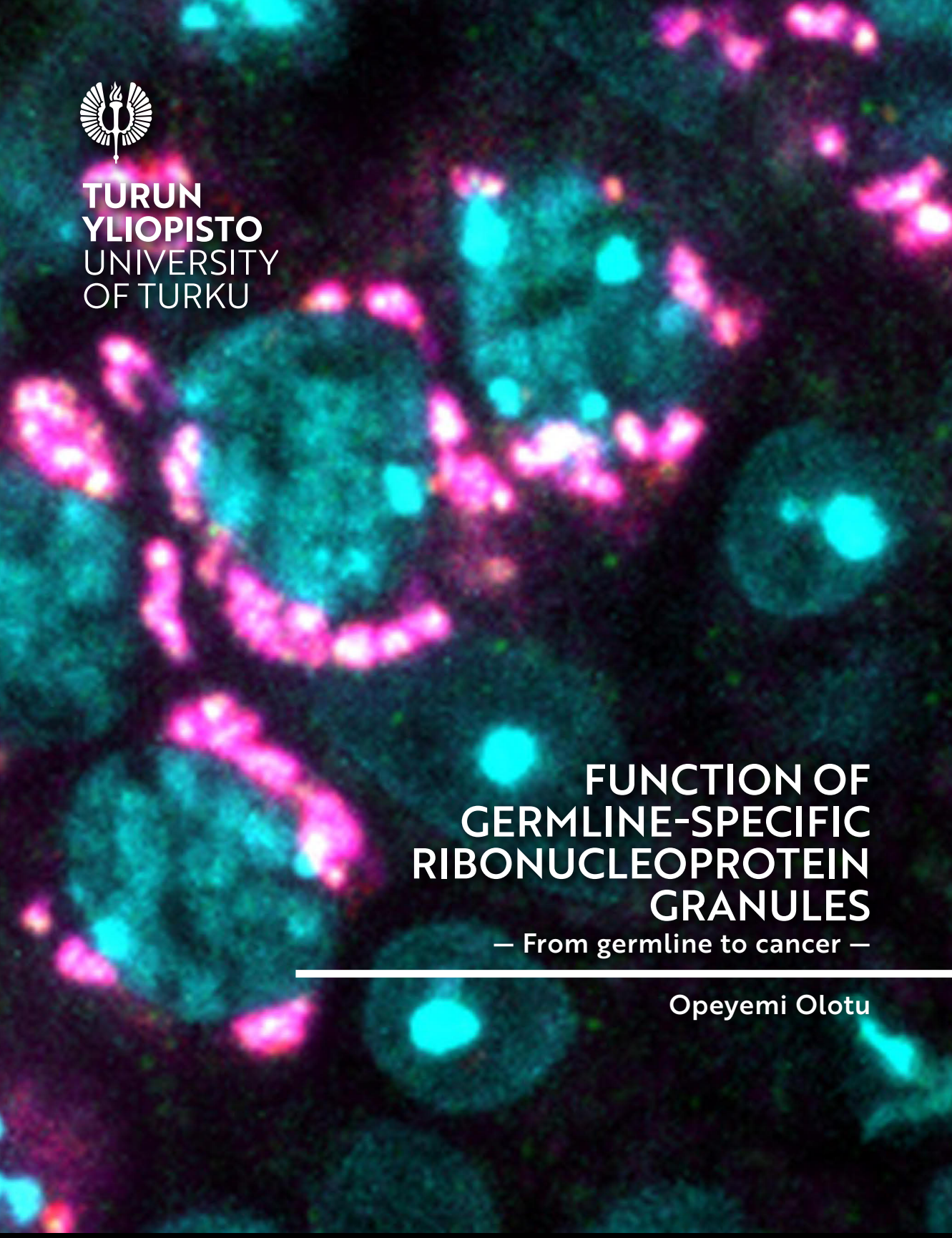

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— From germline to cancer —

Opeyemi Olotu

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ISBN 978-951-29-9548-6 (PRINT)
ISBN 978-951-29-9549-3 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku, Finland 2023

To my family and mentors

UNIVERSITY OF TURKU
Faculty of Medicine
Institute of Biomedicine
Physiology
OPEYEMI OLOTU: Function of germline-specific ribonucleoprotein granules – from germline to cancer
Doctoral Dissertation, 232 pp.
Turku Doctoral Programme of Molecular Medicine (TuDMM)
November 2023

ABSTRACT

Male germ cells undergo a complex differentiation from embryonic germ cells through postnatal spermatogenesis to highly specialized spermatozoa. The precise regulation of gene expression is crucial for meiosis and morphological changes in the differentiating male germ cells. Large ribonucleoprotein (RNP) granules and germ granules appear in the cytoplasm of germ cells to support the high requirements for posttranscriptional gene regulation.

