D. Sperm morphology was assessed by mounting spermatids in Vectashield with DAPI and imaging the spermatids on a Leica SP5 laser scanning confocal microscope.

### 3.2.4 Protein isolation and western blotting

Testes frozen in liquid nitrogen were pulverized into fine powder on dry ice and then suspended in protein extraction buffer (150mM NaCl, 20mMTris-HCl, pH 8.0, 1.0% Triton X-100, 0.1% SDS, and Complete EDTA-free protease inhibitor [Roche]). After complete dissolution of powder, protein concentrations were determined using Bradford reagent (Bio-Rad). For western blot analyses, the following primary antibodies were used: anti-TARBP2

(Abnova, MAB0811), anti-SPATA21 (Abcam, ab173912) and anti-GAPDH (Cell signaling Tech., 14C10).

### 3.2.5 Isolation of basic nuclear proteins and immunoblotting

Basic nuclear proteins, including PRM1 and PRM2 from the testis were isolated as described (Lee, Haugen et al. 1995). Basic proteins were separated on a 15% acetic-acid urea gel and stained with amido black for detection. The samples were run on a separate gel and transferred to a PVDF membrane using 0.7% acetic acid and 1M Urea (Sigma-Aldrich) at 20 volts for 1 h. The membrane was blocked in 5% non-fat milk in 1X PBS (pH 9.0) at room temperature and incubated in Hup1N (PRM1) antibody (1:1000) or Hup 2b (PRM2) antibody (1: 5000) at 4°C overnight (Hup1N and Hup 2b antibodies were purchased from Briar patch Biosciences LLC). The membrane was rinsed 3x in TBST for 20 minutes and incubated with goat anti-mouse IgG-HRP (1:1000) in 5% non-fat milk in TBST for 2 h at room temperature. Following 3 x 10 minute washes in TBST and the membrane was developed using the ECL western blotting detection kit.

### 3.2.6 RNA isolation, high-throughput sequencing and data analysis

Spermatocytes and round spermatids were isolated from testes using FACS-sorting as described previously (Lima, Jung et al. 2017). Total RNA was isolated using Trizol-extraction (Invitrogen, 15596018) followed by analysis on an Agilent Bioanalyzer to determine quality. miRNA and mRNA were separated using AMPure XP beads following the manufacturers protocol. mRNA sequencing libraries were constructed using the Stranded Total RNA LT with Ribo-Zero TM Gold Library Prep kit (Illumina) and single-end 75bp reads sequenced on an Illumina HiSeq 2500 to a minimum depth of 30 million reads per

sample. miRNA sequencing libraries were constructed using the Stranded Total RNA LT with Ribo-Zero TM Gold Library Prep kit (Illumina) and single-end 75 bp reads sequenced on an Illumina HiSeq 2500 to a minimum depth of 30 million reads per sample. mRNA sequencing reads were aligned to a C57BL/6J transcriptome (Ensemble release 80) and TPM counts were calculated via RSEM (Li and Dewey 2011). miRNA sequencing reads were aligned to reference miRBase release 2 and estimated miRNA TPM counts using IsomiRs package in R (Griffiths-Jones, Saini et al. 2008, Morin, O'Connor et al. 2008, Pantano, Estivill et al. 2010). Statistical analysis was performed using the R-Bioconductor package, DEseq2 (Love, Huber et al. 2014).

### 3.2.7 Polysomal fractionation and northern blotting analysis

Testes from *Tarbp2*$^{\Delta/fl}$*; Stra8icre* and suitable control littermates were isolated at 6 weeks of age and immediately placed into lysate buffer and homogenized. Sucrose gradient and Northern blot analysis was performed as described previously (Snyder, Soundararajan et al. 2015).

### 3.2.8 Electron microscopy analysis

Whole testes were fixed in 4% PFA, overnight at 4°C. For chromatin structure analysis samples were post-fixed in 2% aqueous osmium tetroxide, rinsed in PBS, then dehydrated with ethyl alcohol. Samples were then infiltrated and embedded with Embed 812 resin (Electron Microscopy Sciences, Hatfield, PA) and the blocks were polymerized in a 60°C oven for 48 hours. 90nM sections were cut on a Leica UC6 ultra microtome and sections were collected on 300 mesh copper grids. For Immuno-EM, samples were dehydrated up to 70% ETOH, infiltrated with LR White resin (Electron Microscopy

Sciences, Hatfield, PA) and then embedded in the same resin in a 50°C oven for 24 hours. 90nM sections were collected on 200 mesh nickel grids. For PRM1 staining, citrate buffer antigen retrieval was performed on a PCR machine at 94°C for 40min. Staining was performed as described in section 3.2.5, using PRM1 antibody (1:40) followed by secondary antibody coupled with gold particles (1:120). Copper and Nickel Grids were fixed with 2.5% glutaraldehyde and stained with 1% aqueous uranyl acetate and Reynold's lead citrate. After staining with 1% aqueous uranyl acetate/Reynold's lead citrate, grids were viewed on a JEOL JEM 1230 transmission electron microscope and images collected with an AMT 2K digital camera.

### 3.2.9 Sequencing data availability:

All sequencing data uploaded to SRA database of NCBI with a BioprojectID: PRJNA432569**.**

## 3.3 Results

### 3.3.1 Conditional deletion of *Tarbp2* in germ cells arrests spermatid differentiation

To ablate *Tarbp2* expression in spermatogenic cells, we used a *Stra8* promoter-driven cre-recombinase (*Stra8-icre*). By quantitative real-time PCR we confirmed that *Tarbp2* mRNA is absent in pachytene spermatocytes and round spermatids (Figure 3.1.A) isolated from the testes of 6wk old *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ mice. TARBP2 protein was also greatly reduced in whole-testis extracts compared to the GAPDH control (Figure 3.1.B). Immuno staining using a TARBP2 antibody on cross-sections of 6wk old *Tarbp2*$^{\Delta/fl}$*; Stra8icre*+ mice confirmed loss of TARBP2 expression in pachytenes and round spermatids (Figure 3.1.C).

Breeding studies indicated that conditional ablation of *Tarbp2* in male germ cells caused sterility (Table 3.1)

| ♂ | ♀ | # of litter | # of ♂ pups | # of ♀ pups |
|---|---|---|---|---|
| *Tarbp2*$^{\Delta/fl}$;*Stra8icre*+ | *Tarbp2*$^{\Delta/fl}$ | 0 | 0 | 0 |
| *Tarbp2*$^{\Delta/fl}$ | *Tarbp2*$^{\Delta/fl}$;*Stra8icre*+ | 12 | 43 | 59 |

**Table 3.1. *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ males are sterile**

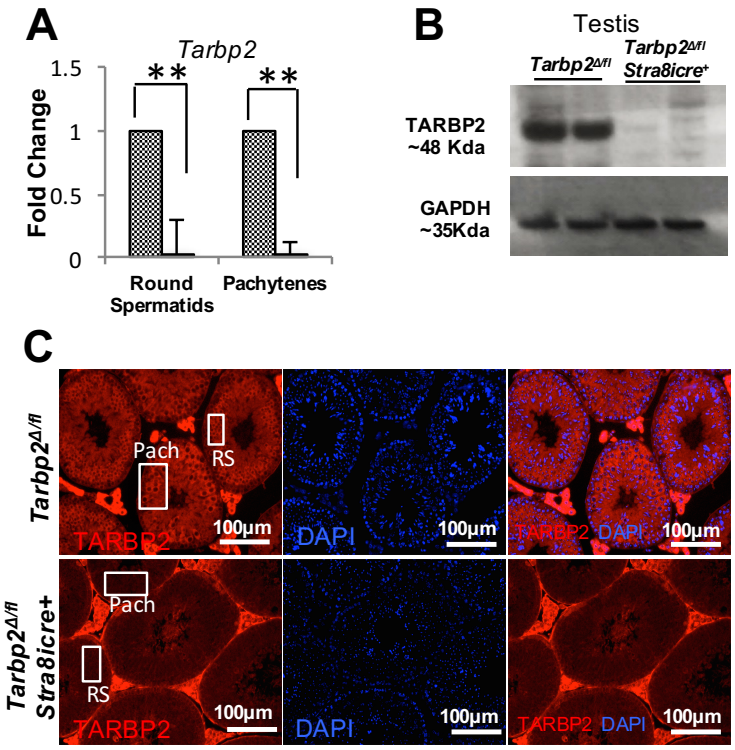



**Figure 3.1-Germ cell specific loss of TARBP2 expression in *Tarbp2*$^{\Delta/fl}$*;Stra8icre*$^{+}$ mice**

(A) qRT-PCR analysis indicating fold-change of *Tarbp2* transcript in pachytenes and round spermatids comparing *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ with *Tarbp2*$^{\Delta/fl}$ control (**p<0.005) (n=3). (B) Immunoblot for TARBP2 (~48kda) protein expression in testes from 6wk old *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ and *Tarbp2*$^{\Delta/fl}$ mice. GAPDH (~35kda) was used as a loading control. (C) Testis cross-sections from 6wk old *Tarbp2*$^{\Delta/fl}$ and *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ indicating TARBP2 expression in Pachytenes (Pach) and Round spermatids (RS) in *Tarbp2*$^{\Delta/fl}$ and loss of TARBP2 expression in these cells in *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ animals (n=6). Sections stained with TARBP2-antibody, counterstained with DAPI.

