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THE ESSENTIAL ROLE OF DICER IN SPERMATOGENESIS AND MALE FERTILITY

Hanna Korhonen

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University of Turku

Faculty of Medicine

Institute of Biomedicine

Department of Physiology

Turku Doctoral Program of Molecular Medicine (TUDMM)

Supervised by

Noora Kotaja, PhD, Docent, Associate Professor
Institute of Biomedicine
University of Turku
Turku
Finland

Reviewed by

Lea Sistonen, Professor
Department of Cell Biology
Faculty of Science and Engineering
Åbo Akademi University
Turku
Finland

Anna-Liisa Levonen, Professor
A.I.Virtanen Institute for Molecular Sciences
University of Eastern Finland
Kuopio
Finland

Opponent

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Université Joseph Fourier
Grenoble
France

Cover image: Hanna Korhonen

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To my Family

ABSTRACT

Hanna Korhonen

The essential role of DICER in spermatogenesis and male fertility

University of Turku, Faculty of Medicine, Institute of Biomedicine, Department of Physiology, Kiinamyllynkatu 10, 20520 Turku.
Turku Doctoral Program of Molecular Medicine (TUDMM) and Turku Doctoral Program of Biomedical Sciences (TUBS).
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Male reproductive health has declined in western countries during recent years. Male infertility is often caused by faults in spermatogenesis, leading to a decreased number of spermatozoa in the semen and/or problems in the sperm morphology and motility. That raises the necessity of understanding the basic molecular mechanisms of the testis function.

Male germ cell differentiation is a complex, precisely organized process that produces mature spermatozoa from spermatogonial stem cells. All the steps during spermatogenesis are governed by strict regulation of gene expression. Small non-coding RNAs, such as microRNAs (miRNAs), are very important gene regulators that function post-transcriptionally to control the stability or translation of their target mRNAs. A crucial player in miRNA pathway is the endonuclease DICER that processes precursor molecules into mature small RNAs.

This study demonstrated the importance of DICER in spermatogenesis and male fertility. By using a conditional knockout mouse model that lacks DICER specifically in post-natal male germ cells, we showed that the absence of functional DICER causes severe defects in male germ cell differentiation. Particularly, the post-meiotic haploid differentiation, including head shaping, chromatin condensation, polarity of developing germ cell, were found to be affected. *Dicer1* KO males produced a greatly reduced number of spermatozoa that were all morphologically abnormal, and the males were completely infertile. More detailed analysis revealed that the problems in haploid differentiation were at least partially due to the defects in cell-cell junctions between spermatids and somatic Sertoli cells. Additionally, we characterized the expression of DICER during spermatogenesis, and showed that the DICER expression peaked in meiotic spermatocytes, where it is localized to germ cell-specific RNA granules, germ granules. This research provides novel, important information about the factors required for the maintenance of spermatogenesis, and helps us to understand those factors that are involved in male infertility.

Keywords: testis, DICER, small RNA, cell-cell junction, germ granule

TIIVISTELMÄ

Hanna Korhonen

DICER-proteiinin rooli siittiön muodostuksessa ja miesten hedelmällisyydessä

Turun Yliopisto, Lääketieteellinen tiedekunta, Fysiologian oppiaine, Kiinamyllynkatu 10, 20520 Turku.
Turun molekyylilääketieteen tohtoriohjelma (TUDMM) ja Turku Doctoral Program of Biomedical Sciences (TUBS).
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Miesten lisääntymisterveys on merkittävästi heikentynyt viime vuosien aikana. Miesten hedelmättömyys johtuu usein ongelmista siittiön muodostuksen aikana ja heikentyneestä siemennesteen laadusta. Tämä lisää tarvetta tarkalle kivesten toiminnan tuntemukselle.

Spermatogeneesi on kivesten siementiehyissä tapahtuva erittäin monimutkainen tapahtumasarja, jossa sukusolulinjan kantasolusta erilaistuu hedelmöityskykyinen siittiö. Spermatogeneesin aikana geenien ilmentyminen on aktiivista ja vaatii tarkkaa geenien ilmenemisen säätelyä. Muiden RNA-säätelymekanismien ohella pienet RNA-molekyylit, kuten mikroRNA-molekyylit (miRNA) osallistuvat siittiönmuodostuksen aikana tapahtuvaan geenisäätelyyn. miRNA:t säätelevät geenin ilmentymistä sitoutumalla lähetti-RNA:ihin ja aiheuttamalla niiden hajotuksen. DICER-endonukleaasi, joka pilkkoo miRNA:iden esiasteista kypsiä lyhyitä miRNA:ita, on keskeinen entsyymi miRNA:iden prosessoinnissa.

Tämä väitöskirjatyö selvittää DICER-proteiinin roolia spermatogeneesin säätelyssä. Työssä käytettiin hyväksi miesten sukusolu-spesifistä poistogeenistä hiirimallia, jossa *Dicer1* -geeni on poistettu sukusolujen kehityksen varhaisessa vaiheessa heti syntymän jälkeen. Näytimme, että DICERin poistaminen aiheuttaa vakavia ongelmia sukusolujen erilaistumisessa, erityisesti meioosin jälkeisissä vaiheissa, kuten siittiön pään muodostumisessa, kromatiinin pakkautumisessa ja solun polarisoitumisessa. *Dicer1*-poistogeeniset urokset tuottivat vain pienen määrän morfologialtaan epänormaaleja siittiöitä ja urokset olivat täysin lisääntymiskyvyttömiä. Tarkempi analyysi osoitti, että virheet haploidien solujen erilaistumisessa ainakin osittain johtuivat viallisista liitoksista somaattisten Sertoli-solujen ja sukusolujen välillä. Lisäksi selvitimme yksityiskohtaisesti DICERin ilmentymistä ja lokalisaatiota erilaistuvissa sukusoluissa, ja näytimme, että DICERin määrä on suurimmillaan meioottisissa spermatosyyteissä, ja näissä soluissa DICER lokalisoituu sukusolu-spesifisiin sytoplasmisiin RNA-rakenteisiin (sukusolurakkula). Tutkimuksen tulokset tarjoavat uutta, merkittävää tietoa niistä tekijöistä, jotka ovat tärkeitä onnistuneelle siittiön muodostumiselle sekä auttaa ymmärtämään miesten hedelmättömyyteen liittyviä tekijöitä.

Avainsanat: kives, DICER, pieni RNA, sukusoluliitos, sukusolurakkula

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ABBREVIATIONS

AGO	argonaute protein
AGO-2	argonaute protein-2
AJs	actin filament-based adherens junctions
aPKC	atypical protein kinase C
aES	apical ectoplasmic specialization
BSA	bovine serum albumin
BTB	blood testis barrier
Brdt	testis-specific bromodomain protein
CB	chromatoid body
CTRL	control
DGCR8	DiGeorge syndrome critical region 8
FSH	follicle-stimulating hormone
DSBs	DNA double-strand breaks
dsRBD	dsRNA-binding domain
DNA	deoxyribonucleic acid
Exp5	exportin-5
endo-siRNA	endogenous small interfering RNA
GnRH	Gonadotropin-releasing hormone
Helica	helicase domain
H1T2	testis-specific histone1 variant 2
HPG	hypothalamic-pituitary-gonadal axis
JAM-C	junctional adhesion molecule-C
KO	knockout
L	leptotene
LH	luteinizing hormone
miRNA	microRNA
OAM	outer acrosomal membrane
PACT	protein activator of protein kinase R
PFA	paraformaldehyde
piRNA	PIWI-interacting RNA
PAZ	PIWI-Argonaute-Zwille domain
PGCs	primordial germ cells
PH	pachytene
pol II	RNA polymerase II
pp	post partum
pre-miRNAs	miRNA precursor
pri-miRNAs	primary miRNAs
PL	preleptotene

rDNA	ribosomal DNA
RISC	RNA-induced silencing complex
RNAi	RNA interference
RS	round spermatids
Sc	spermatocytes
SC	synaptonemal complex
siRNA	small interfering RNA
small RNA	small non-coding RNA
TDRD1	tudor domain-containing protein 1
TFs	transcription factors
TNAP	tissue-nonspecific alkaline phosphatase
TPs	transition proteins
Z	zygotene

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred to in the text by Roman numerals (I-III). Unpublished data are also included.

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. doi: 10.1371/journal.pone.0024821.

II. Korhonen HM, Yadav RP, Da Ros M, Chalmel F, Zimmermann C, Toppari J, Nef S, Kotaja N. DICER Regulates the Formation and Maintenance of Cell-Cell Junctions in the Mouse Seminiferous Epithelium. *Biol Reprod.* 2015 Dec;93 (6):139. doi: 10.1095/biolreprod.115.131938.

III. Korhonen HM, Meikar O, Toppari J, Kotaja N. DICER localization is associated with germ cell-specific RNP granules in mouse male germ cells. Submitted manuscript.

1 INTRODUCTION

Infertility and subfertility are a widespread problem in the Western countries (Sharpe 2012). Although infertility is not a threat to our physical health, it does have a strong impact on the social well-being. Very often, approximately with half of the couples, infertility is caused by a male factor (Poongothai et al. 2009) and alarmingly adverse trends have been reported in male reproduction disorders during past half century (Skakkebaek et al. 2016; Andersson et al. 2008). Insufficient male reproduction is usually due to the faults in spermatogenesis leading to impaired semen quality. The decline in semen quality has been also demonstrated in young Finnish men in recent years (Virtanen et al. 2013). Various genetic and/or environmental factors can cause defects in the regulation of male germ cell differentiation resulting in problems of functional spermatogenesis and male fertility (Anway et al. 2005).

Complex organisms like mammals require gene regulation which is one of the most tightly controlled biological processes. Precise temporal and spatial regulation of gene expression ensures that the correct amount of proteins is translated at the correct time. Any disruption can lead to serious consequences, such as cancer or other severe diseases (Beyersmann 2000; Emilsson et al. 2008) or cause problems in fertility (Bansal et al. 2015). Among the other regulatory mechanisms, small RNAs, which are produced by endonuclease DICER, are the key players in gene regulation. DICER and DICER-dependent pathways are recognized as vital regulatory factors that control the expression of protein coding genes. The absence of *Dicer1* gene leads to an embryonic lethal phenotype in the mice demonstrating the crucial role of DICER in the development (Bernstein E1, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV 2003). The importance of functional DICER in several tissues and cell types has been demonstrated using tissue/cell-specific conditional mouse models (Murchison et al. 2007; Tang et al. 2007; Katsuhiko Hayashi et al. 2008; Davis et al. 2008).

Male germ cell differentiatiation from a spermatogonial stem cell into a mature spermatozoon requires exact, spatio-temporal gene regulation that is assured by several ways. In this study, we focused on the characterization of the role of DICER and DICER-dependent pathways in the regulation of gene expression during spermatogenesis. The general aim of this study was to provide novel, essential information about the molecular mechanisms of male germ cell-specific gene regulation that could be potentially used in the future to develop novel tools for diagnosis and management of male infertility or male contraception.

2 REVIEW OF THE LITERATURE

2.1 DICER

Endonuclease DICER belongs to the RNase III family and is a key player in gene regulation. DICER is a 218-kDa multidomain enzyme comprising of a helicase domain, a DUF283 domain, a dsRNA-binding domain (dsRBD), a PAZ (Piwi-Argonaute-Zwille) domain and two RNase III domains, RNase IIIa and RNase IIIb (Figure 1) (Macrae et al. 2006). The chromosomal localization of *Dicer* gene as well as the number of exons and domains varies between species (Kurzynska-Kokorniak et al. 2015). For example in *Giardia*, DICER contains only three of those domains found in human or murine DICER (PAZ and two RNAseIII domains) (Murchison et al. 2005; Macrae et al. 2006).

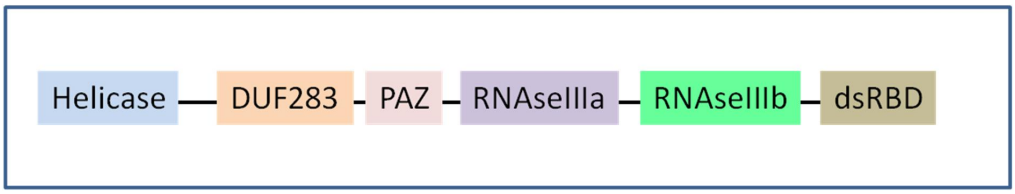

Figure 1 Schematic illustration of the murine DICER. Murine DICER is composed of a helicase domain, a DUF283 domain, a dsRNA-binding domain (dsRBD), a PAZ (Piwi-Argonaute-Zwille) domain and two RNase III domains, RNase IIIa and RNase IIIb. PAZ (Piwi-Argonaute-Zwille) is the domain, which binds the basal end of the double-stranded pre-miRNA (Macrae et al. 2006; Murchison et al. 2005).

The most important function of DICER is considered to be in the small RNA pathway, where DICER cleaves the double-stranded RNA (dsRNA) into short 21–25-nt long double stranded fragments known as small non-coding RNAs (small RNAs) in the cytoplasm (Bernstein et al. 2001). DICER is the critical regulator in the biosynthesis of small RNAs, such as miRNAs, small interfering RNAs (siRNAS), and endo-siRNAs, in several types of tissues and organisms. This is the best known function of DICER, although there is an increasing evidence that DICER has also some other essential functions in biological processes.

The function of DICER is widely studied in different tissues using several mouse models and cell lines. A constitutive DICER knockout mouse leads to an embryonic lethal phenotype and that demonstrates the importance of DICER dependent pathways in biological processes required for the mouse development (Bernstein E1, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV 2003). Tissue-specific deletion of DICER has been studied in adrenal cortex, which is part of the adrenal gland, the bilateral structure on the top of the kidney. These mice showed normal development of adrenal cortex by the embryonic day 14.5 but the adrenal cortex had completely failed later by the embryonic day 18.5 (Krill et al. 2013). Mice lacking DICER in retina had decreased inability to respond to light (Damiani et al. 2008). DICER has also been demonstrated to be required for the normal celebrellar formation in the brain (Zindy et al. 2015). Additionally, the conditional loss of DICER in excitatory forebrain neurons had defective cellular and

tissue morphogenesis of hippocampus and the cortex (Davis et al. 2008). Soukup and co-workers have presented that DICER is important for the inner ear development (Soukup et al. 2009). The ablation of *Dicer1* gene causes problems in the differentiation of the epithelium in mouse epididymis (Björkgren et al. 2012) and additionally, DICER has been demonstrated to control the gene expression in the different segments of epididymis in a human (Belleannée et al. 2012). Recently, DICER-dependent pathways were shown to be maintaining the contractile differentiation and calcium signalling in vascular smooth muscle (Bhattachariya et al. 2015). Furthermore, DICER has been presented to be important for the formation of heterochromatin structure (Fukagawa et al. 2004), and it has also been demonstrated to be involved in the differentiation and centromeric silencing in embryonic stem cells (Kanellopoulou et al. 2005).

Recent studies have revealed the important function of DICER as a tumor suppressor since the decreased DICER activity has been demonstrated to enhance tumorigenic activity of lung and breast cancer cells (Zhang et al. 2014). This role is most probably miRNA mediated since miRNA knockdown has also been demonstrated to enchance tumorigenic activity of human lung adenocarsinoma cells (Kumar et al. 2008). The monoallelic loss of *Dicer1*, that has one wild type allele, was shown not to be involved in the normal retina formation but instead was demonstrated to accelerate the formation of retinoblastoma that is a rare type of cancer in the eye (Lambertz et al. 2010). Taken together, these results demonstrate the fundamental role of DICER and DICER-dependent pathways in various cell types. The lack of functional DICER can cause severe diseases or other consequences.

2.1.1 The role of DICER in small RNA processing

2.1.1.1 miRNA processing pathway

miRNAs are the most studied group of small RNAs and very important players in the post-transcriptional control of the gene expression. Figure 2 illustrates schematically the biogenesis of miRNAs. In the nucleus, miRNAs are synthesized from miRNA genes (Lee et al. 2004). Long, hairpin-looped transcripts (pri-miRNAs) are transcribed by the RNA polymerase II (pol II). Pri-miRNAs contain unique structures such as cap structures and poly -A tails. Pri-miRNAs are initially cleaved to miRNA precursors (pre-miRNAs) by the nuclear microprocessor complex, which is composed of the RNase polymerase III family member DROSHA and DiGeorge syndrome critical region 8 (DGCR8) (Kim et al. 2009). The pre-miRNAs are then exported from the nucleus to the cytoplasm by the Ran-dependent nuclear transport receptor family member EXPORTIN-5 (Exp5), to be further processed by DICER (Lund & Dahlberg 2006; RUI YI 2005).

In the cytoplasm, DICER cleaves the hairpin of the pre-miRNAs and produces miRNA duplex which are loaded with ARGONAUTE (AGO) proteins onto the RNA induced silencing complex (RISC). The catalytic activity of DICER is driven by associated proteins TRBP and protein activator of protein kinase R (PACT)(Lee et al. 2006; Chendrimada et al. 2005). Individual miRNAs share sequence

complementarity to the 3' untranslated region (UTR) of their target mRNAs. Binding RISC-associated miRNAs to their target mRNA usually leads to the translational arrest and/or degradation of the transcript (Pasquinelli 2012) depending on the complementary site of the mRNA target. Thus, miRNAs usually cause repression of their target gene expression (Roberts 2015).