In this study, we elucidated the functions of two types of germ granules, the intermitochondrial cement (IMC) and the chromatoid body (CB). We investigated the protein composition of the IMC and demonstrated its central role in the production of PIWI-interacting RNAs (piRNAs). We also identified two novel IMC components, EXD1 and EXD2, and characterized their association with the germ granules and the piRNA biogenesis machinery. Furthermore, we characterized the collaboration between EXD1 and the Tudor domain-containing protein TDRD12 in piRNA biogenesis in embryogenic testis and also revealed the essential role of this collaboration in male fertility.

We were able to show that the functions associated with germ granules are used in cancer cells, as the germ granule protein DDX4 forms cytoplasmic granules in many tumor types. These DDX4 complexes contained various RNA-binding proteins, including splicing regulators and known germ granule proteins. Deletion of *DDX4* in cancer cells led to an imbalance of the transcriptome and a disrupted splicing landscape of genes that contribute to cancer growth and invasiveness, and consequently retarded tumor growth. Moreover, the presence of DDX4 granules in human cancer samples was shown to correlate with a poor prognosis for the patient.

The results of this study highlight the importance of germ granules in the piRNA pathway during spermatogenesis and provide evidence for the novel role of germ granule components in transcriptome control in cancer cells that promote malignant properties.

KEYWORDS: male fertility, spermatogenesis, germ granules, IMC, CB, piRNA, EXD1, EXD2, DDX4, cancer, cancer-germline antigens

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Biolääketieteen laitos
Fysiologia
OPEYEMI OLOTU: Sukusolu-spesifisten RNA-säätelykeskusten toiminta – sukusoluista syöpään
Väitöskirja, 232 s.
Molekyylilääketieteen tohtoriohjelma (TuDMM)
Marraskuu 2023

TIIVISTELMÄ

Miesten sukusolut käyvät läpi monimutkaisen erilaistumisen alkiovaiheen sukusoluista syntymän jälkeisen spermatogeneesin kautta hedelmöityskykyisiksi siittiöiksi. Täsmällisesti ajoitettu geenien ilmentyminen ja geenituotteiden virheettömyys ovat edellytys meioosin ja morfologisten muutosten onnistumiselle, ja spermaogeneesin aikainen geenien ilmentyminen on tarkasti säädeltyä. Säätelyyn osallistuvat myös sukusoluille ominaiset RNA-säätelykeskukset, iturakeet (germ granules), jotka toimivat erilaistuvien sukusolujen sytoplasmassa koordinoiden posttranskriptionaalista geenisäätelyä.

Tässä tutkimuksessa selvitettiin kahden iturakeen, IMC:n (intermitochondial cement) ja CB:n (chromatoid body) toimintaa. Selvitimme IMC:n proteiinikoostumusta ja vahvistimme sen keskeisen aseman sukusolu-spesifisten pienten RNA:iden (piRNA) tuotossa. Tunnistimme myös kaksi aivan uutta IMC proteiinia, ja selvitimme näiden EXD1 ja EXD2 proteiinien vuorovaikutuksen iturakeiden ja piRNA-koneiston kanssa. Lisäksi selvitimme EXD1:n yhteistyötä TDRD12 proteiinin kanssa alkioin sukusolujen piRNA-tuotannossa, ja näytimme, että tällä yhteistyöllä on tärkeä rooli hedelmällisyyden ylläpidossa. Näytimme myös, että syöpäsolut hyödyntävät iturakeiden toimintaa, ja iturakeiden keskeinen proteiini DDX4 muodostaa sytoplasmisia rakeita monissa kasvaintyypeissä. Nämä DDX4-kompleksit sisältävät useita RNA:ta sitovia proteiineja, kuten silmukoinnin säätelyproteiineja ja tunnettuja iturakeiden proteiineja. *DDX4* geenin poisto syöpäsoluista aiheutti mittavia muutoksia mRNA-tasoissa, ja vaikutti myös vaihtoehtoiseen silmukointiin. Muutoksia tapahtui useissa geeneissä, jotka osallistuvat syövän kasvun ja invasiivisuuden säätelyyn, ja DDX4-poistogeenisten syöpäsolujen kyky muodostaa kasvaimia oli huomattavasti heikentynyt. Lisäksi DDX4-rakeiden ilmentyminen syöpäpotilaiden näytettiin korreloivan potilaan huonon ennusteen kanssa. Tutkimuksen tulokset korostavat iturakeiden keskeistä asemaa piRNA reitin ja spermatogeneesin säätelyssä, sekä paljastavat tiettyjen iturakeiden komponenttien aivan uuden roolin syöpäsolujen RNA-säätelyssä.