To investigate if loss of TARBP2 in germ cells affected spermatogenesis, we compared testes histological sections from 6wk old *Tarbp2*$^{\Delta/fl}$*;Stra8icre+* mice to those from control littermates. We observed normal germ cell development through meiosis. However, unlike controls, spermatid elongation was disrupted and included abnormal nuclear morphogenesis and formation of symplasts (Figure 3.2).

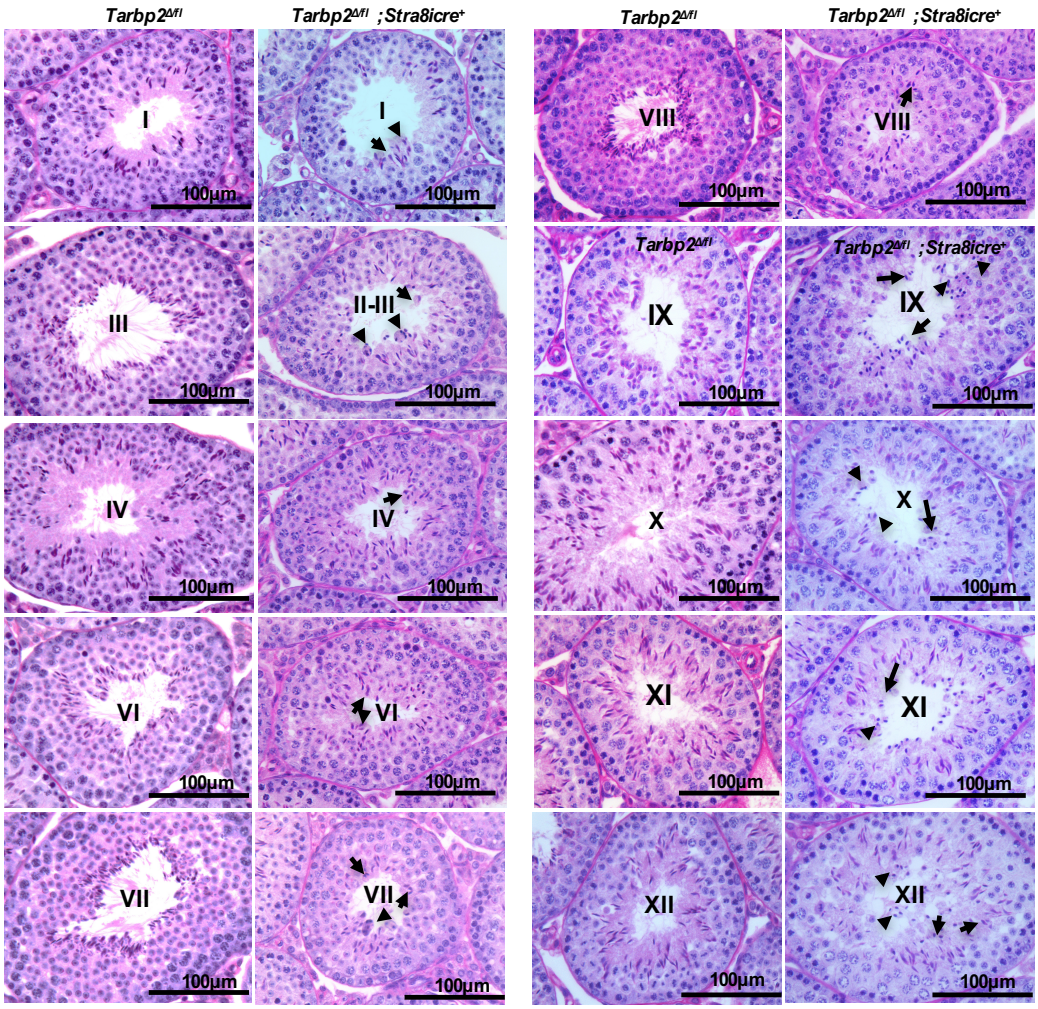



**Figure 3.2-Germ cell specific loss of TARBP2 results in defective spermatid elongation**
Histological analysis of testis cross-sections stained with PAS from *Tarbp2*$^{\Delta/fl}$ *Tarbp2*$^{\Delta/fl}$*;Stra8icre+* mice at 40X magnification corresponding to Stages I-XII of male gamete development. Arrows indicate defective spermatid elongation and arrow heads indicate nuclear symplasts.

Electron microscopy (Figure 3.3.A) and acridine orange staining on isolated spermatids (Figure 3.3.B) confirmed defects in nuclear condensation, while phase contrast microscopy of isolated testicular sperm (Figure 3.3.C) confirmed defects in nuclear morphogenesis including double-headed sperm and tails mis-attached to the mid-region of the head (Fig.S1E).

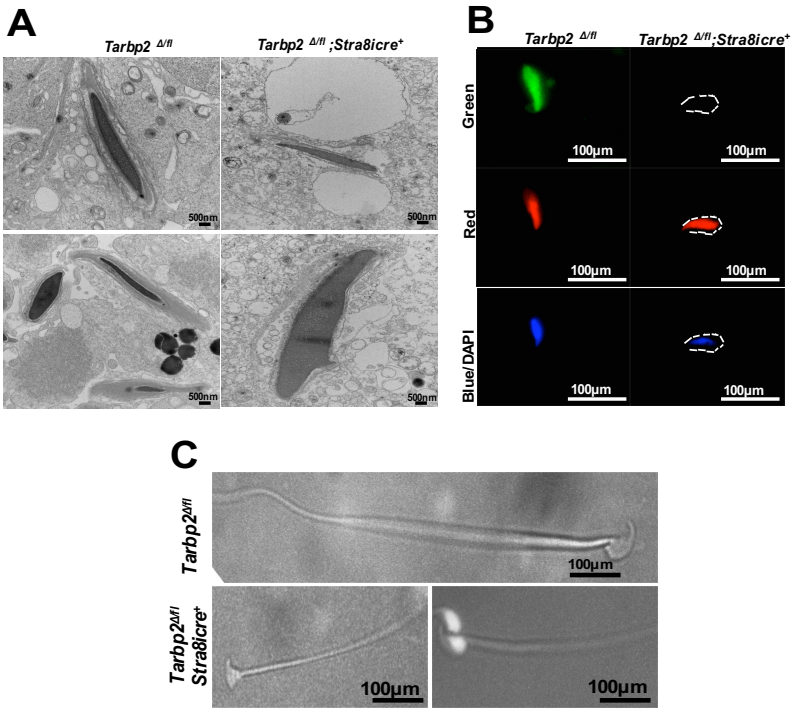



**Figure 3.3 Improper nuclear condensation and morphology in *Tarbp2*$^{-/-}$ sperm**

(A) Electron microscopy images of sperm from *Tarbp2*$^{\Delta/fl}$ (left panel) and *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ (right-panel). Images taken at 20K mag. (B) Acridine orange (AO) staining analysis of elongating spermatids at 40X magnification indicates Red, Green and Blue (DAPI) colored fluorescence in *Tarbp2*$^{\Delta/fl}$ and absence of Green fluorescence in *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ suggesting improper chromatin condensation. (C) Phase contrast microscopy, gray-scale images of sperm stained with DAPI from *Tarbp2*$^{\Delta/fl}$ (top-panel, normal looking sperm) and *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ (bottom-panel, left: abnormal spermatozoa with flagellum attached to equatorial region of head, right: double-headed spermatozoa).

Together these data suggest that TARBP2 is required for post-meiotic spermatid differentiation and that its absence causes multiple defects in nuclear and tail morphogenesis.

### 3.3.2 Altered mature miRNA levels in *Tarbp2$^{\Delta/fl}$;Stra8icre+* mice

TARBP2 has been shown to be a co-factor of DICER (Chendrimada, Gregory et al. 2005), and increased miRNA expression levels overlap with TARBP2 expression during spermatogenesis (Ro, Park et al. 2007). To determine whether loss of TARBP2 in germ cells affected the accumulation of mature miRNAs, we collected total RNA from Hoechst-stained and FACS-sorted spermatocytes and round spermatids (Figure 3.4), and generated libraries of small non-coding RNAs for high-throughput miRNA-sequencing (miRNA-seq).