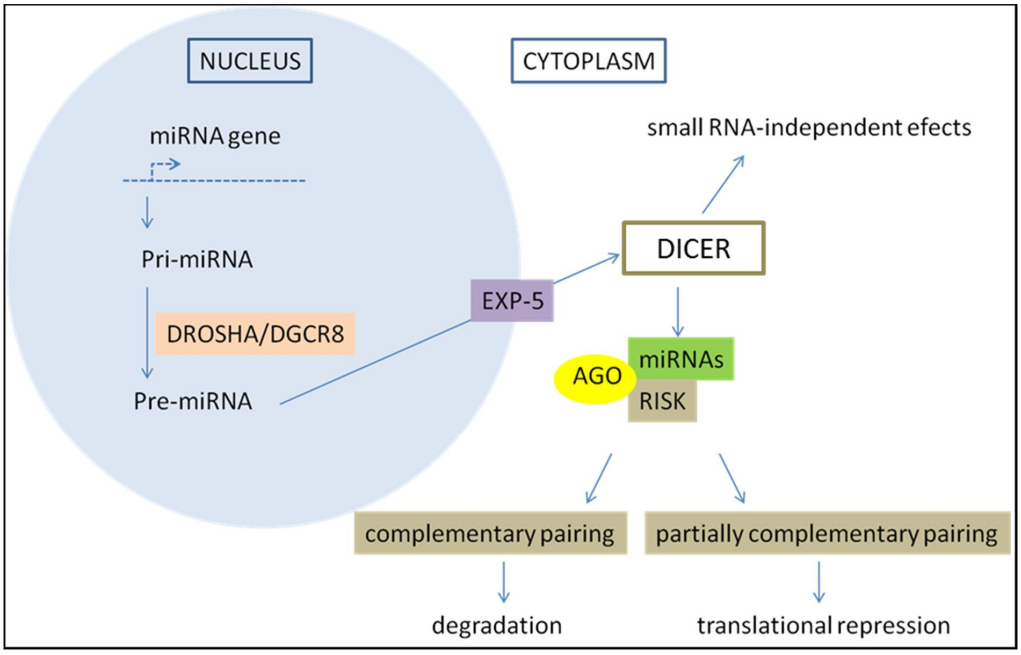

Figure 2 Biosynthesis of miRNAs. MiRNAs are produced first in the nucleus by the small RNA gene and microprocessor complex. Then miRNAS are exported to the cytoplasm, where they are processed by DICER and loaded to the RISC with AGO proteins. Finally they can control the gene regulation by either degradation of target mRNA or translational repression (Goodall et al. 2013).

2.1.1.2 Functions of miRNAs

miRNAs were first discovered in 1993 in *Caenorhabditis elegans* (Lee et al. 1993; Wightman et al. 1993). Later they have been widely studied and hundreds of miRNAs have been discovered. miRNAs are known to be highly conserved across species, and most interestingly, it has been hypothesized already in 2005 that miRNAs could regulate thousands of human genes which would be up to 30% of all the genes in the human genome (Lewis et al. 2005).

For their biosynthesis, miRNAs require endonuclease DICER (Figure 2). After DICER has processed miRNAs, they are loaded on to RISC. Together with AGO proteins they bind to the target mRNA and this way control the gene expression. Usually mRNAs either repress the protein translation or cause the RNA degradation (Valencia-Sanchez et al. 2006). miRNAs have a unique ability to control the

expression of thousands of target mRNAs. Binding to the target mRNAs is derived from the Watson-Crick base pairing. A key player in the recognition and the function of miRNAs is the specificity in the miRNA-mRNA interactions. These interactions can lead to different downstream consequences (Valencia-Sanchez et al. 2006). The first thing which plays a significant role is the 5' end of miRNA. The ability to bind to the target mRNA is determined by the first eight nucleotides of 5' end of miRNAs that is called miRNA "seed" (Doench & Sharp 2004; Hibio et al. 2012). The significant subjects are the thermal balance as well as the complementarity of miRNA seed with the target sequence (Valencia-Sanchez et al. 2006). Perfect pairing of miRNA to its target is often believed to lead the endonucleolytic cleavage of mRNAs. Alternatively miRNAs can recognize partially complementary binding sites located on the 3' UTR of the target mRNA. This mechanism leads to the translational repression and blocks the protein production (Pasquinelli 2012).

By gene repression, miRNAs regulate various cellular processes such as developmental timing, cell death, cell proliferation, hematopoiesis and the nervous system. In addition, the role of miRNAs as a tumor suppressor has been recently demonstrated (C.-C. Sun et al. 2016; Chen et al. 2016; Xu et al. 2016; Wang et al. 2016). Since the misregulation of miRNAs has been connected to several cancers (L. Sun et al. 2016; Samsonov et al. 2016; Báez-Vega et al. 2016), miRNAs have been indicated to have a novel role as a cancer biomarker (Qu et al. 2016; Jiang et al. 2016; Lin et al. 2016). In addition to the carcinomas, the misregulation of miRNAs has a significant role also in various other diseases. miR-146a and miR-221 display high expression in regions of neurons that are important for high cognitive functions and a connection between miRNA dysregulation and neurodegenerative diseases such as autism spectrum disorders, Alzheimer's and schizophrenia have been demonstrated (Hernandez-Rapp et al. 2015; Nguyen et al. 2016; Morrow 2015). A novel role for miRNAs was recently presented as a clinical predictor of pregnancy complications, since placental specific miRNAs are expressed in maternal plasma at different levels. miRNA levels in the maternal plasma varies depending on the state of pregnancy: when pregnancy is normal, when pregnancy is pathological or in the case of no pregnancy (Tsochandaridis et al. 2015).

miRNAs are expressed in various types of tissues and the dysregulation of miRNAs is connected to various types of diseases. In addition to diseases, miRNAs have an important role also in fertility, since several miRNAs are found in the ovary and the testis. miRNAs have been described to be important regulators in male and female gonad development (Grossman & Shalgi 2016). In the testis several miRNAs are found in somatic Sertoli cells and germ cells. However, there is a constant crosstalk between Sertoli cells and germ cells and these interactions are critical. Abnormal miRNA levels in the testis may cause changes in the miRNA levels in Sertoli cells. Therefore some miRNA levels are crucial for establishing the appropriate miRNA levels in Sertoli cells. miR-202-5p is Sertoli cell-specific miRNA, which is expressed at different levels in Sertoli cells in fertile testis and in testis, in which the number of fertile germ cell is depleted (Dabaja et al. 2015). The role of miRNAs in male germ cell differentiation and in spermatogenesis is better described in 2.4.2.1.

2.1.1.3 Endogenous siRNAs

In addition to miRNAs, siRNAS and endo-siRNAs are DICER-dependent small non-coding RNAs. In contrast to miRNAs that are processed form precursors synthesized from the miRNA genes, siRNAs are processed from the long double-stranded RNAs that may enter cells for example by viral infection or transfection (Zeng et al. 2003). The mechanim, by which they function at the gene silencing was first discovered in 1990 (Napoli et al. 1990) and later Mello and coworkers described the mechanism of gene silencing that is called RNA interference (RNAi) (Mello & Conte 2004). This mechanism is the same than in miRNA -mediated regulation. Soon single stranded forms of miRNA and siRNA were found to associate with RISC, miRISC and siRISC, respectively, and both were observed to direct gene expression at the post-transcriptional level. In plants and invertebrates siRNAs can also be derived from virus infection, and by mediating RNAi, they can potentially promote RNA silencing against virus infection resulting in specific antiviral defense (Li et al. 2016; Hammond et al. 2000).

Endo-siRNAs are endogenous short interfering RNAs that are processed similarly to siRNAs but from endogenous double-stranded transcripts. Similarly to miRNAs and siRNAs, endo-siRNAs are processed by DICER and associate with argonaute proteins. endo-siRNAs bind to the AGO complex, which finally defines the biological impact of endo-siRNA pathway (Watanabe et al. 2008). The biosynthesis as well as biological functions of endo-siRNAs varies between species. This includes the number of proteins required for the biosynthesis but there are also some variations in the physiological roles of endo-siRNAs in different species. In *Drosophila,* endo-siRNAs have two distinct pathways involving different isoforms of DICER and AGO proteins. In endo-siRNA pathway, endo-siRNAs are processed by the DICER and loaded onto argonaute protein-2 (AGO-2) (Piatek & Werner 2014).

Functions of endo-siRNAs have been indicated to be involved in the transposon silencing and heterochromatin formation in *Drosophila* (Fagegaltier et al. 2009). In mammals both the synthesis and the function of endo-siRNAs remain unclear but some roles for endo-siRNA have been recently published in a mouse (Piatek & Werner 2014). In mouse oocytes, endo-siRNAs have been described to be formed from naturally occurring dsRNAs and have a role in gene regulation (Watanabe et al. 2008). In oocytes endo-siRNAs have been demonstrated to be involved in the meiotic maturation including spindle formation and chromosome alignment. Knock-in a catalytically inactive allele of *Ago2* in mouse leads to a meiotic failure (Stein et al. 2015). Male germ cells have also been found to express endo-siRNAs that are derived from naturally occurring dsRNAs precursors (Song et al. 2011).

Additional meiotic role for endo-siRNAs have been described in male germ cells (Zimmermann et al. 2014). These examples demonstrate important roles of endo-siRNAs in biological processes including germ cells.

2.1.2 Small RNA-independent functions of DICER

It is well known that DICER has a vital role for various types of tissues and cells, but the production of small RNAs is not the only function of DICER. The miRNA independent functions of DICER are involved in chromatin structure remodelling, inflammation and apoptotic DNA degradation (Kurzynska-Kokorniak et al. 2015). Moreover, in yeast DICER is involved in the RNAi pathway and heterochromatin assembly (Volpe et al. 2002). Recently DICER has been presented to be associated with nuclear rDNA repeats on chromosomes of human and mouse cells (Sinkkonen et al. 2010). However, the role of these associations remains unclear.

Alu elements are the most common repetitive elements in the human genome. They are able to regulate gene expression and increase the number of RNA transcripts. Additionally they can regulate the function of microRNAs, circular RNAs, or long non-coding RNAs. These elements function by influencing diverse RNA processes (Daniel et al. 2015). DICER has been demonstrated to degrade RNA of *Alu* elements, and it has been showed that the dysregulation of *Dicer1* leads to the accumulation of transcripts of *Alu* elements (Kaneko et al. 2011).

The best known function of DICER has long been the production of small RNAs but Rybak-Wolf and co-workers has shown the novel function of DICER (Rybak-Wolf et al. 2014). They have described that DICER -binding sites on mRNA, are not always processed into small RNAs and these sites were named as passive sites. This study was performed by PAR-CLIP/iPAR-CLIP-method (described in Hafner et al. 2010) to identify DICER targets and additionally they used sRNA sequencing to measure the enzymatic activity of DICER. They have presented that these passive sites typically are DICER-bound hairpin structures that structurally and functionally differ from the well-known active DICER sites (Figure 3). Usually these sites stabilize target expression and even reduce miRNA expression but these sites were also found to function by sequestering DICER. Additionally Rybak-Wolf and co-workers have described novel substrates for DICER that are structural RNAs, promoter RNAs, and mitochondrial transcripts. Interestingly, by using RIP RT-PCR, RT-PCR of FLAG/HA-tagged or endogenous DICER IPs, mRNAs with passive sites were demonstrated to be associated with processing-body/RNA granule function, that are known to be essential for germ line (germ granules are described later in 2.4.3). Both human and worm passive targets of DICER were significantly enriched in genes associated with RNP granules but active target transcripts were not enriched in RNP granule associated genes (reviewed from Rybak-Wolf et al. 2014). Moreover, in *C. elegans*, DICER has been known to be required for the assembly of these granules (Beshore et al. 2011).

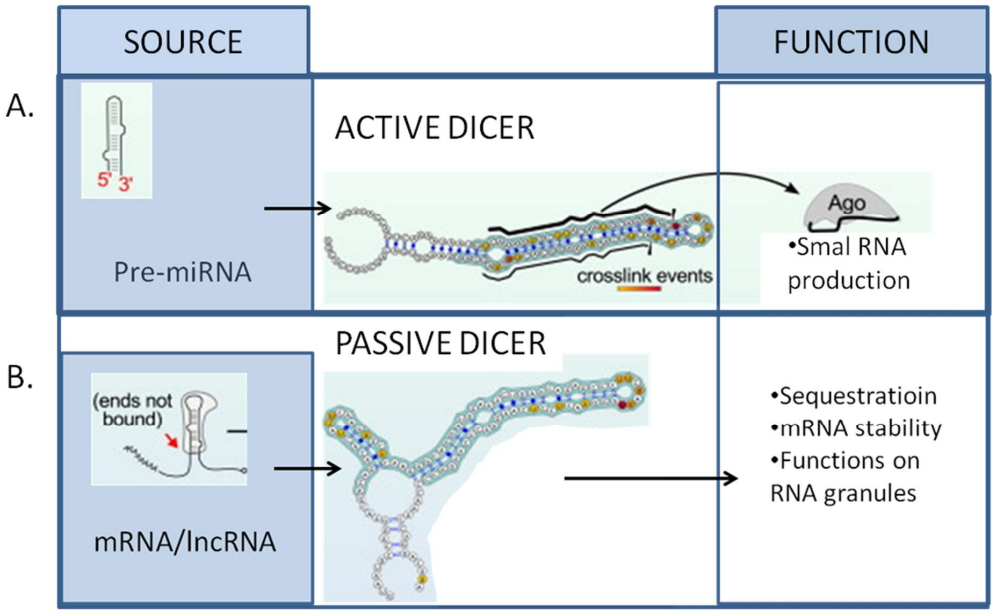

Figure 3 The function of active and passive DICER. A. Active DICER binds to pre-miRNA precursors and function in small RNA biosynthesis. B. the passive DICER transcript harbour on the mRNA or long non-coding RNA (lncRNA), stabilize the miRNA expression, is associated with RNA granules and may also function by sequestering DICER (found in C.elegans)(modified from Rybak-Wolf et al. 2014).

2.2 Spermatogenesis

2.2.1 Phases of spermatogenesis

Spermatozoa, mature male germ cells, are formed from the spermatogonial stem cells in a complex process called spermatogenesis. Germ cells develop and differentiate in the testis, which is composed of seminiferous tubules. Development of germ cells begins with the proliferation of spermatogonia in the basal membrane of the seminiferous tubule. From the basal membrane germ cells migrate across the seminiferous epithelium towards the lumen and meanwhile they undergo all the differentiation steps including dramatic morphological changes. In the spermiation, the final step of spermatogenesis, germ cells are released into the lumen. Finally germ cells are transported to the epididymis where they continue the maturation and become capable for fertilization.

Spermatogenesis begins after birth and continues throughout the male reproductive life (Abou-Haila & Tulsiani 2000). During this fundamental process male germ cell metamorphoses from a primordial germ cell to mature spermatozoa to be able to fertilize an egg. Entire process includes three phases: 1) mitotic proliferation of spermatogonia, 2) two meiotic divisions (meiosis I and meiosis II) of spermatocytes and 3) spermiogenesis, when haploid cells differentiate and elongate to the spermatozoa (Figure 4) (Russell et al. 1993).

The first spermatogonial proliferation phase produces an optimal number of spermatogonia that gives rise to all spermatocytes but also maintains the stem cell pool. Spermatogonial stem cells proliferate by mitosis where two daughter cells are formed of exactly the same DNA and chromosomal content as the original diploid cell. Developing testicular daughter cells have two options: cells either enter the differentiation pathway to develop to mature spermatozoa or alternatively maintain the stem cell status (Abou-Haila & Tulsiani 2000).

Genetic diversity is created via meiotic recombination. Successful meiosis undergoes two rounds of cell divisions, meiosis I and meiosis II. The prophase of meiosis I is long and is further divided into preleptotene (PL), leptotene (L), zygotene (Z), pachytene (PH) and diplotene (D) phases (Abby et al. 2016). Meiotic recombination is performed via formation and repair of DNA double-strand breaks (DSBs), which are necessary to the homologue pairing and meiotic crossing-overs (chromosome exchange via chromosome arm flanking the DSB). Pairing begins in leptotene spermatocytes and the formation of DSBs is catalyzed by topoisomerase II-like protein SPO11 (Milman et al. 2009; Keeney 2008). A hallmark of the meiotic recombination is synapses of two sister chromatids. During the meiosis event prophase I, maternal and paternal homologues pair and condense in *leptonema*. A tripartite protein structure called "synaptonemal complex" (SC), which is composed of two lateral elements and a central element, ensures that homologous chromosomes remain held together during meiosis. Assembling of SC begins in *zygonema* and is finalized in pachynema(Cohen et al. 2006).

As a result of the first meiotic division, the secondary spermatocytes are formed. The secondary spermatocytes then enter to the second phase, meiosis II, to produce haploid spermatids (Russell et al. 1993; Huang et al. 2012; Tan et al. 2015). Round spermatids are haploid cells resulted from meiotic divisions. During the spermiogenesis cells do not divide but instead, in this phase round spermatids differentiate to spermatozoa and undergo extensive deformation of the nucleus and the cytoplasm to receive a condensed nucleus (Russell et al. 1993; Abou-Haila & Tulsiani 2000).

The elongation of spermatids begins in the late stage of spermiogenesis, when spermatids are polarized. At this phase, spermatids are anchored to Sertoli cells until the elongation is completed. After the elongation has ended, spermatozoa are released into the lumen and the excess cytoplasm and cell organelles are discarded by Sertoli cells in the form of a residual body (Huang et al. 2012).

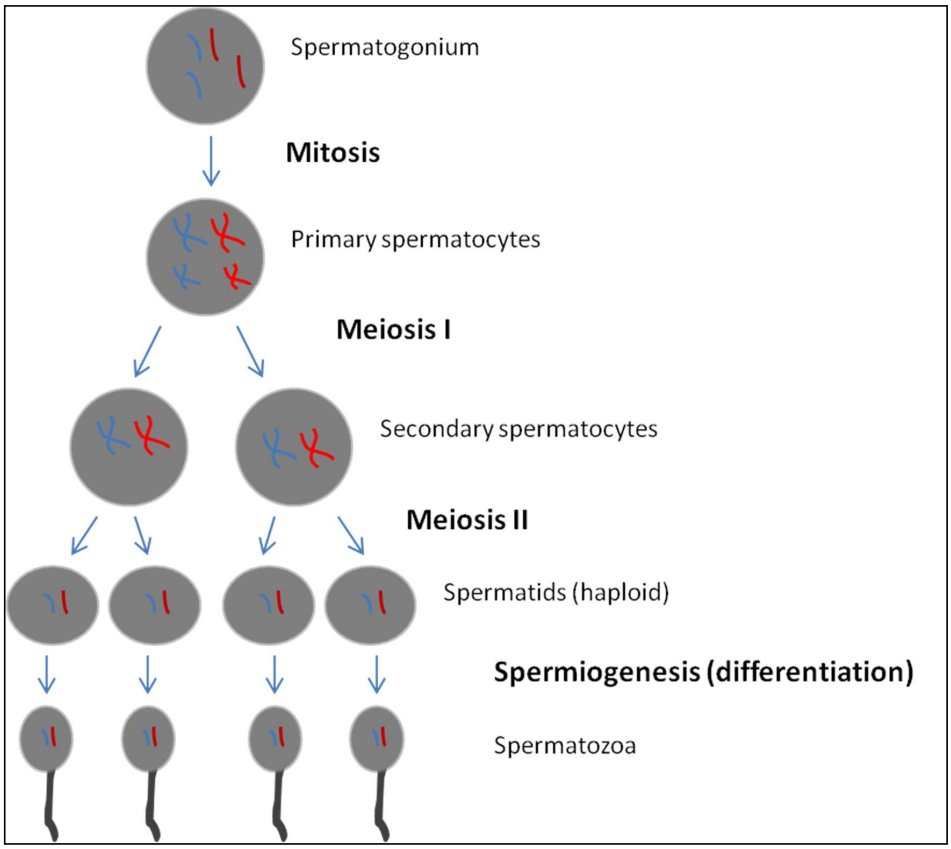

Figure 4 Phases of spermatogenesis. Spermatogenesis involves three phases: the mitotic proliferation of spermatogonia, meiosis with two divisions of reduction of chromatin and spermiogenesis which ends with the release of spermatozoa into the lumen (Russell et al. 1993).