AVAINSANAT: miesten hedelmällisyys, sukusolut, spermatogeneesi, iturakeet, IMC, CB, piRNA, EXD1, EXD2, DDX4, syöpä, syöpä-sukusolu antigeenit

Table of Contents

Abbreviations

2D	Two Dimensional
3D	Three Dimensional
A3SS	Alternating 3' splice site
A5SS	Alternating 5' splice site
A_{al}	$A_{aligned}$
AS	Alternative splicing
A_s	A_{single}
ADAM12	Disintegrin and metalloproteinase domain-containing protein 12
ADT	Androgen dependent therapy
AGO1	Argonaute RISC component 1
AGO2	Argonaute RISC component 2
AGO3	Argonaute RISC component 3
AIPC	Androgen-independent prostate cancer
ALP	Alkaline phosphatase
AMIDA	Automated Morphometric image data analysis
A-MYB	Myeloblast transcription factor
A_p	A_{paired}
AR	Androgen receptor
ATP	Adenosine Triphosphate
BCL2	B-cell leukemia/lymphoma 2 protein
BLIMP1	B-lymphocyte-induced maturation protein 1
BMP4	Bone morphogenic protein 4
BMP8a	Bone morphogenic protein 8a
BPH	Benign prostate hyperplasia
BSA	Bovine serum albumin
BTB	Blood testes barrier
CAMK2N1	Calcium/Calmodulin dependent protein kinase II inhibitor
cAMP	cyclic Adenosine monophosphate
CB	Chromatoid Body
CCND2	Cyclin D 2
CD8	Cluster of differentiation 8

CDH1	Cadherin 1
CDH2	Cadherin 2
CDH6	Cadherin 6
CDH7	Cadherin 7
CDKN1B	Cyclin dependent kinase inhibitor 1B
cDNA	complementary DNA
CEACAM1	Carcinoembryonic antigen- related cell adhesion molecule 1
CGAs	Cancer germline antigens
C-KIT	Mast/stem cell growth factor receptor kit
CLEC2B	C-Type Lectin Domain Family 2 member B
CO_2	Carbon IV Oxide
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated protein 9
CSPG4	Chondroitin sulphate proteoglycan 4
CTAs	Cancer-testes antigens
CTDSPL	Carboxyl Terminal Domain small phosphatase-like protein
CYP26B1	Cytochrome P450 family 26 subfamily B member 1
CYP27C1	Cytochrome P450 family 27 subfamily C member 1
D	Diplotene
DAB	3, 3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-phenylindole
DAZL	Deleted in Azoospermia like
DDK	Dbf4 dependent kinase
DDX4	DEAD box helicase 4
DDX25	DEAD box helicase 25
DHH1	Desert Hedgehog Homologue 1
DIRAS1	GTP binding protein Di-Ras 1
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient mixture F12
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyl transferase 3 alpha
DNMT3B	DNA methyl transferase 3 beta
DNMTL3	DNA methyl transferase 3 like
DTT	Dithiothreitol
E	Embryonic day
ECL	Electrochemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EGCs	Embryonic germ cells
EIF2α	Eukaryotic translation initiation factor 2 alpha
EIF4E	Eukaryotic translation initiation factor 4E
EMBL	European Molecular Biology Laboratory

EMT	Epithelial mesenchymal transition
EXD1	Exonuclease domain containing 1 protein
EXD2	Exonuclease domain containing 2 protein
FAPS	Fluorescence-activated particle sorting
FBS	Fetal bovine serum
FFPE	Formalin-fixed paraformaldehyde embedded
FITC	Fluoresceine isothiocyanate
FYCO1	FYVE and coiled-coil domain autophagy adaptor
GAPDH	Glyceraldehyde-3-Phosphate dehydrogenase
GASZ	Germ cell protein with ankyrin repeats, sterile alpha motif, and leucin zipper
G3BP	Ras GTPase-activating protein-binding protein
G3BP1	Ras GTPase-activating protein-binding protein 1
GJA1	Gap junction alpha 1 protein
GJB2	Gap junction beta 2 protein
GO term	Gene ontology term
GPAT	Glycerol-3-phosphate acetyl transferase
gRNA	guide RNA
HCl	Hydrochloric Acid
HCD	Higher-energy C-trap dissociation
HE	Hematoxylin eosin
HeLa	Henrietta Lacks
HGPIN	High grade prostatic intraepithelial neoplasia
hnRNPs	heterogeneous nuclear RNPs
HNSCC	Head and neck squamous cell carcinoma
HPLC	High performance liquid chromatography
HPV	Human papillomvirus
HRP	Horseradish peroxidase
hRPL19	Human ribosomal protein L19
HSD	Hydrosteroid Dehydrogenase
HSD17B2	Hydrosteroid 17 beta Dehydrogenase 2
HSP72	Heat shock-related 70 kDa protein 2
IDT	Integrated DNA Technology
IGSF9B	Immunoglobulin super family member 9 B
IMC	Intermitochondrial cement
IgG	Immunoglobulin
In	Intermediate
LB	Luria Broth
LC-ESI-MS	Liquid Chromatography-Electrospray Ionization-Mass spectrometry