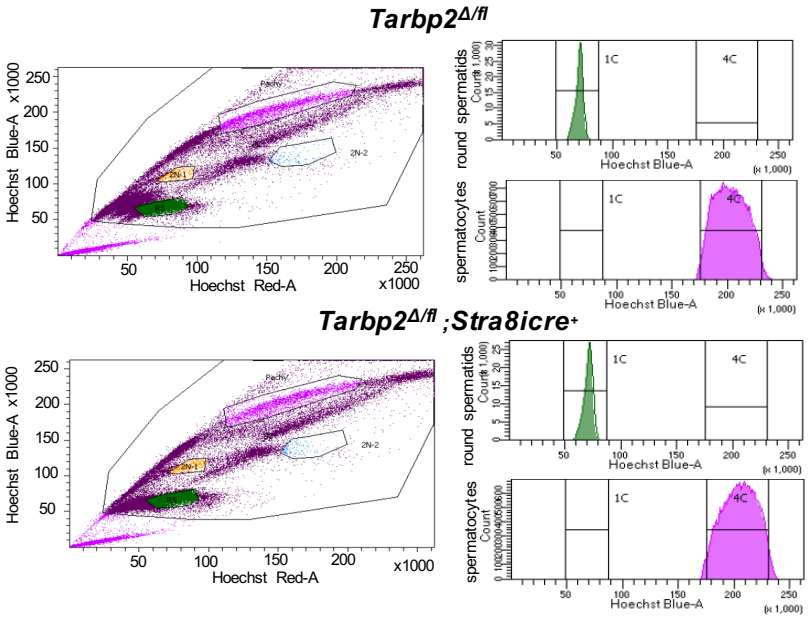



**Figure 3.4- FACS assisted sorting of germ cells based on DNA content**

(Germ cells from testes of 6 week old *Tarbp2$^{\Delta/fl}$* (top-panel) and *Tarbp2$^{\Delta/fl}$;Stra8icre+* (bottom panel) animals were isolated by FACS. Hoechst 3342 dye was used to label the DNA content of cells. Cells were assigned into 4C (spermatocytes) and 1C (round spermatids) depending on the level of staining and size. Propidium Iodide was used to distinguish live and dead cells (n=3)

Using an FDR pass of <0.05, we found that the expression level of 75 mature miRNAs were significantly changed by at least two-fold in *Tarbp2*$^{\Delta/fl}$*; Stra8icre+* spermatocytes (Figure 3.5.A). miRNA transcripts were both decreased (n=47, 62.6%) and increased (n=28, 37.3%). Similar analyses of miRNA-seq data obtained from round spermatids, revealed that the expression level of 74 mature miRNAs were significantly altered (Figure 3.5.B). Among these, 28 miRNAs were down-regulated in both spermatocytes and round spermatids, whereas 19 were unique to spermatocytes and 21 to round spermatids (Figure 3.5.C). Previous studies have shown that loss of TARBP2 results in altered cleavage site selection by DICER and the generation of isomirs (Kim, Yeo et al. 2014, Wilson, Tambe et al. 2015). Loss of TARBP2 in germ cells also resulted in altered Isomirs in spermatocytes (Figure 3.6.A) and round spermatids (Figure 3.6.B). These studies confirm that during spermatogenesis, and as previously shown in early embryos (Chapter 2. Figure 2.5), that TARBP2 is required for the processing of a subclass of pre-miRNAs into different Isomir types.

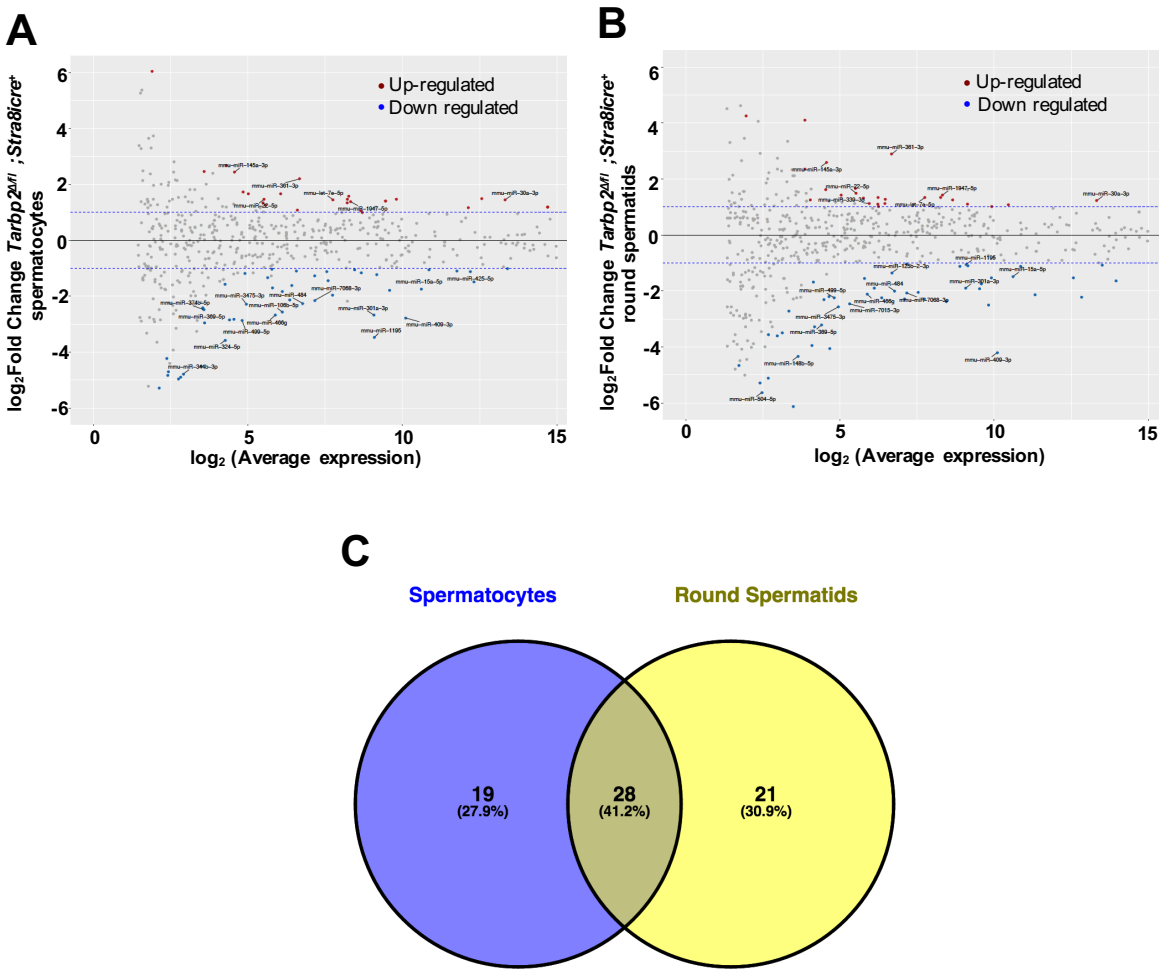



**Figure 3.5-Altered mature miRNA levels in *Tarbp2$^{\Delta/fl}$;Stra8icre+* mice**

(A) MA-Plot of miRNA transcripts in *Tarbp2$^{\Delta/fl}$;Stra8icre+* spermatocytes at 6wks of age and their fold-changes by average expression using miRNA-seq. Upregulated - log2Foldchange>1, FDR<0.05; Down-regulated- $log_2$Foldchange <-1, FDR<0.05. (B) MA-Plot of miRNA transcripts in *Tarbp2$^{\Delta/fl}$;Stra8icre+* round spermatids at 6wks of age and their fold-changes by average expression using miRNA-seq. Upregulated - $log_2$Foldchange > 1, FDR < 0.05; Down-regulated- $log_2$Foldchange <-1, FDR < 0.05. (C) Venn-diagram representing the number of specific and common miRNAs down-regulated in spermatocytes and round spermatids.

