**UNIVERSITY OF TURKU**

# UNCOVERING THE ROLE OF DICER AND PERICENTRIC HETEROCHROMATIN EXPRESSION DURING SPERMATOGENESIS

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*To my family and mentors*

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## ABSTRACT

Male germ cell differentiation is a complex process that includes mitotic proliferation, meiosis and haploid differentiation phase, during which the cell undergoes dramatic morphological changes to produce mature spermatozoa. Male germ cells have unusually diverse transcriptomes that include a broad variety of protein-coding mRNAs and their isoforms, but also a considerable number of non-coding RNAs (ncRNAs). This research focuses on a specific type of ncRNAs, major satellite repeat (MSR) transcripts that originate from the pericentric heterochromatin regions of the chromosomes and their interplay with the endonuclease DICER during mouse spermatogenesis. Using a germ cell-specific *Dicer1* knockout mouse model, I showed that DICER is essential for spermatogenesis and male fertility. The deletion of *Dicer1* mainly affected haploid male germ cell differentiation and resulted in defective chromatin condensation and nuclear shaping of spermatids, leading to severe oligoasthenoteratozoospermia and infertility. I revealed that MSRs are transcribed during normal spermatogenesis, particularly in meiotic spermatocytes. Interestingly, MSR transcripts were shown to be aberrantly induced in DICER-null spermatocytes. Only those transcripts that originate from the forward strand of MSR DNA were misregulated in the absence of DICER, suggesting a strand-specific function for DICER *in vivo*. The forward MSR transcripts and DICER were localized to both the cytoplasm and the nucleus, where nuclear DICER was specifically associated with pericentric heterochromatin regions of chromosomes. Moreover, I showed that MSR transcripts are found in complexes with DICER in the testis, and their processing into small RNAs is compromised in *Dicer1* knockout mice leading to an elevated level of forward MSR transcripts in meiotic cells. I also provided evidence of epigenetic imbalance of the pericentric heterochromatin and meiotic chromosome missegregation in *Dicer1* knockout testes. These results clearly illustrate that DICER is essential for male fertility, and it contributes to the regulation of pericentric heterochromatin during spermatogenesis by direct targeting MSR transcripts. Furthermore, these studies strongly suggest that the expression of pericentric heterochromatin may have a functional role in the regulation of male germ cell differentiation and fertility.

KEYWORDS: germ cells, spermatogenesis, meiosis, DICER, pericentric heterochromatin, major satellite repeats, chromosome segregation.

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TIIVISTELMÄ

Spermatogeneesissä sukusolut jakautuvat meioottisesti ja erilaistuvat haploideiksi hedelmöityskykyisiksi siittiöiksi. Tämän prosessin aikana ne ilmentävät genomiaan laajasti, ja proteiineja koodaavien RNA:iden lisäksi niiden transkriptomi koostuu myös huomattavasta määrästä ei-koodaavia RNA:ita. Tässä tutkimuksessa keskityttiin tiettyihin ei-koodaaviin RNA-molekyyleihin, jotka ovat peräisin kromosomien perisentromeerisella heterokromatiinialueella sijaitsevista satelliittitoistojaksoista (major satellite repeats, MSR). Erityisesti tutkimuksessa selvitettiin DICER-endonukleaasin toimintaa MSR-RNA:iden säätelyssä hiiren spermatogeneesin aikana. Käyttämällä poistogeenistä *Dicer1*-hiirimallia osoitin, että sukusolujen DICER-proteiinilla on keskeinen merkitys hedelmällisyyden ylläpidossa, sillä *Dicer1*-geenin poistaminen esti normaalin siittiötuotannon. Varsinkin haploidi erilaistumisvaihe häiriintyi, mikä johti rakenteeltaan epänormaalien siittiöiden muodostukseen. Tutkimukseni paljasti, että MSR-RNA:ita tuotetaan spermatogeneesin aikana etenkin meioottisissa sukusoluissa, ja että *Dicer1*-poistogeenisen hiiren sukusoluissa MSR-RNA:iden määrä oli huomattavasti kohonnut. Tulosteni mukaan MSR-RNA:t ja DICER sijaitsivat sekä solulimassa että tumassa, ja näytin DICER-proteiinin sitoutuvan perisentromeerisille heterokromatiinialueelle. Lisäksi osoitin, että kiveksessä DICER ja MSR-RNA-molekyylit löytyvät samoista komplekseista, ja että *Dicer1*-poistogeenisessä hiiressä MSR-RNA:iden prosessointi oli häiriintynyt. Tämä viittaa siihen, että DICER säätelee MSR-RNA-molekyylejä osallistumalla niiden prosessointiin. Lopuksi osoitin, että *Dicer1*-geenin poistaminen sai aikaan muutoksia sukusolujen heterokromatiinin epigeneettisessä tilassa. Lisäksi *Dicer1*-geenin poistaminen oli yhteydessä meioottisten jakautumisten häiriöihin ja aneuploidiaan, mikä mahdollisesti johtuu häiriöistä perisentromeerisen heterokromatiinin toiminnassa. Kaiken kaikkiaan tutkimukseni tulokset osoittavat, että DICER-proteiinilla on keskeinen rooli miesten sukusolujen erilaistumisessa ja MSR-RNA:iden säätelyssä. Tulokset myös viittaavat siihen, että perisentromeerinen heterokromatiini on toiminnallisesti tärkeää normaalin siittiötuotannon ja hedelmällisyyden kannalta.

AVAINSANAT: sukusolut, spermatogeneesi, meioosi, DICER, perisentromeerinen heterokromatiini, tärkeimmät satelliittitoistot, kromosomien erottelu.

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# Abbreviations

| | |
|---|---|
| ADP | adenosine diphosphate |
| AGO | argonaute protein |
| ATP | adenosine triphosphate |
| BSA | bovine serum albumin |
| CB | chromatoid body |
| cDNA | complementary DNA |
| ChIP | chromatin immunoprecipitation |
| cKO | conditional knockout |
| CTRL | control |
| DAPI | 4',6-Diamidino-2-Phenylindole |
| DIG | digoxigenin |
| DNA | deoxyribonucleic acid |
| dpp | days post-partum |
| dsRNA | double stranded RNA |
| EDTA | ethylenediaminetetraacetic acid |
| Endo-siRNA | endogenous small interfering RNA |
| ES | elongating spermatids |
| EtBr | ethidium bromide |
| FSH | follicle-stimulating hormone |
| HP1 | heterochromatin protein 1 |
| HT buffer | hepes-triethanolamine buffer |
| IAP | intracisternal A particle |
| IMC | intermitochondrial cement |
| KDa | kilodaltons |
| LH | luteinizing hormone |
| LINE | long interspersed nuclear elements |
| LNA | locked nucleic acid |
| MEF | mouse embryonic fibroblast |
| miRNA | microRNA |
| mM | millimolar |
| mRNA | messenger RNA |

| | |
|---|---|
| MSR-tr | major satellite repeat transcripts |
| ncRNA | non-coding RNA |
| NLS | nuclear localization signal |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PFA | paraformaldehyde |
| PGC | primordial germ cell |
| PIC | protease inhibitor cocktail |
| piRNA | PIWI-interacting RNA |
| PMSF | phenylmethanesulfonyl fluoride |
| Pspc | pachytene spermatocytes |
| qPCR | real-time quantitative-PCR |
| RISC | RNA-induced silencing complex |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| RNAPol | RNA polymerase |
| RNase | ribonuclease |
| RNP | ribonucleoprotein |
| rpm | revolutions per minute |
| RS | round spermatids |
| RT | room temperature |
| RT-PCR | reverse-transcription PCR |
| SDS | sodium dodecyl sulfate |
| SETDB1 | SET domain bifurcated histone lysine methyltransferase 1 |
| Sg | spermatogonia |
| SINE | short interspersed nuclear element |
| siRNA | small interfering RNA |
| Spc | spermatocyte |
| SSC | spermatogonial stem cell |
| SSC | saline-sodium citrate |
| SUV39H2 | suppressor of variegation 3-9 homolog 2 |
| TBE | tris-borate-EDTA |
| TE buffer | tris-EDTA buffer |
| UTR | untranslated region |
| UV | ultra-violet |
| YFP | yellow fluorescent protein |
| μl | microliter |
| μM | micromolar |
| μm | micrometer |

# List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I      Korhonen HM, Meikar O*, **Yadav RP***, Papaioannou MD, Romero Y, Da Ros M, Herrera PL, Toppari J, Nef S, Kotaja N. Dicer is required for haploid male germ cell differentiation in mice. *PloS one.* (2011), 6(9): e24821. https://doi.org/10.1371/journal.pone.0024821 (* equal contribution).

II      **Yadav RP**, Mäkelä JA, Hyssälä H, Cisneros-Montalvo S, Kotaja N. DICER regulates the expression of major satellite repeat transcripts and meiotic chromosome segregation during spermatogenesis. *Nucleic Acids Res.* (2020), 48(13): 7135-7153. https://doi.org/10.1093/nar/gkaa460

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# 1      Introduction

Reproductive disorders are getting increasingly common among human populations, and infertility has been recognized as a worldwide public health issue by the World Health Organization (WHO) (Barazani et al., 2014; Ilacqua et al., 2018). Populations of industrialized countries have experienced a decline in total fertility rates (average number of live births per woman) all over the world (Skakkebaek et al., 2015). According to Statistics Finland's data on population changes, this is also true in Finland, and the total fertility rate has gradually declined from 1.87 to 1.35 during 2010-2019 (Statistics Finland, 2020). The total fertility rate is affected by several factors, including social, economic, and psychological factors, but it is clear that biological factors that influence fecundity also contribute to the lowered total fertility rates. People in Europe and other developed countries are facing alarming challenges due to adverse trends in male reproductive health, which includes reduced semen quality, prevalence of testicular cancers and congenital reproductive malformations (De Jonge and Barratt, 2019; Skakkebaek, 2016). Furthermore, a growing demand for the use of assisted reproductive technologies (ART) has been reported (Ravitsky and Kimmins, 2019), which may reflect the deterioration of male reproductive health. Very often, male subfertility or infertility is because of faults in spermatogenesis, which emphasizes the necessity of in-depth knowledge on basic molecular mechanisms of testis function to be able to better diagnose male infertility. This study contributes to our understanding of the factors that are required for the production of fertile spermatozoa by clarifying the roles of DICER and pericentric heterochromatin expression in differentiating male germ cells. It has become clear that pericentric heterochromatin is transcriptionally active, and it produces major satellite repeat (MSR) transcripts which are required for normal cell physiology. The critical role of MSR transcription has been demonstrated in the early mouse development and differentiation (Probst et al., 2010; Casanova et al., 2013a; Probst and Almouzni, 2008). Importantly, pericentric heterochromatin has been reported to be aberrantly expressed in many human cancers (Ting et al., 2011; Zhu et al., 2011; Slee et al., 2011). Therefore, MSR expression is actively involved not only in development and cell differentiation, but also in cancer progression. DICER is an endonuclease that has a well-characterized function in the processing of small non-

coding RNAs (ncRNAs), such as microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Yadav and Kotaja, 2014a; Kotaja, 2014a). The aim of this study was to clarify the importance of DICER for male fertility in mice, as well as to explore its previously uncharacterized role in the regulation of pericentric heterochromatin expression during spermatogenesis.

# 2 Review of the Literature

## 2.1 Male germ cell differentiation

Male germ cell differentiation, spermatogenesis, is a complex developmental program that produces specialized mature spermatozoa with an ability for independent movement and fertilization of an egg (Oliveira and Alves, 2015; de Rooij, 2017). Germ cells are special in their capability to generate new organisms, and extra caution must be taken to ensure the precise inheritance of genetic and epigenetic information. Male germ cell differentiation is a tightly controlled and timely regulated process that requires several strategies and mechanisms completely unique to these cells (**Figure 1**).
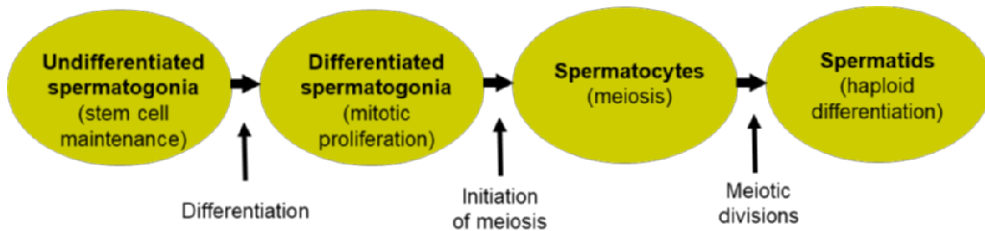


**Figure 1**: Schematic representation of spermatogenesis.

Undifferentiated spermatogonial stem cells (SSCs) undergo self-renewal to maintain spermatogenesis throughout sexual maturity. When SSCs enter the differentiation pathway, they become spermatogonia. Spermatogonia undergo mitotic proliferation phase including stepwise differentiation from type A spermatogonia and intermediate spermatogonia to type B spermatogonia that finally transform to early spermatocytes to start the meiotic phase. Spermatocytes undergo meiotic divisions to produce haploid spermatid that are subsequently differentiatiated to mature spermatozoa during the haploid differentiation phase (Russell et al., 1993; de Rooij, 2017; Hess and de Franca, 2009) (**Figure 1**). This phase includes acrosome and flagellum formation, nuclear reshaping and chromatin compaction (Gaucher et al., 2009). Spermatozoa are released to the lumen of seminiferous tubules and are transported through a complex network of canals in the rete testis to the epididymis.

During epididymal transit, spermatozoa undergo the final maturation and are then temporarily stored in the cauda epididymis before ejaculation (SCIALLI, 1992). All these differentiation steps are governed by strict control of gene expression, and unique regulatory policies are applied to control chromatin organization as well as transcriptional and posttranscriptional events during spermatogenesis (Bettegowda and Wilkinson, 2010).

## 2.1.1 Cellular organization of seminiferous tubule in the mouse testis

Spermatogenesis takes place inside the seminiferous tubules of the testis, which are connected to the epididymis via the rete testis (Russell et al., 1993). Spermatogenic cells are organised in the seminiferous epithelium so that the least differentiated cells are close to the basal lamina, and cells move toward the lumen of the seminiferous tubule as they differentiate (**Figure 2**). Mitotically proliferating spermatogonia form the first layer of cells. Meiotic spermatocytes are found in the second layer, followed by the layers containing haploid round spermatids and elongating spermatids (Russell et al., 1993; de Rooij, 2017). Finally, mature spermatoza are released into the lumen of seminiferous tubules (**Figure 1 & 2**).
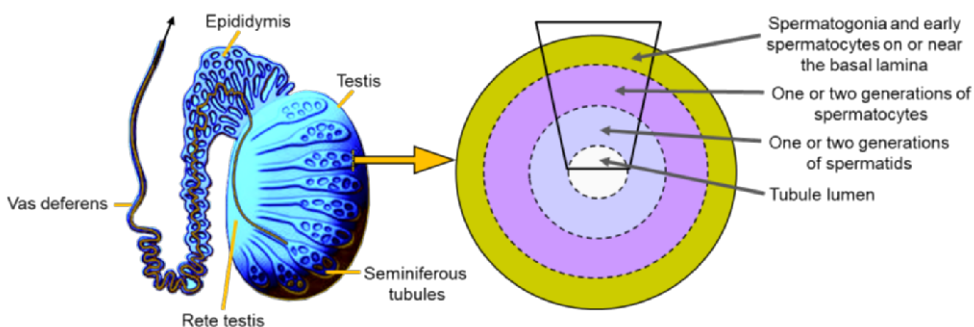


**Figure 2:** Schematic drawings of the mouse testis that consists of long, convoluted seminiferous tubules packed inside a testicular capsule (left). The graphical representation on the right shows a cross section of a seminiferous tubule with layers of differentiating germ cells presented by distinct colors.

Mouse testis contains both somatic cells and germ cells and their cooperation is needed for the production of fertile spermatozoa (Russell et al., 1993; Griswold, 1995) (**Figure 3**). Somatic cells of the testis include Sertoli cells inside the seminiferous tubules and testosterone producing Leydig cells located in the interstitial spaces between the tubules. Sertoli cells reside in the seminiferous epithelium intermingled with male germ cells. Their nuclei are located near the basal

lamina, and their cytoplasmic protrusions expand towards the tubule lumen, embedding differentiating germ cells in the cytoplasmic pockets. The Leydig cells are irregularly shaped, contain fat droplets, pigment granules, and are surrounded by numerous blood vessels, lymphatic vessels, and nerve fibers. In the walls of the seminiferous tubules, there are also other cell types such as peritubular myoid cells and macrophages.
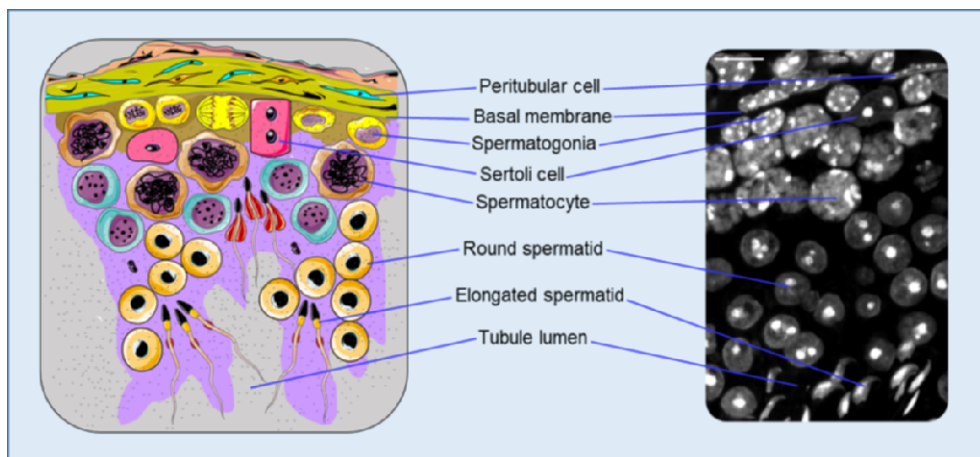


**Figure 3**: Schematic drawings of the mouse seminiferous epithelium (left) showing the organization of different types of cells inside the epithelium. The right panel shows the corresponding cells in a DAPI-stained mouse testis section, scale bar 10 μm.

Sertoli cells act as supporting nurse cells or mother cells for differentiating germ cells (Oliveira and Alves, 2015). Sertoli cells and germ cells are connected to each other via specific cell-cell junctions, and these close contacts are important for the maintenance of spermatogenesis (Griswold, 1995). Besides the mechanical support, Sertoli cells monitor the entry and the exit of nutrients, hormones, and other chemicals. Sertoli cells ingest harmful foreign particles and dead cells by the process of phagocytosis. Spermatogonial stem cell niche is also established and maintained by Sertoli cells, which is critical to ensure the renewal of stem cells (de Rooij, 2017). Furthermore, the conformational changes in the Sertoli cell junctions supports the movement of differentiating germ cells from the basal to the luminal part of the seminiferous tubules, where spermatozoa are finally released from the epithelium in a process known as spermiation (Griswold, 1995). Sertoli cells are regulated by follicle-stimulating hormone (FSH) secreted from the anterior pituitary (O'Donnell et al., 2006). Leydig cells in the intratubular spaces produce testosterone in response to another pituitary hormone, luteinizing hormone (LH) (Oliveira and Alves, 2015; O'Donnell et al., 2006). Testosterone also has an important role in the regulation of Sertoli cell function. The support provided by Sertoli cells enables a specific

organization of spermatogenic cell in the seminiferous epithelium so that a given cross section of the tubule always contains a specific association of different types of germ cells (**Figure 4**) (Russell et al., 1993).
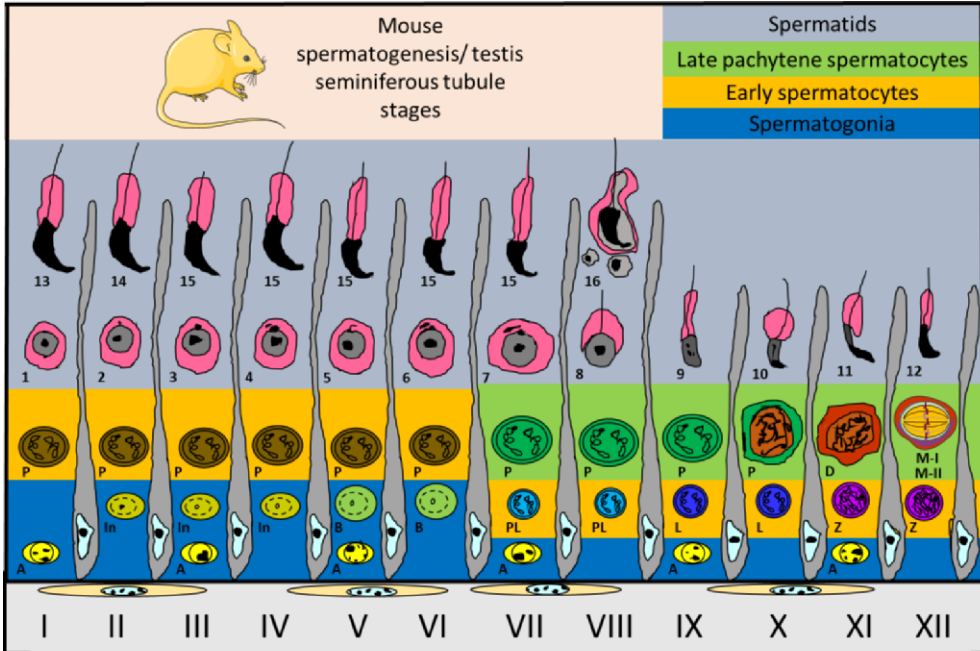


**Figure 4**:  Organization of spermatogenesis inside mouse seminiferous tubules. The stages I-XII of the seminiferous epithelial cycle in the mouse. The least differentiated spermatogonia (type A, Intermediate and type B) forms the bottom layer, followed by preleptotene (PL), leptotene (L), zygotene (Z), pachytene (P) and diplotene (D) spermatocytes. Meiotic divisions (M-I and M-II) are found at stage XII. Steps 1-16 of spermatid differentiation are indicated with roman numerals.