LOX	Lysyl oxidase
L1	Line 1
LNCaP	Lymph node carcinoma of the prostate
lncRNA	Long non-coding RNA
Lsm	N-terminal like-sm
LTR	Long terminal repeat
MAEL	Maelstrom
MAGE-1	Melanoma associated antigen 1
MGP	Matrix Gla protein
mitoPLD	mitochondrial phospholipase D
miRNAs	micro-RNA
MS	Mass spectrometry
MOV10L1	RNA helicase mov10L1
mRNA	messenger RNA
MUC2	Mucin 2
MYLK	Myosin Light chain kinase
NaCl	Sodium Chloride
NDN	Necdin
NE	Neuroendocrine
NEB	New England Labs
NGS	Next Generation Sequencing
NMD	Non-sense mediated decay
NP-40	Non ionic detergent P-40
nt	Nucleotides
OPCML	Opioid binding protein cell adhesion molecule-like
P	Testes time points
PABP1	Poly-A-binding protein 1
PAGE	Poly acrylamide gel electrophoresis
PAS	Period acid schiff
P53	Tumor protein 53
PABP1	Poly-A-binding protein 1
PABPC1	Poly-A-binding protein cytoplasmic protein1
PBs	Processing bodies
PBS	Phosphate buffer solution
PBST	Phosphate Buffer-Tween solution
PCR	Polymerase chain reaction
PD-1	Programmed death protein 1
PD-L1	Programmed death protein ligand 1
PD-L2	Programmed death protein ligand 2
PET	PIWI-EXD1-TDRD12

PFA	Paraformaldehyde
PGCs	Primordial germ cells
Pl	Preleptotene
PI	Propidium iodide
PIN	Prostatic intraepithelial neoplasia
piRNA	PIWI-interacting RNA
PIWIL1	Piwi-like protein 1
PIWIL2	Piwi-like protein 2
PIWIL3	Piwi-like protein 3
PIWIL4	PIWI-like protein 4
PLD6	Phospholipase D family member 6
PMSF	Phenylmethylsulphonyl fluoride
PNLDC1	Poly (A)-specific ribonuclease domain containing exonuclease 1
PPP1R1B	Protein Phosphatase 1 regulatory subunit 1B
PRDM1	PR domain zinc finger protein 1
PRDM14	PR domain zinc finger protein 14
PROX1	Transcription factor prospero homeobox
PRRC2C	Proline rich coiled-coil 2C
PSA	Prostate specific antigen
PTEN	Phosphatase and TENsin homologue
PVD	Poly vinylidene fluoride
QMN	Qiagen sequencing microRNA NGS
rasiRNA	repeat associated siRNA
RBP	RNA binding protein
RBD	RNA binding domain
RBP	RNA binding protein
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNAi	RNA-interference
RNAPol	RNA Polymerase
RNP	Ribonucleoprotein
RS	Round spermatids
RT	reverse transcription
RT-qPCR	reverse transcription quantitative polymerase chain reaction
S	Sertoli cells
siRNAs	small interfering RNA
SOX9	SRY box transcription factor 9
SGs	Spermatogonia
SPc	Spermatocyte
SCP3	Synaptonemal complex protein 3

SSCs	Spermatogonial stem cells
SEPW1	Selenoprotein W1
SFRP1	Secreted frizzled related protein 1
SMOC1	Secreted Modular calcium-binding protein 1
STRA8	Stimulated by retinoic acid 8
TCDM	Turku centre for disease modelling
TDRD1	Tudor domain containing protein 1
TDRD3	Tudor domain containing protein 3
TDRD6	Tudor domain containing protein 6
TDRD7	Tudor domain containing protein 7
TDRD12	Tudor domain containing protein 12
TDRKH	Tudor and KH domain containing
TEs	Transposable elements
TGF-beta	Transforming growth factor-beta
TIA-1	T-cell intracellular antigen-1
TIAR	TIA-1 related protein
TIFF	Tag image file format
TME	Tumor microenvironment
TP53	Tumor protein 53
UMI	Unique Molecular Index
UT-SCC-14	University of Turku squamous cell carcinoma-14
UTR	Untranslated region
UV	Ultraviolet
VCaP	Vertebral-cancer of the prostate
Volts	Voltage
Wnt	Wingless/integrated
WT	Wild-type
YB-1	Y box binding protein 1
Z	Zygotene

List of Original Publications

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

I **Opeyemi Olotu**, Mark Dowling, David Homolka, Magdalena N. Wojtas, Panyi Tran, Tiina Lehtiniemi, Matteo Da Ros, Ramesh S. Pillai, Noora Kotaja. Intermitochondrial cement (IMC) harbors piRNA biogenesis machinery and exonuclease domain-containing proteins EXD1 and EXD2 in mouse spermatocytes. *Andrology*, 2023; 11(4): 710–723.