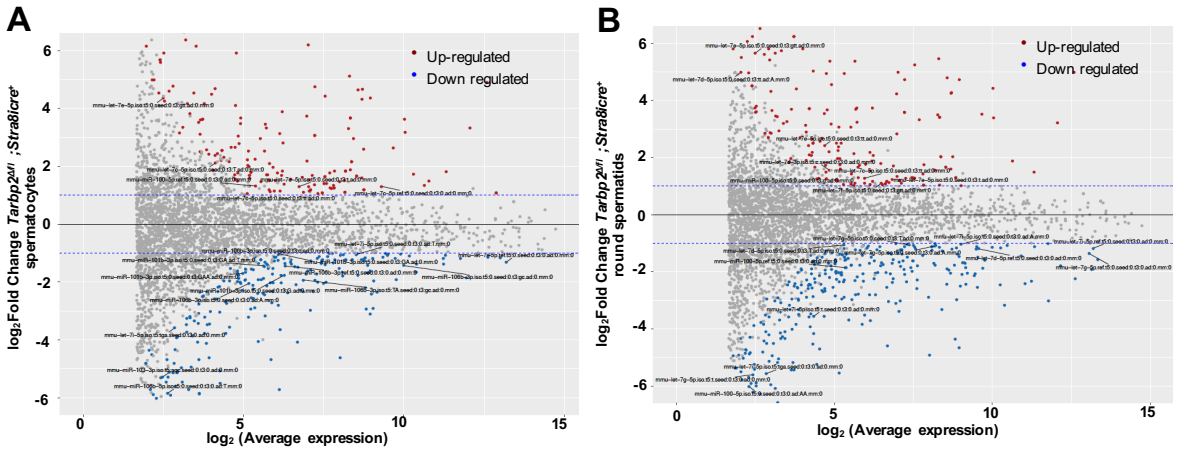



**Figure 3.6- Altered isomiR expression in *Tarbp2$^{\Delta/fl}$;Stra8icre*+ mice**

(A) MA-Plot of Isomirs in *Tarbp2$^{\Delta/fl}$;Stra8icre*+ spermatocytes at 6wks of age and their fold-changes by average expression using miRNA-seq. Upregulated - log2Foldchange>1, FDR<0.05; Down-regulated- $log_2$Foldchange <-1, FDR<0.05. (B) MA-Plot of isomirs in *Tarbp2$^{\Delta/fl}$;Stra8icre*+ round spermatids at 6wks of age and their fold-changes by average expression using miRNA-seq. Upregulated - $log_2$Foldchange > 1, FDR < 0.05; Down-regulated- $log_2$Foldchange <-1, FDR < 0.05.

### 3.3.3 Altered mRNA transcript levels in *Tarbp2$^{\Delta/fl}$;Stra8icre*+ mice

Because we observed deregulation of miRNA processing in spermatocytes and round spermatids with the loss of TARBP2, we investigated if any protein-coding transcripts were deregulated. Using total RNA from which the small RNA libraries were generated, we generated libraries for high throughput mRNA sequencing. Using an FDR pass of <0.05, we found that the expression levels of 346 mRNAs were significantly altered in *Tarbp2$^{\Delta/fl}$;Stra8icre*+ spermatocytes (Figure 3.7.A), and among these, 174 were up-regulated and 172 were down-regulated. In round spermatids, the expression levels of 446 mRNAs were significantly altered and among these, 263 were up-regulated and 183 were down-regulated (Figure 3.7.B). Among the total number of up-regulated transcripts in

spermatocytes and round spermatids, 85 were common to both cell types, whereas 89 were unique to spermatocytes and 178 were unique to round spermatids (Figure 3.7.C).

In-silico analysis predicted a significant negative Pearson's correlation between the expression levels of down-regulated miRNAs and upregulated mRNAs in spermatocytes and round spermatids prepared from *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ testes (data not shown). Altered mRNAs included essential spermatid differentiation factors *Tnp2*, *Prm1*, and *Prm2,* which encode proteins involved in chromatin condensation. Various target-site prediction algorithms (e.g. Target scan, miRANDA, miRSVR, RNA-Hybrid with Δfree-energy>-20)(Kruger and Rehmsmeier 2006, Betel, Wilson et al. 2008, Betel, Koppal et al. 2010, Agarwal, Bell et al. 2015) predict that significantly-altered miRNAs in both spermatocytes and round spermatids target the 3'UTR regions of these same transcripts (Figure 3.7.D), suggesting that TARBP2, acting as a co-factor to DICER to process a subclass of miRNAs, regulates the stability or translation of several key transcripts coding for spermatid differentiation factors, including *Tnp2*, *Prm1* and *Prm2*.

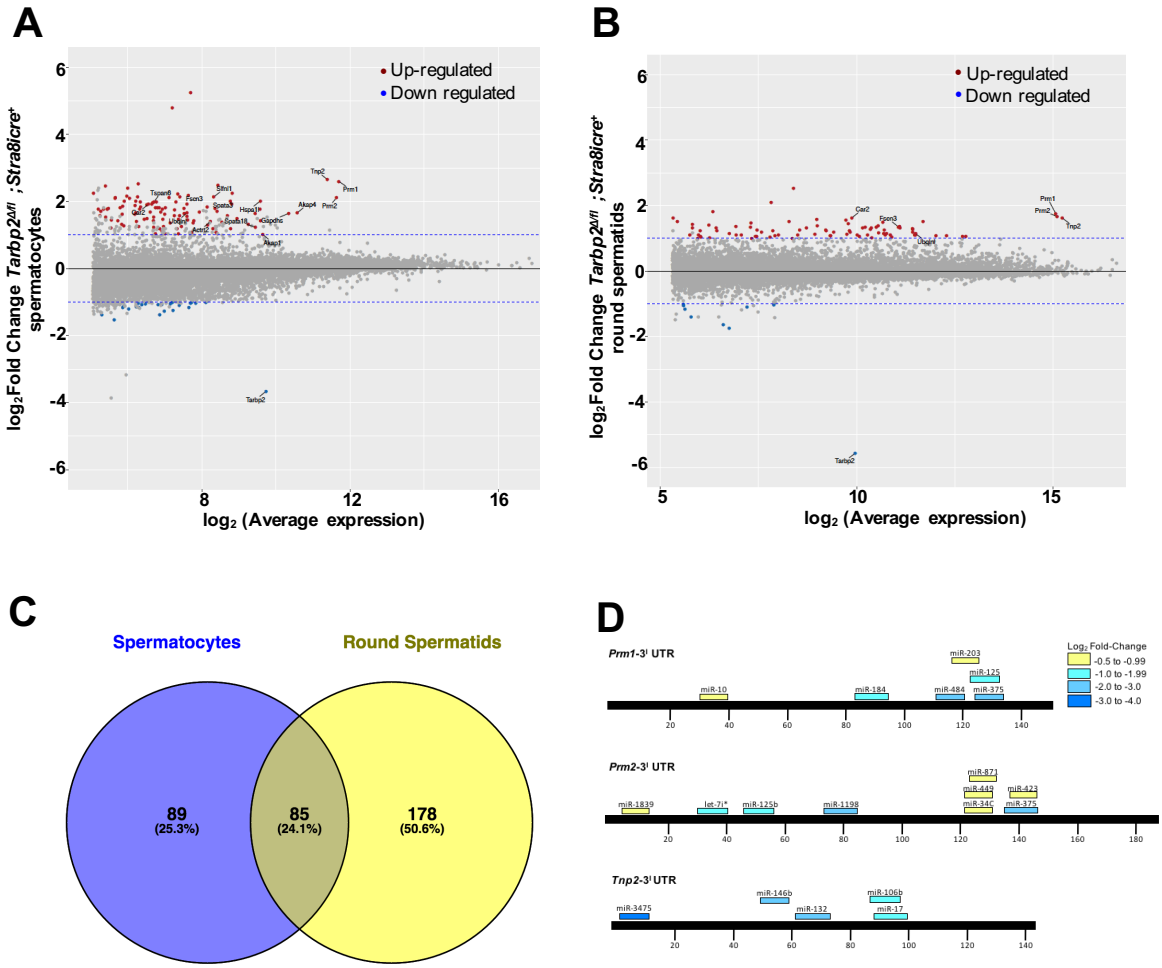



**Figure 3.7- Altered mRNA levels in *Tarbp2$^{\Delta/fl}$;Stra8icre+* mice and miRNA binding sites in 3'UTR regions**

(A) MA-Plot of mRNA transcripts in *Tarbp2$^{\Delta/fl}$;Stra8icre+* spermatocytes at 6wks of age and their fold-changes by average expression using RNA-seq. Upregulated - log2Foldchange>1, FDR<0.05; Down-regulated- $log_2$Foldchange <-1, FDR<0.05. (B) MA-Plot of mRNA transcripts in *Tarbp2$^{\Delta/fl}$;Stra8icre+* round spermatids at 6wks of age and their fold-changes by average expression using mRNA-seq. Upregulated - log2Foldchange>1, FDR<0.05; Down-regulated- $log_2$Foldchange <-1, FDR<0.05. (C) Venn-diagram representing the number of specific and common mRNAs up-regulated (log2Foldchange>0.5, FDR<0.05) in spermatocytes and round spermatids. (D) Diagrammatical representation of 3'-UTR regions of *Tnp2*, *Prm1* and *Prm2* along with in-silico predicted miRNA targeting sites. Each color as indicated in key represents $log_2$fold-change of corresponding miRNA from miRNA sequencing data.