2.2.2 Hormonal control of spermatogenesis

Among the other regulators, a very important level of control is offered by the hormones from the hypothalamic–pituitary–gonadal (HPG) axis during spermatogenesis (Holdcraft & Braun 2004). Gonadotropin–releasing hormone (GnRH) is secreted from the hypothalamus and triggers gonadotropins from the anterior pituitary. There are two gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both are essential for the successful completion of spermatogenesis. Androgens, such as testosterone, are secreted by the influence of LH from somatic Leydig cells, which are located in the interstitial space outside the seminiferous tubules (Shima et al. 2013). Both hormones FSH and testosterone act on Sertoli cells to control their function and effect on germ cells via Sertoli cells.

Testosterone controls all stages during spermatogenesis, which does not progress beyond the meiosis in the absence of testosterone (Walker 2011). It has been noted that testosterone is able to maintain spermatogenesis even at very low levels (Zhang et al. 2003). However, it has been demonstrated that elongated spermatid number is directly related to testicular testosterone concentration in interstitial and seminiferous tubular fluid (Zirkin et al. 1989).

FSH functions by stimulating Sertoli cell mitosis during differentiation (Griswold 1998; McLachlan et al. 1995) but is not essential for fertility. Male knockout mice lacking FSH showed normal sexual development with decreased number of Sertoli cells and mature sperm but did not manifest problems in fertility (Kumar et al. 1997).

2.2.3 Organization of the seminiferous epithelium

Male germ cell differentiates in the semineferous tubules (figure 5). Differentiated germ cells of the same differentiation state form layers consecutively in the seminiferous epithelium. Spermatogonial stem cells and spermatogonia are found at the basal side of the seminiferous epithelium (OAKBERG 1956; de Rooij 1998). The next layer towards the lumen consist of spermatocytes, followed by a layer of round spermatids and finally elongating spermatids that are found closest to the lumen. One wave of spermatogenesis is described as a time that development takes from an A spermatogonium to mature spermatozoa. In mouse testes it takes 34,5 days (OAKBERG 1956).

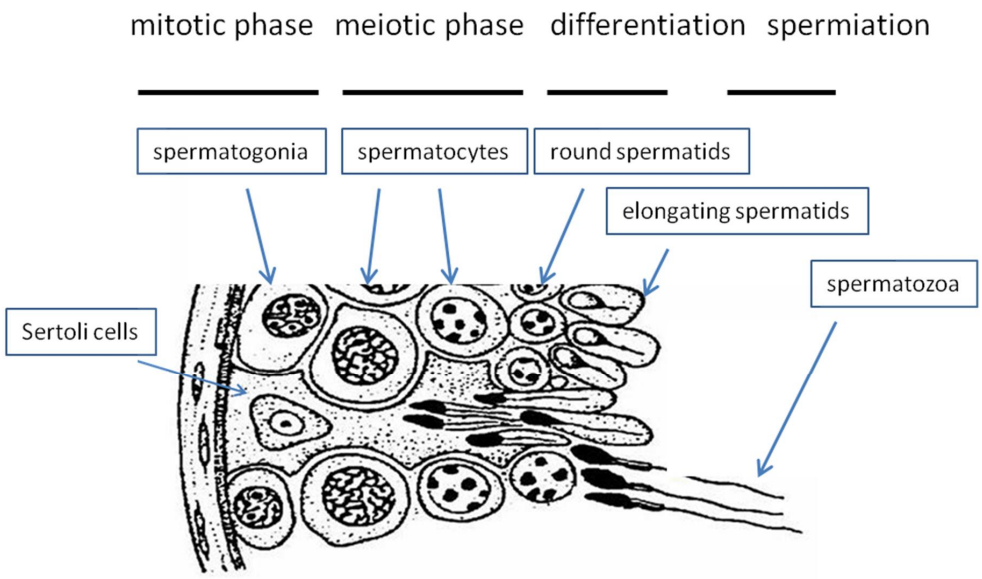

Figure 5 Organization of mouse seminiferous tubules. Schematic drawing illustrates the cross section of a mouse seminiferous tubule with mitotic spermatogonia, spermatocytes, and spermatids surrounded by Sertoli cells. Male germ cells differentiate in layers from basal membrane toward lumen (Modified from Fallahi et al. 2010).

There are synchronized changes in the cell associations in the seminiferous epithelium that occur in repetitious cycle that is called a cycle of seminiferous epithelium. The cycle can be divided into specific stages based on the cell content found in the epithelium (Figure 6). The number of stages within a cycle of the seminiferous epithelium varies between species. For example, a mouse and a human have 12 distinct stages while a rat has 14 stages (Clermont 1972; Muciaccia et al. 2013; Russell et al. 1993). Different stages can be detected and isolated on the basis of their transillumination patterns, which allows the precise characterization of germ cells at specific phase of differentiation and the critical factors involved in the cell cycle, chromatin dynamics, spermatid differentiation, stem cell biology and fertility (Parvinen 1982; Toppari & Parvinen; Kotaja et al. 2004). Differentiating spermatids can be divided in 16 steps on the basis of the morphological changes occurring during their differentiation (Figure 6).

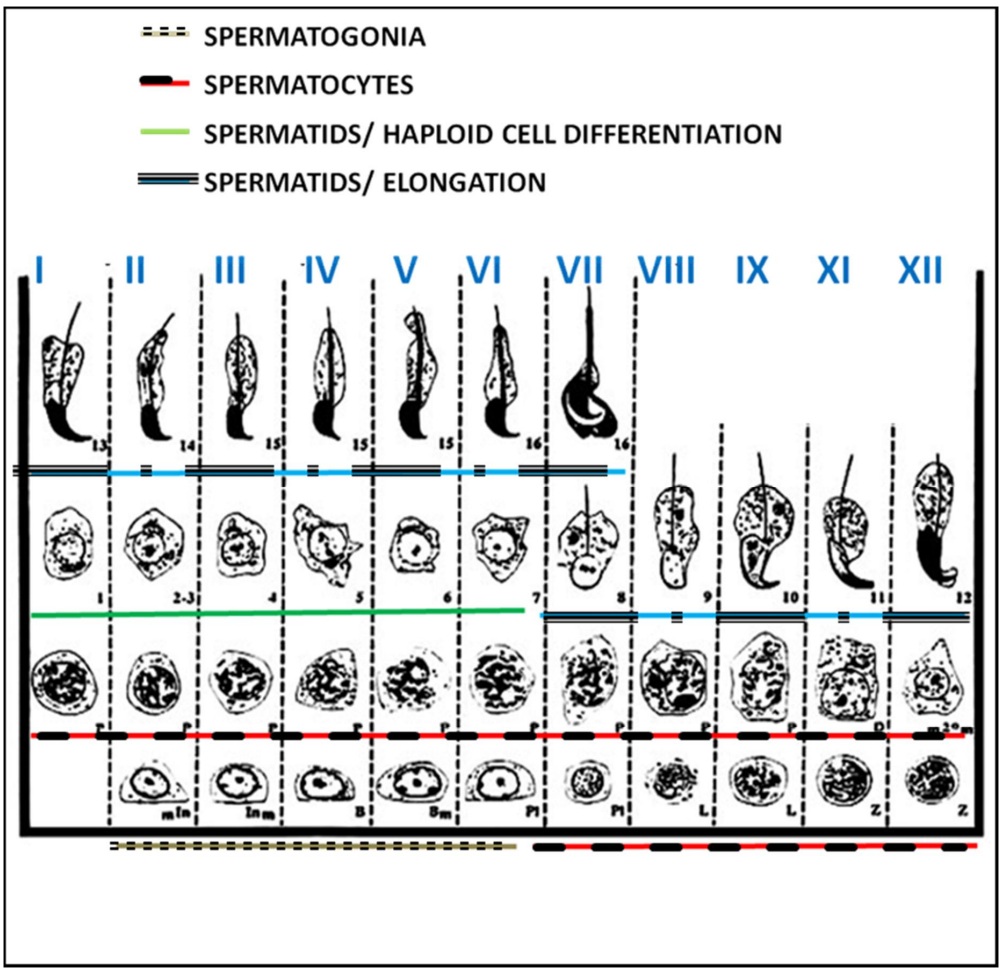

Figure 6 Stages of the cycle of seminiferous epithelium in mice. Precisely organized associations of differentiating cells are found in each stage. The picture illustrates stages of a mouse seminiferous tubule within mitotic spermatogonia, spermatocytes, and spermatids (steps 1-16). Stages are presented with roman numerals (modified from Russell et al. 1993).

2.2.4 Cell-cell junctions in the testis

Sertoli cells are somatic cells that are connected to each other and to the spermatogenic cells, which are going through the meiotic divisions and differentiation. This connection is formed by cell-cell junctions. These junctions can be classified into three major types: anchoring junctions, tight junctions and gap junctions. The anchoring junctions can be further subdivided into actin filament-based adherens junctions, focal adhesions, desmosome-like junctions and cell-matrix hemidesmosomes. Testis has several types of junctions that are in close association with each other in the seminiferous epithelium (Lee et al. 2009). Unique testis-specific junction organization is

formed either between two Sertoli cells or in the spermatid-Sertoli cell interphase. The first mentioned is called blood testis barrier (BTB) and the second mentioned is called apical ectoplasmic specialization (aES). These junction organizations are not passive, rather they are dynamic, specific and complex epithelial organizations that differ from the other epithelial cells in major epithelial marker molecules and structures (Domke et al. 2014).

During extensive morphological modifications of male germ cells including cell polarity, chromatin condensation and the formation of the acrosome and tail, differentiating germ cells migrate across the entire diameter of the seminiferous epithelium until they reach the lumen where mature spermatozoa are released. It is conceivable that there are well organised and strong cell-cell interactions between germ cells and surrounding somatic cells. During the process, spermatids are anchored to the surrounding somatic Sertoli cells. Anchoring of differentiating cells in the seminiferous epithelium and the functional cell interconnections are maintained through junctional organization that takes place in the Sertoli-germ cell interphase. These cell–cell interactions are crucial for the production of several important molecules such as hormones, growth factors, proteases, protease inhibitors and components of the extracellular matrix (Kopera et al. 2010).

2.2.4.1 Junctions between germ cells and Sertoli cells

Several types of junction complexes have been presented in Sertoli cell-spermatid interphase. These include desmosome-like junctions and Ectoplasmic specialization (ES), which includes both apical (aES) and basal Ectoplasmic specialization (bES) (Kopera et al. 2010). The presence of desmosome junctions in an adult mammalian testis is still unclear due to controversial results (Domke et al. 2014).

aES is a unique junction organization to a male germ cell and is formed in the Sertoli cell-spermatid interphase connecting the apical side of nucleus of differentiating spermatids to Sertoli cells (Figure 7). Structural components include nectins (required for the initial Sertoli cells- spermatid contact), the VE-cadherin/catenin complex which is required for the stabilization and the polarization protein, junctional adhesion molecule-C (JAM-C), which is required for the positioning of the spermatid. aES is first visible at stage VIII, immediately when the spermatid nucleus is polarized and attached to Sertoli cell until the differentiation of germ cell is completed. Anchoring of spermatid to the Sertoli cells ensures the correct positioning of head toward to the basal compartment during differentiation as well as prevents the immature release of spermatids (Berruti & Paiardi 2014). This junction organization has been demonstrated to be essential for the correct germ cell differentiation and for the male fertility (Cheng & Mruk 2012).

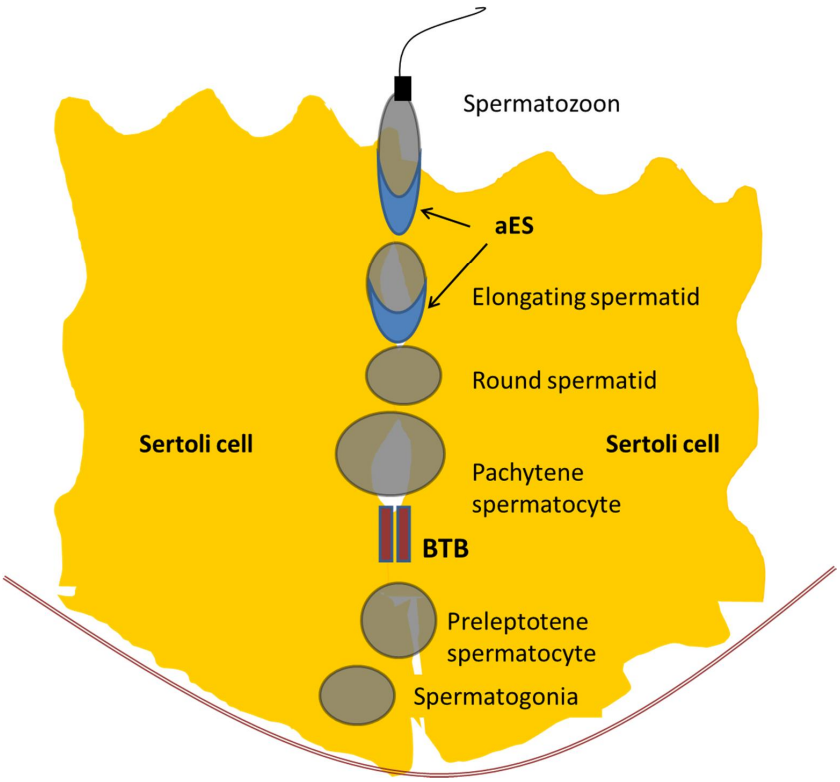

Figure 7 Localization of aES and BTB in the seminiferous tubule.
The picture demonstrates the location of the aES and BTB in the seminiferous tubules. BTB is formed between two Sertoli cell at the basal side of the seminiferous tubule. Junction complex include several junction proteins. Junction proteins of aES are accumulated on the spermatid membrane between the spermatid and Sertoli cell (Cheng et al. 2011).

2.2.4.2 Junctions between Sertoli cells

At the time of puberty, Sertoli cells form specialized tight junctions, which connect them to each other and divide the seminiferous tubular space into apical and basal compartments. This important cell junction structure in testis, BTB, is one of the tightest types of junction. In contrast to the other blood-tissue barriers, such as the blood-brain barrier, which is composed of exclusively tight junctions, BTB is composed of different types of junctions between adjacent Sertoli cells near the basement membrane (Cheng & Mruk 2012). Mammalian BTB is constituted by tight junctions, basal ectoplasmic specializations (a testis-specific adherens junction), gap junctions, and desmosomes. Integral proteins, which are found in the different junction of the BTB, are occludin, claudin-1, -3, -4, -5, -7, -8, -11, JAM-A, JAM-B, CLMP, -cadherin, E-cadherin, nectin-2, and CAR (Mital et al. 2011; Cheng & Mruk 2012).

BTB is physically located between the blood vessels and the luminal compartment of the seminiferous tubules. Beside physical barrier, it controls the entry of chemicals and cells from entering the apical compartment. In general, the main function of the BTB is to create the appropriate microenvironment for regulation of germ cell development (Mital et al. 2011). Meiotic divisions, spermiogenesis, and spermiation all take place at the apical side of the BTB, but all preleptotene spermatocytes preceding processes like spermatogonial renewal and proliferation take place at the basal side of the BTB. At the time of stage VIII, BTB undergoes extensive restructuring when it permits the translocation of preleptotene spermatocytes through the BTB.

2.3 Haploid male germ cell differentiation

A spermatozoon has the most divergent structure and features to the other type of cells. Due to its functions outside the body, the nucleus is compact and well-protected against adverse factors from the environment. In addition to the cell polarization, nuclear shaping and histone replacement by protamines, the last phase of spermatogenesis involves formation of the acrosome and tail.

2.3.1 Acrosome formation

For the successful fertilization, spermatozoon requires the capacitation of spermatozoon in the female reproductive tract, egg binding of capacitated spermatozoon, induction of acrosome reaction, and the fusion of spermatozoon with the egg membrane. Acrosome is a Golgi-derived structure that forms a cap-like structure over half of the sperm's head. Acrosome is composed of an inner and outer membrane and the latter membrane, which fuses with overlaying plasma membrane of the oocyte in fertilization. At the site of the egg binding, the acrosome releases enzymes and proteins that are required for the fertilization (acrosome contents). The reaction is exocytose reaction, also known as acrosome reaction, and it occurs in the acrosome of sperm when the sperm reaches the egg. Only the acrosome reacted sperm can penetrate and thus the acrosome reaction is crucial for the fertilization (Avella & Dean 2011).

The acrosome formation is initiated in the beginning of spermiogenesis, in early round spermatids. Some vesicles in round spermatids are filled with dense, granular materials derived from the trans-Golgi stacks. These vesicles are transported to one pole of the nucleus. Acrosome formation begins when vesicles enlarge and empty material, glycoprotein to the developing acrosome. This continues until late spermiogenesis (Abou-Haila & Tulsiani 2000). The acrosome formation can be divided in four phases (Figure 8): Golgi phase, cap phase, acrosome phase and maturation phase. In the Golgi phase, proacrosomic granules are created from trans-Golgi stacks. During the cap and the acrosome phase, developing acrsome flattens to a cap-like structure on the surface of the nucleus and the structure is spread over the nucleus finally covering half of it. This structure remains until spermiogenesis (Abou-Haila & Tulsiani 2000).