II Radha Raman Pandey*, David Homolka*, **Opeyemi Olotu**, Ravi Sachidanandam, Noora Kotaja, Ramesh S. Pillai. Exonuclease Domain-Containing 1 Enhances MIWI2 piRNA Biogenesis via its Interaction with TDRD12. *Cell Reports*, 2018; 24: 3423–3432.
* These authors contributed equally

III **Opeyemi Olotu**, Anna-Riina Koskenniemi, Lin Ma, Valeriy Paramonov, Sini Leskinen, Elina Louramo, Matthieu Bourgery, Tiina Lehtiniemi, Samuli Laasanen, Adolfo Rivero-Müller, Eliisa Löyttyniemi, Cecilia Sahlgren, Sami Ventelä, Tapio Visakorpi, Matti Poutanen, Paula Vainio, Juho-Antti, Mäkelä, Noora Kotaja. Germline-specific RNA helicase DDX4 forms cytoplasmic granules in cancer cells and promotes tumor growth. *manuscript.*

1 Introduction

For many years, there has been growing concern about the decline in fertility especially in advanced countries (Rolland et al., 2013). The reason behind this adverse trend is largely unknown, but some of the possible causes of infertility are traceable to increased exposure to environmental insults (Ramsay et al., 2023), or unhealthy habits which have been shown to induce changes in the epigenome (Tiffon, 2018). If these changes take place in the germline but do not compromise fertility, the information of the environmental exposures can be transmitted to the next generation, highlighting the important role of the germline in determining transgenerational health (Senaldi & Smith-Raska, 2020).

The germline is a unique lineage of cells that is dedicated to the transmission of genetic and epigenetic information down to successive generation (Han, 2018; Wen & Tang, 2019). In multicellular organisms, such as mice and humans, the germline specification occur during the embryonic development starting from the precursor cells known as the primordial germ cells (Hansen & Pelegri, 2021; Saitou, 2009; Saitou & Yamaji, 2012).

The male primordial germ cells, which are diploid cells, migrate and colonize the gonads, then differentiate into gonocytes and spermatogonial stem cells at birth (Ewen & Koopman, 2010). Some specific populations of these spermatogonial stem cells initiate differentiation and are transformed into matured spermatozoa in the seminiferous tubules through the process of spermatogenesis (Diao et al., 2022). Every step of development from the spermatogonial stem cells through proliferating spermatogonia, meiotic spermatocytes, haploid spermatids until formation of mature spermatozoa, is accompanied by significant changes in gene expression that are controlled at transcriptional and posttranscriptional level by various and different RNA regulatory processes (Almstrup et al., 2004; Kopania et al., 2022).

Ribonucleoprotein granules (RNP granules) are large membrane-less compartments that can be found in the nucleus or cytoplasm. They are enriched with a high proportion of RNAs and proteins, hence, they serve as an important mediator for different posttranscriptional RNA regulatory mechanisms, including RNA metabolism (Tian et al., 2020). There are different kinds of RNP granules and they are functionally linked to growth, development that maintains cellular homeostasis

and are even thought to potentially contribute to the development of diseases (Jeon et al., 2022). Some RNP granules, such as stress granules and processing bodies, can be formed in many different cell types (Riggs et al., 2020), while others are formed only in specific somatic cells as exemplified by neuronal RNP granules (De Graeve & Besse, 2018). Some RNPs are germline-specific, also known as germ granules and their function in the posttranscriptional regulatory processes are the focus of this study (Lehtiniemi & Kotaja, 2017).

The two best-known germ granules are the intermitochondrial cement (IMC) in meiotic spermatocytes, and the chromatoid body (CB) in haploid round spermatids (Lehtiniemi & Kotaja, 2018). Our group previously carried out extensive molecular characterization of the CB (Meikar et al., 2014), and showed that it contains different species of RNAs, including long non-coding RNAs (lncRNAs), mRNAs and germline specific PIWI-interacting RNAs (piRNAs), as well as numerous RNA binding proteins such as RNA helicases, Tudor domain containing proteins, splicing factors and PIWI proteins. On the basis of its molecular composition, the CB also accumulates proteins involved in the nonsense-mediated RNA decay (NMD) pathway (Lehtiniemi et al., 2022b; Meikar et al., 2014). The current study provides an important novel advance into the field by characterizing the molecular composition of the IMC, and thereby elucidating the interrelationship between the IMC and CB.

Interestingly, many germline-specific features appear to be beneficial to cancer cells and germline-specific genes are known to be widely expressed in different somatic malignancies (Nin & Deng, 2023). Even though these cancer germline antigens (CGAs) are epigenetically repressed in somatic tissues, the mechanisms by which their expressions are induced in cancer cells are still unknown, and their *modus operandi* has also remained elusive. Due to their germline-restricted expression pattern, they are considered to be promising therapeutic targets, with limited side effects, for cancer treatment (Kortleve et al., 2022; Meng et al., 2021).

This PhD project aimed at elucidating the role of germ granule-mediated RNA regulation under physiological condition during spermatogenesis, and also the pathological role of germ granule proteins in somatic malignancies.