These cell associations are called the stages of the seminiferous epithelial cycle (stages I-XII in mice). Each stage is regulated differentially, and the stages progress in an ordered manner along the seminiferous tubule (Russell et al., 1993; Hess and de Franca, 2009) (**Figure 4**). Each process during spermatogenesis takes place at a specific stage. For example, the meiosis begins when type B spermatogonia differentiate into preleptotene spermatocytes at stage VII. Leptotene and zygotene phases of the first meiotic prophase are found at stages IX-X and XI-XII, respectively, and the following pachytene phase is very long, spanning almost all stages. Two meiotic divisions occur at stage XII and result in the formation of haploid spermatids at stage I, which are further classified into 16 steps based on their morphological transformations. Within 16 steps, the first 8 steps and the last 8 steps represent round spermatid and elongating spermatid differentiation phases,

respectively. During spermiation, step 16 spermatids are released into the lumen of seminiferous tubules at stage VIII as mature spermatozoa.

## 2.1.2 Meiotic and post-meiotic events during mouse spermatogenesis

Spermatogenesis takes 35 days in the mouse (**Figure 5**), and the majority of this time is dedicated to meiosis and post-meiotic processes (Russell et al., 1993; Hess and de Franca, 2009). Spermatogenesis is initiated very soon after birth, and the progress of spermatogenesis can be followed during the first wave of spermatogenesis with specific cell types appearing at specific time points.
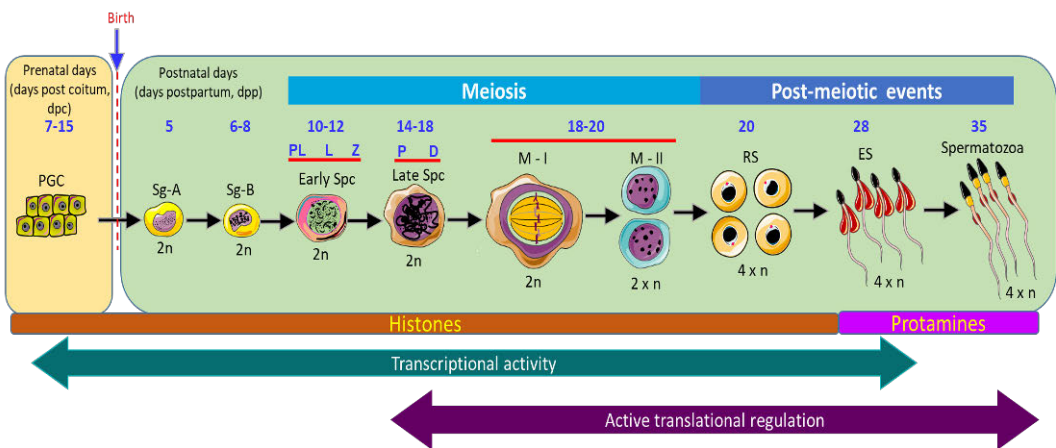


**Figure 5**: Progress of mouse spermatogenesis. The approximate timing when specific cell types appear in juvenile mice during the first wave of spermatogenesis is indicated as days post-partum (dpp). The prenatal testis contains primordial germ cells (PGC), that start differentiation soon after birth by the mitotic proliferation of spermatogonia (Sg). The different phases of the prophase of meiosis I are indicated: PL, preleptotene; L, leptotene; Z, zygotene; P, pachytene; D, diplotene. Haploid round spermatids (RS) then further differentiate to elongating spermatids (ES) and mature spermatozoa.

In the juvenile testis, early meiotic spermatocytes appear at 10 dpp (days post-partum) and late pachytene spermatocytes are found in the testis at 18 dpp. Followed by two meiotic divisions, early round spermatids appear at 20 dpp and the elongation of spermatids is in progress at 28 dpp. Finally, spermatogenesis is completed at 35 dpp and mice are sexually mature (O'Donnell et al., 2011; Nishimura and L'Hernault, 2017; Russell et al., 1993) (**Figure 5**).

Meiosis I is also known as a reductional division where a diploid set of chromosomes is reduced to a haploid set (Griswold and Hunt, 2013). It includes four main stages: prophase I, metaphase I, anaphase I and telophase I. Prophase I of meiosis is the longest phase, which consists of distinctive sub-stages that are called

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preleptotene, leptotene, zygotene, pachytene, diplotene and diakinesis. During preleptotene to leptotene transition, meiotic cells undergo replication of the chromosome. Synapsis between homologous chromosomes starts at the zygotene stage by the formation of the synaptonemal complex (Yanowitz, 2010). The crossing over between non-sister chromatids occurs at the pachytene stage. When synaptonemal complexes disintegrate at the diplotene stage, homologous chromosomes remain attached to the chiasmata. The disintegration of the nuclear membrane and chromosome condensation marks the end of diakinesis and the beginning of metaphase I (Kohl and Sekelsky, 2013). In metaphase I, spindle fibers from opposing centrosomes connect to homologous pairs of chromosomes and align them on the equatorial plane in the middle of the cell. The contraction of spindle fibers starts at anaphase I and homologous chromosomes move to opposite poles of the cell. The chromosome decondensation starts and the nuclear membrane reappears that marks the end of telophase I, followed by cytokinesis to form two haploid daughter cells, known as secondary spermatocytes. Meiosis II occurs very fast after the reductional division, and it is analogous to the mitotic division including prophase II, metaphase II, anaphase II and telophase II followed by cytokinesis (Russell et al., 1993; Hess and de Franca, 2009; Oliveira and Alves, 2015). The second meiotic division is equational where one haploid cell is used to produce two haploids round spermatids (**Figure 5**).

Post-meiotic differentiation process includes several steps to acquire highly specialized structures and morphological features of mature spermatozoa (Russell et al., 1993). During haploid differentiation, a flagellum and an acrosome are constructed. The acrosome is a vesicular structure located on the anterior part of the sperm head, and it has a critical function in augmenting fertilization (Kierszenbaum and Tres, 2004; Lehti and Sironen, 2016). The acrosome formation begins immediately after meiosis in step 1 of the round spermatids. The construction of the flagellum begins in early haploid cells with the development of the axoneme, and continues during the later steps by the assembly of flagellar secondary structures (O'Donnell, 2014; Lie et al., 2010; Pleuger et al., 2020). During the transition from round to elongating spermatids, the nucleus first polarizes to one side of the cell. Then, the nucleus changes its shape with the help of a special microtubular structure called the manchette (Lehti and Sironen, 2016), and finally, the nuclear size is dramatically reduced when the chromatin is tightly compacted. The chromatin compaction is achieved by the process of histone-protamine transition, during which the majority of histones are replaced first by transition proteins and subsequently by protamines (Govin et al., 2004; Kimmins and Sassone-Corsi, 2005a). Because of the chromatin packing, transcription ceases in elongating spermatids, and mature sperms are largely transcriptionally inactive (Bettegowda and Wilkinson, 2010; Meikar et al., 2011; Tanaka and Baba, 2005).

## 2.1.3 Control of gene expression

The complex gene expression programs are triggered during development and differentiation processes or to adapt to new environmental conditions (López-Maury et al., 2008; De Nadal et al., 2011). The basic steps of protein-coding gene expression includes transcription, post-transcriptional RNA processing and translation. All the steps are strictly regulated to generate functional proteins. Genes are transcribed in the nucleus to produce messenger RNAs (mRNA). Eukaryotic cells have three main RNA polymerases (RNAPol I, II, and III), and are involved in the transcription of specific types of genes (Archambault and Friesen, 1993; Cramer et al., 2008). The gene encoding ribosomal RNA, transfer RNA and various small RNAs is transcribed by RNAPol I or RNAPoI III, whereas RNAPoI II represents the general RNA polymerase to transcribe mRNAs (Krishnamurthy and Hampsey, 2009). In addition to RNA polymerase, transcription requires both general and tissue- or cell-specific transcription factors along with different co-regulators that bind to the promoter region to form a transcription initiation complex (Shandilya and Roberts, 2012; Krishnamurthy and Hampsey, 2009). Transcribed pre-mRNA undergoes extensive post-transcriptional processing (see below) that is important for stabilizing mRNAs and determining their fate, and it also increases the efficiency of protein synthesis by allowing only mature mRNA for translation. After transcription and processing, mRNA is transported to the cytoplasm and translated by ribosomes.

The comparative genome analysis shows that humans have more or less a similar number of protein coding genes as compared to phenotypically very different lower organisms (Pertea and Salzberg, 2010). Therefore, the higher complexity of humans compared to lower organisms cannot be explained by the number of protein-coding genes. One explanation for increasing complexity in humans lies in the regulation of their mRNAs at multiple levels, such as production of different variants of a mRNA by alternative splicing, and modifications of mRNAs before translation by RNA editing. It is also known that complex organisms like humans have increased the proportion of non-protein coding DNA in the genome as compared to other organisms (Taft et al., 2007). From an evolutionary perspective, the large percentage of non-coding DNA should have a functional value (Kondrashov, 2005; Taft et al., 2007; Raffaele and Kamoun, 2012), otherwise it would be eliminated from the genome during the course of evolutionary processes. Interestingly, the non-coding genome is also transcribed to produce non-coding RNAs that control the gene expression either at the transcriptional level as components of chromatin remodelling complexes or post-transcriptionally (Rinn and Chang, 2012; Guttman and Rinn, 2012). The large number of non-coding RNAs that modulate the gene expression programs have also been suggested to support the complexity of higher organisms (Mattick and Makunin, 2006; Taft et al., 2010; Soumillon et al., 2013; Kaessmann, 2010; Ashe et al., 2012; Pang et al., 2006; Taft et al., 2007; Jablonka and Raz, 2009; Knoop, 2011; Gommans et al., 2009).

## 2.1.3.1   Epigenetic regulation of gene expression

Epigenetic regulation is defined as heritable changes in gene expression without alterations in the DNA sequence (Bernstein et al., 2007; Jaenisch and Bird, 2003). The epigenetic mechanisms include DNA methylation, covalent modifications of histones and action of ncRNAs. These mechanisms change the chromatin structure that affect the availability of gene regulatory regions and, therefore, the level of transcription (Bernstein et al., 2007; Jaenisch and Bird, 2003). Gene expression patterns are largely governed by these epigenetic modifications, thus uncovering the role of epigenetic processes is crucial to understand cellular functions. Furthermore, the epigenome is sensitive to environmental stimuli and provides the mechanisms to respond to changing environment (Boyce and Kobor, 2015; Sakurada, 2010; Jaenisch and Bird, 2003; Turner, 2009; Pimpinelli and Piacentini, 2020).

DNA methylation is the process of transferring a methyl group to the 5th position of cytosine in DNA. DNA methylation is executed by DNA methyltransferases (DNMTs) DNMT1, DNMT3A and DNMT3B which are responsible for the establishment and maintenance of DNA methylation patterns (Smith and Meissner, 2013). DNA methylation is associated with the silenced status of gene expression, for example, heterochromatin areas are heavily methylated (Nan et al., 1996; Saksouk et al., 2014). The specific amino acids at the N-terminal tails of histones undergo a broad range of modifications such as lysine acetylation, lysine or arginine methylation, serine or threonine phosphorylation, lysine ubiquitination, lysine sumoylation and ADP ribosylation of glutamic acid (Minard et al., 2009; Bannister and Kouzarides, 2011). These modifications can either repress or activate gene expression depending upon gene and physiological context (Bernstein et al., 2007; Margueron et al., 2005). For example, acetylation of specific lysines mostly correlates with transcriptional activity and chromatin accessibility (Hebbes et al., 1988), whereas methylation of the same residue may correlate with the repressive state of chromatin (Lyons and Lomvardas, 2014; Schotta et al., 2004). Higher order chromatin structure can be modulated by several ATP-dependent chromatin remodelling proteins, such as proteins belonging to the ISWI (Imitation Switch), CHD (Chromodomain-Helicase-DNA binding) and INO80 (Inositol Requiring 80)/SWR (SWI2/SNF2 Related) families (Lusser and Kadonaga, 2003). In addition, long non-coding RNAs are more recently discovered players in epigenetic gene regulation (Xu et al., 2019; Sun et al., 2018; Hekimoglu and Ringrose, 2009). They can establish heritable chromatin states and install cell type-specific gene expression patterns by acting as molecular scaffolds for binding and recruiting various epigenetic regulators to the chromatin (Schaukowitch and Kim, 2014; Saxena and Carninci, 2011; Holoch and Moazed, 2015; Magistri et al., 2012; Morris, 2009; Hekimoglu and Ringrose, 2009; Wang et al., 2018; Nishikawa and Kinjo, 2017).

During male germ cell differentiation, germ cells undergo extensive chromatin alterations through various epigenetic transitions (Rousseaux et al., 2011; Kimmins and Sassone-Corsi, 2005a). There is a massive reprogramming of epigenetic marks in primordial germ cells (PGCs), when DNA methylation and other epigenetic modifications are removed to allow the transition from the somatic to the germ cell-specific gene expression program and the establishment of novel male-specific marks, including male-specific imprinting of specific genes (Kota and Feil, 2010a). Epigenetic remodeling is also active during postnatal spermatogenesis, as exemplified by recombination events and meiotic sex chromosome inactivation (MSCI) during meiosis and the tight compaction of the haploid genome during late spermatogenesis by sperm-specific protamines (Kota and Feil, 2010b; Gaucher et al., 2010; Kelly and Aramayo, 2007; Kimmins and Sassone-Corsi, 2005b). Multiple number of chromatin modifying enzymes are temporally regulated during male germ cell differentiation and required for the proper epigenetic transitions and development of a male gamete (Godmann et al., 2009).

### 2.1.3.2    Post-transcriptional regulation of gene expression

After the gene transcription, a series of conserved processing steps are needed to produce mature mRNA. RNA processing steps involve addition of 7-methylguanosine cap at the 5′-end (Ramanathan et al., 2016) and poly A tail to the 3′-end of newly synthesized transcripts in the nucleus (Neve et al., 2017), as well as splicing of introns (Wang and Burge, 2008; Black, 2003) and modifications of RNA by the RNA editing machinery (Pachter, 2012; Gray, 2012; Gott and Emeson, 2000). Along with these processing events, nascent mRNAs are bound by a variety of RNA binding proteins (Keene, 2007; Day and Tuite, 1998; Corbett, 2018). The mRNA-ribonucleoprotein (mRNP) complexes are exported to the cytoplasm via the nuclear pore complex (Natalizio and Wente, 2013). Then, mRNA is targeted to different destinations in the cytoplasm such as ribosomes for translation or cytoplasmic bodies for temporary storage or decay (Buxbaum et al., 2015; Xing and Bassell, 2013; Schoenberg and Maquat, 2012; Garneau et al., 2007). Post-transcriptional regulation of gene expression also includes the action of small non-coding RNAs such as microRNA (miRNAs) which are processed by DICER and act together with argonaute proteins in RNA-induced silencing complex (RISC) to silence gene expression (see below) (Jaskiewicz and Filipowicz, 2008). According to the requirement of a cell, all post-transcriptional processes are regulated to modulate gene expression and support diverse cellular functions.

The fates of RNAs are controlled, particularly by RNA-binding proteins (Paronetto and Sette, 2010; Idler and Yan, 2012). RNA-binding proteins bind their target mRNAs either with their unique motif or via nonspecific interactions. RNA-

binding proteins are known to play important regulatory roles in spermatogenesis, and a variety of testis-specific RNA-binding proteins have been identified particularly in meiotic and post-meiotic cells (Paronetto and Sette, 2010; Idler and Yan, 2012). RNAs and RNA-binding proteins form ribonucleoprotein (RNP) complexes that may further aggregate into larger granules, as exemplified by processing bodies (P-bodies, PBs or GW/P bodies) found in the cytoplasm of various organisms including vertebrate, yeast, plants and trypanosomes. The functions of P-bodies have been associated with many different processes such as mRNA decay, storage, transport and miRNA-mediated pathways (Kulkarni et al., 2010).

Germ cells are characterized by specific cytoplasmic RNP granules that are also known as germ granules. Germ granules are present in the germline of many different organisms, and they share many germline-specific protein components that are essential for RNA regulation (Meikar et al., 2011; Kotaja and Sassone-Corsi, 2007; Chuma et al., 2009). Germ granules provide a means to compartmentalize post-transcriptional regulation and RNA control during spermatogenesis (Meikar et al., 2011, 2013). The most distinguished germ granules are the intermitochondrial cement (IMC) in meiotic cells and the chromatoid body (CB) in haploid round spermatids. The molecular components of germ granules are mainly RNA-binding proteins, RNA helicases, Tudor domain-containing proteins, and other proteins involved in RNA processing. The CB is the largest known cytoplasmic RNP granule, and its appearance right after meiosis suggests that it acts as a molecular switch between the meiotic and post-meiotic phases of differentiation (Kotaja and Sassone-Corsi, 2007; Lehtiniemi and Kotaja, 2018). The characterization of its molecular composition revealed its central role in the PIWI-interacting RNA (piRNA) pathway (Meikar et al., 2011, 2014). The appearance of IMC in pachytene spermatocyte precedes the appearance of the CB, and the IMC is also implicated in the piRNA pathway. The current hypothesis is that the IMC serves as a platform for the processing of piRNAs, and the RNAs are then targeted to the CB for piRNA-mediated degradation of other functions (Lehtiniemi and Kotaja, 2018).

Post-transcriptional regulation of gene expression has an important role during the late steps of male germ cell differentiation. This is because the chromatin compaction in elongating spermatids induces transcriptional silencing (Kimmins and Sassone-Corsi, 2005a; Kimmins et al., 2004). Therefore, genes responsible for the production of spermiogenic proteins have to be already transcribed in meiotic and early haploid cells and temporarily stored and translationally regulated for long periods of time. For example, mRNAs for protamine genes are already transcribed in late meiotic cells and remains inactive for several days until they are needed during histone-protamine transition. The premature translation of these inactive mRNAs results in the impairment of spermiogenic processes and consequences spermatogenic defects (Lee et al., 1995; Kleene, 2001; MONESI, 1964).