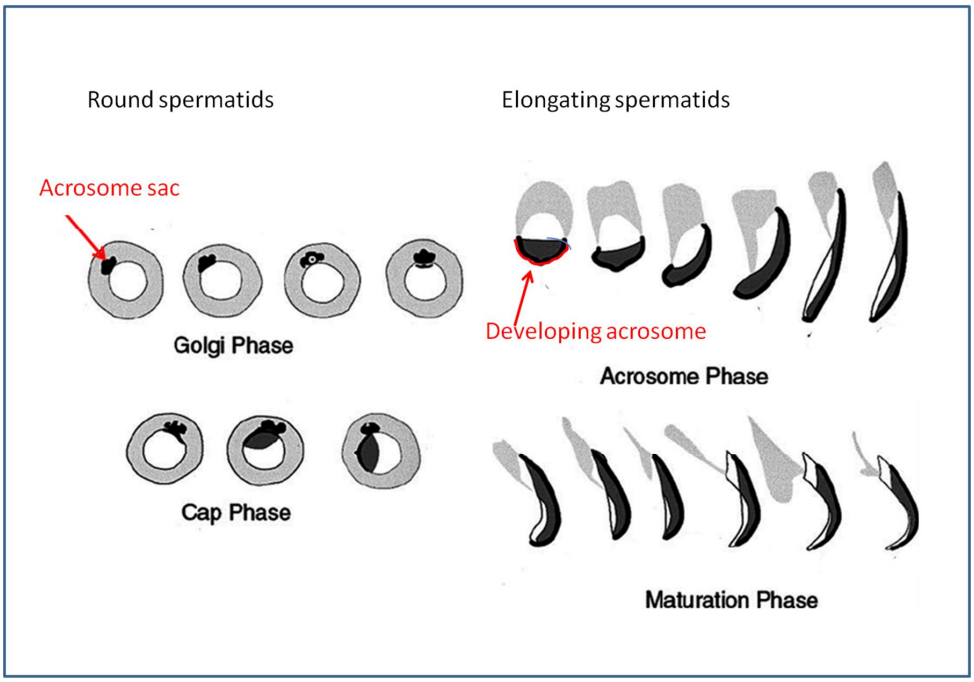

Figure 8 Formation of acrosome. In round spermatids, the acrosome sac is formed from Golgi vesicles. The acrosome develops in four phases: Golgi phase, cap phase, acrosome phase and maturation phase (modified from Abou-Haila & Tulsiani 2000).

2.3.2 Formation of flagellum

Sperm cell is a unique cell since it is able to swim. Motility of sperm cell is a crucial factor in fertilization and it is needed for transportation of male DNA to the egg. One of the main causes of male fertility problems is a malformed tail, which leads to sperm motility problems.

By structure and function, sperm flagellum is similar to cilia (Lodish et al. 2000). Mammalian sperm flagellum is composed of core structure (axoneme), plasma membrane and accessory structures between them, such as mitochondria, outer dense fibers (ODs) and the fibrous sheath (FS). Flagellum comprises proteins, which are involved in creating but also in the regulation of motility. That is based on the active microtubules and motor proteins (Inaba 2011; Lehti & Sironen 2016).

The Formation of the tail begins with the formation of axoneme and continues with combining of accessory structures. ODs are constructed along with the axoneme and then FS is built. All these processes are combined by specific protein delivery system. To gain normal sperm production, specific proteins and protein complexes must be transported to the correct site during tail formation. Some of these proteins have a delivery system between the tail and manchette that is a coat-like structure on the spermatid nucleus and essential for orchestrating protein distribution and

function during elongation. It has been shown that disruption of the protein delivery system during spermiogenesis results in malformations of sperm, but the exact mechanism is not clear (Lehti & Sironen 2016).

2.3.3 Cell polarization

Developing of eukaryotic cells involves rearrangement of cellular structures and polarized redistribution of cell organelles. The nucleus is often the largest organelle that is repositioned during the differentiation (Burke & Roux 2009). An active positioning of the nucleus and the direct association between the nuclear envelope and the cytoskeleton are critical for polarization of the nucleus.

Spermatids are highly polarized cells. Polarization directs the spermatid nucleus to the correct position in the anterior–posterior axis so, that the heads of the elongating spermatids are toward the basal membrane, while the tails are heading to the apical side. The arrangement of polarized spermatids is essential since it enables more space for differentiating spermatids (Gliki et al. 2004) and also lets spermatids to communicate with Sertoli cells, which is crucial for the differentiation. Additionally, the polarization of spermatid nucleus allows the normal formation of cell junction which is required for the attachment of spermatids to the Sertoli cells. Thus the polarized distribution of the spermatid nucleus is critical for the differentiation of spermatids and fertility (Berruti & Paiardi 2014).

Anchoring of the nucleus and nuclear polarization require an assembly of complex of polarity proteins, which are located on the anterior pole of the nucleus, when flagellum is formed on the opposite pole. Polarity proteins are important for orchestrating the polarization but the activity of the polarization, localization of the cytoskeleton and polarity complexes, signaling networks, and membrane trafficking is regulated by the other factors. It has been demonstrated that cyst polarization depends on a typical protein kinase C (aPKC) in *Drosophila.* Accumulation of aPKC is regulated by *Drosophila* CPEB protein Orb2, which has been shown to function in orienting cyst polarization but also process the polarization itself (Xu et al. 2014). Phosphatidylinositol 4,5-bisphosphate (PIP_2) has also shown to regulate the polarization of spermatids in *Drosophila.* The lack of PIP_2 leads to the bipolar cyst and failure in elongation (Fabian et al. 2010).

Martinov and coworkers have presented that changes in chromatin structure can affect the polarization of the nucleus. H1T is a testis specific histone 1 (H1), which is known to have a role in the formation of chromatin structure. H1T2 is a histone variant for H1T, which have been shown to be a component of the polarity complex and therefore reveals the polarity of spermatid nucleus. H1T2 has also been presented as a chromatin-related protein. H1T2 forms a cap-like structure at the nuclear membrane of round spermatids during stages V-VIII. Very weak expression of H1T2 can be detected in round spermatids also at stage IV. In elongating spermatids, polarization can be detected in steps 9–14 (stages IX-II). In both round and elongating spermatids, H1T2 is localized at the apical pole of spermatid nucleus, beneath the acrosome (Martianov et al. 2005). Polarization

has been detected to be affected in the loss of proteins TRF2 or HMGB2 that are involved in organizing of the nuclear chromatin structure. That demonstrates that the polarization of the nucleus is dependent on the normal chromatin structure (Martianov et al. 2002).

Mammalian sperm head shaping requires also the polarization of chromatin structure. Testis specific polarization protein complexes (LINC) are nuclear envelope bridging proteins that are formed to opposite nuclear poles. The anterior polarizing non-nuclear LINC-complex consists of proteins that are mainly located within the outer acrosomal membrane (OAM) and is located in the anterior side of nucleus. This protein complex functions as a connector complex between acrosome and the surrounding manchette. The other LINC complex, the posterior LINC complex, is located on the opposite pole of the nucleus and it can be connected to cytoskeletal elements of spermatids. These protein complexes are critical for chromatin dynamics during meiosis and the formation of head shaping (Göb et al. 2010).

Many of the proteins that are involved in the formation of polarization complex of spermatid nucleus are also involved in the formation of cell junction in germ cells and Sertoli cells. The junction protein described to be involved in the assembly of the polarity complex is a cell-surface protein, JAM-C (Gliki et al. 2004). JAM-C also co-localizes with tight junctions in the epithelium and is a part of the junctional organization of aES (Pellegrini et al. 2011). The protein complex LINC can anchor to the actin filaments of cell junctions by interacting with proteins in OAM (Göb et al. 2010). Rap1 governs the reorganization of actin cytoskeleton, which is required for both aES and spermatic polarization.

2.3.4 Histone-protamine transition

The nucleus of round spermatid elongates and reshapes during spermiogenesis and chromatin structure undergoes dramatic reorganization to become a tightly compacted structure. This process requires DNA breaks to enable chromatin opening and remodelling. In male germ cells histones are replaced by transition proteins and protamines and several histone modifications finally result compact structure of DNA and protamines.

Nucleosome is a basic unit of chromatin and it is composed of DNA wrapped around the core histones H2A, H2B, H3 and H4. In addition to the core histones, histone H1 protects DNA from aggregating with the other nucleosomes (Govin et al. 2004). When DNA is tightly packed around the histones in the nucleosome, the compact structure restricts access to gene regulation components such as transcription factors and RNA polymerase. Therefore distinct mechanisms have evolved to influence the dynamic of the nucleosome and DNA binding factors. The dynamic is regulated by numerous ways. The core histones are targets of a variety of post-translational modifications that alter their interactions with the DNA and the other nuclear proteins, and thus regulate the chromatin organization and the rate of the transcription (Bettegowda & Wilkinson 2010). Canonical histones can also be replaced by histone variants that can alter the structure, stability and dynamics of nucleosome and therefore, have multiple roles in processes like transcription initiation and DNA repair (Clarkson et al. 1999; Weber & Henikoff 2014; Govin et al. 2004).

2.3.4.1 Incorporation of histone variants

Chromatin remodeling includes the incorporation of histone variants. Changes in histone-DNA interaction enable the incorporation. ATP-dependent molecular complexes can induce the reorganization of the chromatin structure (Lusser & Kadonaga 2003) or histone modification enzymes can affect to the chromatin structure by creating " a histone code" via post-translational histone modifications, which cause alterations in the chromatin structure (Govin et al. 2004).

Histone variants for all histones H2A, H2B, H3 and H1 have been identified. Histone variants unique to male germ cells are incorporated into the chromatin during different phases of spermatogenesis (Table 1). H1t and HILS1 are testis specific linker histones. Many variants are incorporated during the meiosis, but for example the linker histone HILS is expressed and incorporated to the chromatin later in spermatogenesis (Govin et al. 2004).

Table 1 Histone variants and testis specific histone variants (Govin et al. 2005; Govin et al. 2007).

Histone	*Histone variants in somatic cells*	*Testis specific variants*
H1	*H1.1- H1.5 H1**	
H2A	*H2A.X,H2A.Z, macroH2A, H2Abbd*	*TH2A, H2AL1, H2AL2*
H2B		*TH2B, TSH2B, H2BFWT*
H3	*H3.1, H3.2, H3.3, CENP-A*	*H3t*

Histone variants are known to be key regulators in the chromatin structure reorganization and chromatin function. H3.3 is known to be incorporated in the first phase of meiosis and is known to concentrate at the chromatin during meiosis and then later disappear with other histones. The function of H3.3 has been analysed in several studies. The function obviously differs between species (Szenker et al. 2011; Govin et al. 2004) but a major role has been described to be in regulating the chromatin dynamics (Yuen et al. 2014). Two variants HAL1, H2AL2 are known to mark pericentric regions in condensing spermatids and thus are involved in the pericentric heterochromatin reprogramming (Govin et al. 2007). Those variants are demonstrated to quickly disappear after fertilization (Wu et al. 2008). Some testis specific variants can function to facilitate the incorporation of other variants such as heterodimers of H2A-H2B and H2A.Z-H2B. Additionally HA2.Z variant have been suggested to alter the surface of nucleosome and alter the chromatin structure and thus prepare the chromatin for remodelling (Govin et al. 2004).

It has been shown that Histone H2AX have a role in condensing Y- and X- chromosomes to form a sex body in meiosis. Moreover, it was described that other sex body proteins, such as macroH2A1.2 and XMR, are not able to localize with the sex chromosomes in the absence of H2AX indicating an important role in chromatin remodelling during meiosis (Fernandez-Capetillo et al. 2003).

2.3.4.2 Histone hyperacetylation

The composition of nucleosome varies from one chromosomal domain to another and that is often caused by the acetylation of core histones (Turner et al. 1992). Histone hyperacetylation is a process when lysine of the core histone is acetylated. There is a correlation between the level of histone acetylation and the transcriptional activity of a chromosomal domain (Jeppesen and Turner 1993) since the hyperacetylated histones accumulate precisely within particular active chromatin domains and the hypoacetylated histones accumulate within the transcriptionally silenced domains (Braunstein et al. 1993). This correlation is consistent with the structural alterations between the nucleosome with hyperacetylated histones and the nucleosome with hypoacetylated histones. Hyperacetylation of core histones directs the allosteric changes in the nucleosome that destabilizes higher-order structure of the nucleosome and makes nucleosomal DNA more accessible to transcription factors (Wolffe & Pruss 1996).

Hyperacetylation of core histones precedes histone-protamine transition and results in a reduction of DNA-histone interaction. Hyperacetylation of histone H4 occurs in elongating spermatids when the entire complement of histones is replaced by protamine-like proteins and protamines. Therefore hyperacetylation is one of the key steps in histone-protamine transition (Grimes & Henderson 1984). Hyperacetylation of histones takes place in step 9 to 11 elongating spermatids and disappears later in condensing spermatids when protamines replace histones. It is known to be essential for chromating remodelling and for all the later steps in spermatogenesis including elongation of spermatids. Failure in hyperacetylation can reflect to problems in fertility (Sonnack et al. 2002).

Incorporation of histone variants creates an unstable nucleosome, which then undergoes hyperacetylation. Acetylation at critical lysines destabilizes the nucleosome and generates the platform for the recruitment of Bromodomain protein (BRDT). BRDT is a key molecule in guiding the chromatin remodelling (Dhar et al. 2012). It recognises and binds to an hyperacetylated histone H4 that enables the histone replacement by transition proteins (TPs) (Jonathan Gaucher et al. 2012; Goudarzi et al. 2014). BRDT is a crucial regulator of both meiotic divisions and post-meiotic genome repackaging. It directs the developmental timing of a testis-specific gene expression by activating essential genes and repressing the others. BRDT is therefore an important regulator of male germ cell differentiation that controls a specific spermatogenic gene expression program as well as packaging of the male genome (Gaucher et al. 2012; Dhar et al. 2012).

The role of BRDT has been studied by creating a mouse (Brdt(ΔBD1/ΔBD1) lacking the first bromodomain of BRDT. Mice showed sterile phenotype and exhibited profound defects in chromatin remodelling during spermiogenesis. Consequently they suggested that the first bromodomain of BRDT might be crucial for the formation and/or maintenance of an intact chromocenter and needed for remodelling of the chromatin architecture of the sperm head (Berkovits & Wolgemuth 2011).

2.3.4.3 Transition proteins and protamines

In mammals histones are replaced by sperm-specific protamines to enable tight packing of the chromatin. This replacement does not occur directly from histones to protamines, instead, there is a group of TPs that replace histones and bind to the DNA in the transition state (Balhorn 2007). Actually, TPs constitute 90% of the basic chromatin proteins during this specific phase (Meistrich et al. 2003).

There are two major transition proteins TP1 and TP2 in testis that have a role in histone displacement, sperm nuclear shaping, chromatin condensation, and reducing the number of DNA breaks prior the histone protamine exchange. Mice with null mutations in the genes for TP1 and TP2 have shown normal histone displacement and sperm nuclear shaping. However, problems in nuclear condensation and an elevated number of DNA breaks have been demonstrated in the lack of TP1 and TP2 (Zhao et al. 2004; Meistrich et al. 2003).

Protamines are the arginine-rich proteins that are the major group of nuclear proteins in sperm. The human sperm nucleus contains two types of protamine: protamine 1 (P1) and the family of protamine 2 that includes family members P2, P3 and P4. These proteins are with a high content of positively charged amino acids, particularly arginine. Protamines condensate the nucleus into a compact hydrodynamic shape, protect of the genetic message in spermatozoa, are involved in the processes that maintain the integrity and repair of DNA during or after the protamine transition and are involved in the epigenetic imprinting of the spermatozoa. Protamines are showed to be crucial to the male fertility. Changes in the expression of P1 and P2 protamines and also the mutations in the protamine genes have been found to be associated with infertility in man (Oliva 2006; Jiang et al. 2015). Protamines have a critical role in the compaction of chromatin and changes in the expression of protamines can lead to the structural defects in the sperm nucleus leading to variable degrees of infertility (Aoki & Carrell 2003). Some histones are retained in humans. Thus, the persistent histones are not always the result of inefficient protamine replacement. (Weber et al. 2007).

2.4 RNA regulation of spermatogenesis

Complex organisms like mammals require tissue-specific gene regulation, which is one of the most tightly controlled biological processes. Precise temporal and spatial regulation of gene expression ensures that the correct amount of functional RNAs and proteins are produced at the correct time. A disruption in the regulation system can lead to severe diseases or other consequences (Beyersmann 2000; Emilsson et al. 2008), such as problems in fertility (Bansal et al. 2015). The gene expression as a response to stimuli is strictly regulated at the transcriptional level, but in addition, posttranscriptional RNA regulation plays a major role.

2.4.1 Transcriptional activity during spermatogenesis

The first step of gene regulation takes place at the transcriptional level when genes are transcribed from DNA to RNA in the nucleus. This step is regulated by several transcription factors, including homeobox, zinc-finger, heat-shock, and cAMP-response family members (Maclean & Wilkinson 2005). Transcription factors function by binding onto a DNA sequence and either activating or silencing genes. Chromatin is the key player in the control of transcription factor accessibility to the promoter regions of genes. In general, chromatin can be presented in the two states, euchromatin or heterochromatin. Euchromatin is open for transcriptional control, whenever heterochromatin is compact and tightly packed, out of the transcriptional activation (Meikar et al. 2014). Additionally, euchromatin can undergo extensive remodelling that either facilitates or inhibits the binding of transcription factors, the gene regulatory elements (Luo & Dean 1999).

Chromatin modification is also active in differentiating male germ cells, which widely alters the level of the transcription at specific stages during spermatogenesis (Sassone-Corsi 2002). When the transcription is activated, the gene promoter is open for transcription factors and other regulators.

The trimethylation of H3 at the lysine 4 (H3K4met3) is a histone modification that can activate transcription, whereas the dimethylation of H3 at lysine 9 (H3K9met2) is a process, which generally prevents transcription factor binding by changing the chromatin structure and represses transcription (Stewart et al. 2005; Eissenberg & Shilatifard 2010; Lim & Maher 2010). However, these processes rarely function without complex communication between other modifications, including acetylation, ubiquitylation and methylation (Rose & Klose 2014).