### 3.3.4 TARBP2 regulates translation of spermatid differentiation factors

Because we detected an increase in *Prm1* mRNA in *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ germ cells, we also investigated PRM1 protein expression. In wild type animals, PRM1 was first detected in elongating spermatid nuclei in Stage XII seminiferous tubules (Figure 3.8.A, top right). Strong immunostaining of PRM1 was also found in residual bodies of elongated spermatids in Stage VII tubules prior to the release of the spermatids into the lumen at spermiation (Figure 3.8.A, top left), confirming previous studies on the temporal expression pattern of PRM1 during spermiogenesis (Fajardo, Haugen et al. 1997). In *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ mice, PRM1 was also first detected in elongating spermatids in Stage XII tubules (Figure 3.8.A, bottom right). However, immunofluorescence was also detected in elongating spermatids in Stage VII and Stage IX tubules (Figure 3.8.A, bottom left and center), and in elongating spermatids in other staged-tubules (data not shown), but not in residual bodies in Stage VII (Figure 3.8.A, bottom left), confirming that spermatid differentiation is abnormal. The presence of PRM1-positive elongating spermatids in Stage IX (Figure 3.8.A, bottom center) and Stage XII tubules (Figure 3.8.A, bottom right) also suggests that spermiogenesis is incomplete and that undifferentiated elongating spermatids are retained at spermiation.

To further investigate the expression of the basic nuclear proteins involved in chromatin condensation, we analyzed basic proteins extracted from *Tarbp2*$^{\Delta/fl}$ and *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ testes . Surprisingly, we were unable to detect PRM1 with either amido-black staining (Figure 3.8.B) or by western blotting (Figure 3.8.C) in either the sonication-sensitive or sonication -resistant fractions. We also failed to detect TP2, PRM2 and any of the PRM2 precursor proteins in *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ mice (Figure 3.8.B).

The failure to detect PRM1 protein in whole-testis extracts could be due to the lack of sensitivity of amido-black staining and western blotting compared to immunofluorescence of individual cells in sections, coupled with an arrest in spermatid differentiation prior to the completion of the normal temporal window of PRM1 expression, which would result in less protein in whole-testis extracts. To address this possibility, we also assayed by western blotting the presence of SPATA 21 (Iida, Yamashita et al. 2004), which is normally expressed during spermiogenesis and whose mRNA levels were not altered (Figure 3.8.C). The ability to detect SPATA 21 protein in extracts suggest that the failure to detect PRM1 is not due to an early block in spermatid differentiation that prevents the accumulation of sufficient protein for detection.

To further investigate the failure to detect PRM1 by western blotting and amido-black staining, we used polysome profiling to determine the extent to which *Prm1* mRNA was recruited from the mRNP fraction to actively translating ribosomes. Prior to its translation in elongating spermatids, *Prm1* mRNA is normally stored in round spermatids as an mRNA ribonucleoprotein particle (mRNP) that sediments with the 40S ribosomal protein subunit (Kleene 1989). Upon recruitment to polysomes and translation in elongating spermatids, *Prm1* mRNA is reduced in size from approximately 550 nucleotides (nts) down to 400 nts due to shortening of its poly(A) tail (Kleene 1989). As expected, in polysomes prepared from control testes, we detected the bulk of the stored *Prm1* mRNA in fractions 2 and 3, corresponding to the 40S and 60S ribosomal subunits, and the deadenylated forms of the mRNA in fractions 8-11, corresponding to actively translated mRNAs containing ribosomes spaced ~ 50 nts apart (Cataldo, Mastrangelo et al. 1999) (Figure 3.8.D, left). In polysomes prepared from *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ testes, we also detected the majority of the

*Prm1* mRNA in fractions 2 and 3. However, unlike the control, the deadenylated forms of *Prm1* RNA were found in fractions 4-6, corresponding to the 80S monosome (fraction 4) and single or small polysomes (fractions 5 and 6). The presence of the deadenylated forms or *Prm1* mRNA in fractions 4-6, which normally is a signature of active translation in fractions 8-11, suggests that the initiation of translation of *Prm1* mRNA was inefficient, or that translation elongation was blocked or stalled. Interestingly, the mRNAs encoding the other basic proteins that we failed to detect by amido-black staining, were also shifted from the higher to lower fractions in the gradient, where they were also deadenylated, suggesting that translation initiation or elongation was also stalled on those mRNAs (Figure 3.7.D).

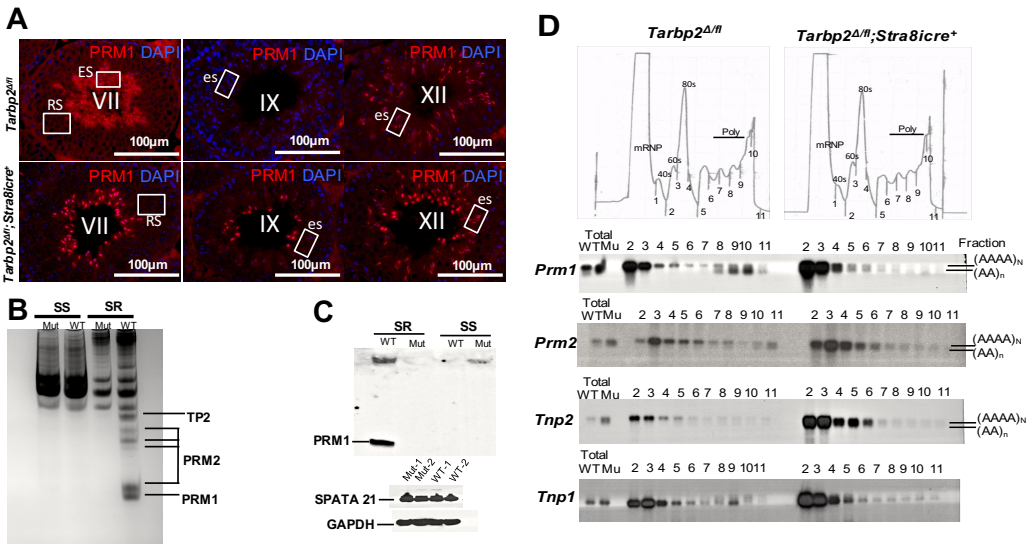



**Figure 3.8- Altered PRM1 expression and defective translation elongation in *Tarbp2$^{\Delta/fl}$; Stra8icre*+ mice**

(A) Testis cross-sections stained with PRM1-antibody and counterstained with DAPI.PRM1 expression in elongating spermatids (es) of stage XII but not stage IX and elongated spermatids (ES) of stage VII tubules in *Tarbp2$^{\Delta/fl}$* (top panel), where as PRM1 expression is observed in every staged tubule in *Tarbp2$^{\Delta/fl}$;Stra8icre*+ (bottom panel) (n=4) PRM1 expression is absent in round spermatids (RS) of mutant and wild-type control tubules. (B) Amido-black staining analysis of total basic proteins in sonication resistant (SR) and sonication sensitive (SS) nuclear fractions from testes of *Tarbp2$^{\Delta/fl}$;Stra8icre*+ and *Tarbp2$^{\Delta/fl}$* mice (n=2). The positions of TNP2, PRM1 and PRM2 (precursor, mature, processed form) are indicated. (C) Western-blot analysis of PRM1, SPATA21 and GAPDH (loading control). (D) Absorbance profiles of sucrose gradient fractionation of lysate extracted from 6wk old *Tarbp2$^{\Delta/fl}$;Stra8icre*+ and *Tarbp2$^{\Delta/fl}$* testes. Each peak represents corresponding fractions as indicated. Numbers 1-11 represent collected fractions and their corresponding location in the absorbance profile. Northern blot analysis of *Prm1*, *Prm2, Tnp2, and Tnp1* mRNA extracted from fractions collected in polysomal analysis of *Tarbp2$^{\Delta/fl}$;Stra8icre*+ and *Tarbp2$^{\Delta/fl}$* testes. Total RNA extracted from samples before loading onto sucrose gradient and fraction numbers are indicated. Adenylated [$(AAAA)_N$] and de-Adenylated [$(AA)_n$] forms of mRNA are indicated.

### 3.3.5 Altered PRM1 localization in TARBP2 deficient elongating spermatids

As discussed above, the ability to detect PRM1 by immunofluorescence but not by amido-black staining or by western blotting, could be due to differential sensitivity of the methods. However, the polysome analysis suggested the additional possibility that the failure to detect full-length protein could also be due to a failure to complete translation elongation. The antibody used to detect PRM1 recognizes the amino terminus of the protein. A prediction of this hypothesis should be the detection of the amino terminal region of PRM1 outside of the nucleus. To test this hypothesis, we performed immunogold staining of PRM1. In control animals, the gold particles were mostly detected with the condensed chromatin or with the nuclear membrane (Figure 3.9.A, left). However, in *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ mice, immunogold staining was detected in the cytoplasm as well as in the nucleus (Figure 3.9.A, right). Statistical analysis confirmed a differential distribution of gold particles between the control and mutant (Figure 3.9.B). In wildtype sperm, PRM1 immunogold staining was either localized near the nuclear envelope (at least 2-5 gold particles in 23 out of 30 cells) or on chromatin (1-7 gold particles in 28 out of 30 cells), but never in cytoplasm. In *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ mice, PRM1 was consistently found in the cytoplasm (at least 2-9 gold particles in 22 out of 25 cells), on chromatin in few incidences (at least 1-12 gold particles in 14 out of 25 cells), but rarely near the nuclear membrane structures (1-3 particles in 3 out of 25 cells). Together these data suggest that the absence of TARBP2 results in a block in translational elongation and retention of partially synthesized PRM1 in the cytoplasm where it is presumably associated with stalled ribosomes.