### 2.1.3.3    Unique gene expression patterns in male germ cells

The progress of spermatogenesis is accompanied by a wide transcriptional activity of the genome (Bettegowda and Wilkinson, 2010). All steps of spermatogenesis are governed by temporally regulated waves of gene expression, each cell type has its specific transcriptome that supports the given differentiation phase (Grive et al., 2019; Hermann et al., 2018; Chen et al., 2018). Interestingly, the testicular transcriptome is unusually diverse compared to other organs (Soumillon et al., 2013; Xia et al., 2020). Especially late spermatocytes and early haploid round spermatids express their genome widely and have complex transcriptomes that includes a broad spectrum of non-coding RNAs and intergenic transcripts besides the numerous protein-coding mRNAs and their isoforms (Elliott and Grellscheid, 2006; Soumillon et al., 2013; Laiho et al., 2013). While some studies suggest that this broad genome expression originates as an unspecific consequence of the epigenetic remodelling in meiotic cells (Soumillon et al., 2013), a recent study revealed that it is utilized for transcriptional scanning that can shape germline mutation signatures and therefore modulate mutation rates in a gene-specific manner (Xia et al., 2020). In this study, it was shown that when the testicular expression level of a gene increases, the overall mutation rate drops, and this drop can be mostly attributed to the template strand, supporting a transcription-dependent DNA repair in the germline (Xia et al., 2020). Thus, the widespread transcription of the genome during spermatogenesis helps with maintaining DNA sequence integrity by correcting DNA damage through transcriptional scanning (Xia et al., 2020). After the broad genome expression in meiotic and early postmeiotic cells, late postmeiotic cells face a different kind of challenge in the regulation of gene expression when RNA storage and translational regulation becomes essential due to transcriptional silencing upon chromatin compaction (Kimmins and Sassone-Corsi, 2005a; Kimmins et al., 2004). Therefore, differentiating male germ cell needs specific regulatory mechanisms to control the cell type-specific gene expression waves, the unusually broad genome expression in meiotic and early postmeiotic cells, as well as the active translational regulation during the late step of spermatogenesis. These processes are supported by the presence of a high number of testis-specific RNA-binding proteins (Paronetto and Sette, 2010; Idler and Yan, 2012) and as well as germline-specific RNA-regulatory mechanisms such as piRNAs and germ granules (Meikar et al., 2011; Kotaja and Sassone-Corsi, 2007; Chuma et al., 2009; Meikar et al., 2013).

Spermatogenesis is well-conserved between species, including humans (Schlatt and Ehmcke, 2014; White-Cooper and Bausek, 2010; Chalmel et al., 2007). Abnormalities at any stage of spermatogenesis can cause human infertility. Currently, there is insufficient knowledge on the genetic basis of male infertility that minimize treatment and therapeutic options. It has turned out to be very challenging to model the entire spermatogenesis processes *in vitro*, and therefore, animal models,

in particular mouse models, provide a valuable alternative for understanding postnatal germ cell development and infertility studies (Stukenborg et al., 2014; Jamsai and O'Bryan, 2011; Cooke and Saunders, 2002).

## 2.2　　Pericentric heterochromatin

In eukaryotic cells, DNA is organized into a chromatin structure with the help of chromatin proteins. The basic unit of the chromatin is a nucleosome. It contains 146 bp of nuclear DNA that has been wrapped 1.7 times around the octameric protein complex containing two copies of each basic histone protein H2A, H2B, H3 and H4 (Kornberg, 1974; Luger et al., 1997). During evolution, different variants of histones have evolved, and germ cells also express several testis-specific histone variants that serve specialized functions during spermatogenesis (Hoghoughi et al., 2018; Rousseaux et al., 2011). The nucleosomes further fold into higher order structures to facilitate DNA compaction and are associated with various non-histone proteins (**Figure 6**).
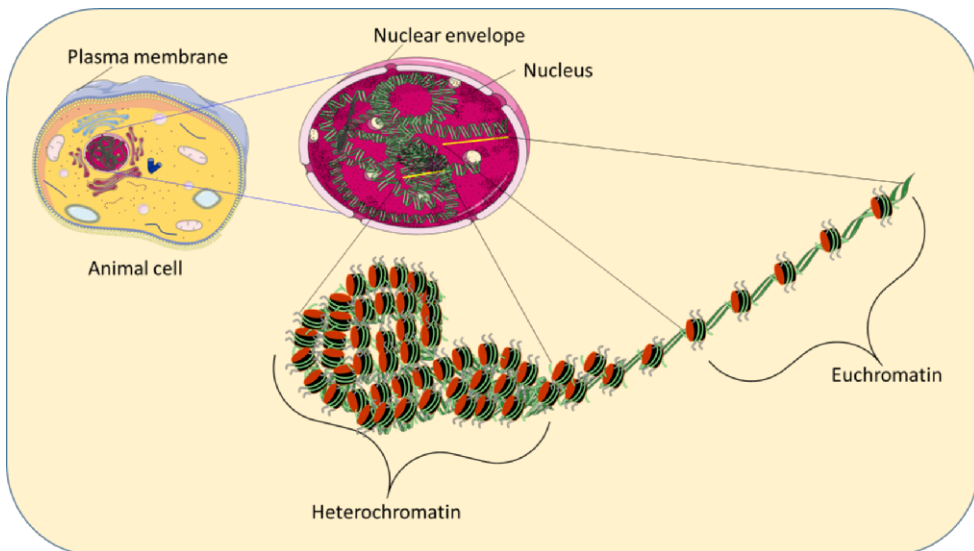


**Figure 6**:　An animal cell with the nucleus highlighted to show loosely packed euchromatin and densely packed heterochromatin regions of the genome.

The chromatin therefore contains DNA, histone proteins as well as non-histone proteins (Flemming, 1882). Many epigenetic mechanisms are involved in the regulation of chromatin structure and organization, including methylation of DNA, covalent modifications of histone proteins, exchange of histones with histone variants, ATP-dependent chromatin remodeling and RNA-mediated mechanisms

such as RNAi-dependent silencing of heterochromatin (Becker and Hörz, 2002; Bernstein, 2005; Jones, 2012; Kouzarides, 2007). On the basis of the degree of compaction, chromatin is divided into two forms, mainly euchromatin and heterochromatin (**Figure 6**) and (**Table 1**). Euchromatin is a loosely packed part of chromatin (Babu and Verma, 1987). Uncompacted organization allows access to gene regulatory protein complexes and RNA polymerase to initiate transcription. Actively transcribed genes are found in the euchromatin, for example, so-called housekeeping genes that are always turned on to produce proteins for cell survival and maintenance of basic functions. Firmly packed form of chromatin, which is not readily transcribed but remains inactive for most of the time, is termed as heterochromatin (Babu and Verma, 1987; Janssen et al., 2018). It forms dense, irregular particles distributed throughout the nucleus or adjacent to the nuclear envelope. Heterochromatin can be distinguished from euchromatin in light microscopy by a simple DNA staining that stains heterochromatin much more intensively than euchromatin (**Table 1**).

**Table 1**:    The main difference between euchromatin and heterochromatin region.

| Categories | Euchromatin | Heterochromatin |
|---|---|---|
| Coiled or uncoiled | Loosely uncoiled form of DNA is termed as euchromatin | Tightly coiled form of DNA is termed as heterochromatin |
| Density | Low DNA density | High DNA density |
| Stain | Light stain | Dark stain |
| Location | Both prokaryotic & eukaryotic cells, found in the inner part of the nucleus | In the eukaryotic cells, found mostly at the periphery of the nucleus |
| Replication | Replicate early | Replicate late |
| Transcription | Actively transcribed region of the chromosome | Most of the time remains inactive or shows transcriptional activity at the specific part of the chromosome. |

## 2.2.1    Repetitive DNA

Repetitive DNA is defined as DNA sequences that are repeated in the genome. These sequences do not code for proteins. Repetitive DNA is estimated to constitute about 30% of the genome. Repetitive DNA is subdivided into two categories: tandemly repetitive sequences (satellite DNA) which are localized to specific location of the genome and interspersed repeats which are distributed throughout the genome

(Nishibuchi and Déjardin, 2017; Munoz-Lopez and Garcia-Perez, 2010) (**Figure 7**). Many of the tandemly repeated sequences are localized either at the centromeric regions (major satellite repeats and minor satellite repeats) or telomeric regions (telomeric repeats). The dispersed repeats include transposable elements (TEs) which are mobile genetic elements having the ability to change their position within a genome. Examples of transposable elements include Long Interspersed Nuclear Elements (LINEs), Short Interspersed Nuclear Elements (SINEs), Intracisternal A Particles (IAPs).
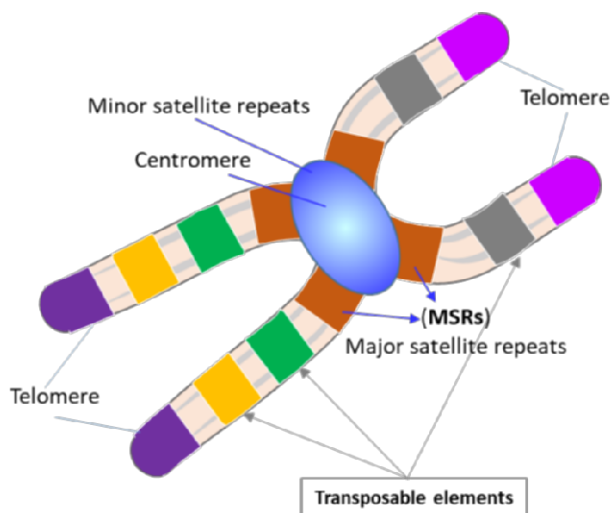


**Figure 7**:  Distribution of repetitive elements in the mouse genome.

Most of these non-coding repetitive sequences appear to be located in heterochromatic regions (Janssen et al., 2018; Nishibuchi and Déjardin, 2017). Repetitive DNA is not "junk" DNA, but it is an essential component of the genome. Repetitive DNA is known to a play important role in the structural and functional evolution of the genome and to facilitate the genome to express precisely and transmit accurate information to the next generations (Shapiro and Von Sternberg, 2005; Lee et al., 2019; Encode Consortium et al., 2013; Hartley and O'neill, 2019). During evolutionary processes, repetitive transposable elements and satellite repeats are also known to be involved in the adaptation to stress and other extreme conditions (Shapiro and Von Sternberg, 2005; Biscotti et al., 2015; Lanciano and Mirouze, 2018; Horváth et al., 2017).

## 2.2.2 Organization of pericentric heterochromatin

Pericentric heterochromatin forms at the specific part of the chromosome, just near to the centromere. The organization of pericentric heterochromatin is compact in nature, and it consists of repetitive sequences called major satellite repeats (MSRs) in the mouse. Pericentric regions are constitutively heterochromatinized and contain several characteristics that define its repressive functional state, including high level of DNA methylation, specific histone modifications, nuclease inaccessibility and association with specific heterochromatin proteins (Yue et al., 2014; Filion et al., 2010; Kharchenko et al., 2010). The repressive histone marks at the pericentric heterochromatin include trimethylation of lysine 9 in histone H3 (H3K9me3), and trimethylation of lysine 20 in histone H4 (H4K20me3) (Probst and Almouzni, 2008; Yue et al., 2014) (**Figure 8**). The formation and maintenance of pericentric heterochromatin are largely mediated by heterochromatin-associated proteins and enzymes responsible for repressive epigenetic modifications. These include histone methyltransferases SUV39H2 (Suppressor of Variegation 3-9 Homolog 2) and SETDB1 (SET Domain Bifurcated Histone Lysine Methyltransferase 1) that catalyze H3K9me2 to H3K9me3 methylation and Suv4-20h that catalyze H4K20me3 formation at the pericentric heterochromatin (Hahn et al., 2013; Schotta et al., 2004, 2008; Peters et al., 2001; Loyola et al., 2009; Pinheiro et al., 2012). These enzymes are recruited to pericentric heterochromatin via interactions with heterochromatin proteins or epigenetic modifications. For example, SUV39H enzymes can interact with core heterochromatin proteins (HP1) via their chromodomains (Bannister et al., 2001; Lachner et al., 2001). After the establishment of specific heterochromatin marks, other proteins can be recruited through binding to either HP1 or H3K9me3 (Hediger and Gasser, 2006; Kwon and Workman, 2011; Nozawa et al., 2010; Vermeulen et al., 2010; Fodor et al., 2010). The oligomerization of HP1 on the H3K9me3-modified chromatin domain is known to lead to the recruitment of H3K9 methyltransferases and further extension of H3K9me3-modified chromatin, therefore causing an efficient spread of the heterochromatin (Canzio et al., 2011; Hall et al., 2002; Hathaway et al., 2012; Verschure et al., 2005; Cheutin et al., 2003).
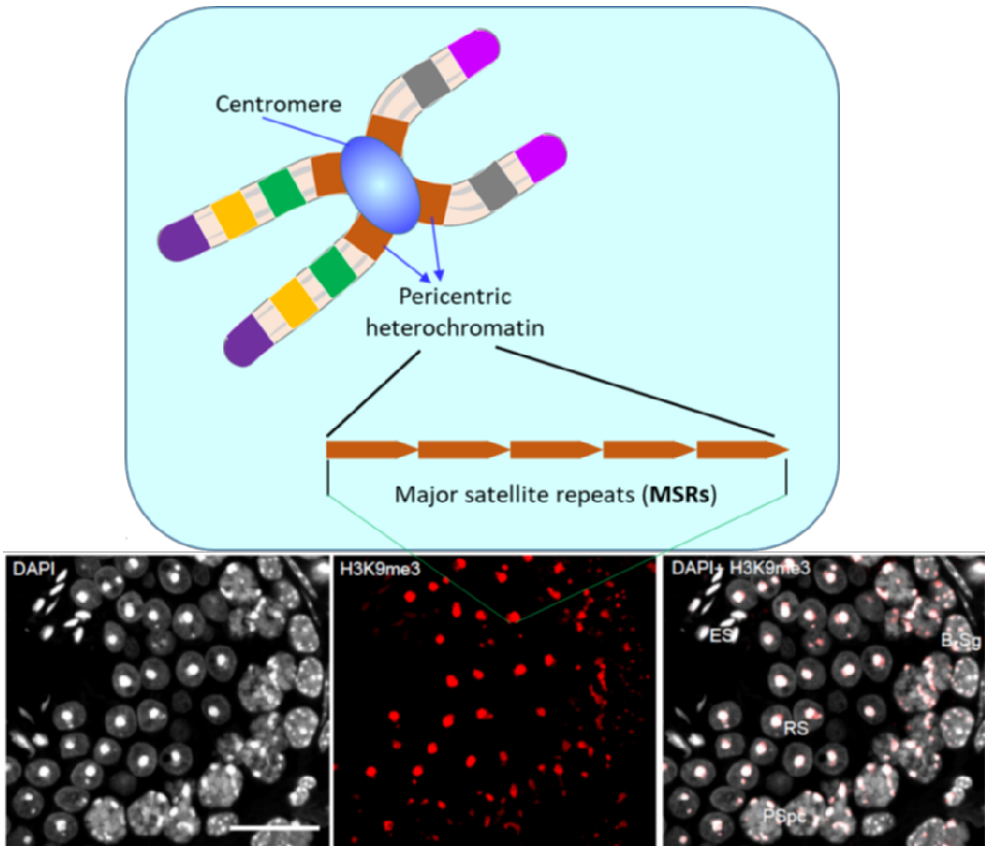
**Figure 8**: Pericentric heterochromatin organization in the mouse testis. Pericentric heterochromatin can be visualized by immunostaining using H3K9me3 antibody, as exemplified here on the mouse testis section. Scale bar: 25 µm. B-Sg: type B spermatogonium, PSpc: pachytene spermatocyte, RS: round spermatid, and ES: elongating spermatid.

The functional role pericentric heterochromatin is not completely understood, but satellite DNA has been reported to be involved in many cellular processes. These include specific events during male germ cell differentiation, such as histone-protamine transition, meiotic segregation of achiasmatic chromosomes, X chromosome dosage compensation and formation of lampbrush-like loops on the Y chromosome during male meiosis (Mills et al., 2019; Yunis and Yasmineh, 1971; Bonaccorsi et al., 1990; Dernburg et al., 1996; Menon et al., 2014; Joshi and Meller, 2017).

## 2.2.3 Pericentric heterochromatin is expressed under various physiological conditions

Initially, centromeric and pericentromeric regions were thought to be transcriptionally silenced because of their repressive epigenetic status. However, now we know that centromeric and pericentromeric domains are transcriptionally active and capable of producing RNA transcripts with functional roles (Ugarkovic, 2005; Eymery et al., 2009b; Pezer et al., 2012). RNA transcripts produced from the pericentric heterochromatin are known as MSR transcripts (**Figure 9**).
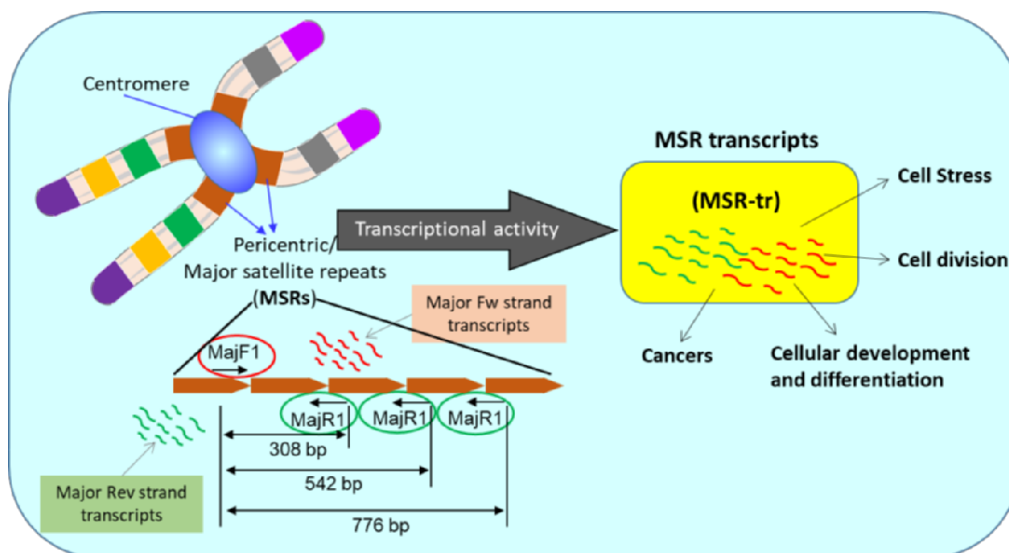


**Figure 9**: Major satellite repeats (MSRs) expression has been reported under different physiological and pathological conditions. MajF1 and MajR1 indicate the primers that can be used in PCR to amplify MSRs in the mouse. The amplification product size (308 bp, 542 bp and 776 bp) varies depending on how many repeats are amplified.

Pericentromeric heterochromatin is present in all chromosomes, including sex chromosomes, but pericentromeric heterochromatin in the Y chromosome has different sequences. The activity of RNA polymerase II (RNAPol II) was seen during ongoing transcription in the centromeric and pericentromeric regions (Hsieh et al., 2011b; Chan et al., 2012; Kato et al., 2005; Pezer and Ugarković, 2008). It is not clear how the transcription of pericentric heterochromatin occurs, and how RNA polymerase is able to recognize the promoter of repeat sequences. However, pericentric heterochromatin has been reported to be transcribed under different physiological conditions such as during cellular stress, cell division, early mouse development and differentiation, and aberrant expression of MSR transcripts has

been also demonstrated in many cancers (Ferreira et al., 2015; Probst and Almouzni, 2008; Eymery et al., 2009b; Briers et al., 2009) (**Figure 9**). MSRs are known to be transcribes from both forward and reverse strands, and these transcripts have been shown to have differential expression dynamics during the early mouse development (Casanova et al., 2013a; Probst et al., 2010). Furthermore, reducing the MSR transcripts level by injecting Locked Nucleic Acid (LNA)-DNA gapmers to zygotes led to developmental  arrest at the G1/S stage of the cell cycle, suggesting the important functional role for MSR transcripts in the early mouse development (Casanova et al., 2013a; Probst et al., 2010).

Due to the repetitive nature of pericentric heterochromatin, it makes a scaffold-like structure that is able to recruit many transcription factors and other regulators such as histone methyl transferases with possible regulatory role in MSR expression. The binding of transcription factors PAX3 (Paired Box 3) or PAX9 (Paired Box 9) is known to facilitate heterochromatin formation (Bulut-Karslioglu et al., 2012). Another pericentric heterochromatin regulator is LRWD1, a leucine-rich protein containing a WD40 repeat domain that associates with the origin replication complex (ORC) for pericentric heterochromatin silencing in mouse embryonic fibroblast (MEF) cells. Interestingly, loss of H3K9me3 leads to defective localization of LRWD1 and ORC2 and increased levels of MSR transcripts (Chan and Zhang, 2012). WD40-domain and HMG-domain containing protein, WDHD1 was also shown to localize to pericentric heterochromatin in NIH-3T3 mouse fibroblasts cells, and to stabilize the association of DICER with centromeric RNAs for post-transcriptional processes (Hsieh et al., 2011a). Lymphoid-specific helicase (HELLS or LSH) has been shown to be a master regulator of the repetitive genome. HELLS belongs to a family of chromatin remodeling proteins, and it associates with pericentromeric heterochromatin and regulates pericentric heterochromatin expression and H3K9me3 levels (Huang, 2004; Lungu et al., 2015). Knockout of *Lsh* in MEFs or mouse embryos showed increased levels of MSR transcripts (Huang, 2004).