Spermatogenesis is a carefully orchestrated differentiation program. Transcriptional activity of the genome is exceptionally diverse and extensive during most of the steps of spermatogenesis from spermatogonia to early elongating spermatids. Especially meiotic and post-meiotic male germ cells have a more diverse transcriptome than other cell types due to a broad expression of their genome (Soumillon et al. 2013). Despite the high transcriptional activity, meiotic cells undergo transcriptional silencing of specific chromosomal regions, particularly sex chromosomes. During the pachytene stage, the X and Y chromosomes form the XY body, where they achieve partial synapsis in the pseudoautosomal region. The transcription of genes linked to unsynapsed axes is silenced in a process that is called meiotic sex chromosome inactivation. Within the context of chromatin, transcriptional silencing includes several types of histone modifications (An et al. 2010).

Transcription is mostly silenced in late elongating spermatids and in mature spermatozoa due to histone-protamine transition, which makes the genome transcriptionally incompetent (Kimmins et al. 2004). RNA regulation is very active during this time of spermatogenesis. Range of proteins is required for the formation of specific structures of sperm cells but transcription of mRNA is silenced because of the condensed nucleus of elongating spermatids. Therefore it is important that mRNA transcripts are transcribed earlier and stored for the later necessity (Bettegowda & Wilkinson 2010).

Cyclic AMP response element (CRE) modulator (CREM) is one of the master transcription factors involved in the control of spermatogenesis. Several isoforms of CREM are expressed at different times of spermatogenesis, and CREMτ, an activator isoform, is specifically accumulated in postmeitoic round spermatids. CREM knockout mice have been described to be infertile with a complete block of spermatogenesis at the round spermatid stage (Blendy et al. 1996; Nantel et al. 1996). Several important genes required for haploid differentiation are misregulated in CREM knockout testis (Martianov et al. 2010). The CREM mediated transcriptional control is regulated by a follicle stimulating hormone which is secreted from Sertoli cells (Gupta 2005).

2.4.2 Small non-coding RNAs

The best known and the most studied group is miRNAs. Together with piRNAs, miRNAs represent a very important group of gene regulators in differentiating male germ cells. The small RNA groups, their biogenesis, sorting and function are presented in Table 2.

Table 2. Four main classes of small RNAs and their difference in biogenesis, sorting and function (Sasidharan & Gerstein 2008; Piatek & Werner 2014).

Small RNA	miRNA	siRNA	endo-siRNA	piRNA
Source	miRNA genes	Exogenous dsRNA	Pseudogenes, Double stranded intrinsic transcripts	Single stranded RNA
DICER dependent/independent	DICER dependent	DICER dependent	DICER dependent	DICER independent
length	22 -25nt	21 nt	20-23 nt	24-30 nt
Argonaute effector within RISC	AGO1	AGO2	AGO2	PIWI
Cellular role	Gene regulation	Viral defence, gene regulation	Gene regulation	Transposon silencing

2.4.2.1 miRNA expression and function during spermatogenesis

In mammals, numerous miRNAs have been identified to be expressed in testis and miRNAs have recently been shown to regulate spermatogenesis in many different ways. miRNA pathways have been demonstrated to be crucial for germ cell development starting from pri-mordial germ cells to germ cell differentiation. That has been shown using several testis-specific conditional knockout mouse models. (K Hayashi et al. 2008; Maatouk et al. 2008; Romero et al. 2011; Korhonen et al. 2011; Wu et al. 2012; Greenlee et al. 2012). The expression of testis-specific miRNAs has been studied by using techniques such as microarray and high-throughput sequencing. The results have shown clear accumulation of miRNAs in differentiating germ cells instead of the undifferentiated ones indicating the important role of miRNAs in germ cell differentiation (Kotaja 2014).

In *Drosophila*, miRNAs are demonstrated to be important for the proper control of germline stem cells by using a *Dicer1* mutant. The results indicate that miRNAs are required for the division of germline stem cells and for the normal bypass the G1/S checkpoint in the cell cycle (Hatfield et al. 2005). The role of DICER in mammalian primordial germ cells (PGCs) has been studied using a mouse model with a germ cell-specific deletion of *Dicer1* performed by a *Tnap-Cre* transgene. The mouse model demonstrated the defects in PGC proliferation and led to male fertility (K Hayashi et al. 2008). This PhD thesis has importantly contributed to the characterization of the role of DICER in postnatal germ cells, which will be discussed in more detail in the Result and Discussion sections.

2.4.2.2 piRNAs

PIWI-interacting RNAs (piRNAS) are the male germline -specific, DICER-independent group of small RNAs found in different species such in flies, fish and mammals. In mouse, piRNAs can be divided into two groups: pre-pachytene and pachytene piRNAs. Pre-pachytene piRNAs are expressed in pro-spermatogonia and early postnatal spermatogenesis (Figure 9) and are known to target transposable elements (TEs). These piRNAs have an important role in transposon silencing and therefore the protection of male germline genome (Han et al. 2015). Mouse pachytene piRNAs are transcribed from specific pachytene piRNA loci and processed in the nuage. Different mechanisms for biosynthesis of piRNAs have been described in a mouse, *D. melanogaster* and *C.elegans* (Weick & Miska 2014). Pachytene piRNAs are highly abundant during the pachytene stage of meiosis and in round spermatids (Figure 9). Their production and function is closely associated with germline-specific ribonucleoprotein (RNP) granules (described detailed in 2.3.4).

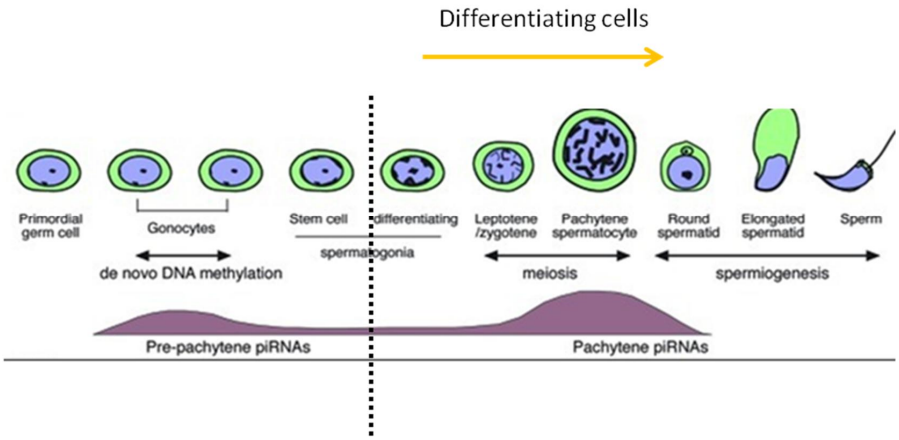

Figure 9 piRNA expressions during the male germ cell differentiation. Pre-pachytene piRNAs are abundant in pro-spermatogonia and the expression remains until the onset of meiosis, whereas pachytene piRNA are expressed from leptotene/zygotene to round spermatids and thire expression is highest at pachytene stage of differentiation (modified from Fu & Wang 2014).

In mouse testis piRNAs interact with PIWI proteins that are: MIWI (PIWIL1), MILI (PWIL2) and MIWI2 (PWIL4). The expression of these proteins is different in at different stages. Pre-pachytene piRNAs associate with MILI and MIWI2, whereas pachytene piRNAs associate with MILI and MIWI (Aravin et al. 2008). The function of pachytene piRNAs is still not fully understood, but they have shown to be involved in the post-transcriptional regulation of different transcripts, including mRNAs and lncRNAs during spermatogenesis (Goh et al. 2015). Pachytene piRNAs are also linked to LINE1 transposon silencing (Reuter et al. 2011; Schubert 2015). These piRNAs have also demonstrated to protect meiosis trough silencing genes that prevent meiosis from progressing. Thus pachytene piRNAs are important for maintaining meiotic processes and give rise to post-meiotic events (Zheng & Wang 2012; Schubert 2015).

Although piRNAs are predominantly expressed in the germline, some studies have reported piRNAs also in somatic cells in *Drosophila* ovaries (Li et al. 2009) (Malone et al. 2009), principal cells in epididymis of macaque (Yan et al. 2011), some tissues such as sea slug central nervous system (Lee et al. 2011) (Rajasethupathy et al. 2012) and rat brain (Dharap et al. 2011).

2.4.3 Germ granules

2.4.3.1 Germ granules as characteristic features of germ cells in different species

An additional level in the regulation of the germ cell development is offered by the RNA-rich, non-membranous cytoplasmic particles that are called germ granules. Due to their cloudy-like composition in microscope, mammalian germ granules are also called “nuage”, which is a French word “cloud”. These granules are similar to polar granules in insects as well as a germinal plasm in amphibians (Eddy 1974). A term ‘‘germ granules,’’ is commonly used, but they differ in their structure and function. For example, six types of nuage have been identified in rat spermatocytes (Sengupta & Boag 2012).

Germ granules are cytoplasmic aggregates formed from the fibrous material in the cytoplasm that contain proteins and RNA. Germ granules have been described in several organisms from fly to

mammals. A common feature of germ granules in all organisms is that they are necessary for germ cell function (Voronina et al. 2011). In some organisms, like *Drosophila melanogaster* and *Caenorhabditis elegans,* germ granules function in germ cell specification in egg cell (Trcek et al. 2015). Mammalian germ granules first appear in the primordial male germ cells after the germline has been specified, and undergo extensive morphological changes during postnatal germ cell differentiation.

The exact function for germ granules has long been poorly understood but during the last 20 years, the molecular events in the germ granule function and formation in several organisms have begun to be revealed and some hypothesis exists. Germ granules are usually considered as "RNA storage" (Trcek et al. 2015), involving proteins and mRNA required for germ cell development and for post-transcriptional gene control. Those proteins are often components of the miRNA and piRNA pathways (Vasa, Aub, AGO3, Tud, Spn-E, Bel, Squ, Cuff and AGO1) or associate with the piRNA mediated gene control (Kibanov et al. 2011).

Germ granules from different organism vary in their function, but they share some common features for all species. Comparison has shown that many regulatory proteins from diverse pathways in RNA regulation are concentrated in germ granules including small RNA pathways (Seydoux & Braun 2006), DEAD-box RNA helicases, PIWI family member protein, Tudor-domain containing proteins and a wild range of other proteins involved in various roles of RNA regulation (Goh et al. 2015b; Tsai-Morris et al. 2012; Toyooka et al. 2000; Hay et al. 1988; Meikar et al. 2014). The abundance of RNA-associated proteins strongly indicate that germ granules have an important role in the control of cell functions via post-transcriptional regulation.

2.4.3.2 Intermitochondrial cement (IMC)

The most prominent germ granules during mammalian spermatogenesis are the intermitochondrial cement (IMC) and the chromatoid body (CB). IMC forms in between mitochondrial clusters and CB first appear in the cytoplasm of pachytene spermatocytes as small granules. However, both disappear after the first meiotic division but CB appears again in the step 1 and 2 spermatids, begins to condense and is closely adjacent to the germ cell nuclear envelope (Morroni et al. 2008). IMC is not present anymore in haploid round spermatids (Fawcett et al. 1970). In late spermatids, CB is present as a late CB and enters the residual body at the base of flagellum (Yokota 2008).

The function of IMC and the possible functional interplay between the mitochondria and IMC has been unclear. In recent years, the improved techniques and instrumentation, for instance for histology and microscopic studies of tissues and cells, have improved our knowledge on of the composition and function of germ granules. Currently, the function of IMC has been linked to piRNA biogenesis (Huang et al. 2011; Kibanov et al. 2011). In silkworms PNLDC1, which is the pre-piRNA 3′

Trimmer, is described to bind to Tudor Papi/Tdrkh protein on mitochondrial surface (Izumi et al. 2016).

2.4.3.3 Chromatoid body (CB)

CB material begins to assembly in the cytoplasm of late pachytene spermatocytes. During meiotic divisions, CB precursor granules appears to disintegrate, but in secondary spermatocytes, the CB material aggregates again into large dense granules that are further aggregated in early round spermatids to form a single large structure per cell (Meikar et al. 2011).

CB was first discovered already more than hundreds of years ago by von Brunn (1876) and Benda (1891). However, knowledge of its composition and function remained unclear, until recent years. The breakthrough in the CB research was the biochemical isolation of CB (Meikar et al. 2010). It allowed better characterization of the function and the content of the CB. By mass spectrometry of isolated CBs, main protein components of the CB were described to be: MVH/DDX4, MIWI/ PIWIL1, TDRD6, TDRD7, GRTH/ DDX25, PABP, HSP72/HSPA2, DDX3L, and MILI/PIWIL2. In addition, tens of other proteins were identified as CB components, and the majority of them were shown to be RNA binding proteins or other proteins involved in RNA regulation pathways (Meikar et al. 2014). piRNA pathway represents the predominant functional pathway in the CB, and the current hypothesis is that the CB functions in piRNA-targeted RNA regulation.

Earlier studies showed that CB is an RNA-rich particle (Walt & Armbruster 1984; Kotaja, Bhattacharyya, et al. 2006; Soderstrom 1976), which includes mRNAs and some miRNAs (Kotaja & Sassone-Corsi 2007) and therefore CB was described as an RNA-processing centre that controls stability and translation of mRNA (Kotaja, Lin, et al. 2006; Oko et al. 1996; Tsai-Morris et al. 2004; Toyooka et al. 2000). However, the analysis of isolated CBs revealed that these granules accumulate mostly pachytene piRNAs and transposon piRNAs, whereas the other small RNAs such as miRNAs are only underrepresented. In addition CB has been shown to involve structural RNAs, transposable elements and moreover, both mRNAs and intergenic RNA transcripts have demonstrated to be enriched in the CBs (Meikar et al. 2014).

CB is a constantly moving, dynamic structure that moves in the cytoplasm of round spermatids bouncing close to the nuclear envelope. In the beginning of the elongation, the CB reduces in size and moves toward the base of the flagellum forming a ring around the annulus. At later steps of elongation, the ring becomes smaller and CB dissociates in two smaller parts. Finally the ring moves down the tail with annulus and the globular body is discarded with the excess of cytoplasm and cell organelles in the form of a residual body (Shang et al. 2010; Onohara et al. 2010).

3 AIMS OF THE STUDY

The general aim of this study was to characterize the factors involved in the control of spermatogenesis and male fertility. Especially this study focused on the role of DICER and DICER-dependent small RNAs in the regulation of male germ cell-specific gene expression. A germ cell-specific *Dicer1* knockout mouse line was created by using the loxP/Cre-recombinase system in which *Dicer1* gene was deleted specifically in early postnatal spermatogenesis in spermatogonia. Due to the lack of in vitro culture model for spermatogenesis, this mouse model provides an important tool to study the Role of DICER-dependent small RNA pathways in male germ cell differentiation. This study clarifies three specific aims:

1. The importance of DICER in spermatogenesis and male fertility
2. The role of DICER in the control of cell-cell junctions in the seminiferous epithelium
3. The germ granule-associated functions of DICER

4 MATERIALS AND METHODS

4.1 Laboratory animals (I-III)

All mice used in these experiments were mixed of C57Bl/6J and SV129 genetic background. Mice were housed at the University of Turku Animal Facility under controlled environmental conditions. Deletion of the *Dicer1* gene in postnatal spermatogonia was performed by crossing floxed *Dicer1* mice (Brian D Harfe et al. 2005) with transgenic animals carrying Neurogenin 3 (*Ngn3)* (Herrera 2009) promoter-driven Cre expression. A detailed description of the *Dicer1(fx/fx);Ngn3Cre* mouse line and genotyping is published (Korhonen et al. 2011).

4.1.1 Genotyping of Dicer1 KO mouse

All mice were genotyped using primers F(DICER) and R(DICER) for the floxed allele (Table 3). Detailed description is shown in (B D Harfe et al. 2005). Genotyping primers for *Ngn3Cre* mice were: F(Ngn3Cre), R(Ngn3Cre), F(pTimer) and R(pTimer). Detailed description of primer sequences is published in (Herrera 2009) and (Korhonen et al. 2011).

4.1.2 Dicer1 KO males are infertile with disrupted spermatogenesis

Dicer1 KO mice grew normally, just like control littermates, and did not show any visible physiological or anatomical abnormalities in adulthood. However, *Dicer1 KO* males were not able to sire any pups and therefore considered as infertile. Closer analyses showed that testis size was reduced approximately 50 % of control testis and the number of mature spermatozoa was dramatically reduced in the cauda epididymis. That was confirmed by HE- staining of the cauda epididymis as well as by the sperm count analysis. FACS analysis revealed reduced number of haploid cells in knockout testes, which was at least partially explained by clearly increased number of apoptotic spermatocytes as observed by TUNEL staining. Periodic acid-Schiff (PAS) and HE-staining of knockout testis sections demonstrated severe disruption of spermatogenesis with unbalanced cellular composition and somewhat disrupted organization of the stages of the seminiferous epithelial cycle.

4.2 Ethics statement

All animal handling and animal experimentation were carried out in accordance with the institutional animal care polices of the University of Turku and Finnish laws. All the suffering of animals was minimized. Persons involved in research using animals are educated and trained before they perform procedures to animals. Protocols for the use of animals were approved by the Committee on the Ethics of Animal Experimentation at the University of Turku in accordance with the Guide for Care and Use of Laboratory Animals (National Academy of Science) (license number: 2009-1206-Kotaja).

4.3 Antibodies (I-III)

All the primary antibodies used in this study are listed in the Table 3.

Table 3 Primary antibodies used in the experiments.