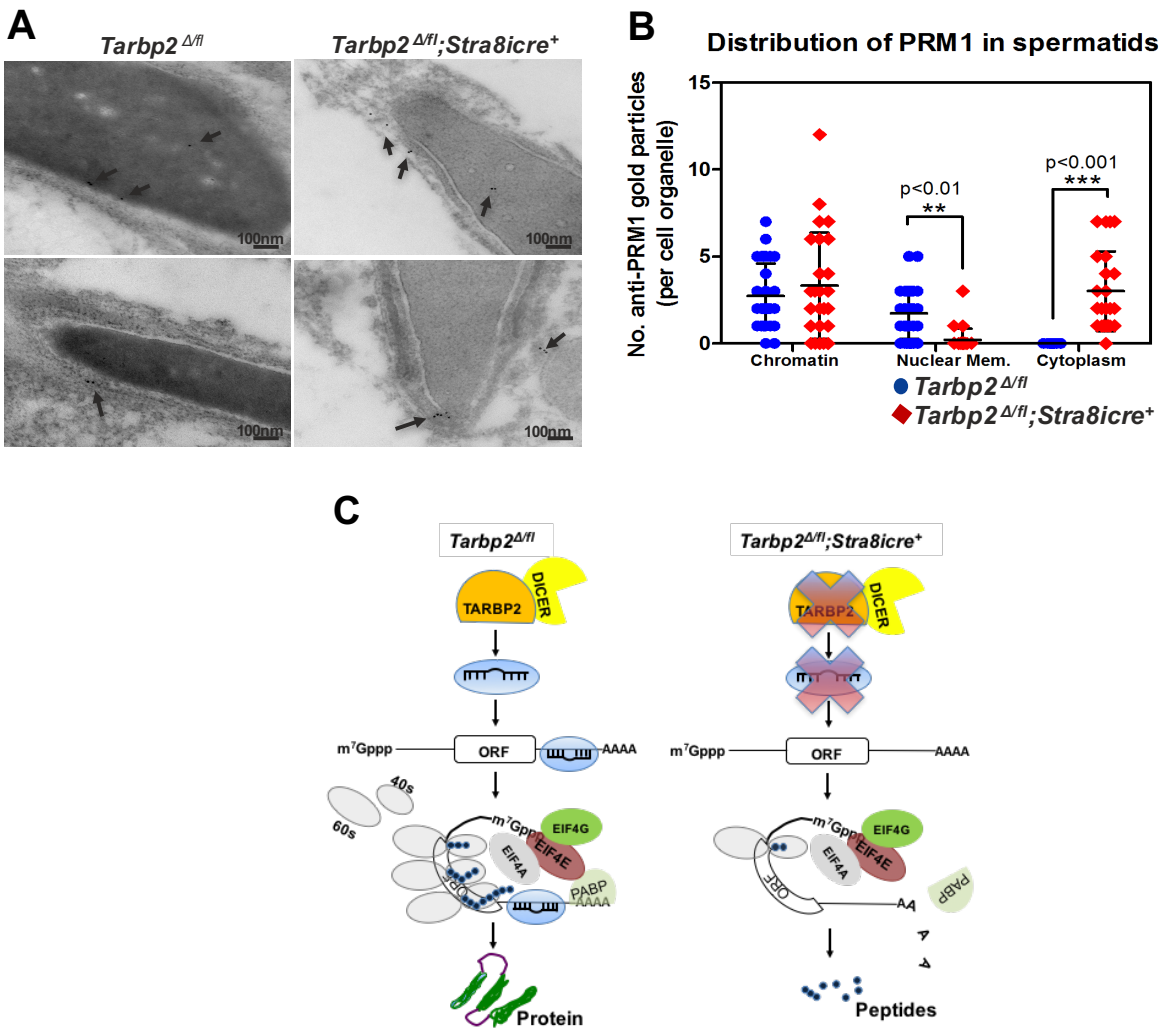



**Figure 3.9 - Altered PRM1 localization in TARBP2 deficient elongating spermatids and TARBP2 dependent miRNAs in translation elongation**

(A) Electron microscopy images of sperm from *Tarbp2*$^{\Delta/fl}$ (left panel) and *Tarbp2*$^{\Delta/fl}$*;Stra8icre+* (right-panel) mice showing gold-particles (as indicated by arrows) representing localization of PRM on chromatin, nuclear membrane and cytoplasmic structures. Images taken at 20K mag. (B) Graphical representation of number of gold particles localized to three different structures as observed in each sperm cell from *Tarbp2*$^{\Delta/fl}$ (n=30) and *Tarbp2*$^{\Delta/fl}$*;Stra8icre+* (n=25) testes. p-value indicate results from students t-test. (C) Diagrammatical representation of the possible role of TARBP2 in regulating translation of spermatid differentiation factors through miRNAs binding to 3'UTR region.

## 3.4 Discussion

Previous studies have shown that DICER is required in germ cells for murine spermatogenesis (Korhonen, Meikar et al. 2011, Romero, Meikar et al. 2011, Greenlee, Shiao et al. 2012) and that male mice lacking TARBP2 on a mixed genetic background are infertile (Zhong, Peters et al. 1999). We extended these previous studies here by generating a germ cell-specific knockout of *Tarbp2* on a pure C57BL/6J background and investigated the molecular mechanism underlying the sterility. Cell-specific ablation of *Tarbp2* resulted in a change in the abundance and isomer type of a subclass of miRNAs in both meiotic and post-meiotic cells. Predicted mRNA targets of the miRNAs were both elevated and reduced, including transcripts encoding the highly basic protamines required for chromatin condensation. mRNAs were recruited for translation however there was a reduction in polyribosomes and the accumulation of detectable PRM1 protein in the cytoplasm, suggesting that TARBP2-dependent miRNAs mediate translation elongation.

Germ cell-specific ablation of *Dicer* using *Stra8* or *Ngn3* promoter-driven cre recombinases results in both meiotic and post meiotic defects (Romero, Meikar et al. 2011, Greenlee, Shiao et al. 2012). As might be expected, mutation of *Dicer* has a stronger effect on spermatid differentiation than ablation of *Tarbp2*. DICER ablation results in the loss of all, or nearly all, miRNAs, and endogenous short interfering (endo-siRNAs), whereas as loss of TARBP2 affects only a sub-class of miRNAs. In $Tarbp2^{fl/fl}$ *; Stra8-icre* mice, spermiogenesis is blocked after initiation of PRM1 expression, whereas in $Dicer^{fl/fl}$ *; Stra8-icre* mice, spermatogenesis halts prior to the onset of protamine synthesis (Greenlee, Shiao et al. 2012).

Several independent studies have reported a role for TARBP2 as a cofactor for the RNAse III enzyme DICER in canonical miRNA biogenesis in cell culture (Chendrimada,

Gregory et al. 2005, Wilson, Tambe et al. 2015). In the testis, TARBP2 is expressed in a cell type-specific manner and overlaps with increased miRNA expression levels (Lee, Fajardo et al. 1996, Ro, Park et al. 2007). Loss of TARBP2 resulted in altered processing of a subset of miRNAs in the same cell populations leading us to conclude that TARBP2 also functions as a co-factor of DICER during male gamete development. The absence of TARBP2 during embryonic development also results in altered processing of a sub-set of miRNAs (Pullagura, Buaas et al. 2018). The miRNAs that are affected in *Tarbp2*$^{-/-}$ embryos differ from those in spermatids, suggesting that other cell-type specific factors control either the expression or selectivity in processing of TARBP2-dependent miRNAs.

miRNAs are essential for post-transcriptional regulation of gene expression in meiotic and haploid cells, either for regulating transcript levels or by regulating translation of spermatid differentiation factors (Yu, Raabe et al. 2005, Dai, Tsai-Morris et al. 2011). Consistent with this we observe a decrease in a sub-set of miRNA transcripts with an up-regulation of their target mRNA transcripts. Most of these mRNA transcripts are known to play a role in several key events including nuclear compaction, chromatin condensation and tail morphogenesis.