## 2.3    DICER

### 2.3.1    Small RNA processing by the endonuclease DICER

DICER is a central RNA processing endonuclease that is involved in the production of small interfering RNAs (siRNAs), microRNAs (miRNAs) and endogenous siRNAs (endo-siRNAs) (Yadav and Kotaja, 2014b) (**Figure 10**). siRNA pathway and RNA interference (RNAi) have been evolved for responding to exogenously introduced double-stranded RNA (dsRNA) molecules to protect our genome from foreign invaders such as viruses, transposons and transgenes, and to maintain genome integrity (Mello and Conte, 2004; Carthew and Sontheimer, 2009). In

contrast, miRNAs are produced from endogenous genes, and they regulate the cell's own transcriptome (Kim et al., 2009; Carthew and Sontheimer, 2009). miRNA genes are transcribed first by RNA polymerase II or III in the nucleus to generate longer hairpin loop primary transcripts (pri-miRNA) which are first processed by nuclear microprocessor complex (Drosha/DGCR8) (Siomi and Siomi, 2010; Krol et al., 2010; Winter et al., 2009) to produce precursor miRNAs (pre-miRNAs). These pre-miRNAs are transported through nuclear pores to the cytoplasm with the help of Exportin-5 in a Ran-GTP dependent manner. Pre-microRNAs are then processed by DICER into 18-25 nt double-stranded RNAs (dsRNAs), which then unwind into mature single-stranded miRNAs. These mature miRNAs associate with Argonaute proteins to form RNA-induced silencing complexes (RISCs). With the help of miRNA sequence complimentary, the RISC complex binds to their target mRNAs. miRNA binding sites are usually located at the 3' untranslated region (3'-UTR) of mRNAs. The nature of the base pairing between mature miRNA and mRNA determines the fate of mRNA; imperfect base pairing leads to repression of translation of target mRNAs whereas perfect base pairing leads to exonucleolytic mRNA decay. Both mechanisms negatively regulate gene expression by ultimately reducing their respective protein levels (**Figure 10**).
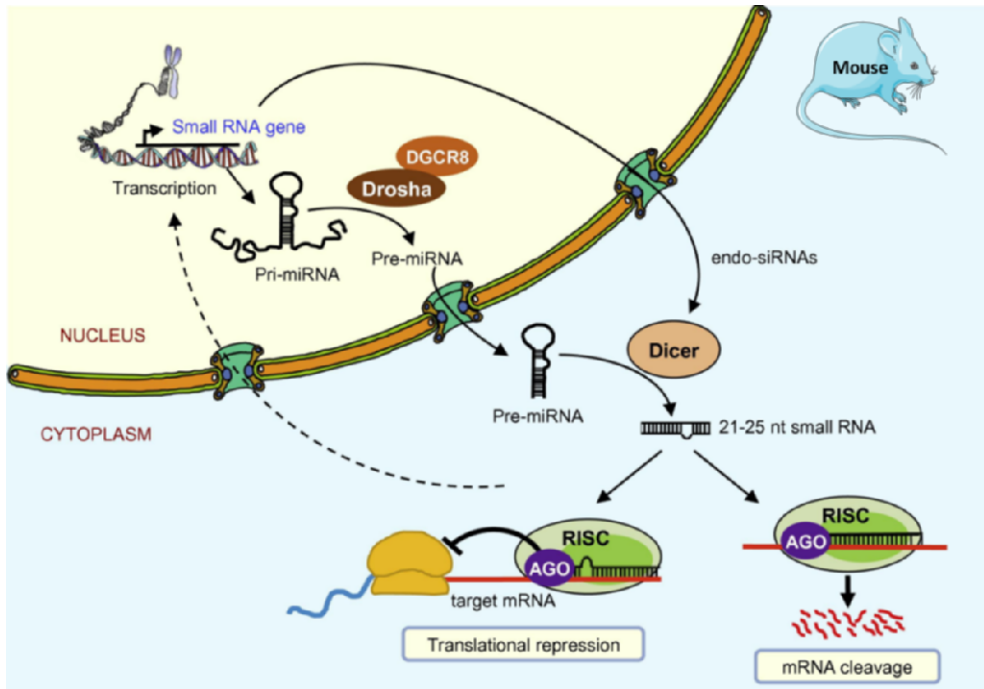


**Figure 10**: Biosynthesis and functions of DICER-dependent small RNAs, adopted from (Yadav and Kotaja, 2014a), with some modifications.

miRNAs play vital critical role in regulating many physiological processes such as proliferation, apoptosis and cellular differentiation, including spermatogenesis (Sun et al., 2010; Yadav and Kotaja, 2014b). Using different techniques such as miRNA microarrays, RT-PCR or small RNA sequencing, several miRNAs are identified which are highly, exclusively or preferentially expressed in the testis or at specific phases of male germ cell differentiation (Papaioannou and Nef, 2010; Mciver et al., 2012). The differentially expressed miRNAs were observed between immature and mature mouse testes (Yan et al., 2007) as well as miRNA profiles during the first wave of spermatogenesis on post-natal days 7, 10 and 14 (Buchold et al., 2010). The enriched populations of pre-meiotic and meiotic cells such as spermatogonia, spermatocytes and spermatids expressed some common miRNAs but some cell type specific miRNAs (Marcon et al., 2008; Smorag et al., 2012). The transient inhibition of the miRNAs such as miR-21, expressed in the spermatogonial stem cell (SSC) enriched population, leads to increased apoptosis and reduced the SSC potency (Niu et al., 2011). The functional importance of miRNAs also revealed by deleting the Mir-17-92 cluster in a mouse undifferentiated spermatogonia, resulted in small testes and lower number of epididymal sperm (Tong et al., 2012).

In addition, male mouse germ cells express a large number of endogenous siRNAs (endo-siRNAs) (Song et al., 2011), which are processed by DICER but independent of the microprocessor complex (Drosha-DGCR8). To trace the origin of these testicular endo-siRNAs, these endo-siRNAs were mapped to several different sites on multiple chromosomes in contrast miRNAs that normally derive from a unique locus or very few loci (Song et al., 2011). The target sites for these endo-siRNAs were as mRNAs (~92%), non-coding RNAs (~4%), transcripts of pseudogenes (~3%) and retrotransposons (~1%) (Song et al., 2011). Endo-siRNAs were shown to act at the post-transcriptional level to degrade mRNA efficiently. However, they were also shown to have nuclear effects on chromatin modifications (Song et al., 2011).

## 2.3.2    Conditional *Dicer1* knockout mouse models

The ablation of the *Dicer1* gene at the embryonic stage in mice leads to embryonic lethality, mice dying at embryonic day 7.5 with a lack of detectable multipotent stem cells, and severe developmental abnormalities (Bernstein et al., 2003; Murchison et al., 2005b), which demonstrates the importance of DICER-dependent processes in the maintenance of stem cell population and embryonic development. The embryonic lethal phenotype hinders the assessment of the specific role of DICER in different cell types and tissues and, therefore, conditional knockout mouse models are required. The conditional knockout mouse models are usually generated using a Cre/loxP system to delete a gene of interest at a specific tissue or cell type using cell

type-specific promoters to drive the expression of Cre recombinase (Walrath et al., 2010; Smith, 2011). *Dicer1* has been deleted in many different tissues using this approach, and its critical role in many physiological processes has been proven.

To understand the role of DICER in male and female fertility, different conditional *Dicer1* knockout mouse models have been generated (Yadav and Kotaja, 2014a; Luense et al., 2009; Björkgren and Sipilä, 2015). For example, the conditionally inactivation of *Dicer1* in the somatic cells of the female reproductive tract, that is, using Cre recombinase under the control of anti-Müllerian hormone receptor type 2 promoter in the mesenchyme of the developing Müllerian ducts, resulted in female sterility with multiple reproductive defects including decreased ovulation rates and shorter uterine horns (Nagaraja et al., 2008). The deletion of *Dicer1* in the mouse oocytes using the *Zp3* promoter consequences blockage of meiosis I with severe chromosomal defects, disorganized spindles and misregulation of transposable elements (Murchison et al., 2007). Interestingly, removal of *Dicer1* by *Stra8* promoter-driven *Cre* expression in the post-natal spermatogonia showed significant reductions in testis mass and sperm number, infertile due to disrupted spermatogenesis (Greenlee et al., 2012; Wu et al., 2012). In addition to male germ cells, DICER was shown to be important for the function of Sertoli cells, and Sertoli cell-specific *Dicer1* knockout mice were completely infertile with severe defects in testicular functions (Papaioannou et al., 2009; Kim et al., 2010) (**Table 2**).

**Table 2**:    *Dicer1* knockout mouse models in the testis.

| Mouse Model | Cre expression | Phenotype | References |
|---|---|---|---|
| *TnapCre-Dicer1* | Primordial germ cells (PGCs) | Defects in PGC proliferation and post-natal spermatogenesis | (Hayashi et al., 2008; Maatouk et al., 2008) |
| *Ddx4Cre-Dicer1* | Prospermatogonia (E18) | Meiotic defects, reduced number of haploid cells, drastic abnormalities in head and tail morphology of the remaining spermatozoa | (Liu et al., 2012; Romero et al., 2011b) |
| *Stra8-iCre-Dicer1* | Spermatogonia | Morphological disruptions of spermatocytes and round spermatids, impaired meiotic progression | (Wu et al., 2012; Greenlee et al., 2012) |
| *Prm1Cre-Dicer1* | Round spermatids | Defects mainly in post-meiotic differentiation, abnormal head morphology | (Chang et al., 2012) |
| *AmhCre-Dicer1* | Sertoli cells | Defective Sertoli cell maturation, massive apoptosis | (Papaioannou et al., 2009; Kim et al., 2010) |

*Dicer1* ablation in the mouse epididymal epithelium using the *Defb4* promoter also caused infertility due to dedifferentiation of the epithelium and imbalance in sex steroid signaling (Björkgren et al., 2012). Altogether, these studies support the essential role of DICER in the maintenance of reproductive functions. In our study, we also used the Cre/loxP approach to delete *Dicer1* conditionally in male germ cells using Cre recombinase under the control of Neurogenin 3 (*Ngn3*) promoter that drives the Cre expression specifically in early undifferentiated spermatogonia (Yoshida et al., 2004). This allowed the dissection of the role of germ cell-intrinsic DICER in postnatal spermatogenesis. Some *Dicer1* knockout mouse models for male reproductive studies are listed in the **Table 2**.

DICER is an endonuclease for both miRNAs and endo-siRNAs. To understand the roles of miRNAs and endo-siRNAs in mouse spermatogenesis, testicular phenotypes of both *Dicer1* and *Drosha* germ cell-specific knockout mice (Wu et al., 2012) were compared. Interestingly, *Drosha cKO* testes showed to be more severe in spermatogenic disruptions than *Dicer cKO* testes, highlighting the vital role of miRNA pathways during spermatogenesis. In the transcriptome analysis, mRNA profiles of *Drosha-* or *Dicer-*null pachytene spermatocytes and round spermatids were altered (Wu et al., 2012). This indicates that both miRNAs and endo-siRNAs have a role in regulating protein-coding mRNAs during post-natal spermatogenesis (Wu et al., 2012). The importance of miRNA pathway in the male germline was also demonstrated by deleting the microprocessor component DGCR8 (Zimmermann et al., 2014).

### 2.3.3    Non-canonical functions of DICER

Besides the canonical functions of DICER in miRNA/siRNA biogenesis, several non-canonical functions of DICER have emerged (Pong and Gullerova, 2018). Particularly, DICER has been implicated in the control of repeat sequences that derive from non-coding regions of the genome (Alexander et al., 2010; Xing et al., 2007), such as transposons. Transposons are mobile genetic elements that have to be strictly regulated to prevent their aberrant expression and therefore to protect genomic integrity (Jurka et al., 2007; Druker and Whitelaw, 2004; Huang et al., 2012). DICER was shown to be involved in the regulation of Alu transposable elements in human cells (Kaneko et al., 2011a). The conditional ablation of *Dicer1* in human retinal pigmented epithelium (RPE) cells led to accumulation of Alu transposable RNAs, which resulted in cytotoxicity and age-related macular degeneration in the geographic atrophy (Kaneko et al., 2011a). The expression of transposable elements such as SINE (short interspersed nuclear element) was also upregulated in mouse oocytes lacking *Dicer1* (Murchison et al., 2007), as well as in the *Ddx4Cre Dicer1* knockout male mice (Romero et al., 2011a), where *Dicer1* was

deleted in the prospermatogonia of the mouse, signifying the possible roles of DICER in regulating transposons in the germline.

Another type of repeats that are regulated by DICER are the satellite repeats found at the centromeric and pericentric chromosomal regions (Guenatri et al., 2004). DICER has been shown to have a role in the control of these domains in the fission yeast, and this DICER-dependent silencing of heterochromatin is dependent on the RNA interference pathways (White and Allshire, 2008). Some studies suggest the involvement of DICER in the regulation of satellite repeat expression in mammalian cells as well. For example, *Dicer1* deficient mouse embryonic stem cells showed severe defects in centromeric silencing (Kanellopoulou, 2005). Furthermore, conditional deletion of *Dicer1* resulted in cell death with an increased number of abnormal mitotic cells and high accumulation of human centromeric repeat transcripts in a chicken-human hybrid cell line (Fukagawa et al., 2004a).

Although DICER is mainly found in the cytoplasm, some of its non-canonical roles require nuclear localization (Burger and Gullerova, 2018; Gagnon et al., 2014; Skourti-Stathaki et al., 2014). The nuclear activity of DICER is mediated by the C-terminal double-stranded RNA-binding domain, which contains a nuclear localization signal (NLS) (Doyle et al., 2013). NLS allows DICER to shuttle from the cytoplasm to the nucleus. DICER is transported to the nucleus with the help of a nuclear pore complex component (Ando et al., 2011), where it is involved in the vital nuclear activities such as regulating transcription and splicing (Kalantari et al., 2016), maintenance of RNA homeostasis between cytoplasmic and nuclear compartment in response to DNA damage or stress (Burger et al., 2017; White et al., 2014; Neve et al., 2016; Turunen et al., 2019). The nuclear DICER along with RNAi components is required for heterochromatin formation and silencing which is well-studied in lower organisms such as fission yeast, plants, *C. elegans* and flies (Djupedal and Ekwall, 2009; Cam et al., 2005; Volpe et al., 2002; Zilberman et al., 2003; Pal-Bhadra et al., 2004; Vastenhouw et al., 2006) but not well-studied in the mammals. However, recently it was found that DICER associates with chromatin in the mammalian system (Sinkkonen et al., 2010; White et al., 2014), clearly suggesting that DICER operates in the nuclear compartment in addition to its well-known cytoplasmic functions. In summary, growing evidence suggests that DICER has chromatin-associated functions (Sinkkonen et al., 2010; White et al., 2014; Nesterova et al., 2008; Giles et al., 2010), and it is involved in the regulation of satellite repeats in mammalian cells (Kanellopoulou, 2005; Fukagawa et al., 2004a), but the direct involvement of DICER in the regulation of pericentric heterochromatin has remained unclear.

# 3    Aims of the Present Study

Due to a high demand for transcriptome regulation within male germ cells, several specific regulatory mechanisms are required for the development of fertile spermatozoa. The endonuclease DICER produces miRNAs and siRNAs which mainly act post-transcriptionally to control the mRNA stability or translation. In addition, DICER is known to have other miRNA-independent functions. This study investigated the importance of DICER-dependent pathways during mouse spermatogenesis, particularly focusing on the non-canonical functions of DICER in the regulation of the non-coding genome. The specific aims of the study were:

1. To explore the role of DICER in male germ cell differentiation and male fertility using a germ cell-specific conditional knockout mouse model.

2. To investigate the expression of pericentric heterochromatin during spermatogenesis.

3. To reveal the function of DICER in the regulation of pericentric heterochromatin expression in male germ cells.

# 4 Materials and Methods

## 4.1 Mouse breeding and maintenance (I & II)

Mice were housed under controlled environmental conditions at the central Animal Laboratory of the University of Turku, Finland. Germ cell-specific conditional *Dicer1* knockout (*Dicer1* cKO) mice were generated as previously described using Neurogenin3 (*Ngn3*) promoter-driven Cre expression (Yoshida et al., 2004) (**Figure 11**).
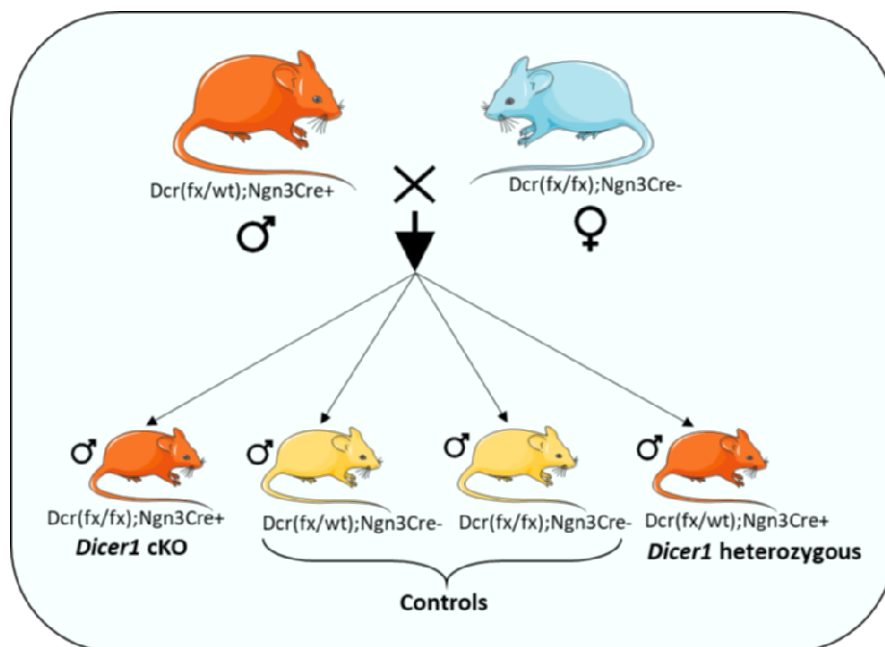


**Figure 11**: A schematic representation of mouse breeding strategy. For knockout breedings, male mice carrying *Dcr(fx/wt);Ngn3Cre* were mated with females *Dcr(fx/fx)* to produce pups containing genotypes: *Dcr(fx/fx);Ngn3Cre (Dicer1* cKO), *Dcr(fx/wt);Ngn3Cre (Dicer1* heterozygous), *Dcr(fx/fx);Ngn3Cre-* and *Dcr(fx/wt);Ngn3Cre-*. The male littermates without Cre expression (*Dcr(fx/fx);Ngn3Cre-* and *Dcr(fx/wt);Ngn3Cre-*) were used as controls and these control male mice were shown to be phenotypically similar to the wild type mice.

The second RNase III domain of the *Dicer1* gene (Exon 24) was flanked between two loxP sites. Cre-expressing transgenic mice were crossed with floxed *Dicer1* mice to induce Cre recombinase and mediated the recombination events between two flanked loxP sites leading to the conditional removal of exon 24 of *Dicer1* in undifferentiated spermatogonia. The mice were of mixed genetic background (C57BL/6J and SV129). For wild type (WT) studies, we used either C57BL/6J or C57BL/6N mouse strain (Simon et al., 2013).