By providing insight into the molecular mechanisms of gene regulation during spermatogenesis, this study has an important contribution to make to our understanding of the etiology of male fertility problems. In addition, by translating the results from the male germline-related studies to cancer cells, this research also provides important novel information on the mechanisms involved in cancer transcriptome regulation; therefore, opening new avenues for the utilization of germ granule components as biomarkers for cancer classification and prognosis.

2 Review of the Literature

2.1 Male germ cell differentiation

The male germ cell differentiation is a complex physiological process, which produces mature spermatozoa capable of fertilizing an oocyte. The process of male germ cell differentiation is initiated at the point of germ cell specification during embryonic development and finalized postnatally during spermatogenesis and epididymal sperm maturation. These processes are quite well conserved between different mammalian species, but some differences exist. This PhD thesis project used mice as models and therefore the focus here will be on mice, if not otherwise mentioned.

2.1.1 Germline specification

During embryonic development in mammals, the mouse embryo (blastocyst) has three cell types, the outer trophectoderm cells, epiblast cells and the inner endoderm cells which differentiate from the epiblasts. Just before implantation, during embryonic day 6.25 (E6.25), cells from the epiblasts begin to respond to signal induction by the bone morphogenic factors (BMP4 and BMP8b) which are produced in the surrounding extraembryonic walls and the adjacent cells (Ying et al., 2000). This process of induction is called germ cell specification and the induced cells are called primordial germ cells (PGCs) (Fig.1) (Roelen & Chuva de Sousa Lopes, 2022; Yao et al., 2022). The process of germ cell specification is well characterized in mouse species using different methods to monitor the changes at different time points during development. By single-cell gene expression analysis it has been shown that the formation of PGC is already initiated during embryonic day E6.25 (Tam & Zhou, 1996; Wrighton, 2018).

The bone morphogenic proteins belong to transforming growth factor-β family of proteins and they are mostly involved in the transformation and differentiation of cells (Bragdon et al., 2011; T. Fujiwara et al., 2001; Wrana, 2013). The bone morphogenic proteins 4 and 8a induced the PGCs expression of germline specific genes such as B lymphocyte-induced maturation protein 1 coding genes (*Blimp1* or *Prdm1*), expressed during E6.25 (Ohinata et al., 2005). The *Prdm1* function is

to repress genes associated with somatic cell differentiation (Ohinata et al., 2005) and also enhance the expression of germline specific genes. Apart from *Prdm1,* another gene that is crucial for germ cell specification is the *Prdm14* and both are considered master regulators required for germ cell specification (Sybirna et al., 2020). Other genes that have been shown to provide support for PGCs differentiation and development include *DAZ-like* (*Dazl*) which encodes for the RNA binding protein, DAZL (H. Li et al., 2019). The PGCs also starts to express alkaline phosphate (ALP) during E7–7.25; positive staining of ALP is used as marker for detection of the PGCs (Chiquoine, 1954; Ginsburg et al., 1990; Magnúsdóttir & Surani, 2014).

The PGCs undergo further proliferation and migration towards the gonadal ridge. The PGCs of both male and female embryos are very similar in their characteristic features, however the male gonadal ridge can already be distinguished by microscopic observation during embryonic day E9.5 in mice. The migration of the PGCs to the male gonads is enhanced by factors such as c-kit (De Miguel et al., 2002), integrin- β1 (R. Anderson et al., 1999), as well as E-cadherin (Di Carlo & De Felici, 2000). The male gonad is characterized by the expression of sex determining-genes, for example *Sox9* and Chromodomain Y-like protein, on the Y-chromosomes (Okashita et al., 2023).

2.1.2 Embryonic male germ cells

The Embryonic male germ cells (EGCs) are derivatives of the PGCs and are also known as gonocytes (Culty, 2009). During their development within the gonads, the gonocytes begin to express germline specific genes such as *Ddx4/mvh* in mouse (S S Tanaka et al., 2000). The male gonads contain supporting somatic cells and a basement membrane, which forms the seminiferous cords enclosing the EGCs (gonocytes) during embryonic day E10.5. The somatic cells eventually form the supporting Sertoli cells and together with peritubular myoid cells and the male EGCs, they form the seminiferous tubules. The male EGCs are transformed into prospermatogonia in the periphery of the seminiferous tubules. The male EGCs undergo mitotic division, and are then arrested in the G0 phase of the cells cycle during day E13.5. The mitotic arrest of the male EGCs continues until immediately after birth when postnatal development of the spermatogonial stem cells resumes.