The failure to detect full-length PRM1 protein and other spermatid differentiation factors suggests that TARBP2-dependent miRNAs regulate translation. To characterize the effect of the loss of TARBP2 on the translation, we analyzed RNA collected from polysomal fractions. mRNAs for *Tnp1, Tnp2, Prm1 and Prm2* were elevated in monosomal fractions in *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ testes, supporting a role for TARBP2 in translation elongation. This was further supported by the presence of both full-length and shortened transcripts, which suggests that the transcripts sedimenting with the monosomal fractions had undergone

translation initiation, as deadenylation normally accompanies translation in male germ cells(Kleene 1989). Our studies are consistent with a recent report suggesting a role for miRNAs in the movement of transcripts with short 3'UTRs from mRNP fraction to polysomes (Zhang, Tang et al. 2017).

While our study clearly demonstrates changes in mature miRNA levels and up-regulation of their corresponding mRNA targets in *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ mice, the failure to detect full-length protein was unlikely to be a consequence of the cells failing to differentiate to the stage at which the proteins are normally synthesized, as we could detect PRM1 by immunofluorescence in Stage XII elongating spermatids. It is also unlikely to be due to transcript levels, as mRNAs for *Prm1*, *Prm2*, *Tnp1* and *Tnp2* were all elevated in *Tarbp2*$^{\Delta/fl}$*;Stra8icre*+ mice. The ability to detect PRM1 by immunofluorescence could simply be due to the increased sensitivity of immunofluorescence, or it could be due to the failure to synthesize the full-length protein in the mutant. The antibody used recognizes the amino terminus, which would be able to detect newly synthesized PRM1 on sections but not in total protein extracts. The detection of PRM1 in the cytoplasm by immuno-EM also suggests a function for TARBP2-dependent miRNAs in translation elongation. The pioneer round of translation, or an early block in translation elongation, can generate defective ribosome products (DRiPs) that are usually rapidly degraded (Bourdetsky, Schmelzer et al. 2014). The detection of PRM1 in the cytoplasm by immuno-gold staining could be due to the detection of PRM1 DriPS.

*Dicer* ablation in haploid cells using a *Prm1*-driven cre-transgene results in defective translation due to sequestration of *Prm1* mRNA by elevated levels of ARPC5 (Chang, Lee-Chang et al. 2012). Elevation of ARPC5 is due to a down regulation of miR-

22/883-5p that targets *Arpc5*. In our study, we did not observe a deregulation of miR-22/883-5p miRNA. Instead, we observed deregulation of in-silico predicted *Arpc5* targeting miRNAs (miR-9-5p, miR-471-3p, miR-425-3p, miR-466g, miR-677-5p). Differences in these studies could be due to the temporal window of *Dicer* and *Tarbp2* ablation, or to the selectivity of TARBP2 in processing a subclass of miRNAs.

Posttranscriptional control plays a major role in the successful development of male gametes (Schafer, Nayernia et al. 1995, Laiho, Kotaja et al. 2013). In the case of chromatin compaction, the mRNAs encoding *Prm1* and *Prm2* are synthesized in round spermatids and stored as mRNPs for up to 7 days before being recruited for translation in elongating spermatids (Kleene, Distel et al. 1983, Kleene, Distel et al. 1984). Translational repression of *Prm1* is mediated by sequences in its 3'UTR (Fajardo, Haugen et al. 1997) and temporal translational delay is essential for completion of spermatid differentiation (Lee, Haugen et al. 1995). The mechanism of translational repression is not fully understood but requires the Y box proteins YBX2 and YBX3 proteins (Snyder, Soundararajan et al. 2015). Our previous studies of a whole body knockout of *Tarbp2* revealed a role for *Tarbp2* in translational activation of *Prm1* (Zhong, Peters et al. 1999), although the mechanism underlying the defect was not known, in part because miRNAs had not yet been discovered. The results reported here show that TARBP2-miRNAs are required for translation elongation of mRNAs that are subject to temporal translational delay. It has yet to be determined if the mechanism of translational depression is initiated by miRNAs, or if miRNAs are simply required for translation elongation following translational activation.

# CHAPTER 4. SUMMARY AND FUTURE DIRECTIONS

## 4.1 Summary of key findings

The study described in this dissertation sought to identify *in vivo,* the role of TARBP2 during post-transcriptional regulation of gene expression. Using two different C57BL/6J strain mouse models, one with constitutive null allele of *Tarbp2* and another with a germ cell specific knock out of *Tarbp2,* we analyzed miRNA expression levels during embryonic development and spermatogenesis. In my research findings, TARBP2 was shown to regulate the expression of a sub-set of miRNAs at embryonic day 15.5 (E15.5) during murine embryonic development and in post-meiotic cells during spermatogenesis. Based on my findings, I propose a role of TARBP2 in processing unique pre-miRNAs into mature miRNAs.

I observed TARBP2 expression along with DICER at every stage of murine embryonic development. However, I found a discordance in the *Tarbp2*$^{-/-}$ (lethality at E18.5) phenotype compared to the *Dicer* $^{-/-}$ (lethality at E7.5) (Bernstein, Kim et al. 2003) phenotype. Based on this observation, I propose a role of TARBP2 dependent miRNAs in regulating murine embryonic development of multiple organs at stages later than E7.5. Similarly, as determined by using the same germ-cell specific cre, there is a discordance in the effect on spermatid differentiation stage from TARBP2 ablation compared to DICER ablation (Greenlee, Shiao et al. 2012). This observation further strengthens my hypothesis that TARBP2 regulates developmental stage-specific and cell-specific miRNA mediated post-transcriptional regulation.

Germ-cell specific ablation of TARBP2 altered mature miRNA levels coupled with aberrant translation elongation of mRNAs. Along with *Prm1*, these abnormally translated mRNAs, including *Tnp1*, *Tnp2*, and *Prm2*, have multiple target sites for TARBP2-dependent miRNAs in their 3'UTR regions. Together, these observations suggest that TARBP2-dependent miRNAs regulate translation elongation of spermatid differentiation factors during murine spermatogenesis. Furthermore, by generating *Tarbp2*$^{-/-}$ *Prkra*$^{-/-}$ double mutant mice, I uncovered the existence of possible redundant function between TARBP2 and PRKRA, independent of miRNA biogenesis.

## 4.2 Future Directions

### 4.2.1 TARBP2-dependent miRNA biogenesis

*In vitro* studies have shown that TARBP2 binds to dsRNA formed both by perfectly matched GC rich sequences or structures formed by mis-match pairing (Lee, Fajardo et al. 1996, Parker, Maity et al. 2008, Kini and Walton 2009, Gredell, Dittmer et al. 2010, Zhu, Kandasamy et al. 2018). Future work should be focused on identifying features of miRNAs specific to TARBP2 binding *in vivo*. In part, by utilizing *in silico* motif and structure analysis for deregulated miRNAs from *Tarbp2*$^{-/-}$ embryos and spermatids, conserved features among these miRNAs can be evaluated. In addition, miRNA sequencing of *Tarbp2*$^{-/-}$ embryos from stages E7.5 to E18.5, combined with mRNA sequencing will reveal transcriptional networks dependent on TARBP2 mediated post-transcriptional regulation of gene expression during murine embryonic development.

### 4.2.2 miRNA mediated translation regulation of spermatid differentiation factors

miRNA-mediated post-transcriptional regulation has been shown to be characterized by inhibition of translation followed by mRNA degradation. An independent study suggests a role of miRNAs in activating translation of mRNA after they bind to ARE elements of mRNA in human cells (Vasudevan, Tong et al. 2007). In my study, I failed to observe TNP1, TNP2, PRM1 and PRM2 in *Tarbp2* $^{-/-}$ testes. The transcripts encoding these proteins contain multiple TARBP2-dependent miRNA target sites in their 3'-UTR regions. I hypothesized that TARBP2 dependent-miRNAs bind to *Tnp1*, *Tnp2*, *Prm1* and *Prm2* mRNAs in their 3'UTR regions and promote their translation during murine male gametogenesis. *In silico* prediction has provided a list of miRNAs that could target these mRNA with high confidence. *In vitro* experimental analysis can be performed to verify these predictions. One type of experiment involves using miRNA mimic versions of these miRNAs *in vitro*. These miRNA mimics could be expressed in HeLa cells along with a luciferase reporter gene to which a 3'UTR sequence of each *Tnp1*, *Tnp2*, *Prm1* and *Prm2* mRNA is attached. By comparing with suitable controls and analyzing the amount of luciferase reporter protein expressed in the presence and absence of each miRNA mimic, a relation between the miRNA expression and translation of its corresponding putative mRNA target can be evaluated. One caveat for this proposed experiment is that HeLa cells do not express regulatory elements in the same way that germ cells would express those factors. This difference would itself affect the translation profiles of *Tnp1*, *Tnp2*, *Prm1* and *Prm2* along with TARBP2-dependent miRNAs in germ cells. Hence, these findings cannot be translated directly to *in vivo* observations. A complementary approach to address this issue would be to generate a germ cell specific

knock out of each individual TARBP2 dependent miRNA and analyze the effect on translation of each of their corresponding mRNA targets.