## 4.2     Genotyping of *Dicer1* cKO mice (I & II)

Ear samples were taken to extract DNA using standard procedures followed by ethanol precipitation. The genotyping of all samples were done using primers F(DICER) and R(DICER) for the detection of floxed *Dicer1* allele (Harfe et al., 2005) and F(Ngn3Cre), R(Ngn3Cre), F(pTimer) and R(pTimer) to monitor the presence of transgenic Ngn3Cre (Desgraz and Herrera, 2009; Korhonen et al., 2011) as previously described. The genotyping primers are listed in the **Table 1.**

## 4.3     Ethics statement (I & II)

All animal experiments and animal husbandry were performed following the guidelines of the Ethics of the Animal Experimentation at the University of Turku in accordance with the Guide for Care and Use of Laboratory Animals (National Academy of Science). In all our studies, we incorporated the 3Rs principle and minimized the animal suffering during experiments. All experiments were authorized by the National Animal Experiment Board.

## 4.4     Collection of testis samples

The testes samples were collected from adult mice or on postnatal days 8, 12, 14, 18, 20, 24, 28 and 34 dpp. Testes were immediately frozen in liquid nitrogen and stored at -80 ˚C.

## 4.5     Preparation of testicular cell suspension and enrichment of mouse germ cells (I & II)

To enrich pachytene spermatocytes and round spermatids from mouse testes, we prepared testicular cell suspension by digesting seminiferous tubules with 0.5 mg/ml Collagenase Type I (Worthington Biochemical Corporation) in the 0.1% glucose in Phosphate-Buffered Saline pH 7.4 (PBS) for 1 hour. The cell suspension was first filtered with 100 μm filter then with 40 μm filter to remove cell debris, and cells

were pelleted by centrifugation at 1500 rpm for 5 min. Finally, cell pellets were resuspended in 10 ml of PBS. Spermatocytes and round spermatids were enriched using centrifugal elutriation by Beckman JE-6B rotor using specific flow rates and centrifugal forces as previously described (Barchi et al., 2009a). Each fraction was stained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) and subjected to microscopical examination to check the purity of fractions. Cells were pelleted at 1500 rpm for 10 min at +4 °C and immediately frozen in liquid nitrogen and stored at -80 ˚C.

## 4.6 Total RNA isolation and semi-quantitative RT-PCR (I & II)

Total RNAs were extracted from testes or enriched germ cells using TRIzol (Invitrogen) and isopropanol precipitation. Turbo-DNase (Invitrogen) was used to remove DNA contamination. The cDNA synthesis was performed by DyNamo cDNA synthesis kit (Finnzymes) using random hexamers. *L19* was used as a reference gene. For strand-specific MSR expression analysis, we used either MSR forward or reverse strand-specific primers to synthesize cDNAs. *U1* was used as a reference gene for strand-specific RT-PCR (Probst et al., 2010). PCR conditions were: initial denaturation, 96°C for 3 min, and subsequently for 26-27 cycles, 95°C for 30 sec, 57°C for 20 sec, 72°C for 1 min, followed by final elongation at 72 °C for 10 min. PCR products were run into the 3-3.5% agarose gel containing ethidium bromide (EtBr) to visualize amplified products. The quantification of expression was done using ImageJ software by measuring the peak intensity of 308 bp band amplified from MSR transcripts (MSR-tr). The signal intensity was then normalized by dividing the MSR-tr signal with the reference gene expression in the same sample. A selected sample was used as a calibrator and set as '1', and the expression of MSR-tr in other samples was presented relative to the calibrator expression. All the primers are listed in the **Table 3.**

**Table 3.** Primers used in the studies I and II.

| Name | Primer sequences (RT-PCR and ChIP-PCR) | Ta (°C) |
|---|---|---|
| Dicer1 | Fw, 5′-CTTGACTGACTTGCGCTCTG-3′<br>Rev, 5′-AATGGCACCAGCAAGAGACT-3′ | 60 |
| L19 | Fw, 5′- GGACAGAGTCTTGATGATCTC -3′<br>Rev, 5′- CTGAAGGTCAAAGGGAATGTG -3′ | 57 |
| Ppia | Fw, 5′- GCCATGGTCAACCCCACCGT -3′<br>Rev, 5′- TGCAAACAGCTCGAAGGAGACG -3′ | 57 |
| Major satellite | Fw, 5′- GACGACTTGAAAAATGACGAAATC -3′<br>Rev, 5′- CATATTCCAGGTCCTTCAGTGTGC -3′ | 57 |
| Minor satellite | Fw, 5′- CATGGAAAATGATAAAAACC -3′<br>Rev, 5′- CATCTAATATGTTCTACAGTGTGG -3′ | 57 |
| Line1 | Fw, 5′- TTTGGGACACAATGAAAGCA -3′<br>Rev, 5′- CTGCCGTCTACTCCTCTTGG -3′ | 60 |
| SineB1 | Fw, 5′- GTGGCGCACGCCTTTAATC -3′<br>Rev, 5′- GACAGGGTTTCTCTGTGTAG -3′ | 60 |
| IAP | Fw, 5′- AGCAGGTGAAGCCACTG -3′<br>Rev, 5′- CTTGCCACACTTAGAGC -3′ | 62 |
| Gapdh | Fw, 5′- AGTGCCAGCCTCGTCCCGTA -3′<br>Rev, 5′- AGGCGCCCAATACGGCCAAA -3′ | 57 |
| rDNA | Fw, 5′- GTAGTCGCCGTGCCTACCAT -3′<br>Rev, 5′- TTTTCGTCACTACCTCCCCG -3′ | 60 |
| **Name** | **Primer sequences (Genotyping)** | **Ta (°C)** |
| Dicer | Fw, 5′-CCTGACAGTGACGGTCCAAAG-3′<br>Rev, 5′-CATGACTCTTCAACTCAAACT-3′ | 57 |
| Ngn3Cre | Fw, 5′-CCTGTTTTGCACGTTCACCG-3′<br>Rev, 5′-ATGCTTCTGTCCGTTTGCCG-3′ | 52 |
| pTimer | Fw, 5′-ACGGCTGCTTCATCTACAAGG-3′<br>Rev, 5′-TTGGTGTCCACGTAGTAGTAG-3′ | 52 |

$T_a$, annealing temperature.

## 4.7 Histology and immunohistochemistry (I)

Tissues were fixed in 4% paraformaldehyde (PFA) (immunohistochemistry) or in Bouin's fixative (histology) for 4–20 hours at room temperature (RT). Tissues were dehydrated by a series of increasing concentrations of ethanol and embedded in paraffin. Paraffin-embedded tissues were sectioned and rehydrated. For histology, sections were stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS) according to standard protocols. For immunostaining, sections were proceeded to antigen retrieval by cooking in a pressure cooker in 10 mM Sodium Citrate buffer (pH 6.5) or TE buffer (pH 9.0) for 20 min. Testis sections were blocked with the blocking solution (3-10% normal donkey or goat serum and 3-10% bovine serum

albumin [BSA]) in PBS containing 0.1% Triton X-100, followed by incubating with primary antibodies solution (**Table 2**) for overnight at 4°C or 1 hour at 37°C. Testis sections were washed in the PBS with 0.1% Tween-20 (PBST) 3 x 10 min. Subsequently, sections were treated with 3% $H_2O_2$ to block the endogenous peroxidase activity. A biotinylated secondary antibody (1:750, Vector Laboratories) was diluted with the blocking solution and incubated for 1 hour at 37°C. Rabbit and mouse IgG were used as negative controls (Vector Laboratories). For the immunohistochemical detection, VECTASTAIN ABC (peroxidase) system (Vector Laboratories) and 3,3′-diaminobenzidine (liquid DAB+, DAKO) were used to detect antibody localization. Then, stained with hematoxylin, dehydrated and, finally, mounted in PERTEX medium.

## 4.8    TUNEL assay (I)

To check DNA fragmentation in apoptotic cells, TdT-mediated dUTP nick end labelling (TUNEL) was done on Bouin's-fixed paraffin-embedded testis sections. Testis sections were subjected to deparaffinization, rehydration and antigen retrieval in 10 mM Sodium Citrate (pH 6.5) buffer for 20 min in a microwave. Subsequently, sections were treated with 3% $H_2O_2$ (Sigma) to block endogenous peroxidase activity at RT for 15 min. Then, sections were placed in the reaction mix containing 1× TdT buffer, Terminal transferase (0.6 U, Roche), biotin-16-dUTP (10 µM, Roche) and 10 µM $CoCl_2$ for 60 min at 37°C. The reaction mix without an enzyme was used as a negative control. Testis sections were blocked with the blocking solution (3-10% normal donkey or goat serum and 3-10% BSA) in PBS, followed by incubating with ExtrAvidin®–Peroxidase (1:50, Sigma) for 30 min at 37°C. To detect apoptotic cells, sections were incubated with 3,3′-diaminobenzidine (liquid DAB+, DAKO), stained with hematoxylin, dehydrated and mounted with PERTEX medium.

## 4.9    Sperm counts and sperm slides (I)

To evaluate epididymal sperm counts, spermatozoa were released from cauda epididymides and ductus deferens of adult male mice (60 dpp or 120 dpp). The sperm concentration was measured as previously described (Guerif et al., 2002). Epididymal spermatozoa were also spread on glass slides and stained with hematoxylin for morphological analysis.

## 4.10    Squash preparations and drying down preparations (I)

Testes were placed in PBS, decapsulated and the specific stages of the seminiferous epithelial cycle were identified on the basis of their transillumination patterns. Squash preparations were done according to a previously published protocol (Kotaja et al., 2004). For drying down preparations, cells were released from stage-specific seminiferous tubules with forceps, and cell suspension was spread on slides and fixed according to previously published protocol (Kotaja et al., 2004). Phase contrast microscopy was used to visualize the cells during the drying down preparations. For immunofluorescence, both drying down and squash slides were post-fixed in 4% paraformaldehyde, followed by permeabilization in 0.2% Triton-X 100 for 15 min at RT, and immunofluorescence was done as described below. To evaluate the mitochondria, 200 nM Mitotracker (Invitrogen) was added in PBS and incubated with slides for 15 min. Wide field fluorescence images were captured with a Leica DMRBE microscope connected to the Olympus DP72 digital color camera using cellSens Entry 1.5 (Olympus) digital imaging software or Zeiss AxioImager M1 microscope. Laser scanning confocal microscope (Zeiss LSM510 META) was used to acquire high resolution confocal images.

## 4.11    Immunofluorescence (I & II)

For immunofluorescence, testis samples were fixed overnight in the 4% PFA in PBS and embedded in paraffin. The deparaffinization of testis sections were done as follows: Xylene 3 x 3 min, 100% EtOH 2 x 3 min, 96% EtOH 2 x 3 min, 70% EtOH 2 x 3 min and Milli-Q water 1 x 5 min. The antigens were retrieved by cooking in the pressure cooker containing the citrate buffer (10 mM sodium citrate, pH 6.0) for 20 min. Testis sections were quenched with 100 mM $NH_4Cl$ for 5 min, followed by washing in PBS and permeabilization with 0.5% Triton X-100 PBS for 10 min. Testis sections were blocked with the blocking solution (2-3% normal donkey or goat serum, 2-5% BSA, 0.1% Triton X-100 in PBS) followed by incubating with primary antibodies solution (**Table 2**) for overnight at 4°C. Subsequently, testis sections were washed in PBST for 3 x 10 min. The secondary antibodies (AlexaFluor488 or 594 anti-mouse, anti-rabbit and anti-rat antibodies produced in a donkey or goat, 1:1000 dilutions, Invitrogen) were incubated for 1 hour at RT. The testis sections were washed again with PBST for 3 x 10 min followed by staining with DAPI (Sigma-Aldrich, 0.25 mg/ml) for 10 min at RT. Finally, slides were mounted with ProLong Diamond Antifade Mountant (Invitrogen) and scanned by laser scanning confocal microscopy (Zeiss LSM780).

## 4.12    Electron microscopy (I & II)

For electron microscopy, testis samples were fixed in 5% glutaraldehyde followed by treating with the potassium ferrocyanide-osmium fixative. The epoxy resin (Glycidether 100, Merck) was used for embedding. Then, testis blocks were sectioned, post-stained with 5% uranyl acetate and 5% lead citrate, followed by a visualization on the JEOL 1400 Plus (JEOL Ltd., Tokyo, Japan) transmission electron microscope.

## 4.13    Nuclear and cytoplasmic fractions (II)

Nuclear and cytoplasmic fractions were done according to the published protocol (Nilsen, 2014) with some modifications. 18 days old mouse testes were digested with Collagenase Type I to prepare a testicular cell suspension as described above. Germ cells were mildly crosslinked in 0.1% PFA in PBS for 10 min at the RT. The crosslinking reaction was stopped by adding 125 mM glycine (Sigma) at the RT for 5 min. Cells were resuspended in the hypotonic lysis buffer (40 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.005% NP-40) supplemented with fresh 1x Protease Inhibitor Cocktail (PIC, Roche) and 1 mM phenylmethanesulfonyl fluoride (PMSF) for 2 min in ice. The cell suspension was centrifuged at 2000 rpm for 2 min to collect supernatant as a cytoplasmic fraction. The pellets containing nuclear fraction were washed twice with wash buffer I (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 3 mM $MgCl_2$, 0.25% NP-40, 0.25% Triton-X-100, 1xPIC) and once with wash buffer II (1% Triton-X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris HCl pH 8.0, 1xPIC). Subsequently, high salt buffer (40 mM Tris-HCl pH 7.4, 400 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 1 mM DTT, 1xPIC) was used for 15 min in ice to dissolve the nuclear pellet. Then, the extracts were diluted 5 times with the dilution buffer (20 mM HEPES pH 7.4, 0.2 M NaCl, 0.5% Triton X-100, 5% glycerol, 1 mM EDTA, 1 mM EGTA, 3 mM $MgCl_2$, 1xPIC) and centrifuged at 10000 rpm for 5 min. The top supernatant was collected as a nuclear fraction. Both cytoplasmic and nuclear fractions were heated with Laemmli buffer at 90°C for 10 min and used in western blotting for loading the samples as described below.

## 4.14    RNA *in situ* hybridization (II)

Testes were fixed at 4% PFA to prepare cryosections for RNA *in situ* hybridization. DIG-labelled (5') LNA probe for MSR forward transcript (5'-TCTTGCCATATTCCACGTCC-3', (II) (Probst et al., 2010) was purchased from Exiqon. DIG labeled Scramble probe was used as a negative control (Exiqon). Cryosections of adult testes were post-fixed and permeabilized with freshly prepared solution containing 4% PFA, 0.5% Triton X-100 and 2 mM vanadyl ribonucleoside

complex (VRC, New England BioLabs) in PBS for 20 min on ice with slow agitation. Then, sections were incubated with proteinase K (1.5 µg/ml) in PBS containing 35 mM EDTA for 20 min at 37°C, followed by resuspending with 0.2% glycine in PBS for 5 min at 37°C. Cryosections were post-fixed in 4% PFA in PBST (0.1% Triton X-100) for 10 min on ice and equilibrated in 100 ml pre-acetylation (2.6 ml triethanolamine in 200 ml of DEPC-treated MQ water, pH 8) mix for 10 min. After adding 500 µl of acetic anhydride and incubating for 10 min, slides were washed with PBS. RNase treatment (Riboshredder, 50 U/ml, Epicenter Biotechnologies) was done in Tris buffer (pH 7) for 90 min in a humidified chamber at 37°C. Pre-hybridization was done in pre-hybridization mix containing 50% formamide, 2x saline sodium citrate (SSC), 10 mM VRC and 2 mg/ml BSA (New England BioLabs) at 37°C for 1 hour. Hybridization was performed in the hybridization mix (50% formamide, 2xSSC, 10% dextran sulfate, 10 mM VRC, 0.1% Tween-20, 2 mg/ml BSA, 10 mM citric acid, 500 µg heparin) mixed with heat-denatured MSR forward LNA probe (0.035 µM) in the sealed humidified chamber at 60°C for overnight. The post-hybridization washes were done with 2xSSC for 10 min, 2xSSC for 2x30 min and 0.1x SSC for 2x10 min at 66-68°C. Sections were blocked with 4% BSA in the reaction buffer (100 mM Tris-HCl, 150 mM HCl, pH 7.5) at 37°C for 40 min, and incubated with alkaline phosphatase (AP)-conjugated anti-Digoxigenin-AP antibody (1:750, 11093274910, Roche) in 1% BSA in the reaction buffer at 37°C for 60 min. For combined immunostaining, anti-Digoxigenin-AP (1:1000) was incubated with other primary antibodies (**Table 2**) in 1% BSA in PBS overnight at 4°C. After washing, secondary antibody incubations were done for 30 min at RT, the slides were then equilibrated with the detection buffer (100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 8) for the detection of the DIG-labelled probes with the HNPP Fluorescent Detection Set (11758888001, Roche) according to the manufacturer's instructions. Nuclei were stained with DAPI (0.25 mg/ml) and sections were mounted with ProLong Diamond Antifade Mountant (Invitrogen). Images were scanned by Laser scanning confocal microscopy (Zeiss LSM780). Average cytoplasmic intensity of MSR forward transcript was quantified using ImageJ from CTRL and *Dicer1* cKO pachytene spermatocytes (three independent technical replicates of 15-20 cells each) as shown in (II, Fig. S1G).

## 4.15    Chromatin fractionation (II)

The fractionation of chromatin was carried out according to the published protocol (Herrmann et al., 2017) with some modifications. Testicular germ cells were prepared from 18 dpp mouse testes as described above. The cell pellets were resuspended in Buffer A containing (10 mM HEPES pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1xPIC, 1 mM PMSF and 0.3 U/µl RNasin

Plus RNase Inhibitor [Promega]) and incubated for 15 min on ice. Cell suspension was loaded on the top of 0.8 M sucrose and centrifuged at10000 rpm for 15 min. Cytoplasmic and nuclear fractions were collected from the top and bottom of the tube respectively. The bottom nuclear pellet was gently resuspended and washed three times with Buffer A containing 3 mM EDTA and 0.3 mM EGTA. Nuclear pellet was subjected to the chromatin fractionation with increasing salt concentrations (150 mM, 300 mM and 600 mM NaCl) prepared in nuclear Buffer B (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 3 mM $MgCl_2$, 1 mM DTT, 1xPIC, and 0.2 U/µl RNasin Plus RNase Inhibitor) at +4°C for 15 min. Subsequenctly, collected fractions were diluted 5 times with dilution buffer (20 mM HEPES pH 7.4, 0.2 M NaCl, 0.5% Triton X-100, 5% glycerol, 1 mM EDTA, 1 mM EGTA, 3 mM $MgCl_2$, 1 mM DTT, 1xPIC and 0.15 U/µl RNasin Plus RNase Inhibitor) and centrifuged at 10000 rpm for 5 min to collect supernatant. The part of the fractions was subjected to western blotting for detecting the proteins as described below. Remaining part of the fractions was used for isolating the small and long RNA by mirVana miRNA Isolation Kit (Invitrogen) and RNA dot blot was performed as described below.

## 4.16 Co-immunoprecipitation and western blotting (II)

Testicular cell lysate was prepared from 18 days of mouse testes. Testis were decapsulated and incubated with slow rotation in a lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Trition X-100, 0.1% NP-40, 1 mM PMSF, 1xPIC and 1x phosphatase inhibitors cocktail (PhosSTOP, Roche) for 50 min at +4°C. Cell lysate was centrifuged at 10000 rpm for 10 min, and collected top fraction as a supernatant lysate. The supernatant was precleared by incubating with Dynabeads Protein G (Invitrogen) for 1 hour at +4°C. 2-3 µg of antibodies (**Table 2**) were incubated with precleared lysates overnight at +4°C, followed by 2 hours incubation with beads pre-blocked with 5% BSA in PBS. Immunoprecipitated beads complexes were washed first with wash buffer I (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 0.2 % Trition X-100, 0.2% NP-40) and subsequently with wash buffer II (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 0.1 % Trition X-100, 0.1% NP-40). For western blotting, protein complexes were eluted in the Laemmli buffer by heating at 90°C for 10 min then loaded in 4–20% polyacrylamide gel (Mini-PROTEAN, Bio-Rad) to separate proteins, followed by transferring onto PVDF membrane overnight on ice at 60 V. Primary antibodies (**Table 2**) and all secondary antibodies [Anti-rabbit HRP-conjugated, Cell Signaling (7074S), Anti-mouse HRP-conjugated, Cell Signaling (7076S) and Anti-rabbit light chain HRP-conjugated, Millipore (MAB201P)] were used in 1:1000 dilution, and prepared in 4% skimmed milk powder in TBST (0.05% tween) and incubated for 1 hour at RT.