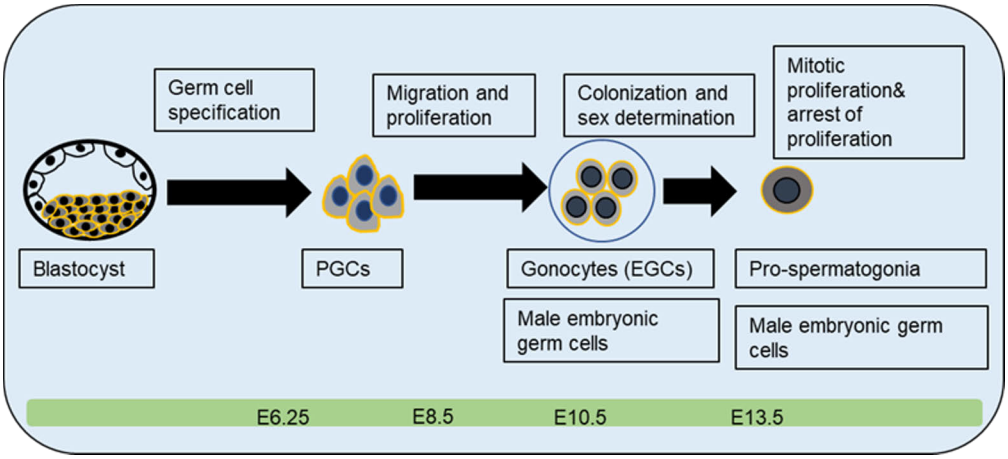

Figure 1. The trajectory of male germ cell differentiation during prenatal male germ cell development. These events include: germ cell specification occuring during E6.25, PGC migration and proliferation during E8.5, transformation into embryonic male germ cells of gonocytes during embryonic day E10.5, which further differentiates into pro-spermatogonia in the male gonads during E13.5.

2.1.3 Postnatal spermatogenesis

Spermatogenesis is a highly specialized differentiation program leading to the formation of mature spermatozoa in the seminiferous tubules of the testis (Renkawitz-Pohl et al., 2005). Spermatogenesis begins with the mitotic proliferation of spermatogonia, continues with the meiotic division of spermatocytes and finally with the haploid differentitaion of spermatids (spermiogenesis), during which the round spermatids morphologically transform into elongating spermatids and finally into spermatozoa. The first wave of spermatogenesis is initiated shortly after birth in mice, when spermatogonial stem cells (SSC) on the basement membrane of seminiferous tubules enter the differentiation pathway (Montoto et al., 2012). This process is temporally regulated, and different postnatal time points reflect the progress of the first wave of spermatogenesis: spermatogonia predominate on postnatal day 7 (P7), early spermatocytes appear at P9, pachytene spermatocytes at P14, late pachytene and diplotene spermatocytes at P18, and the first haploid round spermatids at P20 (Laiho et al., 2013). Spermatogenesis is a continuous process in adult male mouse testes, where about 62–80 million spermatozoa are produced per day (Cordeiro et al., 2021), while in humans it is 45 million per day (Griswold, 2016). The ultimate goal of spermatogenesis is the production of spermatozoa that are able to transmit both genetic and epigenetic information from fathers to the progeny when fusing with a mature oocyte (female gamete) in fertilization (Rando, 2016).

2.1.3.1 Organization of spermatogenesis in the testis

Spermatogenesis is a complex physiological process which occurs throughout the entire seminiferous tubules of the testes (Fig.2). The seminiferous tubule consists of Sertoli cells which have been described as the "nurse" cells due to their role in nurturing and supporting the germ cells (Bhandary et al., 2021). Developing cells within the seminiferous tubules are arranged in such a way that the less differentiated cells are very close to the basement membrane, other cells migrate towards the luminal part as they differentiate to form spermatozoa which is finally released into the lumen. The duration of spermatogenesis varies across different mammalian species. In mice, it takes approximately 35 days, and in humans it takes approximately 64 days (Heller & Clermont, 1963; Oakberg, 1956). The whole process of spermatogenesis is tightly regulated by both intrinsic factors (within the Sertoli cells and germ cells) as well as extrinsic stimulating factors (e.g androgens and retinoic acid).