One key observation during my research is the detection of amino-terminus of PRM1 in the cytoplasmic region of *Tarbp2*$^{-/-}$ elongating spermatids. This could be due to the release of defective ribosome products as a consequence of a pioneer round of translation or an early block of translation elongation. This observation can be confirmed by a direct approach, immunoEM analysis using an PRM1 antibody raised against its C-terminal epitope. It would be interesting to perform an immunoEM analysis using antibodies against other aberrantly translated mRNAs in *Tarbp2*$^{-/-}$ germ cells, including those encoding PRM2, TNP2 and TNP1. This would help to determine if DriPs due to loss of TARBP2 function are specific to PRM1 or if it is a general phenomenon observed in germ cells. If my observation holds true, a germ cell specific proteomic analysis in *Tarbp2* $^{-/-}$ germ cells, using mass spectrometry, will help to identify the accumulation of DriPs from mRNAs, whose translation elongation is dependent on TARBP2 mediated miRNA biogenesis.

### 4.2.3 miRNA dependent translation elongation in germ cells

In the absence of TARBP2 expression, I observed defective translation elongation of *Tnp1*, *Tnp2*, *Prm1* and *Prm2*. Previously, it was reported that during murine gametogenesis, miRNAs bind to mRNAs that have short 3'UTR regions and control their movement from mRNP onto polysomes as haploid germ cell differentiation unfolds (Zhang, Tang et al. 2017). Interestingly, *Tnp1*, *Tnp2*, *Prm1* and *Prm2* contain shorter 3'UTR regions. Based on this, I hypothesize that miRNA dependent translation elongation, can be unique to mRNAs under temporal translation regulation during haploid germ cell differentiation. As these cells are transcriptionally inactive, there are limited ribosomal proteins available. Hence, there is a

need for controlled use of these proteins. I hypothesize that miRNA mediated translation elongation regulation can happen through controlled recruitment of ribosomal proteins or by stabilization of ribosomes on translating mRNA.

To address this *in vivo*, a ribosomal profiling assay using a RPL22 ribo-tag strategy as described earlier (Sanz, Yang et al. 2009) could be performed to analyze the occupancy of ribosomes in the presence and absence of TARBP2. Considering the embryonic lethality of a constitutive null allele of *Tarbp2,* multiple genetic crosses would have to be made to obtain males with germ cell-specific depletion of *Tarbp2* and expression of ribo-tag. If this strategy is pursued, its success will be dependent on the efficiency of germ cell specific cre-recombinase in recombining *lox-p* sites in two allelic regions corresponding to $Tarbp2^{fl/fl}$ and $Rpl22^{fl/fl}$.

It is proposed that another variable affecting results is the identity of the cells in the system being analyzed. This is based on recent studies which suggest that the cellular or sub-cellular interactome can regulate the occupancy of ribosomal proteins on to translating mRNAs (Simsek, Tiu et al. 2017). Because the ribo-tag strategy was developed to study translation in brain, one has to account for the fact that they will be utilizing this strategy in a different organ system-the testis-which will have a different ribosomal interactome from that found in the brain. In particular, the profile of ribosomal proteins essential for translation of *Tnp1*, *Tnp2*, *Prm1* and *Prm2* can be different from that of RPL22. If this will be the case, in particular, if ribo-tagged RPL22 is not expressed at the same time that ribosomes were translating the mRNA for each of these targets, then it might be difficult to label the translating complex with ribo-tagged RPL22a and determine the ribosome occupancy. In such a scenario, there would be a need to identify the ribosomal proteins required for

translation of these transcripts. One approach would be to analyze the expression of transcripts coding for ribosomal proteins from mRNA-seq data derived from germ cells during murine spermatogenesis, and then perform an RNA immunoprecipitation of these transcripts using antibodies against those proteins. Once a suitable ribosomal protein is identified, then a mouse model would have to be generated using a strategy similar to that used for the ribo-tag mouse model. This would be challenging due to the unavailability of working antibodies against ribosomal proteins, and the subsequent need to generate monoclonal antibodies against the several ribosomal proteins that would be tested.

*In vitro* studies cannot be applied to study translation of these transcripts due to the difference in Ribo-interactome proteins expressed *in vitro* compared to *in vivo*. In addition, the expression of proteins from these transcripts, which would be derived from germ cells, is lethal to somatic cells, which would be used to express those transcripts. The major impediment to study translation in germ cells directly is the lack of proper germ cell culture techniques. Therefore, future work should be focused on developing a strategy to culture germ cells, perhaps in 2D or 3D culture systems.

### 4.2.4 Role of PRKRA *in vivo*

The role of PRKRA in miRNA biogenesis still remains an open question due to a low level of PRKRA expression in the *Prkra*$^{lear1j}$ mouse model (Figure 2.3.C). To address this issue, miRNA expression levels in E15.5 embryos bearing a constitutive null allele of *Prkra* would have to be analyzed and compared to suitable controls including *Tarbp2* $^{-/-}$ embryos. This would reveal the role of PRKRA in miRNA biogenesis and if any, the uniqueness of PRKRA dependent miRNAs compared to TARBP2 dependent ones. These miRNAs should be analyzed in terms of their sequence or structural features.

### 4.2.5 Redundancy between TARBP2 and PRKRA

Several *in vitro* studies have reported that an RNAi independent mechanism of TARBP2 in translation regulation occurs through inhibition of PRKRA mediated activation of PKR (Daher, Laraki et al. 2009). Even though these studies have shown PRKRA as a translation inhibitor, *in vivo* study focused on understanding the role of PRKRA in anterior pituitary lobe development suggests its role in that context as a translation enhancer (Dickerman, White et al. 2015). My research on murine embryonic development suggests possible redundancy between these two proteins-TARBP2 and PRKRA- independent of miRNA biogenesis. However, failure to observe the co-expression of TARBP2 and PRKRA among individual cell populations in tissue systems including heart, brain and testis poses a major challenge for addressing redundancy between these proteins. I observed that loss of TARBP2 in germ cells leads to translation elongation defects. These cells do not express PRKRA. To address redundancy between TARBP2 and PRKRA in regulating translation in germ cells, a *Prkra* transgene under the control of an *Hspa2* promotor (Inselman, Nakamura et al. 2010) can be expressed in *Tarbp2*$^{-/-}$ germ cells and the effect on translation of transcripts derived from *Prm1*, *Prm2* and *Tnp2* can be monitored. Another approach to investigate this redundancy would be to replace the *Tarbp2* allele in its genomic region with the *Prkra* allele using a strategy similar to that described previously (Tvrdik and Capecchi 2006) and investigate the effects on miRNA biogenesis, mRNA stability and translation regulation during spermatogenesis.

### 4.2.6 RNAi independent role of TARBP2

My research did not rule out the possibility of RNAi-independent mechanisms for TARBP2 in post-transcriptional regulation of gene expression. An independent study using breast cancer cell lines showed that TARBP2 directly binds to secondary structures of mRNA and helps in the degradation of these mRNAs (Goodarzi, Zhang et al. 2014). Future work should be focused on investigating the existence of such direct interactions during murine germ cell development. Cross-linking immunoprecipitation (CLIP) of TARBP2-bound RNA sequences combined with high through put sequencing, would help to identify the exact sequences preferred by TARBP2. Sequence analysis combined with transcriptome wide mapping would reveal if TARBP2 can bind directly to mRNA sequences or if TARBP2 mediated post-transcriptional regulation is exclusively mediated through miRNAs *in vivo*. This study would also provide information regarding sequence or structure specificity for TARBP2 binding. A major challenge in this approach would be to standardize a CLIP-seq protocol suitable for germ cells, which express high levels of RNA and dsRBPs. So far, CLIP-Seq has been standardized for brain tissue or neuronal cells and for human cell lines, but not for germ cells.

### 4.2.7 Strain dependency of TARBP2 related phenotype

As discussed earlier, constitutive null allele of *Tarbp2* on C57BL/6J was embryonic lethal whereas on a B6129S4 background, mice were able to survive up to adulthood. Similarly, germ cell specific loss of TARBP2 leads to exhibition of severe phenotype in C57BL/6J compared to a milder germ cell related phenotype in the B6129S4 strain. My experiment to identify genetic modifiers on mixed background revealed the possible existence of multiple modifiers dispersed all over the genome (unpublished data). This raises

a possibility that these modifiers could exist in the genomic region coding for miRNAs. Using computational approaches, a thorough investigation to identify these loci and careful characterizing, including their SNPs, will provide useful insight into the role of strain dependent SNPs in miRNA expression and efficiency. Further investigation using diversity outbred mice to identify SNPs in miRNA coding regions and in genes encoding miRNA biogenesis factors across several strains of house mice would provide a possible model to study miRNA-mediated post-transcriptional regulation and possibly a model to evaluate RNAi therapeutics for pre-clinical studies.

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