Western lightning ECL Pro (NEL122001EA, PerkinElmer) reagent was used to visualize the signal and image captured by ImageQuant LAS 4000 Biomolecular Imager (GE Healthcare).

## 4.17    Mass spectrometry (II)

18 days mouse testes were used to prepare testicular lysate and DICER was immunoprecipitated as described above. Beads were washed with Tris buffer (pH 8.0), and proteins were digested with trypsin at the Turku Proteomics Facility according to the standard protocol. 0.1% formic acid was added to dissolve the digested peptides and samples were loaded to LC-ESI-MS/MS analysis, on a nanoflow HPLC system (Easy-nLC1200, Thermo Fisher Scientific) coupled to the Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ionization source. MS data were obtained automatically by using Thermo Xcalibur 3.1 software (Thermo Fisher Scientific). Orbitrap MS survey used the information dependent acquisition method and scanned the mass range 300-2000 m/z followed by HCD fragmentation for 10 the most intense peptide ions. Proteome Discoverer 2.2 software (Thermo Fisher Scientific) was used to search data files and for protein identification that was connected to an in-house server running the Mascot 2.6.1 software (Matrix Science). Data was searched against SwissProt database (version 2018_04).

## 4.18    RNA binding and processing assays (II)

Plasmid DNA for mouse MSR DNA (Maj9-2 plasmid) (Lehnertz et al., 2003) was obtained and digested with MssI (PmeI) according to Thermo Scientific instructions. SP6/T7 Transcription Kit (Roche) along with DIG RNA Labeling Mix (Roche) was used to produce DIG-labelled MSR transcripts *in vitro* from linearized plasmid (Maj9-2) at 37°C for 3 hours. DIG-labelled control transcripts were obtained from SP6/T7 Transcription Kit that contained a mixture of pSPT18- and pSPT19-neo-DNA cleaved with EcoRI. After DNase digestion, DIG-labeled RNAs were purified by acid-phenol chloroform (pH 4.5) extraction (Invitrogen), followed by isopropanol precipitation. For MSR transcript binding and processing assay, 5 µg of DIG-labelled MSR and control transcripts were incubated with DICER complexes (immunoprecipitated from 18 dpp mouse testes as described above) in the binding buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$ and 0.3% glycerol) at 35°C for 60 min. For the binding assay, bound RNAs were isolated from washed beads containing immunoprecipitation complexes by using TRIsure (Bioline). Isolated RNA was used either for dot blotting on nylon membrane (Hybond-N+, Amersham Biosciences, Little Chalfont, UK) or run into a 2.75% denaturing

formaldehyde agarose gel in HT buffer (Mansour and Pestov, 2013) and capillary transferred overnight onto a nylon membrane in 10xSSC at +4°C. For the processing assay, total RNA was extracted from whole reaction mixture, loaded into a denaturing 15% polyacrylamide-urea gel in TBE buffer. Semi-dry transfer system (Trans-Blot Turbo, Bio-Rad) was used to transfer the RNA from polyacrylamide-urea gel to the nylon membrane in the 0.5xTBE buffer, 20V for 90 min at +4°C. The crosslinking of the RNA onto the Nylon+ membrane was done using UVP CL-1000 Ultraviolet Cross linker (400 mJ for 40 sec). Membranes were blocked at 4% BSA in Maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5) for 60 min at RT and subsequently incubated with the alkaline phosphatase (AP)-conjugated anti-DIG-AP antibody (1:10000 dilution, 11093274910, Roche) prepared in Maleic acid buffer containing 1% BSA for 30 min. Membranes were washed in Maleic acid buffer with 0.3% Tween 20, equilibrated with DIG detection buffer (0.1 M Tris-HCl, 0.1M NaCl, pH 9.5) and detected by the Chemiluminescent alkaline phosphatase substrate (CSPD ready-to-use, Roche) according to the manufacture instructions. The signals were detected with ImageQuant LAS 4000 Biomolecular Imager (GE Healthcare).

## 4.19    Chromatin immunoprecipitation (II)

Testicular germ cells were prepared from 18 dpp mouse testes as described above. Germ cells were crosslinked in 1% PFA in PBS for 20 min at RT. Crosslinking reaction was stopped by adding 125 mM glycine (Sigma) at RT for 5 min and pelleted by centrifuging at 500 x $g$ for 10 min. ChIP assay was done according to the published protocol (Lin et al., 2012) with minor modifications. Pellets were dissolved in the cytosol lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, 1 mM PMSF, 1xPIC) at 4°C for 5 min. Pelleted nuclei were dissolved in the nuclei lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1x PIC) and snapped frozen twice in liquid nitrogen. Subsequently, diluted 5 times with ChIP dilution buffer (1.1% triton, 0.01% SDS, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris HCl pH 8.0, 1 mM PMSF and 1xPIC), sonicated with the BioRuptor sonicator (Diagenode) to generate 500-700 base pairs of chromatin fragments for 45 min on ice. Sonicated nuclear lysate was centrifuged to 13000 rpm for 10 min to get supernatant and precleared with Dynabeads protein G for 90 min at + 4°C. 2-3µg of ChIP antibodies (**Table 2**) were added in the precleared lysates and rotated overnight at 4°C. Simultaneously, beads were also pre-blocked overnight with a blocking solution containing 5% BSA, 0.6% cold fish gelatin, 0.2µg/ml yeast tRNA, 1.5µg/ml mouse Cot1 DNA, 0.05% Triton X-100, 1 mM PMSF and 1xPIC in PBS. Then, pre-blocked beads were incubated with lysate containing ChIP antibody complexes for 2 hours at RT. ChIP complexes were washed 3 times with low salt wash buffer

(150 mM NaCl, 0.1% SDS, 20 mM Tris–HCl pH 8, 2 mM EDTA, 1% Triton X-100), 3 times with high salt wash buffer (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 20 mM Tris–HCl pH 8, 2 mM EDTA), 3 times with LiCl wash buffer (0.25 M LiCl, 1% IGEPAL CA-630, 10 mM Tris–HCl pH 8, 1 mM EDTA, 1% sodium deoxycholate) and 2 times with TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA). The elution buffer (1% SDS, 0.1 M NaHCO$_3$) was used to elute protein-nucleic acid complexes from the beads. Cross-links were reversed by incubating at 65°C overnight. Subsequently, added 6 µl of 0.5 M EDTA (pH 8), 10 µl of 1 M Tris-HCl (pH 6.5), 6 µl of 20 mg/ml proteinase K and incubating for 60 min at 42°C. TRIsure (Bioline) was added to extract DNA according to the manufacturer's instructions, followed by isopropanol precipitation. ChIP PCR was carried out with the primers listed in **Table 1**.

## 4.20 *In vivo* DICER-RNA immunoprecipitation (II)

Testicular germ cells were prepared from 17-18 dpp mouse testes as described above. Germ cells were crosslinked in 1% PFA in PBS for 20 min at RT. The crosslinking reaction was stopped by adding 125 mM glycine (Sigma) at RT for 5 min and pelleted by centrifuging at 500 x *g* for 10 min. RNA immunoprecipitation assay was done according to the published protocol (protocol PROT28 at https://www.epigenesys.eu/en/) with minor modifications. Pellets were dissolved in lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1xPIC and 0.8 U/µl RNasin Plus RNase Inhibitor) for 60 min on ice. Then, diluted 5 times with the dilution buffer (1.1% triton, 0.01% SDS, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris HCl pH 8.0, 1 mM PMSF and 1x PIC). Lysate was mildly sonicated in ice cold condition using a BioRuptor sonicator (Diagenode) for 10 min and centrifuged at 13000 rpm for 10 min to collect the supernatant. The supernatant was used for the immunoprecipitation (4 µg of Anti-DICER) using the same protocol as for the ChIP (see above). The elution buffer (1% SDS, 0.1 M NaHCO$_3$, and 0.8 U/µl RNase Inhibitor) was used to elute protein-RNA complexes from the beads. Crosslinking between protein-RNA complexes was reversed by incubating in 300 mM NaCl at 65 °C for 2 hours. After adding 6 µl of 0.5 M EDTA (pH 8), 10 µl of 1 M Tris-HCl (pH 6.5), 6 µl of 20 mg/ml proteinase K and 0.25 U/µl RNase Inhibitor, the samples were further incubated for 45 min at 42°C. RNA was extracted from the samples by TRIsure (Bioline) followed by isopropanol precipitation. DNA contamination was removed by treating with Turbo-DNase (Invitrogen). For cDNA synthesis, we used DyNamo cDNA synthesis kit (Finnzymes) along with random hexamers and the M-MLV reverse transcriptase to perform the cDNA synthesis. PCR was carried out with the primers listed in **Table 1**.

## 4.21 Dot blotting and northern blotting (II)

Total RNA was isolated by TRIzol (Invitrogen) and isopropanol precipitation. DNA contamination was removed by treating with Turbo-DNase (Invitrogen). For RNA dot blotting, RNA was directly applied onto the nylon membrane (Hybond-N+, Amersham Biosciences, Little Chalfont, UK) and cross-linked using UVP CL-1000 Ultraviolet Cross linker (400 mJ for 40 sec). Northern blotting was performed separately for long RNA and small RNAs to detect MSR derived RNA transcripts using DIG-labelled forward MSR transcript LNA probes. Northern blotting for long RNAs, total RNA (30 μg) was loaded in 2% denaturing formaldehyde agarose gel prepared in HT buffer (Mansour and Pestov, 2013) and separated RNA was transferred onto nylon membrane by capillary method using 10xSSC at +4°C overnight. Northern blotting for small RNAs, small RNAs were isolated from adult testes using mirVana miRNA Isolation Kit (Invitrogen). Small RNA (40 μg) was loaded in 18% polyacrylamide-urea gel in TBE buffer. Then, separated RNA was transferred onto nylon membrane by semi-dry transfer system (Trans-Blot Turbo, BioRad) using 0.5x TBE buffer (18 V, 3 hours, +4°C), and cross-linked the transferred RNAs onto nylon membrane using UV (400 mJ for 60 sec). Pre-hybridization was done to block the nonspecific interactions by preparing a pre-hybridization mix containing 50% formamide, 2x SSC, 10 mM VRC, 2 mg/ml BSA (New England BioLabs) and 1 μg/ml mouse Cot1-DNA (Invitrogen) at 37°C in a sealed plastic bag for 1 hour. Hybridization was performed in prehybridization mix plus 0.1% Tween-20, 0.25% CHAPS, 10 % dextran sulfate and 0.065 μM of DIG-labelled forward MSR transcript LNA probes denatured by heating at 85°C for 10 min. Hybridization was carried out in the sealed humidified chamber overnight at 38°C for dot blots, at 59°C for long RNA northern blots and at 40°C for small RNA northern blots. The post-hybridization washes were done with 2xSSC for 10 min, 2xSSC for 2x30 min and 0.1x SSC for 2x10 min at 62°C for long RNAs and at 42°C for small RNAs. Membranes were blocked and incubated with anti-Digoxigenin-AP antibody, and DIG detection was performed as described above.

## 4.22 Flow cytometry (I & II)

To evaluate the proportion of haploid (1C), diploid (2C) and tetraploid (4C) cells in adult *Dicer1* KO and control testes, flow cytometric assay (FACS) was done as described earlier (Rotgers et al., 2015) with some modifications. Testes were decapsulated and seminiferous tubules were pulled apart in PBS and transferred to 15-ml Falcon tube containing 10 ml PBS on ice. The seminiferous tubules were digested with Collagenase I solution (0,1 mg/ml [Worthington, USA, #LS004196] in DMEM/F12) by incubating at 37ºC for 5+5 min with mild shaking. Then, 10 ml of PBS was added to inactivate the collagenase activity. After the sedimentation of

tubules on the bottom of the tube, tubules were digested with Trypsin (0.6 mg/ml in PBS; Worthington, USA, #LS003703) plus DNase I (8 μg/ml, Sigma Aldrich, #DN25) at 37ºC for 5 min. 10% FBS was added to inactivate the activity of trypsin. Cells were filtered with 100 μm filter and centrifuged at 600 x g for 5 min at 4ºC, and cell pellets were resuspended in PBS. For the gating strategy, epididymal cells from adult WT mice were used as controls (II, Fig. S7A, B). Single cell suspensions were prepared by dissecting cauda epididymis using mechanical dissociation, followed by enzymatic digestion as described above. Finally, single cells from testes and epididymis were resuspended in buffer containing 0.2% BSA and 5 mg/ml RNase A in PBS and incubated at 37ºC for 15 min. Hoechst 33342 solution (12.5μg/ml, Thermo Fisher, #62249) was added to the samples and incubated at RT for 10 min. Cells were analyzed with LSRFortessa flow cytometer (Becton Dickinson) equipped with a high-throughput sampler (HTS) in a 96-well plate format. Band pass filter for Hoechst 33342 was fixed by setting wavelengths for excitation at 405-nm laser and emission at ranges 450/50-nm. Data were acquired and analyzed with the FlowJo software (FlowJo LLC).

## 4.23   DNA *in situ* hybridization (II)

For DNA *in situ* hybridization, cell suspension was prepared from adult CTRL and *Dicer1* cKO (n=2) testes, cells were pelleted, and cell pellets were resuspended in 75 mM KCl solution for 20 min at 37°C. Subsequently, cells were fixed in the fixing solution (1% PFA and 0.15% trition-X-100 in PBS, pH 9.2). Fixed cells were spread on slides and incubated overnight in a humidified chamber at RT. The fixed slides were air-dried, washed with 0.4% Photo-Flo (Kodak) and air-dried again. Mouse Aneuploidy Kit (FMAC-01, Creative Bioarray) was used to detect X and Y chromosomes aneuploidy in *Dicer1* cKO testes. DNA *in situ* hybridization was carried out using probes detecting X and Y chromosomes according to the manufacturer's instructions. Slides were stained with DAPI (Sigma-Aldrich, 0.25 mg/ml) for 10 min at RT. Finally, slides were mounted with ProLong Diamond Antifade Mountant (Invitrogen) and images were captured by 3i spinning disk confocal microscopy. Round spermatids were identified based on their size and a typical heterochromatin pattern visualized by DAPI staining. The Y and X chromosome signals were manually calculated and quantified from at least 500 round spermatids per mouse.

## 4.24   Statistical analyses (I & II)

All data are presented as mean values ± SEM. Statistical significances between groups were determined using two-tailed t-test. P-values < 0.05 were considered to be statistically significant.

## 4.25 Primary antibodies used in the study (I & II)

The primary antibodies used in the study are listed in the Table 4. The specificity of the DICER antibody has been validated by immunofluorescence and western blotting using Dicer1 cKO testes (Korhonen et al., 2015).

**Table 4.** Primary antibodies used in the study I & II.

| Antibodies | Company (Cat. No.) | Applications |
|---|---|---|
| CENP-A (rabbit) | Santa Cruz (sc-22814) | IF (1:1000) |
| GATA4 (rabbit) | Santa Cruz (sc-9053) | IF (1:50) |
| phosphorylated histone H3 Ser10 (rabbit) | Millipore (06-570) | IF (1:100) |
| SCP3 (rabbit) | Santa Cruz (sc-33195) | IF (1:100) |
| PRM1 (rabbit) | Santa Cruz (sc-30174) | IF (1:100) |
| αTubulin (mouse) | Thermo Scientific | IF (1:1000) |
| dimethylated histone H3 Lys9 (mouse) | Millipore | IF (1:500) |
| Cre (mouse) | Covance | IF (1:100) |
| CBX1/HP1β (mouse) | Abcam | IF (1:100) |
| acetylated histone H3 Lys9 (mouse) | Millipore | IF (1:500) |
| GFP (rabbit) | Invitrogen (A11122) | IF |
| DDX4/MVH (rabbit) | From T. Noce | IF (1:1000) |
| AKAP4 (mouse) | BD Biosciences | IF (1:200) |
| H1T2 (mouse) | From I. Davidson | IF (1:500) |
| DICER (rabbit) | Sigma (SAB4200087) | IF (1:100), WB (1:200) |
| DICER (rabbit) | Bethyl Laboratories, Inc. (A301-936A) | IP, ChIP |
| SUV39H2 (rabbit) | Abcam (EPR18495) | ChIP |
| SUV39H2 (rabbit) | Proteintech Group, Inc. (11338-1-AP) | IP, WB (1:500) |
| SETDB1 (rabbit) | Proteintech Group, Inc. (11231-1-AP) | IP, WB (1:500) |
| HP1 beta (mouse) | Millipore (MAB3448) | IF (1:25) |
| H3K9me3 (rabbit) | Millipore (07-442) | IF (1:100), ChIP |
| MILI/PIWIL2 (mouse) | Sigma (MABE363) | IF (1:300) |
| TDRD1 (rat) | R&D Systems (MAB6296) | IF (1:200) |
| DDX25 (goat) | Santa Cruz Biotechnology (sc-51271) | IF (1:100) |
| phospho-Histone H2A.X (Ser139) (mouse) | Millipore (05-636) | IF (1:500) |
| phospho RNA Polymerase II (S2) (rabbit) | Bethyl Laboratories, Inc. (A300-654A) | WB (1:500) |
| Beta-TUBULIN (rabbit) | Cell Signaling (2128S) | WB (1:1000) |
| GAPDH (mouse) | Hytest Ltd. (5G4-6C5) | WB (1:3000) |
| Cytochrome C (mouse) | BD Biosciences (556433) | IF (1:300) |
| DDX4 (rabbit) | Abcam (ab13840) | WB (1:500) |
| YY1 (rabbit) | Bethyl Laboratories, Inc. (A302-779A-M) | WB (1:500) |
| Histone H3 (rabbit) | Cell Signaling (4499S) | WB (1:300) |
| TRIM33 (rabbit) | Bethyl Laboratories, Inc. (A301-060A-M) | WB (1:500), IP, IF (1:50) |
| TRIM28 (rabbit) | Bethyl Laboratories, Inc. (A304-145A-M) | IP |
| TRIM28 (rabbit) | Bethyl Laboratories, Inc. (A300-274A-M) | WB (1:500), IF (1:100) |
| Negative control IgG (mouse) | Santa Cruz Biotechnology (sc-2025) | IP,ChIP |
| Negative control IgG (rabbit) | Neomarkers (NC100-P1) | IP, ChIP |

# 5 Results

## 5.1 DICER is required for male fertility (I)

To investigate the role of DICER in mouse spermatogenesis, we generated a conditional *Dicer1* cKO mouse model. The deletion of *Dicer1* was induced by *Ngn3* promoter-driven Cre transgene expression. *Ngn3* promoter is activated at 5 dpp in the male germline, therefore leading to Cre recombinase-mediated deletion of exon 24 containing the second RNase III domain of the *Dicer1* gene in undifferentiated spermatogonia (I, Fig. 1B). The efficiency of Cre-recombination was evaluated by crossing the transgenic mice with the reporter mice expressing the transgenic ROSA26YFP gene. The Cre-mediated removal of the stop codon in the transgenic ROSA26YFP gene lead to a wide expression of YFP in all differentiating male germ cells of the seminiferous epithelium (I, Fig. 1D). Genomic PCR using primers located on both sides of the deleted region validated the deletion of the *Dicer1* gene (I, Fig.1E). RT-PCR also showed dramatically reduced *Dicer1* mRNA levels in *Dicer1* cKO testis as compared to the control (I, Fig. 1F).

The phenotypic analysis of *Dicer1* cKO mice revealed that DICER is essential for normal spermatogenesis and male fertility. *Dicer1* cKO mice were completely infertile. The testis size was reduced compared to the control littermates (I, Fig. 2A), and histological analysis revealed abnormal spermatogenesis (I, Fig. 2B) with major problems in haploid differentiation. These included the defective morphology of elongating spermatids and their abnormal organization in the seminiferous epithelium. The number of haploid cells were reduced in *Dicer1* cKO testes with respect to control (I, Fig. 2C), and we also observed an increased number of apoptotic spermatocytes (I, Fig. 2D). In addition, sperm counting revealed a drastic reduction in the numbers of mature spermatozoa in the cauda epididymis, illustrating the defective spermatogenic processes in *Dicer1* cKO mice (I, Fig. 2E).