Primary antibody	Dilution	Manufacturer
anti-GFP (R)		Invitrogen
anti-DDX4/MVH (R)	1:1000	Abcam
anti-GATA4 (R)	1:50	Santa Cruz
anti-CENP-A (R)	1:1000	Santa Cruz
anti-phosphorylated histone H3 (R)	1:100,	Millipore
anti-SCP3 (R)	1:100	Santa Cruz
anti-PRM1 (R)	1:100	Santa Cruz
anti-DICER (R)	1:200 IF, WB	Sigma
anti-DICER (R)	1:200 IF, WB	Santa Cruz
anti-Claudin-5 (R)	1:100, IF, 1:200, WB	Invitrogen
anti- BRDT (R)	1:200, IF	Abcam
anti-Hyperacetylated histone H4 (R)	1:500, IF	Millipore
anti-Histone H2B, testis variant (R)	1:200, IF	Millipore
Phospho-histone H2A.X (Ser139) (R)	1: 500, IF	Cell Signaling Technology
anti-phosphorylated histone H2AX (M)	1:100, IF	Millipore
anti-αTubulin (M)	1:1000, IF	Thermo Scientific
anti-dimethylated histone H3 Lys9 (M)	1:500, IF	Millipore
anti-Cre (M)	1:100, IF	Covance
anti-CBX1/HP1β (M)	1:100, IF	Abcam
anti-acetylated histone H3 Lys9 (M)	1:500, IF	Millipore
anti-AKAP4 (M)	1:200, IF	BD Biosciences
anti-H1T2 (M)	1:500, IF	from I.Davidson
anti-Espin (M)	1:500, IF	BD Biosciences
anti-b-Actin (M)	1:1000, WB	Sigma
anti-Vimentin (G)	1:100, IF	Santa Cruz
anti-DDX25 (G)	1:200, IF	Santa Cruz
anti-TDRD1 (Rat)	1:50, IF	Sigma
(R)= Made in rabbit	IF (immunofuorescence)	
(M)= Made in mouse	WB (Western Blot)	
(G)=Made in goat		
(Rat)= Made in rat		

The following secondary antibodies were used in these experiments: a biotinylated secondary antibody (1:750, IHC, Vector Laboratories), AlexaFluor488 and AlexaFluor594 conjugated secondary antibodies (1:750, IF, Invitrogen, Thermo Fisher Scientific Inc., Waltham, US). HRP-conjugated anti-rabbit or anti-mouse IgG (1:5000, WB, Promega).

4.4 Histological samples

4.4.1 Histology (I) and Immunohistochemistry (I-III)

For histological analyses, tissues were collected and directly fixed in 4% paraformaldehyde (PFA) or in Bouin's fixative for 4–20 hours at room temperature. PFA was then washed briefly in water, whereas Bouin's fixative was washed for 5-7 days in water. Tissues were dehydrated in a series of ethanol washes and embedded in paraffin. Paraffin-embedded testis sections were rehydrated and antigens were retrieved by pressure cooking in 10 mM Sodium Citrate buffer (pH 6.5) or TE buffer (Tris + EDTA, pH 9.0) for 2 hours. Non-specific binding sites were blocked in 10% normal goat serum, 10% BSA or 10% normal donkey serum. Primary antibodies were diluted in blocking solution and incubated for 1 hour at 37°C, or over-night at 4°C. Rabbit and mouse IgG were used as negative controls (Vector Laboratories). The secondary antibody was diluted in blocking solution and incubated for 1 hour at 37°C. For the immunohistochemical detection, antibody localization was detected with VECTASTAIN ABC (peroxidase) system (Vector Laboratories) and 3,3′-diaminobenzidine (liquid DAB+, DAKO), stained with hematoxylin, dehydrated and mounted in PERTEX medium. On the contrary for immunofluorescence detection the sections were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories, Burlingame, US). Drying down cell spreads and squash preparations (Kotaja et al. Nat Methods 2004) were post-fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton-X 100 for 15 minutes at room temperature, and immunofluorescence was performed as described above. For the detection of mitochondria, slides were incubated with 200 nM Mitotracker (Invitrogen) in PBS for 15 minutes. Wide field fluorescence images were acquired with a Leica DMRBE microscope with Olympus DP72 digital color camera using cellSens Entry 1.5 (Olympus) digital imaging software or Zeiss AxioImager M1 microscope. A Zeiss LSM510 META laser scanning confocal microscope was used for the confocal images. All images were processed using Photoshop (Adobe). 3D rendering was performed with BioimageXD version 1 (II).

4.4.2 TUNNEL assay (I)

For the evaluation of apoptotic germ cells TdT-mediated dUTP nick end labelling (TUNEL) was used on Bouin's fixed, paraffin embedded testis sections to detect DNA fragmentation in apoptotic cells. Paraffin was removed and sections were rehydrated and permeabilized in 10 mM Sodium Citrate (pH 6.5) for 20 minutes in a microwave. Endogenous peroxidase activity was blocked in 3% H_2O_2 (Sigma) at room temperature for 15 minutes.

Sections were incubated in 1× TdT buffer, Terminal transferase (0.6 U, Roche), biotin-16-dUTP (10 µM, Roche) and 10 µM $CoCl_2$ for an hour at 37°C. A negative control was incubated without an enzyme. Non-specific sites were blocked with 3% BSA and 10% Normal Goat Serum for 15 minutes at room temperatures and subsequently incubated with ExtrAvidin®–Peroxidase (1:50, Sigma) for 30 minutes at 37°C. Apoptotic cells were detected with 3,3′-diaminobenzidine (liquid DAB+, DAKO), and cells were stained with hematoxylin, dehydrated and mounted with PERTEX medium.

4.4.3 Electron microscopy (I-III)

For electron microscopy analysis, a piece of mouse testis at the specific age or epididymal sperm was fixed in 5% glutaraldehyde and then treated with a potassium ferrocyanide-osmium fixative. Sections (70 nm) were empedded with Epoxy resin (Glycidether 100, Merck Co., Darmstadt, FRG), stained with 5% uranyl acetate and 5% lead citrate, and visualized on a JEM-1400 plus TEM transmission electron microscope (JEOL, Tokyo, Japan).

4.5 Stage-specific analyses, cell content and sperm analyses

4.5.1 Squash preparation (I)

Squash preparations were prepared as descriped in (Kotaja et al. 2004). Testes were dissected and decapsulated and the specific stages of the seminiferous epithelial cycle were identified on the basis of the transillumination patterns. Live cell microscopy was performed by using phase contrast optics (Leica DMRBE microscope).

4.5.2 Drying down analyses (I)

For drying down preparations, stage-specific segments of seminiferous tubules were isolated and cells were released and fixed on slides as previously described (Kotaja et al. 2004). Preparations were visualized by phase contrast microscopy or used for immunofluorescence.

4.5.3 Flow cytometric analysis (I)

To study the cell content in *an adult Dicer1 KO and control mouse* testes, flow cytometric assay (FACS) was performed as described in (Malkov 1998). For the FACS analysis, cell suspensions were prepared from adult mouse testes and cells were separated on a FACScalibur machine according to their DNA content, which is haploid (1C), diploid (2C) or tetraploid (4C).

4.5.4 Sperm counts (I)

Epididymal sperm from cauda epididymis and ductus was extracted from adult (60 dpp or 120 dpp) male mice, counted and analyzed as previously described (GUERIF et al. 2002).

4.5.5 Morphological analyses (I)

For morphological analysis, epididymal sperm released in PBS from cauda epididymides was spread on glass slides, air dried and stained with hematoxylin. A Morpghologycal analysis was done by light field microscope.

4.6 Western Blotting (I-III)

Testes from *Dicer1* knockout and control mice were collected and frozen in liquid nitrogen. Testes were homogenized in a RIPA lysis buffer (50 mM Tris-HCl (pH 7.5), 1% Nonidet P40, 0 .5% sodium deoxycholate, 0.05% SDS, 1 mM ethylenediaminetetraacetate (EDTA), 150 mM NaCl, 1:25 Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche), 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and the lysates were centrifuged at full speed for 15 minutes. Enriched populations of spermatocytes, round spermatids and elongating spermatids were collected from adult C57Bl/6J mice testes using centrifugal elutriation and the lysates were prepared as described above. Western Blotting samples for Chromatoid body isolation analysis were collected as described (Meikar et al. 2010). Protein (20 µg) was loaded onto commercial precast Tris-HCl polyacrylamide gels (Mini-PROTEAN® Dodeca™, Bio-Rad Laboratories, Helsinki, Finland). After electrophoresis, proteins were transferred to an Amersham Hybond hydrophobic polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK). The membrane was blocked with 5% milk in phosphate buffered saline (PBS), incubated with a primary antibody (Table 4) in 5% milk in PBS at 4°C, washed three times in PBST (PBS with 0.1 % Triton X-100), incubated with HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:5000 diluted in 5% milk) at room temperature for 1 hour, and washed three times for 15 minutes in PBST with a final PBS rinse. Detection was performed with Western Lightning ECL Pro, luminol-based enhanced chemiluminescence HRP substrate (Perking Elmer, Turku, Finland) and a LAS4000 (Fujifilm) detection system.

4.7 Isolation of spermatocytes by BSA gradient (II)

Spermatocytes were isolated from the total testis cell suspension for the RNA extraction and quantitative RT-PCR analysis. Control and *Dicer1* knockout testes were decapsulated and digested in 0.05% (w/v) collagenase and 0.1% glucose in Kreb's buffer (85 mM $NaHCO_3$, 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM K_2HPO_4, 5.5 mM Glucose, 1 mM $CaCl_2$, pH 7.4) at 34°C for 10 min and centrifuged at 500 x g at room temperature for 2 minutes. The pelleted tubules were resuspended

in Kreb's buffer, washed twice and subsequently digested in 0.6 mg/ml of trypsin and 5 µg/µl of DNAse in rotation at 34°C for 10 minutes. Cells were filtered and centrifuged at 500 x g at room temperature for 5 minutes. Cells were suspended in 0.5% BSA. Different cell types were separated from cell lysate through BSA gradient from 1% BSA to 6% BSA. The purity of spermatocyte cell fraction (80%) was analyzed with DAPI staining and fluorescent microscopy.

4.8 Gene expression analyses

4.8.1 RNA extraction

For the gene expression analysis testes from 18-, 24-, 30-day-old and adult (more than 60 days old) *Dicer1* knockout and control mice were collected, placed in 1 ml of TRIsure reagent solution (Bioline USA Inc.) and homogenized. Alternatively 500 ml of TRIsure reagent solution was added to the cells suspension collected by elutriation centrifugation or BSA-gradient-based spermatocyte isolation. To extract RNA from isolated CBs, all RIPA buffer was removed from the CB immunoprecipitation beads and 500 ml of TRIsure reagent solution was added. Total RNA was extracted by chloroform and isopropanol precipitation. Purity was confirmed by the ratio of absorbance at 260 and 280 nm measured by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, US).

4.8.2 Quantitative RT-PCR (I-II)

RNA was treated with DNase (Sigma-Aldrich Co, US) and cDNA was synthesized from 1 µg of total RNA using a commercial DyNAmo™ cDNA synthesis kit (Thermo Fisher Scientific Inc, Waltham, US). Real-time quantitative PCR was performed with DyNAmo™ Flash SYBR® Green qPCR Kit (Thermo Fisher Scientific Inc., Waltham, US). Each assay was performed in three independent technical and biological replicates. A geometric mean of validated reference genes was used for normalization. *TATA-binding protein* (*TBP)*, peptidylprolyl isomerase A (PPIA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were validated as the best reference genes for normalization of the data from a panel of 12 genes analyzed by RefFinder (http://www.leonxie.com/referencegene.php). The $2^{-\Delta\Delta CT}$ – method (Livak & Schmittgen 2001) was used to analyze the relative changes in gene expression between the knockout and the control testes. Each assay was performed in three independent experiments with three technical replicates.

4.8.3 Primers (I-II)

Primers for quantitative RT-PCR used in this study (I-II) are described in the Table 4.

Table 4 Primers and their sequences used in this study.

Mouse gene	Primer name	Sequence (5' to 3')
Dicer	*F(Dicer)*	CTTGACTGACTTGCGCTCTG
Dicer	*R(Dicer)*	AATGGCACCAGCAAGAGACT
L19	*F(L19)*	GGACAGAGTCTTGATGATCTC
L19	*R(L19)*	CTGAAGGTCAAAGGGAATG-TG
TBP	*TBP (forward)*	AGAGCCACGGACAACTGCGT
TBP	*TBP (reverse)*	TGTTCTTCACTCTTGGCTCCTGTGC
PPIA	*PPIA (forward)*	GCCATGGTCAACCCCACCGT
PPIA	*PPIA (reverse)*	TGCAAACAGCTCGAAGGAGACG
GAPDH	*GAPDH (forward)*	AGTGCCAGCCTCGTCCCGTA
GAPDH	*GAPDH (reverse)*	AGGCGCCCAATACGGCCAAA
Claudin 5	*Cldn5 (forward)*	CTGGACCACAACATCGTGAC
Claudin 5	*Cldn5 (reverse)*	CGCCAGCACAGATTCATACA
Ezrin	*Ezrin(forward)*	TCGGAGATTATAACAAGGAAATGC
Ezrin	*Ezrin(reverse)*	GAGCTTGTGTTGGTCCATGA

Additionally primers for SINE B1 and LINE L1 RNA are described in (Martens et al. 2005), IAP primers are described in (Rowe et al. 2010), primers for centromeric major and minor repeat transcripts are described in (Lehnertz et al. 2003). Normalization was performed with L19 mRNA levels for repetitive elements and mRNA transcripts.

4.8.4 Statistical analyses (II)

Statistical data was analyzed with JMP Pro 11 software (SAS Institute Inc., Cary, NC, USA). For the comparison of two groups, unpaired *t* Test was used to determinate the P-value. P-value is less than 0.05 was considered significant. All the results are presented as mean±SD of fold change.

4.9 Isolation of Chromatoid body

CB immunoprecipitation was performed for the RNA sequencing analysis as described in (Meikar et al. 2010). All together six mice were used for the experiment. In all individual experiment two *Dicer1 KO* and control mice testes were decapsulated and digested in collagenase (0.5 mg/ml in PBS). Cells were then fixed in 0.1% PFA (Electron Microscopy Sciences, Hatfield, PA, USA) diluted in PBS. Cell were lysed in 1,5 mL of RIPA buffer [50 mM Tris-HCl, pH 7.5, 1% Nonidet P40, 0 .5%

sodium deoxycholate, 0.05% SDS, 1 mM ethylenediaminetetraacetate, 150 mM NaCl, 1:25 Complete Mini EDTA-free Protease Inhibitor Cocktail, (Roche, Basel, Switzerland), 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride] by sonication (UCD-200, Diagenode, Liege, Belgium). CB enriched pellet faction was isolated by low speed sentrifugation at 300 x g 10 min. The CBs were immunoprecipitated by Dynabead Protein G (Thermo Fisher Scientific, Waltham, MA, USA) coupled to anti-MVH antibody (homemade). Progression of isolation was analyzed with detecting of CBs by western blot using anti-mvh antibody (commercial). The quality of RNA was analyzed by RNA gel and Bioanalyzer. Isolation protocol was performed three independent times.

5 RESULTS

5.1 DICER is required for haploid male germ cell differentiation (I, II)

5.1.1 Generation of male germ cell-specific Dicer1 KO mouse line (I)

Dicer1 KO mouse was generated to study the role and the importance of *Dicer1* in spermatogenesis. A mouse carrying a floxed *Dicer1* allele (Dcr(fx/fx) (B D Harfe et al. 2005) was crossed with a transgenic mouse expressing the Cre recombinase under the promoter Ngn3Cre (Herrera 2009). Ngn3 is shown to be expressed in type A spermatogonia starting from day 5 dpp (Herrera 2009). The deletion was generated by inserting two loxP-sites around the exon 24 that encodes the second RNase III domain of DICER (I, Fig.1 B). The Cre-recombination generates a mutated allele that encodes a truncated, functionally inactivated, DICER protein.

The efficiency of Cre-recombination was confirmed in three ways: 1) The *Ngn3Cre* line was crossed with a transgenic mouse line expressing a Cre-recombination sensitive *ROSA26YFP* reporter gene. The analysis of the testes from *Ngn3Cre; ROSA26YFP* mice demonstrated the Cre-dependent YFP expression in differentiating male germ cells throughout the meiotic and postmeiotic stages (I Fig. 1D). 2) A genomic PCR amplifying the deleted *Dicer1* allele showed a deletion-specific 600 bp band in *Dicer1 KO* mouse but not in the control. 3) The deletion of *Dicer1* in adult knockout testes was confirmed with the amplification of *Dicer1* mRNA using one primer hybridizing within the deleted area. The analysis showed the significant decrease of *Dicer1* mRNA in an adult *Dicer1 KO* mouse testis (Korhonen et al. 2011).

5.1.2 Meiosis appear normal in Dicer1KO mice (I)

The meiosis gives the rise to the haploid cells and it is one of the most critical steps in the sexual life cycle. Because of the increased apoptosis of spermatocytes in the *Dicer1* knockout testis, we wanted to study the progress of meiosis in more detail. However, we were not able to identify any severe meiotic abnormalities in *Dicer1 KO* seminiferous epithelium. SC are highly ordered protein complexes between aligned homologous chromosomes during meiotic prophase in spermatocytes. The formation of SC in knockout pachytene spermatocytes did not show any abnormalities when compared to the littermates manifesting the functional meiosis. SC formation was analysed by immunostaining with an antibody against synaptonemal complex protein SCP-3 as well as in electron microscopy analyses. Silencing of X and Y chromosomes in the sex body was also detected by an antibody against histone variant H2AX (Table 1), which revealed normal appearance of sex bodies in knockout pachytene spermatocytes. One important step in the meiosis is the formation of metaphase plates at the metaphase stage. In *Dicer1 KO* mouse metaphase plate composition was

analysed by immunostaining of phosphorylated Serine 10 of histone H3. The immunostaining revealed the presence and normal number of metaphase plates in the *Dicer1 KO* spermatocytes.

5.1.3 Abnormal haploid differentiation in Dicer1 KO mice (I, II)

Our histological analysis revealed that the most dramatic spermatogenic failure in *Dicer1*KO testis took place during haploid cell differentiation. Round spermatids appear more or less normal, however, severe defects were observed in elongating spermatids, as demonstrated by the reduced number and abnormal organization of elongating spermatids in the seminiferous epithelium. The phase contrast microscopy of stage-specific drying down preparations revealed morphologically abnormal elongating spermatids in knockout mouse testes. Less than ten normal appearing step 15–16 spermatids were detected among all spermatogenic cells analyzed. Nearly all late spermatids showed an abnormal head shape and chromatin condensation and a disrupted organization of tail structures.

Mature spermatozoa were isolated from cauda epididymides of knockout mice and littermates (control mouse from the same litter) and stained with hematoxylin to study the morphological status of fully developed gametes. The number of knockout spermatozoa was greatly reduced compared to the control. Furthermore, knockout spermatozoa showed clearly abnormal features including small head shapes and thin, fragile-looking tails. Barely any unharmed spermatozoon was detected. In addition, immunostainings with a fibrous sheath marker (anti-AKAP4) and mitochondrial sheet marker (Mitotracker) demonstrated that the fibrous and mitochondrial sheaths were present only in some but not all of the spermatozoa. Electron microscopic analyses revealed also small abnormal heads but in addition an excess of cytoplasm (I, Fig. 6 D).