## 5.2 Elongation of spermatids is disrupted in *Dicer1* cKO mice (I)

Defective spermiogenesis in the knockout testis pushed us further to analyse the progress of haploid cell differentiation. Morphological analysis revealed severe

defects in *Dicer1* cKO elongating spermatids, such as abnormal head shapes and problems in chromatin condensation, as well as disrupted organization of tail accessory structures (I, Fig. 4A). To study the problems in chromatin condensation, we first analyzed histone hyperacetylation, which appears around step 9–11 of spermatid differentiation prior to chromatin condensation, and disappears later when histones are replaced with protamines (Hazzouri et al., 2000). Histone hyperacetylation appeared normally in early elongating spermatids in the knockout testes, but interestingly, hyperacetylation was retained in the subsequent stages that should have undergone histone-protamine transition (I, Fig. 4B). Furthermore, protamine staining revealed that the protamine incorporation was greatly reduced in knockout elongating spermatids as compared to controls. This clearly indicates the defects in spermatid elongation before histone-protamine transition (I, Fig. 4C).

To clarify the reason for the abnormal head shape of elongating spermatids, we studied the localization of some proteins responsible for chromatin architecture. A testis-specific histone H1 variant, H1T2/H1FNT, appears as a cap-like structure right beneath the nuclear membrane at the apical pole in the wild type round and elongating spermatids (Martianov et al., 2005). Interestingly, bipolar localization pattern was observed at both the apical and basal side of the nucleus in the absence of DICER (I, Fig. 5A), indicating defects in cell polarization. Manchette is a microbular stucture involved in the shaping of sperm head (Lehti and Sironen, 2016; Kierszenbaum and Tres, 2004), which is located in an organized fashion on the basal side of wild type nuclei. Intrestingly, the manchette appeared disorganized in knockout elongating spermatids, suggesting that the defective head shaping may originate from defective structural organization of elongating spermatids (I, Fig. 5B). Disruption of microtubule organization was also confirmed with electron microscopy in late elongating spermatids (I, Fig. 5C).

## 5.3 Major satellite repeat (MSR) expression is elevated in the absence of DICER in male germ cells (I & II)

### 5.3.1 MSRs are transcribed during spermatogenesis (II)

Recently, transcriptional dynamics of pericentric heterochromatin have been reported during early mouse development (Probst et al., 2010), which prompted us to analyze MSR expression during mouse spermatogenesis. Pericentric heterochromatin that is composed of MSRs has a distinct dynamic organization during spermatogenesis. After visualizing pericentric heterochromatin by immunofluorescence with anti-H3K9me3 antibody, we observed that the H3K9me3 signal was enriched in distinct nuclear foci in spermatogonia and spermatocytes, and

the signal was shown to concentrate on one single chromocenter in round spermatids and finally disappear in condensing elongating spermatids (II, Fig. 1A). We used semi-quantitative RT-PCR to detect MSR transcripts originating from the pericentric heterochromatin. Due to the repetitive nature of MSRs, RT-PCR results in different-sized products depending on how many repeats are amplified (II, Fig. 1B, S1A). Expression analysis was performed on juvenile mouse testes collected at different time points corresponding to the appearance of specific types of differentiating germ cells during the first wave of spermatogenesis (II, Fig. 1C). We were able to detect MSR transcripts in all time points. Interestingly, the MSR expression peaked at time points 14-18 dpp corresponding to the appearance of pachytene spermatocytes (II, Fig. 1C, D), which suggests that a high level of MSR transcripts is transcribed in these cells. Motivated by the reported strand-specific expression dynamics of MSRs during early mouse development (Probst et al., 2010), we also used strand-specific primers for RT-PCR to separately detect transcripts originating from the forward or reverse strands (II, Fig. 1E). We observed differential expression patterns for forward and reverse strands during spermatogenesis, and only forward strand transcripts peaked at 18 dpp whereas reverse strand transcripts were slightly elevated in early meiotic cells (II, Fig. 1F, G).

## 5.3.2 MSR transcripts are elevated in *Dicer1* cKO testes (I & II)

DICER has a well-characterized role in the posttranscriptional regulation of mRNAs through its function in the processing of miRNAs (Kotaja, 2014b; Yadav and Kotaja, 2014b). However, it has also been reported to have non-cannonical miRNA-independent fucntions (Pong and Gullerova, 2018), and we were particularly interested in studying if transposable elements and satellite elements are affected in *Dicer1* cKO testes. We screened the expression of transposable elements such as LINE1 (Long Interspersed Nuclear Element 1), SINEB1 (Short Interspersed Nuclear Element 1), SINEB2 (Short Interspersed Nuclear Element 2), IAP (Intracisternal A-Particle) and satellite repeats in knockout testes as compared to control. We did not find any significant differences in the expression of transposable elements in *Dicer1* cKO testes (I, Fig. 7A), but interestingly, we did observe a clear induction of pericentric MSR transcripts (I, Fig. 7B). To study this enchanting finding in more detail, we performed a detail analysis of MSR expression during the first wave of spermatogenesis in *Dicer1* cKO mice. Interestingly, we observed a relatively higher level of MSR transcripts at the 18 dpp time point in the *Dicer1* cKO testes as compared to the controls (II, S1B, C). 18 dpp testes are enriched with pachytene spermatocytes, suggesting that the elevated level of MSR transcripts in *Dicer1* cKO testes at this time point could originate from defects in these cell types.

### 5.3.3 MSR transcripts are elevated in *Dicer1* cKO spermatocytes and round spermatids (II)

To study if MSR transcript expression is affected in pachytene spermatocytes in the absence of DICER, we isolated enriched fractions of pachytene spermatocytes and round spermatids (>85% pure) by centrifugal elutriation (Barchi et al., 2009b) from both control and *Dicer1* cKO adult mouse testes (II, Fig. S1D). Then, we quantified the MSR expression in isolated cells by RT-PCR. As expected, MSR transcripts were readily detected in both spermatocytes and round spermatids (II, Fig. 2A, B). Moreover, MSR transcripts were highly elevated in *Dicer1* cKO pachytene spermatocytes. We also quantified the strand-specific expression of MSRs in spermatocytes, and interestingly, only forward strand derived MSR transcripts were elevated in *Dicer1* cKO pachytene spermatocytes, while reverse strand-derived MSR transcripts were not affected (II, Fig. 2C, D). The induction of forward strand-derived MSR transcript was also validated during the first wave of spermatogenesis in *Dicer1* cKO testes (II, Fig. S1E, F). This data clearly shows that the deletion of *Dicer1* specifically affects the levels of the forward MSR transcripts in meiotic spermatocytes.

## 5.4 DICER binds and processes MSR transcripts in the mouse testis (II)

Due to the aberrant induction of MSR transcripts in the *Dicer1* cKO testis, we wanted to study if this induction is mediated by direct action of DICER in the post-transcriptional processing of MSR transcripts. We first studied whether DICER directly binds and processes MSR transcripts *in vitro* by performing a binding and processing assay with DICER complexes immunoprecipitated from testes and *in vitro* transcribed MSR transcripts. Interestingly, we found that DICER complexes bound and processed MSR transcripts to produce small RNAs (II, Fig. 3A, B). The randomly selected control transcript (*Neomycin* mRNA) was similarly processed, suggesting the broad substrate specificity. To verify the *in vitro* results *in vivo*, we first carried out an anti-DICER-RNA immunoprecipitation assay from testes. Importantly, MSR transcripts but not control transcripts (*L19* and *Ppia*) were immunoprecipitated with DICER, thus confirming a specific association of DICER with MSR transcripts *in vivo* (II, Fig. 3C). To study if the processing of MSR transcripts is affected in the absence of testicular DICER, we performed the northern blot analysis of control and *Dicer1* cKO testicular RNAs. Interestingly, we revealed that in addition to the normal MSR transcript products sized around 300-800 nucleotides, *Dicer1* cKO testes contained additionally longer product that was not detected in the control testes (II, Fig. 3D). Furthermore, we found a significant decrease in forward MSR transcript derived small RNAs (II, Fig. 3E). These results

indicate that the processing of forward MSR transcripts is defective in *Dicer1* cKO testes, therefore, suggesting a direct role for DICER in the post-transcriptional regulation of MSR expression.

## 5.5 DICER and MSR forward transcripts associate with chromatin (II)

In order to understand the DICER-mediated control of MSR expression, we explored the subcellular localization of DICER and MSR transcripts in more detail. First, we carried out *in situ* hybridization on testis sections using a probe specifically detecting the forward MSR transcript that was shown to be upregulated in *Dicer1* cKO germ cells. We detected a prominent cytoplasmic signal as well as some distinct foci inside the nucleus in late pachytene spermatocytes (II, Fig. 4A). The disappearance of the signal after RNase treatment confirmed that the signal was derived from RNA. A scramble LNA probe was used as negative control (II, Fig. 4B). In line with the RT-PCR results, we detected relatively stronger cytoplasmic MSR forward transcript signal in the *Dicer1* cKO late pachytene spermatocytes as compared to the control (II, Fig. 4C, S1G). Immunofluorescence analysis was carried out to study DICER localization during spermatogenesis. As expected, most of the DICER signal was observed in the cytoplasm of differentiating male germ cells. The highest expression of DICER was found in pachytene spermatocytes, *i.e.* the same cell type where the expression of forward MSR transcripts also peaked (II, Fig. S2). Interestingly, we showed that the cytoplasmic DICER co-localized with PIWIL2/MILI (Piwi-like protein 2) and TDRD1 (Tudor domain-containing protein 1) in pachytene spermatocytes (II, Fig. S3), indicating that DICER localize to a specific germ granule, intermitochondrial cement (IMC) that is involved in the processing piRNAs (Meikar et al., 2011). In addition to the cytoplasmic localization, we found nuclear DICER-positive foci in late pachytene spermatocytes at stages VII to X of the seminiferous epithelial cycle (II, Fig. 5A, Fig. S2A). These DICER-positive nuclear foci appeared in close association with DAPI-bright and HP1-positive heterochromatin areas (II, Fig. 5A, B, Video S1, S2). The existence of DICER in the nuclear compartment was further confirmed by nuclear-cytoplasmic fractionation (II, Fig. S2B). Interestingly, *in situ* hybridization using a probe for forward MSR transcript combined with immunofluorescence using an anti-DICER antibody showed that both nuclear and cytoplasmic DICER partially overlapped with MSR transcript signal (II, Fig. 5C), further supporting the association of DICER with MSR transcripts *in vivo* that was also shown by DICER-RNA immunoprecipitation (II, Fig. 3C).

Nuclear localization of DICER and MSR transcripts prompted us to study if they associate with chromatin in the testis. To this end, we executed chromatin

fractionation from 18 dpp testicular cells by increasing concentrations of NaCl and separated long (>200 nt) RNAs and small RNAs (<200 nt) from the cytoplasmic and chromatin fractions. RNA dot blotting was performed with DIG-labeled MSR forward probe and scramble LNA probe as negative control. Long MSR forward transcripts were found in both cytoplasmic and chromatin fractions whereas MSR forward transcript-derived small RNA were enriched in the chromatin fraction (II, Fig. 4D), implying that these small RNAs produced from MSR forward transcripts mainly localize to the chromatin. In addition, we showed that DICER was also present in the chromatin fraction by immunoblotting of the same subcellular fractions (II, Fig. 4F). Interestingly, DICER signal was also detected when chromatin complexes were eluted with the highest NaCl concentration (600 mM), suggesting a tight association of DICER with chromatin along with MSR forward transcripts in male germ cells.

## 5.6 DICER targets pericentric heterochromatin in male germ cells (I & II)

### 5.6.1 DICER associates with pericentric heterochromatin and heterochromatin regulators (II)

The association of DICER with the chromatin fraction pushed us further to explore the chromosomal regions that are targeted by DICER. We performed chromatin immunoprecipitation using an anti-DICER antibody followed by PCR using primers to specifically detect different repetitive regions in the genome. Strikingly, we found that DICER interacted only with MSR regions but not with other genomic regions such as minor satellite repeats and transposons LINE1, SINEB1, SINEB2 and IAP (II, Fig. 6A), which is in line with our earlier RT-PCR results that did not show any significant differences in the expression of these transcripts in *Dicer1* cKO as compared to control testes (I, Fig. 7A).

We then wanted to clarify how DICER could be recruited to the pericentric heterochromatin. To identify potential interaction partners that could function in the recruitment of DICER into the pericentric heterochromatin, we performed DICER immunoprecipitation from testes followed by mass spectrometric analysis. In addition to several cytoplasmic interaction partners, we identified some proteins with known nuclear localization (Table S1), such as KHDRBS1/SAM68 (KH domain-containing, RNA-binding, signal transduction-associated protein 1), snRNP200 (U5 small nuclear ribonucleoprotein 200 kDa helicase), hnRNP-M (Heterogeneous nuclear ribonucleoprotein M), PSPC1 (Paraspeckle component 1), SFPQ (Splicing factor, proline- and glutamine-rich), TRIM28 (Tripartite motif-containing 28) and TRIM33 (Tripartite motif-containing 33). Remarkably, TRIM28 and TRIM33 act as

scaffolding proteins to recruit a variety of epigenetic modifiers to chromatin, including histone methyltransferases that are responsible for trimethylation of histone H3 at lysine 9 (Rivero-Hinojosa et al., 2017; Leseva et al., 2016; Iyengar and Farnham, 2011; Xi et al., 2011; Briers et al., 2009; Jang et al., 2018). We validated the interaction of DICER with TRIM28 and TRIM33 by co-immunoprecipitations followed by western blotting (II, Fig. 6B). We also confirmed the nuclear localization of TRIM28 and TRIM33 in late pachytene spermatocytes by immunofluorescence analysis (II, Fig. S5A). Furthermore, co-immunoprecipitation experiments revealed that DICER also interacts with H3K9 methyltransferases SUV39H2 (Suppressor of variegation 3-9 homolog 2) and SETDB1 in the mouse testis (II, Fig. 6C). Altogether, this data shows that DICER associates with pericentric heterochromatin, and suggests that the association is mediated by the interaction with heterochromatin regulators.

## 5.6.2 Epigenetic imbalance at pericentric heterochromatin in *Dicer1* cKO testis (I & II)

Next, we wanted to understand if defective MSR transcript processing has any consequences on heterochromatin organization and epigenetic status of pericentric heterochromatin in *Dicer1* cKO testis. We first carried out immunofluorescence to study the localization of heterochromatin proteins and epigenetics marks such as HP1 beta, H3K9me3, H3K9me2 and centromeric protein CENP-A in *Dicer1* cKO testis. We do not find any obvious changes in their localization patterns in adult *Dicer1* cKO testes (I, Fig. S5; II, Fig. 6D, S5B, C). In contrast, chromatin immunoprecipitation using specific antibodies followed by PCR with MSR region-specific primers showed a significantly reduced association of SUV39H2 with MSR chromatin in adult *Dicer1* cKO testes (II, Fig. 6E, F). Moreover, the level of H3K9me3 also appeared reduced in the MSR chromatin in adult *Dicer1* cKO testes, although this difference did not reach the statistical significance (II, Fig. 6E, F). In conclusion, the lack of DICER-dependent activities appears to imbalance the epigenetic status of pericentric heterochromatin by reducing the recruitment of SUV39H2 and the level of H3K9me3.

## 5.7 Meiotic chromosome segregation is defective in *Dicer1* KO mice (I & II)

### 5.7.1 Meiotic progression in *Dicer1* cKO spermatocytes (I)

The implication of centromeric and pericentric regions in the chromosome segregation during cell division (Probst and Almouzni, 2008; Hall et al., 2012;

Jagannathan and Yamashita, 2017) and misregulation of pericentric heterochromatic expression in the *Dicer1* cKO spermatocytes prompted us to analyze meiotic progression in more detail. During the original phenotypic analysis, we studied the synaptonemal complexes formation in the *Dicer1* cKO pachytene spermatocytes by staining with an anti-SCP3 (Synaptonemal complex protein 3) antibody combined with anti-phosphorylated histone γH2AX antibody in the mouse testis. SCP3 is one of the major components of synaptonemal complexes (Bisig et al., 2012) and phosphorylated histone γH2AX is a marker to visualize unsynapsed X and Y chromosomes in the sex body of pachytene spermatocytes (de Vries et al., 2012; Fernandez-Capetillo et al., 2003). The synaptonemal complex and sex body formation were detected normally in the *Dicer1* knockout testes as compared to the control (I, Fig. 3A). Furthermore, we confirmed this finding by detecting synaptonemal complexes with electron microscopy of *Dicer1* knockout testes (I, Fig. S4). Furthermore, we studied the meiotic metaphase plates in the knockout tubules by immunostaining with anti-phosphorylated Serine 10 of histone H3 (H3S10p) combined with anti-tubulin. H3S10p is a marker of mitotic and meiotic chromatin condensation (Huang et al., 2006; Nowak and Corces, 2004) and tubulin is used to visualize meiotic spindles and general organization of microtubular network in the seminiferous epithelium. We found that meiotic metaphases were normally present at stage XII of the seminiferous epithelial cycle in the *Dicer1* knockout tubules (I, Fig. 3B). Thus, based on these results, we concluded that meiotic progression proceeds normally without any gross abnormalities during *Dicer1* knockout spermatogenesis.

## 5.7.2    Chromosome missegregation in *Dicer1* cKO spermatocytes (II)

Our original analysis revealed that meiotic divisions take place in the absence of DICER and haploid round spermatids are produced. However, when we took a closer look at the appearance of *Dicer1* cKO early haploid round spermatids right after the meiotic division (stage I-II), we observed more unevenly sized round spermatid nuclei in *Dicer1* cKO testis compared to the control at the same stage (II, Fig. 7A). Therefore, we further wanted to analyze the haploid cell population in *Dicer1* cKO testes by using flow cytometry. In line with the observed histological phenotype, we noticed that elongating spermatids were virtually absent from *Dicer1* cKO testes (II, Fig. 7B, S6). More detailed analysis of haploid round spermatids on the basis of their DNA staining by Hoechst 33342 revealed that their staining intensity had shifted slightly but significantly, indicating a higher DNA content in *Dicer1* cKO round spermatids as compared to the control (II, Fig. 7C, D). Consequently, this finding pushed us further to investigate possible meiotic chromosome mis-segregation and

aneuploidy in *Dicer1* cKO testis. We carried out DNA *in situ* hybridization with sex chromosomes specific to X and Y probes. In the control, haploid cells contained either X or Y chromosome, as expected. Interestingly, we observed a significant higher number of round spermatids with abnormal number of X and Y signals in *Dicer1* cKO round spermatids, illustrating elevated level of meiotic chromosome mis-segregation leading to aneuploidy in *Dicer1* cKO spermatids (II, Fig. 7E, F). Thus, our results show that the induction of MSR expression in *Dicer1* cKO spermatocytes is accompanied by clear defects in meiotic chromosome segregation.

# 6    Discussion

Male germ cell differentiation includes a broad range of specific processes, such as chromosome pairing, synaptonemal complex formation, and homologous recombination in meiotic cells and histone-protamine transition and chromosome condensation in haploid male germ cells. These events are accompanied by dynamic changes in gene expression that have to be strictly controlled to facilitate the production of fertile spermatozoa (Soumillon et al., 2013) (Griswold, 1995; O'Donnell et al., 2006). This study provided important novel insight into the field by exploring specific RNA regulatory mechanisms that operate to control the complex process of spermatogenesis.

DICER is an endoribonuclease that produces small non-coding RNAs such as miRNAs, and therefore, participates in the regulation of a large number of mRNAs (Rybak-Wolf et al., 2014; Winter et al., 2009; Davis and Hata, 2010; MacRae, 2006; Song et al., 2011). DICER is ubiquitously expressed in a variety of cell types and deletion of *Dicer1* alters the global processing of small RNAs (Hobert, 2005). Therefore, conditional *Dicer1* cKO mouse models are required to elucidate the function of DICER specifically in certain cell types (Kawase-Koga et al., 2010; Maatouk et al., 2008; Harfe et al., 2005; Bernstein et al., 2003; Wienholds et al., 2003; Murchison et al., 2007, 2005a). We studied the role of DICER during male germ cell differentiation by generating *Dicer1* cKO mouse model, where the *Dicer1* gene was deleted in early postnatal spermatogonia. Interestingly, we found that mice were infertile with dramatically reduced sperm counts and major problems in haploid differentiation. The most prominent defects included abnormal nuclear shaping and chromatin condensation during the late stages of spermatogenesis (Korhonen et al., 2011, 2015). These results revealed a vital role of DICER-dependent pathways in the posttranscriptional regulation of gene expression during male germ cell differentation.

In addition to miRNA-dependent functions, DICER has also non-canonical functions, and it can directly bind and process many different kinds of substrates (Pong and Gullerova, 2018). In this study, we specifically focused on the characterization of these non-canonical functions of DICER. In other cellular systems, DICER has been implicated in the regulation of transposon expression.

DICER was shown to control the levels of *Alu*-like B1 and B2 RNAs in mouse retinal pigmented epithelium (RPE) cells (Kaneko et al., 2011b). Furthermore, the deletion of *Dicer1* in mouse oocytes (Murchison et al., 2007) or in prospermatogonia (Romero et al., 2011b) induced aberrant expression of transposons. Interestingly, we did not find any significant changes in the expression of transposable elements in *Dicer1* cKO testes, suggesting tissue or developmental stage-specific target preferences for DICER. In contrast, we found that the level of MSR transcripts originating from the pericentric heterochromatin was increased in *Dicer1* cKO mice as compared to controls. This lets us study in detail the expression of pericentric heterochromatin and possible mechanisms behind aberrant levels of MSRs in *Dicer1* cKO male germ cells. The deletion of *Dicer1* in mouse embryonic stem cells (Kanellopoulou et al., 2005) and chicken-human hybrid DT40 cell lines (Fukagawa et al., 2004b) have previously been shown to induce the expression of centromeric satellite repeat transcripts, suggesting the involvement of DICER in the maintenance and silencing of centromeric heterochromatin (Kanellopoulou et al., 2005).

## 6.1 Effects of *Dicer1* deletion on the expression of pericentric heterochromatin during spermatogenesis

Pericentric heterochromatin has a distinct chromocenter organization and its dynamic reorganization is a prerequisite for normal cellular differentiation early development (Probst et al., 2010; Probst and Almouzni, 2008) and the function of primordial germ cells (Hajkova et al., 2008; Probst and Almouzni, 2008; Guenatri et al., 2004). Pericentric heterochromatin has a typical highly compacted, inaccessible architecture that suggests transcriptional inactivity. However, pericentric heterochromatin is known to be transcribed for example during cellular stress, cell cycle, cellular differentiation, early development and in cancer cells (Ferreira et al., 2015; Probst and Almouzni, 2008; Eymery et al., 2009b; Briers et al., 2009). We showed for the first time that pericentric heterochromatin is also expressed during spermatogenesis and, moreover, the forward and reverse strands showed differential expression dynamics, suggesting autonomous mechanisms to regulate their expression. Forward strand-specific (MSR) transcripts were found to be highly expressed in the late meiotic phase, whereas the reverse strand was more equally expressed at all-time points, with a slightly higher level found in the early meiotic phase of spermatogenesis.

Differential expression of forward and reverse MSR strands has also been observed in specific mouse tissues and human cells (Eymery et al., 2009a; Rudert et al., 1995), and particularly, during early mouse development (Probst et al., 2010). In early embryos, the forward strand was shown to be expressed in a parent of origin

manner at the early two-cell stage of development (Probst et al., 2010), emanating only from the paternal chromosome. In contrast, the reverse strand transcription burst towards the end of the two-cell stage when chromocenters have just formed prior to the second mitotic division, appearing from both maternal and paternal chromosomes (Probst et al., 2010). Interestingly, while the forward MSR transcripts were localized to the nucleus and the cytoplasm in early mouse embryos, the reverse MSR transcripts were confined within the nucleus, demonstrating that the nuclear retention is also strand-specific (Probst et al., 2010). In our study, we also localized the forward strand transcripts both in the nucleus and cytoplasm, which is in line with its localization in early mouse embryos.

Interestingly, we found that only forward MSR transcripts, but not reverse strand transcripts, were induced in *Dicer1* cKO germ cells. A relatively stronger signal of cytoplasmic MSR forward transcripts was also detected in the *Dicer1* cKO late pachytene spermatocytes by *in situ* hybridization. This finding suggests that DICER targets selectively forward MSR transcripts by yet unknown mechanisms. Previously, selective misregulation of forward MSR transcripts has been demonstrated in cells deficient in a chromatin remodeling protein LSH (Huang, 2004), indicating that reducing the transcripts derived from one strand does not affect the levels of other complementary strand transcripts (Casanova et al., 2013a; Probst et al., 2010). This justifies the existence of independent mechanisms for the regulation of the forward strand and its transcripts *in vivo*.

Our detailed analysis of the first wave of spermatogenesis revealed that the most profound induction of MSR expression in *Dicer1* cKO testes takes place at time points (14-18 dpp) corresponding to the appearance of meiotic pachytene spermatocytes in juvenile testes. Further, using an enriched population of meiotic spermatocytes we confirmed that these cells have aberrantly elevated levels of MSR transcripts, indicating that DICER operates in spermatocytes to regulate MSR transcript levels. While the MSR expression was induced at 14-18 dpp during the first wave of spermatogenesis, we found that the transcript levels were normally downregulated at 20 dpp time point, corresponding to the appearance of haploid round spermatids. This implies that separate mechanisms operate in spermatocytes and round spermatids to control MSR transcripts, and the one active in meiotic cells is dependent on DICER.

## 6.2　　Direct role of DICER in the regulation of pericentric heterochromatin expression

MSR transcript binding and processing assays confirmed that DICER-containing complexes can directly bind to MSR transcripts and process them into small RNAs. Very importantly, we also demonstrated that DICER associates with MSR

transcripts *in vivo* in the testis, and we revealed accumulation of longer MSR forward transcripts and defective production of small RNAs derived from MSR transcripts in *Dicer1* cKO testes. These findings strongly suggest that DICER forms complexes with MSR transcripts *in vivo* and is involved in their posttranscriptional processing. One potential player that could be involved in the function of DICER in MSR processing is WDHD1. The knockdown of *Wdhd1* in NIH-3T3 mouse fibroblasts cells resulted in accumulation of satellite transcripts and the reduction of small RNAs derived from them similar to the *Dicer1* cKO testes, and authors suggest the interplay between WDHD1 and DICER in these cells (Hsieh et al., 2011b). It remains to be characterized if WDHD1 and DICER could also co-operate in male germ cells.

Our detailed analysis of subcellular localization of DICER and MSR transcripts revealed that they both were prominently found in the cytoplasm, but also in distinct nuclear foci in pachytene spermatocytes. In mammals, DICER is known to be mainly localized to the cytoplasm, where its main function in miRNA processing takes place. This is in line with the prominent cytoplasmic localization detected in male germ cells in this study. A previous study that characterized the mouse model expressing HA-tagged DICER suggested that the localization of DICER is restricted to the cytoplasm during mouse spermatogenesis (Much et al., 2016). However, as validated by several different methods, we clearly detected the endogenous DICER also in the nucleus of meiotic cells.

In lower organisms, nuclear DICER and DICER-dependent small RNAs are known to be involved in the maintenance of heterochromatin (Djupedal and Ekwall, 2009; Reyes-Turcu and Grewal, 2012). Chromatin-associated DICER has also been detected under various physiological contexts in the mammalian system (Sinkkonen et al., 2010; Burger and Gullerova, 2018; Ando et al., 2011; Gagnon et al., 2014; Skourti-Stathaki et al., 2014; Cheng et al., 2017). Because of the emerging evidence on chromatin-associated functions of DICER, and because we observed DICER-positive nuclear foci to be located in very close vicinity of heterochromatin areas, we were very interested in further characterize the chromatin localization of DICER and MSR transcripts. We indeed validated their association with the chromatin fraction, and MSR forward transcript-derived small RNA even appeared to be enriched in the chromatin fraction compared to other cellular compartments. Co-localization analysis demonstrated that both cytoplasmic and nuclear signals of DICER were partially ovelapped and with MSR forward transcripts in spermatocytes. Finally, chromatin immunoprecipitation revealed that DICER specifically interacts with MSR regions but not with other repetitive genomic regions such as minor satellite repeats and transposable elements (LINE1, SINEB1, SINEB2 and IAP). This data provides evidence that the nuclear DICER could regulate pericentric heterochromatin directly at the site of MSR transcription.

One important question is by which mechanisms DICER is recruited to the pericentric heterochromatin. In this study, we managed to uncover some of the molecular components potentially included in the heterochromatin-associated DICER complexes. In mass spectrometric analysis, we found several intriguing DICER-interacting proteins such as TRIM28 and TRIM33 that can act as scaffolding proteins to recruit a variety of epigenetic modifiers to the chromatin (Rivero-Hinojosa et al., 2017; Leseva et al., 2016; Iyengar and Farnham, 2011; Xi et al., 2011; Briers et al., 2009; Jang et al., 2018). These modifiers include histone methyltransferases that are responsible for trimethylation of histone H3 at lysine 9, epigenetic modification that is considered as a hallmark of heterochromatin (Schotta et al., 2004; Loyola et al., 2009). Furthermore, co-immunoprecipitation experiments revealed that DICER interacts with H3K9 methyltransferases complexes such as SUV39H2 (Suppressor of variegation 3-9 homolog 2) and SETDB1 in the mouse testis. Therefore, the recruitment of DICER to the pericentric heterochromatin could be mediated via specific association with these heterochromatin regulators. Interestingly, although the general organization of pericentric heterochromatin, as detected by the localization of HP1β, H3K9me3, H3K9me2 and centromeric protein CENP-A, were not affected in the absence of DICER, we found reduced association of SUV39H2 and reduced level of H3K9me3 at the MSR chromatin in *Dicer1* cKO testes. This finding indicates that the deletion of *Dicer1* compromises the epigenetic status of pericentric heterochromatin, thus signifying the role of DICER in the regulation of pericentric heterochromatin.

Because DICER and MSR forward transcripts localize to both the cytoplasm and nucleus, it is challenging to make clear conclusions about the exact subcellular location of the DICER-dependent MSR transcript regulation. Interestingly, while MSR forward transcripts were observed in both cellular compartments, MSR transcript-derived small RNAs were enriched in the nuclear compartment. Therefore, it is possible that MSR transcripts are first transported to the cytoplasm but are moved back to the nucleus either before or immediately after their processing. The cytoplasmic processing is supported by the accumulation of MSR forward transcripts in the cytoplasm of spermatocytes in the absence of DICER. At the same time, there is a general reduction in the level of MSR forward transcript-derived small RNAs that we observed to be mainly found in the chromatin fraction. Interestingly, SUV39H2 needs major satellite RNA transcripts to stabilize its association with chromatin (Velazquez Camacho et al., 2017). The recruitment of SUV39H2 to the pericentric heterochromatin was reduced in *Dicer1* cKO testes, and it is tempting to speculate that this could be due to reduced level of DICER-processed MSR small RNAs in the chromatin.

## 6.3 Aberrant meiotic chromosome segregation in *Dicer1* cKO spermatocytes

In plants and yeast, the role of pericentric heterochromatin transcription and its involvement in the regulation of heterochromatin maintenance by RNA interference mediated mechanisms is well-established (White and Allshire, 2008; Djupedal and Ekwall, 2009; Reyes-Turcu and Grewal, 2012). However, the mechanisms of how pericentric heterochromatin is transcribed, as well as the functional consequences of its transcription are still largely unclear in mammals. Some evidence exists to support the functional role of MSR transcription in early mouse development. The depletion of MSR transcripts by injecting LNA-DNA gapmers into zygotes resulted in developmental arrest at the two-cell stage (Probst et al., 2010). Furthermore, the infection of gapmers specifically targeting either forward or reverse transcripts revealed that the depletion of reverse transcripts resulted in developmental arrest at the two-cell stage and problems in chromocenter formation, illustrating the importance of MSR transcripts in the regulation of the genomic regions they are derived from (Casanova et al., 2013b). Interestingly, despite high expression of the forward strand in early two-cell stage, its depletion did not affect early development (Casanova et al., 2013a; Probst et al., 2010). These results suggest that the transcripts derived from different strands of MSRs have differential functions.

Centromeric and pericentric regions are known to have important roles during cell division and chromosome segregation (Probst and Almouzni, 2008; Hall et al., 2012; Jagannathan and Yamashita, 2017), and therefore, we were interested to find out if the MSR transcript accumulation in *Dicer1* cKO spermatocytes affect meiotic progression. We showed that synaptonemal complexes and sex body formation appeared normally without any gross abnormalities, and metaphase plates were identified at stage XII of the seminiferous epithelial cycle. Therefore, the meiosis appeared to progress in pachytene spermatocytes without any visible defects. However, when we took a closer look at haploid cells produced as a result of meiotic division, we observed unevenly sized round spermatid nuclei, and flow cytometric analysis suggested a higher DNA content of round spermatids in *Dicer1* cKO testes, suggesting problems in meiotic chromosome segregation. This was confirmed by chromosomal *in situ* hybridization, which revealed that haploid spermatids containing abnormal number of X and Y chromosomes were significantly increased in *Dicer1* cKO testes. These findings confirmed the defective meiosis I in the *Dicer1* cKO spermatocytes, which may result as a consequence of the aberrant control of pericentric heterochromatin.

Due to the important regulatory roles of DICER-dependent miRNAs in various cellular processes, we cannot rule out the possibility that some of the defects we observe in *Dicer1* cKO mice, such as the meiotic chromosome mis-segregation, are secondary consequences of the defective miRNA processing. However, we provide

strong evidence on the association of DICER with pericentric heterochromatin and MSR forward transcripts, and the functional consequences of DICER ablation on the expression and processing of MSR transcripts, as well as on the epigenetic status of pericentric heterochromatin. These findings strongly suggest that DICER has a direct regulatory role on MSR expression in male germ cells in mice. Our results are further supported by the studies showing that RNA interference machinery is involved in the chromatin compaction in *C. elegans* germ cells (Fields and Kennedy, 2019; Gushchanskaia et al., 2019), demarcating that similar type of mechanisms may occur in mouse germ cells. Furthermore, seeing the important roles of centromeric and pericentric chromosomal regions during chromosome segregation (Probst and Almouzni, 2008; Hall et al., 2012; Jagannathan and Yamashita, 2017), it is likely that the defective MSR expression in *Dicer1* cKO spermatocytes is functionally linked to the observed problems in chromosome segregation. Importantly, a recent study that was published concurrently with our study supports our findings by showing that over-expression of satellite repeat RNAs in cultured mouse spermatocytes triggers chromosome mis-alignment and chromosome mis-segregation in meiosis I (Hsieh et al., 2020).

This study provides important novel information about DICER-mediated RNA/chromatin regulatory mechanisms that take place in the male germline. Our results clearly indicate that these mechanisms are critical for the maintenance of male fertility in mice. Due to the conserved nature of spermatogenesis in mice and humans (Schlatt and Ehmcke, 2014; White-Cooper and Bausek, 2010; Chalmel et al., 2007), our results provide also important molecular insights into human male infertility. The future studies will reveal if defective DICER function or aberrant MSR expression could contribute to abnormal spermatogenesis and meiotic chromosome segregation leading to male sub/infertility.

# 7 Summary and Conclusions

The main findings of this doctoral dissertation are:

1. DICER is required for normal progress of spermatogenesis in mice. The deletion of *Dicer1* in early spermatogenic cells results in defective haploid male germ cell differentiation with major defects in chromatin condensation and nuclear shaping of spermatids leading to severe oligoasthenoteratozoospermia and infertility.

2. Pericentric heterochromatin originated major satellite repeats (MSR) are transcribed during spermatogenesis, with the expression peaking in meiotic spermatocytes. The forward and reverse strands of MSRs have differential expression dynamics, and the meiotic expression peak was shown to derive mostly from the forward strand.

3. The expression of MSR transcripts are induced in the testis in the absence of DICER, while the expression of other repetitive elements such transposons is unaffected. The level of MSR transcripts is elevated, particularly in spermatocytes. Only the expression of the MSR forward strand, but not the reverse strand, is aberrantly induced in *Dicer1* null spermatocytes.

4. DICER associates with MSR transcripts *in vitro* and *in vivo*, and the processing of MSR forward transcripts is defective in *Dicer1* cKO testes, resulting in reduced levels of MSR forward transcript-derived small RNAs. This suggests that the elevated levels of MSR forward transcripts in *Dicer1* cKO testis originate from their defective processing.

5. In addition to its prominent cytoplasmic localization, DICER localizes to the nuclear foci and partially co-localizes with MSR transcripts. DICER specifically associates with pericentric heterochromatin and heterochromatin regulators, such as TRIM28, TRIM33 and the H3K9 methyltransferase SUV39H2 in the testis. The recruitment of SUV39H2 and the level of H3K9me3 at the pericentric heterochromatin is compromised in *Dicer1* cKO testes.

6. Meiosis progresses without any gross abnormalities until the meiotic divisions in the *Dicer1* cKO testes. However, chromosome segregation is abnormal resulting in chromosome aneuploidy in haploid spermatids, therefore compromising the genomic integrity of the germline.

In summary, this study revealed the essential role of DICER in spermatogenesis in mice, and its novel function in the regulation of the expression of pericentric heterochromatin through direct targeting of MSR transcripts in male germ cells. Altogether, these results provide novel important information about the regulatory mechanisms that contribute to the production of fertile spermatozoa and, therefore, the maintenance of male fertility.
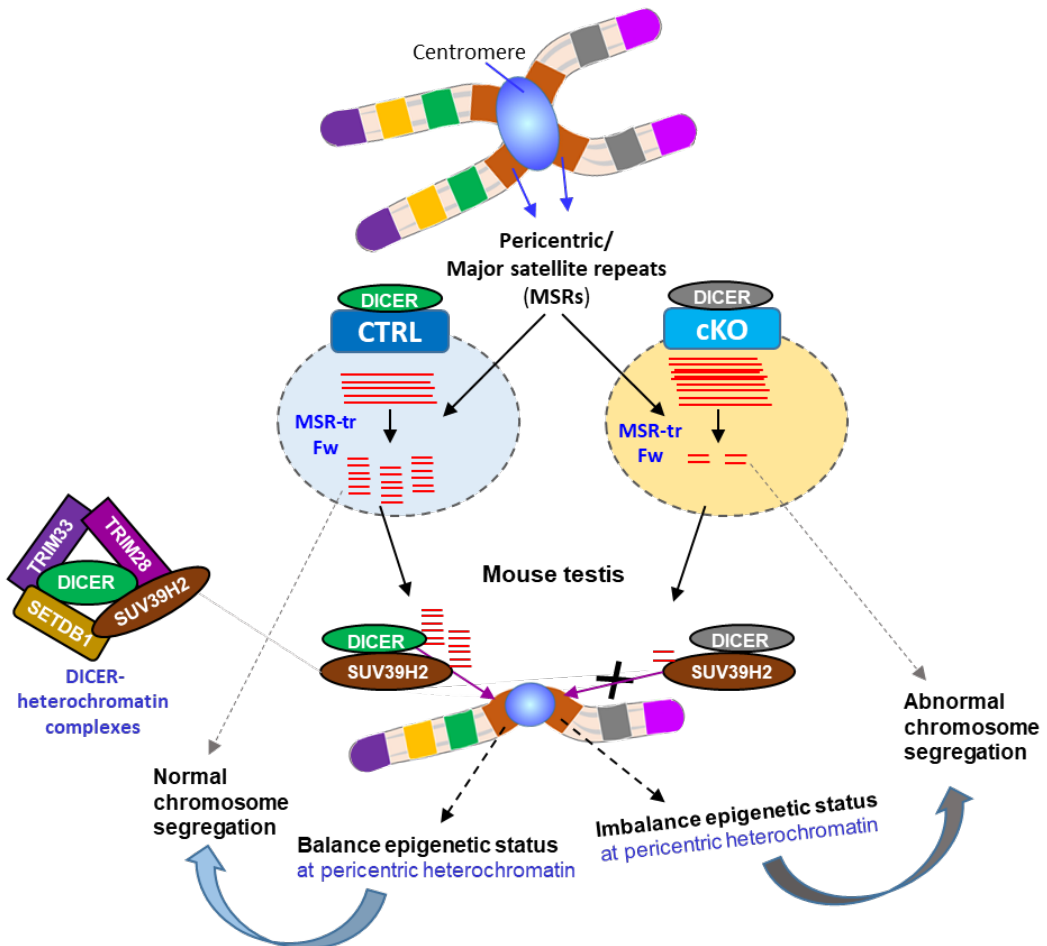


**Figure 12**: A model summarizing the main results of this study, about the interplay of DICER with MSR transcripts and pericentric heterochromatin in mouse germ cells as well as possible functional consequences of the defective in MSR transcripts processing in the absence of DICER.

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*Ram Prakash Yadav*

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