5.1.3.1 Processes preceding histone-protamine transition appear normal (I, II)

After the meiosis during the spermatid elongation, the majority of the core histones are replaced by transition proteins and protamines facilitating chromatin hyper-compaction. Because of the previously described problems in spermatid elongation, histone-protamine transition was analysed by immunostainings with protamine marker anti-PRM1. Interestingly, a lowered number of elongating spermatids was detected, which were positive for protamine PRM1. A decrease number of anti-PRM1 positive spermatids in *Dicer1* KO testes and impaired chromatin condensation in *Dicer1*KO elongating spermatids prompted us to analyze if the processes preceding the histone-protamine transition were affected. Histone hyperacetylation is known to destabilize the nucleosomes before histone-protamine transition (Gaucher et al. 2010; Ausio & Van Holde 1986). Acetylated forms of H2A, H2B, H3 and H4 appear in the step 9–11 elongating spermatids, and disappear later in condensing spermatids. We determined the dynamics of H4 hyperacetylation in

Dicer1 KO spermatids. The analyses showed a correct appearance of hyperacetylation in early elongated spermatids, and this modification was also correctly retained in the chromocenters of step 12 (stage XII) elongating spermatids just before the protamine incorporation. However, we observed a delay in the transition from hypearcetylated histone-containing spermatid to protamine-containing spermatids in *Dicer1* KO seminiferous epithelium, as demonstrated by the presence of hyperacetylated spermatids in stages II-IV of the seminiferous epithelial cycle that normally contain protaminated spermatids (I, fig. 4 B).

The other important step preceding histone-protamine transition is the bromodomain testis-specific protein (BRDT) binding on hyperacetylated histones. It is also known to be involved in a global remodelling of acetylated chromatin (Gaucher et al. 2012b). Confocal immunofluorescence microscopy revealed normal association of BRDT with hyperacetylated chromatin in *Dicer1 KO* spermatids. Furthermore, we did not detect any major defects in the level of double strand breaks that appear in elongating spermatids before the histone-protamine transition.

5.1.3.2 Defects in cell polarization and cell organelles (I, II)

During the haploid cell differentiation and the elongation, nucleus and several other cellular organelles (ER, Golgi apparatus, mitochondria, centriole, etc.) undergo structural and biochemical changes when spermatid metamorphoses into a spermatozoon. At the end, the plasma membrane of sperm has also differentiated into specialized areas (Abou-Haila & Tulsiani 2000). Because of the observed misshaped sperm heads of *Dicer1 KO*, we clarified the acrosome morphology in *Dicer1 KO* spermatids by electron microscopy and immunofluorescence staining using rhodamine conjugated PNA as a marker. Interestingly, *Dicer1 KO* round spermatids frequently showed a fragmented acrosome.

Manchette is a transient microtubular structure that surrounds the posterior region of the nucleus in elongating spermatid, and is involved in the shaping of sperm head. Immunostaining against anti-tubulin demonstrated disorganized manchettes in *Dicer1 KO* mouse spermatids, in which anti-tubulin staining was distributed randomly in the cytoplasm of spermatids, often surrounding the whole nucleus, whereas a normal manchette was located only at the basal side of the control nuclei. Furthermore, electron microscopy of late elongating spermatids confirmed the disrupted microtubule organization and defects in the nuclear morphology.

The polarization of spermatid is critical for chromatin condensation, acrosome formation and nuclear elongation. We used an antibody against H1T2/H1FN as a marker to study the general chromatin status and cell polarization in Dicer1 KO spermatids. H1T2 is a testis-specific histone H1 variant, which has been shown to have a polarized localization within the round and elongating spermatid nuclei. H1T2 is normally concentrated in a cap-like structure at the apical pole of the nuclear membrane right beneath the acrosome. H1T2 is a chromatin-related protein that has been shown to confer to the polarity of the spermatid nucleus during spermiogenesis and is known to be crucial for chromatin packing. The absence of H1T2 causes infertility (Martianov et al. 2005). Interestingly, *Dicer1 KO* spermatids did not show the polarized localization of H1T2 but H1T2 signal was bipolarized and found on the both apical and basal sides of the nucleus (I, fig. 5A).

5.2 DICER regulates the formation of cell-cell junctions (II)

5.2.1 Defective apical ectoplasmic specialization in Dicer1 KO testis (II)

Our finding of defected polarity in the *Dicer1 KO* spermatid nucleus prompted us to study further this step of differentiation. Specialized cell-cell junctions between somatic Sertoli cells and spermatids are known to be important for the cell polarization (Wong et al. 2008). These junctions that are formed at the apical side of spermatid nucleus are called apical ectoplasmic specializations (aESs).

One of the major functions of aES is to orientate developing spermatids properly in the epithelium during their development and differentiation. We analyzed the orientation and the organization of aES by immunofluorescence staining using anti-ESPIN antibody. ESPIN is an actin binding protein and the component of aES. To detect the cell orientation we stained acrosome with PNA. Immunofluorescences staining revealed the ESPIN signal both in *Dicer1 KO* and control spermatids. However, in *Dicer1* KO seminiferous tubules, aES was clearly fragmented and disrupted. In most of the cases, only small pieces of junctions were found between spermatids and Sertoli cells and the location of junctions was irregular. Due to this, most of the spermatids were disoriented and poorly connected to the Sertoli cells (II fig.3 A, B). Sometimes the connection to Sertoli cells was completely lost leading to the immature release of spermatids into the lumen, which explains the reduced number of elongating spermatids in the *Dicer1 KO* seminiferous epithelium, and a very low number of mature sperm in cauda epididymides.

5.2.2 Blood testis barrier is abnormal in the absence of germ cell DICER (II)

Having been interested in the organization of cell-cell junctions in *Dicer1 KO* seminiferous tubules, we analyzed the other type of germ cell junction, blood testis barrier (BTB). BTB is formed between two Sertoli cells and during the seminiferous epithelial cycle of spermatogenesis it undergoes extensive restructuring in a process that includes an active remodeling of cell-cell junctions allowing spermatocytes to pass through the BTB (Wong & Cheng 2005). BTB in *Dicer*KO seminiferous tubules and the control was detected by immunofluorescence staining and confocal microscope using an antibody against ESPIN, which is also the component of BTB. ESPIN signal was strong and detectable but the composition of BTB was abnormal. Knockout BTB was thicker, like many layers as a massive bed, when compared to the control BTB (II fig.4 A, B).

5.2.3 Cell adhesion-related genes are misregulated in Dicer1 KO testis (II)

To better understand the role of DICER in the regulation of germ cells transcriptome, we analysed the recently published RNA sequencing data of *Dicer1* knockout (*Ddx4Cre-Dicer1 knockout*) pachytene spermatocytes (Zimmermann et al. 2014). Transcriptome analyses were performed by selecting all the genes that were upregulated 1.1-fold or more from the *Ddx4Cre-Dicer1* knockout and the control spermatocytes and analysing the enriched gene ontology (GO) terms usings software called GOrilla (*Gene Ontology enRIchment anaLysis and visuaLizAtion tool, http://cbl-gorilla.cs.technion.ac.il/)*. Very interestingly, GO terms related to cell-cell junctions and cellular adhesion, including terms “regulation of cell adhesion”, “biological adhesion” and “cell adhesion”, were strongly enriched among the upregulated genes. The enriched GO terms also included GO terms connected to cell motility and locomotion. These results suggest an important role for DICER-dependent processes in the maintenance of epithelial architecture in the testes.

In the transcriptome data from *Ddx4Cre-Dicer1* knockout mouse*, Claudin5* (*Cldn5*) was one of the most upregulated gene. We confirmed the significant upregulation of *Cldn5* mRNA expression in the *Ngn3Cre*-driven *Dicer1* knockout testes at P18 by the RT-qPCR. Not all the cell adhesion-related genes were upregulated in *Dicer1* knockout testes, and for example the expression of EZRIN, which localizes also to the BTB and aES (Gungor-Ordueri et al. 2014), was not affected in *Dicer1* KO spermatocytes when analysed by RNA sequencing (Zimmermann et al. 2014), or *Ngn3Cre*-driven *Dicer1* KO testes. Interestingly, we also demonstrated that *Cldn5*, *Itga5* (integrin alpha 5), *Itga3* (integrin alpha 3) and Lgals9 mRNAs were upregulated in isolated *Ngn3Cre-Dicer1*KO spermatocytes, which validated that the two mouse models (*Ddx4Cre-Dicer1* knockout and *Ngn3Cre-Dicer1 knockout*) showed similar upregulation of cell adhesion related genes (II, table 2).

5.3 Expression of DICER during spermatogenesis (I-II)

5.3.1 DICER expression peaks in pachytene spermatocytes (I-II)

To better understand the role of DICER in the control of spermatogenesis, we wanted to reveal the exact expression pattern of DICER during male germ cell differentiation. We started by using RT-qPCR analysis of juvenile mouse testes collected at different time points during the first wave of spermatogenesis. The first wave of spermatogenesis is synchronized and the different time points represent the appearance of different germ cell types in the seminiferous epithelium: spermatogonia just prior to or at the onset of meiosis (8 dpp), pachytene spermatocytes (14dpp), round spermatids (24dpp) and elongation spermatids (28dpp). *Dicer1* expression was highest in testes collected at 14 dpp, and the level of *Dicer1* mRNA decreased in the samples of older animals, suggesting that the *Dicer1* expression peaks at pachytene spermatocytes (I, Fig 1 A). The expression of DICER was studied at the protein level by immunoblotting. For this purpose, testis samples were collected at 14 dpp, 18 dpp, 24 dpp and 28 dpp and blotted using two different DICER antibodies. The results showed strong DICER expression in 14 dpp and 18 dpp testes that are enriched with pachytene spermatocytes (II, Fig 2A).

This result was confirmed by immunofluorescence staining with two different commercial antibodies. DICER was found to be expressed in all types of differentiating male germ cells, including spermatogonia, spermatocytes, and spermatids, but the expression increased in pachytene spermatocytes and reached a peak in late pachytene and diplotene spermatocytes present at stages IX– XI of the seminiferous epithelial cycle. DICER was expressed at lower levels during haploid cell differentiation, the expression being relatively higher in step 1–5 round spermatids (at stages I–V) than in step 6–8 round spermatids (at stages VI–VIII) (II, Fig 1 A). Signal was predominantly cytoplasmic and appeared to be concentrated at specific regions in the cytoplasm (III, Fig 1 A). Taken together, even though DICER is expressed in all cell types, the level of expression changed during spermatogenesis being the most prominent in the pachytene spermatocytes.

5.3.2 Higher molecular weight DICER appears during the late steps of spermatogenesis

While we were analyzing the expression of DICER during the first wave of spermatogenesis by immunoblotting, we noticed that in P28 and adult testes, a higher molecular weight (MW) DICER band (250 kDa) appeared (II, Fig.2 A). At P18 and P28 knockout testes showed dramatically a reduced expression of both forms of DICER compared to the control. To validate the expression of an extra DICER band, we analyzed the different forms of DICER in enriched populations of specific cell types isolated by centrifugal elutriation. The higher MW form of DICER was found in round spermatids (70% pure fraction with elongating spermatids as major contaminants) and was the predominant form in elongating spermatids and spermatozoa isolated from cauda epididymis (II,

Fig 2 B). Interestingly, the higher MW form was not detected in spermatocytes, even though spermatocytes revealed the strongest expression of DICER.

5.3.3 DICER localization is associated with germ granules (III)

Knowing the central role of cytoplasmic germ granules in the germ cell-specific RNA regulation, we wanted to clarify if DICER localization is associated with germ granules, specifically intermitochondrial cement (IMC) in spermatocytes and chromatoid body (CB) in round spermatids. By immunofluorescence staining using an antibody against MILI, one of the three murine PIWI proteins, we detected granular staining pattern in pachytene spermatocytes that is known to correspond to the IMC (Thomson & Lin 2009). Co-staining with anti-DICER antibody and anti-MILI antibodies revealed that DICER signal predominantly associated with MILI signal both in mid-pachytene spermatocytes (stages VII-VIII) and late pachytene spermatocytes (stages IX-X) (III, Fig 2 A, B). The result was confirmed by using additional IMC markers (TDRD1, Cytochrome C). All markers clearly showed the same results: The DICER signal is predominantly associated with the IMC in spermatocytes.

Although the cytoplasmic expression of DICER was weaker in round spermatids than in spermatocytes, a clear signal was still detected and it appeared granular. We used anti-DDX25 as a CB marker to study if DICER localization is associated with the haploid germ cell-specific germ granule. We showed that DICER-positive granules were frequently found in close association with the CB, but DICER signal did not completely overlap with the CB (III, Fig 3 A).

The association of DICER with the CB was supported by the finding that DICER was sometimes, but not always (two out of four experiments) co-immunoprecipitating with CBs. These results suggest that there is interplay between DICER and the CB in round spermatids, but DICER is not a stable component of the CB.

5.3.4 Germ granule morphology is unaffected in Dicer1 KO germ cells (III)

To study if DICER is required for the formation or maintenance of germ granules, we analysed the morphology and the composition of IMC and CB in *Dicer1 KO* mouse by electron microscopy as well as immunohistochemical and immunofluorescence methods using germ granule markers. Immunostaining with anti-MVH and anti-DDX25 revealed the normal appearance and number of CBs in *Dicer1* KO testis. Furthermore, the IMC signal appeared unaffected as detected by antibodies against MILI, TDRD1 and Cytochrome C. In electron microscopy analysis of the CB and IMC in the *Dicer1 KO* and the control germ cells conclusion, our results demonstrated that even though DICER localization is associated with the germ granules, the integrity and morphology of IMC and CB were

not affected in *Dicer1* KO mice and therefore, DICER is not required for the formation/maintenance of the germ granule structures (III, Fig. 4 B, C) .

Because of the intact integrity of CBs in *Dicer1 KO* round spermatids, we were able to isolate CBs from knockout and control testes using the protocol we have developed earlier (Meikar & Kotaja 2014). Immunostaining of the samples collected during the protocol showed that CBs were correctly enriched during the low-speed centrifugation step (Figure 10). 20µl of lysate was collected during the isolation from the different isolation steps and analyzed by the immunoblotting using anti-MVH antibody. The result validated the successful isolation in both *Dicer1* KO and control samples (Figure 11). IP-MVH sample include the isolated CBs and the band was visible in both *Dicer1* KO and control samples. Because DICER is an RNA-binding protein that has a central role in RNA regulation, we are interested to clarify if DICER is involved in the targeting of specific RNAs into germ granules. Therefore, the CB isolation will be optimized to get a high quality RNA for sequencing, which will allow us to analyze if the CB RNA content is affected in the absence of DICER.

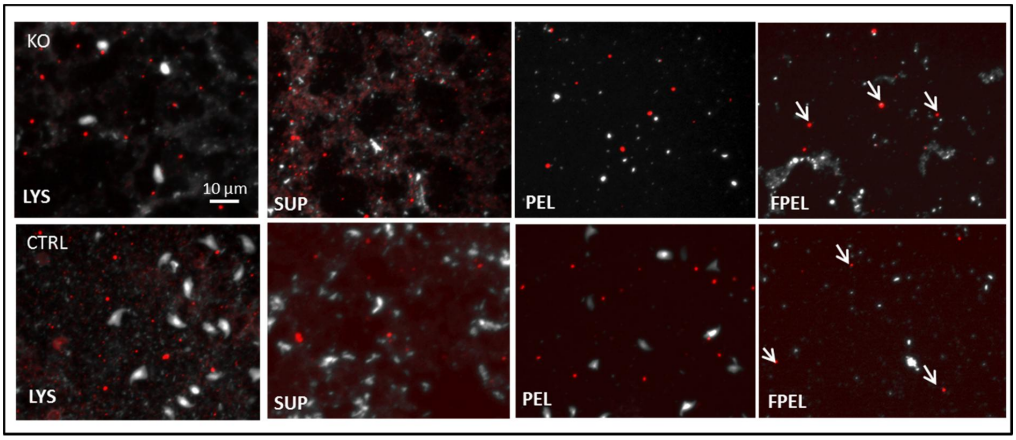

Figure 10 Isolation of CBs. Immunostaining of the samples collected different steps during the CB isolation protocol showed that CBs were correctly enriched in Dicer1 KO and control samples. LYS= lysate, SUP=supernatant, PEL=pellet, FPEL= filttered pellet. Some of CBs are pointed with an arrow. Samples are described in Meikar & Kotaja, 2014.

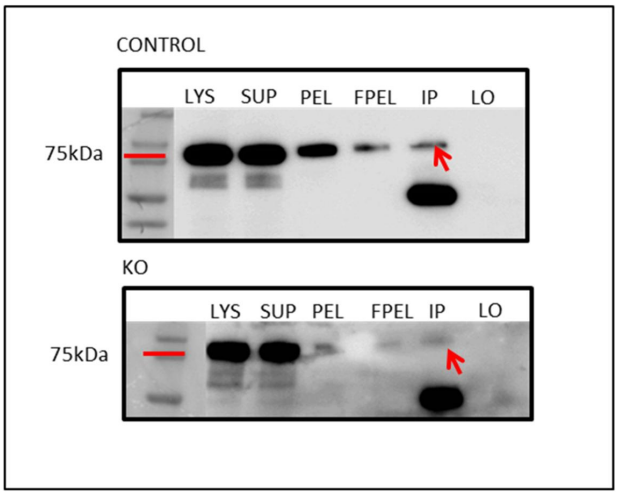

Figure 11 Immunoblotting of CB isolation. Immunoblotting picture to show the successful CB isolation from Cntrl and Dicer1 KO testis. Cell lysates from different steps of CB isolation were blotted and detected with anti-MVH antibody. LYS= lysate, SUP=supernatant, PEL=pellet, FPEL= filttered pellet, IP= immune precipitation (pure CB), LO= left over. CB enriched fraction is pointed with an arrow. Samples are described in Meikar & Kotaja, 2014.

6 DISCUSSION

6.1 *Dicer1* KO mouse

Dcr(fx/fx);Ngn3Cre mouse line was created to study the function of DICER in male germ cell differentiation. Using the *Ngn3* promoter to drive Cre expression, DICER was removed specifically in postnatal spermatogonia. The deletion of DICER in these cells does not interfere in embryonic development of primordial germ cells. This mouse line was the first testis specific mouse line, in which DICER was deleted in the postnatal germ cells, and it gave us valuable information about the function of DICER-dependent pathways during later stages of male germ cell differentiation. Although Ngn3-transgene is known to be expressed also in the brain and the pancreas (Gradwohl et al. 2000; Song et al. 2010), no other abnormalities in addition to the disrupted spermatogenesis was observed in the development of *Dcr(fx/fx);Ngn3Cre* mice.

6.2 Importance of DICER in gene regulation during male germ cell differentiation

Today, several germ cell-specific *Dicer*1 KO mouse models have been published in addition to our Dcr(fx/fx);Ngn3Cre line (Maatouk et al. 2008; Romero et al. 2011; Zimmermann et al. 2014; Greenlee et al. 2012) and all the results support the finding that DICER is required for spermatogenesis and male fertility. In this study, the deletion of DICER in postnatal spermatogonia was shown to cause major defects in haploid cell differentiation and elongation of spermatids indicating the crucial role of DICER in haploid cell differentiation. The differentiation of spermatids showed clear problems in the early elongation phase, as demonstrated by the accumulation of abnormal elongating spermatids prior to the nuclear condensation and chromatin packing on spermatogenic cell spreads. The delay in the transition from non-condensed to condensed spermatids was also demonstrated by the retention of hyperacetylated spermatids at stages that normally contain protaminated spermatids. The number of elongating spermatids decreased during differentiation and only a few protamine-positive nuclei were detected in the seminiferous epithelium of *Dicer1* KO mouse. The number of mature spermatozoa in cauda epididymides was also dramatically reduced, and all spermatozoa had small misshaped heads and fragile tails. Immature spermatids were frequently found in the epididymis.

Our analysis did not reveal any major problems during the early phases of spermatogenesis in spermatogonial proliferation or meiosis. Because DICER was removed in spermatogonia and miRNAs are known to be stable for some days after the inactivation of DICER, we cannot draw any strong conclusions about the role of DICER in early postnatal germ cells, and we focused our analysis on the haploid phase where clear defects were manifested.

The parallel analysis of a Dcr(fx/fx); *Ddx4* conditional knockout mouse model, in which the deletion of DICER occurs a few days earlier already in embryonic germ cells right before birth, revealed that DICER has also a meiotic role. The haploid phenotype of this mouse model was similar to Ngn3 knockout mouse model (Romero et al. 2011). However, meiosis was also severely affected. One hypothesis is that when DICER is removed later in postnatal cells, differentiating germ cells are able to escape a meiotic failure due to miRNAs that remain longer in cells after the deletion. Alternatively, DICER-dependent pathways may participate in the epigenetic reprogramming of male germline that takes place during the time when *Ddx4* promoter is activated. Defects in the epigenetic reprogramming are known to cause meiotic problems (Bao et al. 2014).

In addition to the misregulation of mRNAs, we showed that pericentromeric repeat transcript expression in *Dicer1* KO testes was increased. DICER has been linked to centromeric silencing also in other studies, such as in DICER-null embryonic stem cells (Kanellopoulou et al. 2005; Murchison et al. 2005). It is still unclear how DICER is involved in the regulation of these pericentromeric heterochromatin-derived repeat transcripts, whether it occurs by direct post-transcriptional regulation or at the chromatin level. The mammalian DICER-dependent small RNA pathways are known to function mainly at post-transcriptional level. DICER-dependent small RNA–mediated regulation of heterochromatin formation and maintenance has been convincingly described in plants and some evidence exists to support the role of DICER -dependent pathways in these processes in vertebrates (Giles et al. 2010; Fukagawa et al. 2004). The nuclear localization of DICER has been detected in human cells, where DICER was localized to ribosomal chromatin (Sinkkonen et al. 2010). All these results support the regulatory role of DICER dependent pathways at the chromatin level. Further studies are required to clarify the role of DICER in the formation and regulation of heterochromatin or chromatin organization in differentiating male germ cells.

6.3 DICER controls both aEs and BTB formation and cell adhesion genes

At the onset of meiosis, spermatocytes migrate through the BTB and translocate into the adluminal compartment. That process requires an active remodeling of cell-cell junctions between Sertoli cells (BTB) and between Sertoli cells and germ cells (aES). Spermatozoa are strongly polarized cells and cell polarization requires specific protein complexes that are also involved in the formation of aES. Thus cell polarization is critical for the normal formation of aES as well as for the normal elongation of differentiating haploid cells.

Interestingly, the nuclear polarization of spermatid was disrupted in *Dcr(fx/fx); Ngn3Cre* mice. That was demonstrated by abnormal bipolar localization of a testis-specific linker histone variant H1T2 in the absence of DICER. The similar mislocalization of H1T2 was also detected in spermatids lacking the expression of Trf2, a transcriptional regulator involved in the nuclear chromatin organization (Catena et al. 2006; Martianov et al. 2002). The defects in spermatid polarization prompted us to analyze the status of cell-cell junctions in *Dcr(fx/fx); Ngn3Cre* mice. The examination was done by immunofluorescence staining of anti-ESPIN, an Actin-binding protein that is a component of both aES and BTB. The analysis revealed that the junction organization in

spermatid-Sertoli cell interphase was clearly disrupted. aESs were discontinuous, and fragments of junctions were found around spermatids but rarely at the correct location. The problems in aES caused disorientation of spermatids in the epithelium, as well as immature release of spermatids into the lumen, which explains the greatly reduced number of mature spermatozoa produced in the *Dicer1* KO testis.

BTB was analyzed likewise by the immunofluorescence staining with anti-ESPIN. The results showed abnormally thick BTB at the interphase of two Sertoli cells. The BTB permeability test was not performed, and therefore, it remained unclear whether BTB is functional in *Dcr(fx/fx); Ngn3Cre* mice or not. However, the general appearance of BTB was clearly affected. It was somewhat unexpected that defects were also seen in junctions related to Sertoli cells, because in this mouse model DICER is specifically deleted in germ cells. This can be explained by the close cross talk between Sertoli and germ cells, which allows Sertoli cells to respond to the defects in germ cells.

The deletion of *Dicer1* induced general misregulation of mRNA transcriptome in spermatocytes (Zimmermann et al. 2015) and we showed that mRNAs that are involved in the processes of cell adhesion and the formation of cell-cell junctions were enriched among the upregulated ones. We focused our analysis on *Cldn5* that was one of the most upregulated mRNAs in the original RNA sequencing. Interestingly, despite the upregulation at mRNA level, the expression of the CLAUDIN5 protein showed downregulation in the western blot analysis of *Dcr(fx/fx);Ngn3Cre* testis. It is still unclear how the upregulation at the mRNA level is translated to a reduction at the protein level, but it is possible that posttranscriptional regulation and translation are disrupted due to the overall imbalance at the mRNA level and general disruption of the epithelial organization. Importantly, the CLAUDIN5 localization on aESs was also disrupted in *Dcr(fx/fx); Ngn3Cre* mice, and the polarized localization of CLAUDIN5 at aESs was lost.

The mechanistic conclusion about the regulation of the junction organization via DICER dependent pathways is difficult to give since the function of DICER is diverse; in addition to miRNAs, it also processes endo-siRNAs, and moreover, DICER has been demonstrated to have miRNA/siRNA-independent roles, for example in the regulation of protein-coding mRNAs and Alu transposon transcripts. Some evidence exists to support the hypothesis that many of the defects in *Dicer1* KO testis are caused by disrupted miRNA processing (see below). However, further studies are still required to clarify the mechanistic background.

6.4 miRNA or endo-siRNA-mediated regulation?

miRNA production requires two endonucleases, DROSHA and DICER, but endo-siRNAs are produced in the cytoplasm in a DROSHA-independent but DICER-dependent manner. Endo-siRNAs have been identified in the mouse testis and reported to control a variety of protein encoding mRNAs in male germ line (Song et al. 2011) and therefore, both miRNAs and endo-siRNAs participate in the regulation of male germ cells' transcriptome. The roles of these two pathways during spermatogenesis have been dissected by comparing two different mouse models with the Ddx4 promoter-driven inactivation of DICER or DCGR8 microprocessor complex. The deletion of DCGR8, which leads to a lack of miRNAs but intact biosynthesis of endo-siRNAs, induced similar defects in spermatogenesis as the deletion of DICER; but the defects were less severe (Zimmermann et al. 2015).

Since both miRNAs and endo-siRNAs are produced by DICER, the question is whether the specific defects in *Dcr(fx/fx); Ngn3Cre* mice are due to the lack of miRNAs or endo-siRNAs, or possibly due to the small RNA-independent functions of DICER. In this study, it was shown that the germ cell-specific deletion of DCGR8 caused similar, but not as severe, defects in the testicular cell-cell junctions as the deletion of DICER. It was also shown that *Cldn5* and several other adhesion related genes were upregulated in *both* knockout spermatocytes. All these results support the importance of miRNA-mediated regulation in the control of cell junctions and cell adhesion genes. Although conclusive direct evidence about the direct miRNA-mediated regulation of testicular junction protein expression is missing, some studies exist that show the involvement of miRNAs. For example, miR-34c that is highly expressed in spermatids (Zhao et al. 2015) has been shown to be regulated BTB through MAZ, that in turn, regulates the transcription of BTB proteins ZO-1, Occludin, and Claudin5 (Zhao et al. 2015). In addition, by TargetScan target prediction software (TargetScanHuman 6.2), we demonstrated that several potential miRNAs such as miR-93, miR-22, miR-199a and miR-17, are predicted to target Claudin5 mRNA. All these miRNAs are expressed in round spermatids (Kotaja 2014).

6.5 A novel isoform for DICER in mouse testis?

Recently, a novel isoform for DICER was found in mouse oocytes. An oocyte-specific DICER isoform is driven by an intronic retrotransposon promoter and it is shown to be required for female fertility (Flemr et al. 2013). Interestingly, in this study, two different bands were detected in the Western blot analysis of testis extracts that indicated the presence of a long and a short form of DICER in the testes. The novel high MW DICER was abundant but did not appear in all types of germ cells. The western blot results showed that this form of DICER was not detected in spermatocytes, but instead it appeared in haploid cells and was the predominant form in the epididymal spermatozoa.

In this study we did not further characterize the high MW DICER form and additional studies are required to find out whether the haploid cell-expressed DICER form is produced from a novel mRNA isoform or whether it corresponds to a post-translationally modified DICER. In any case, it provides intriguing opportunity for functional diversity of the DICER-dependent pathways in distinct spermatogenic cell types.

6.6 DICER associates with IMC and CB

In this study, the expression of DICER during spermatogenesis was studied in detail. It was shown that although DICER was expressed throughout male germ cell differentiation, the highest level of expression was detected in pachytene spermatocytes. DICER was not localized diffusely in the cytoplasm, but a granular signal was detected in spermatocytes and round spermatids. This granular expression pattern has long taken our attention while studying the expression of DICER in testes. Interestingly, we found out that the granular DICER signal in spermatocytes corresponded to IMC as detected by co-immunofluorescence staining using several IMC markers, such as MILI, TDRD1 and mitochondrial cytochrome C. Furthermore, DICER-positive granules in round spermatids were shown to frequently associate with another type of germ granule, the CB. The co-localization of DICER with CB has been previously described (Kotaja, Bhattacharyya, et al. 2006). However, our current results suggest that DICER is not a CB component, but there is interplay between DICER-positive granules and the CB. The close contact between these two types or granules explains the earlier results showing the localization of DICER to the CB on germ cell spreads (Kotaja et al. 2006), and also the detection of DICER in some of the mass spectrometric analyses of isolated CB (Meikar et al. 2014 and data not shown).

DICER has been reported to be required for the assembly of RNA granules in the germ line of *C.elegans* (Beshore et al. 2011). However, we showed that the IMC and CB morphology was intact in *Dicer1* KO germ cells, therefore, suggesting that the DICER function is not necessary for the assembly of germ granules in mice. The studies in human cells and *C. elegans* revealed the so-called passive DICER can sequester mRNAs without processing them into small RNAs (Rybak-Wolf et al. 2014). Interestingly, these mRNA transcripts that are targets for passively bound DICER were reported to be significantly enriched in genes whose mRNAs and translated proteins are located in germ granules and associate with the germ granule function. This is a very interesting finding to our research group, since our group has designed the unique isolation method, which enables the specific isolation of CBs from the testis (Meikar & Kotaja 2014). In addition we have earlier demonstrated the expression of DICER in CBs (Kotaja, Bhattacharyya, et al., 2006).

All the existing evidence supports the hypothesis that DICER is functionally linked to germ granules, and future studies will reveal if DICER is required for example targeting specific RNAs to germ granules in mouse male germ cells. The CB isolation protocol developed in our laboratory could

provide a useful tool to characterize the CB RNA composition in *Dicer1* KO vs. control round spermatids.

Germ granules accumulate piRNAs and proteins functioning in the piRNA processing and piRNA-targeted RNA regulation. The best characterized function of piRNA is the silencing of transposable elements (Castañeda et al. 2011). The most extensive transposon derepression takes place in germ cells from embryonic day 15 to postnatal day 3 during the time of massive epigenetic reprogramming when sex-specific epigenetic marks of the genome are being established (Castañeda et al. 2011), and piRNAs are active in transposon silencing during this time period. DICER-dependent pathways have also been linked with the regulation of transposon expression in several studies (Tam et al. 2008; Kaneko et al. 2011; Watanabe et al. 2008; Catena et al. 2006). The level of transposable element transcripts seemed unaffected in *Dcr(fx/fx);Ngn3Cre* mice but the transposon expression was increased in *Dcr(fx/fx); Ddx4Cre* mice, indicating at least some role for DICER-dependent pathways in transposon silencing. Therefore, it is also possible that the germ granule localization supports the function of DICER in the regulation of transposable elements.

Taken all together, DICER clearly associates with RNA-rich germ granules, which further expands the role of germ granules in RNA regulation beyond piRNA-targeted mechanisms. However, these results are raising many questions to be addressed in further studies. Is the germ granule associated DICER active or passive DICER? What is the role of germ granules in DICER-dependent pathways? Does DICER have a role in the piRNA pathway? We know that DICER is not required for the formation of germ granules as normal IMC and CB was detected in *Dcr(fx/fx);Ngn3Cre* mice. However, it remains to be characterized how DICER-dependent pathways are affected in the absence of functional germ granules.

6.7 Future prospectives

The results of this study clearly demonstrated that DICER is required for haploid germ cell differentiation in mice and particularly in the formation and maintenance of cell-cell junctions in the seminiferous epithelium. Mechanistic details of the DICER-mediated regulation of the haploid cell differentiation as well as cell-cell junctions in the seminiferous tubule require further analyses. A high throughput transcriptome analysis in combination with quantitative proteomics of *Dcr(fx/fx);Ngn3Cre* mice would uncover the genes from the DICER-dependent regulatory pathway during male germ cell differentiation. A high throughput transcriptome analysis of spermatocytes has been performed for *Dcr(fx/fx);Ddx4Cre* mice (Zimmermann et al. 2015), which shows severe defects in gene expression during meiosis. A similar analysis with *Dcr(fx/fx);Ngn3Cre* mice could reveal more specific information about the role of DICER in the control of haploid cell differentiation.

Our intriguing finding, the novel high MW form of DICER in haploid cells, definitely requires further analysis. The characterization of a possible novel isoform by RT-PCR, Northern blot and sequencing analyses would enable designing of an isoform-specific mutant mouse to clarify the role of this novel isoform in male fertility or in epigenetic inheritance.

For further analyses of the function of DICER-dependent regulatory pathways in germ granules, CBs can be isolated using the specific CB isolation protocol (Meikar & Kotaja 2014) from both adult *Dcr(fx/fx);Ngn3Cre* and control testis. The detailed examination of the CB RNA composition in the absence of DICER would reveal if DICER is required for the targeting of specific population of RNAs into the CB. Because the localization of DICER is even more prominently overlapping with the IMC in spermatocytes, it would be interesting to isolate IMC for RNA analysis. However, the protocol for IMC isolation is not currently available and therefore this approach would require protocol development.

7 SUMMARY AND CONCLUSIONS

The importance of DICER and DICER-dependent small RNA pathways in spermatogenesis and male fertility has been characterized in this work. A male germ cell-specific *Dicer1* KO mouse model, in which the deletion of *Dicer1* gene takes place during the early phases of spermatogenesis in spermatogonia, was created to clarify following: 1) The importance of DICER in spermatogenesis, 2) the role of DICER in the regulation of cell-cell junctions in the seminiferous epithelium, and 3) the association of DICER with germ granules.

The key findings based on this work are presented below:

I. Due to the lack of *in vitro* culture model for spermatogenesis, mouse models provide a valuable tool for the identification and characterization of genes associated with spermatogenesis and male infertility. The endonuclease protein DICER has an essential role in the male germ differentiation and in the absence of DICER mice were infertile with major spermatogenic failure. Haploid differentiation was drastically affected, and elongating spermatids had severe defects in nuclear shaping and chromatin compaction. The amount of epididymal sperm was drastically reduced and the remaining spermatozoa had abnormal morphology with small misshapen heads and disrupted tail structures.

II. The origin of these defects was characterized in this work and the results showed that the processes preceding the histone-protamine transition were largely unaffected in *Dicer1* KO mouse testes. Instead, the deletion of functional DICER in differentiating male germ cells induced disorganization of the cell-cell junctions in the seminiferous epithelium. Discontinuous and irregular apical ectoplasmic specializations between elongating spermatids and Sertoli cells were detected. The defective anchoring of spermatids to Sertoli cells caused a premature release of spermatids into the lumen. These findings explain the abnormal elongation process of remaining spermatids because these junctions and the correct positioning of germ cells in the epithelium are critically important for the progression of spermiogenesis.

III. Interestingly, transcriptome analysis revealed that cell adhesion-related genes were generally upregulated in *Dicer1* KO germ cells. *Claudin5 (Cldn5)* was among the most upregulated genes. These results suggest that DICER-dependent pathways control the formation and organization of cell-cell junctions in the seminiferous epithelium via the regulation of cell adhesion-related genes.

IV. DICER expression co-localizes with germ granules in the testis. The localization is apparent in intermichondrial cement (IMC) and DICER-positive granules are also associated with chromatoid body (CB). Although DICER has clear interaction with germ granules, it is not required for the formation and architecture of IMC or CB.

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