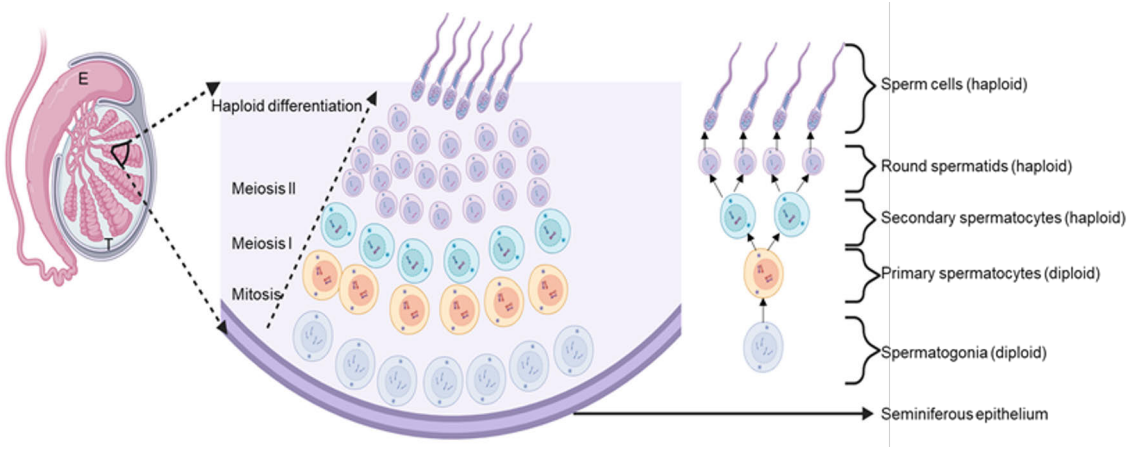

Figure 2. Cross section of seminiferous tubule of adult mouse testis (T). Diploid spermatogonia stem cells near the basement of the seminiferous tubule undergo proliferation (mitosis) and differentiate into primary spermatocytes (diploid cells) which undergo two meiotic cell divisions into haploid round spermatids and elongating spermatids. Matured haploid sperm cells are delivered into the lumen of the seminiferous tubule where they are propelled to their temporary storage in the epididymis (E). The entire population of the developing germ cells are suspended in the *milieu* of the Sertoli cells which nurtures and support them in the seminiferous tubule.

Spermatogenic cells are organized in a complex but highly coordinated way within the seminiferous epithelium. This epithelial arrangement was resolved over a century ago by Clermont and Perey, when they observed and showed that different cross sections or regions of the seminiferous tubule appeared to contain a regularly occurring germ cell population (specific populations of spermatogonia,

spermatocytes, haploid rounds spermatids and elongating spermatids) in a cyclic manner (Clermont & Perey, 1957). This organized regular occurrence of certain types of differentiating germ cells at a given cross section defines the stage of the epithelial differentiation (Figure 3). Furthermore, these stages that occur sequentially in a wave-like manner along the length of seminiferous tubules (I–XII in mice and I–XIV in rats) form the cycle of seminiferous epithelium (Clermont & Perey, 1957). In mice, one cycle takes approximately 8.6 days, while in humans it lasts16 days (Heller & Clermont, 1963; Oakberg, 1956).

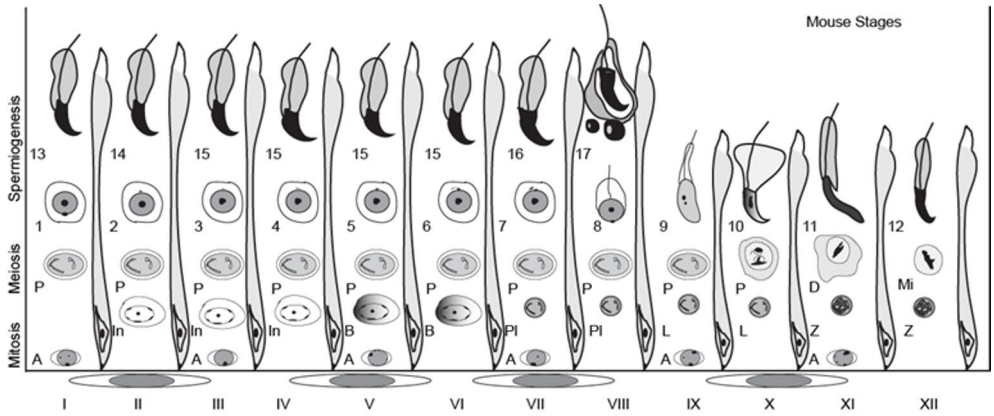

Figure 3. Drawing showing the stages (I–XII) in the seminiferous epithelial cycle in mice. The developing germ cells are compartmentalized within the cytoplasmic pockets of supporting Sertoli cells in a way that germ cell types at specific phases of differentiation are always found together within the same stage of the cycle. Type A- spermatogonia (A), In- intermediate spermatogonia (In), and Type B spermatogonia (B) at the basement of the epithelium undergo mitotic division and then differentiate by mitosis into Preleptotene spermatocytes (Pl) initiating the beginning of prophase of the first meiotic division. The prophase progresses with leptotene (L) and zygotene (Z) pachytene (P) and diplotene (D) phases, and after meiotic division at stage XII, haploid round spermatids (steps 1–8) are formed, which further differentiate to elongated spermatids (step 9–17). This figure is modified from the book chapter, Spermatogenesis and cycle of seminiferous epithelium (Hess & De Franca, 2008).

2.1.3.2 Proliferation of spermatogonia

Spermatogonia are the first population of germ cells that initiate spermatogenesis. The SSCs have the ability to self-renew in order to maintain the stem cell pool required for constant production throughout sexual maturity (Fayomi & Orwig, 2018). The spermatogonia also continuously differentiate into another population of germ cells that is required for sperm production (de Rooij, 2001).

The Spermatogonia undergo rapid clonal expansion at the basement membrane of the seminiferous tubules. The first population is the A_{single} (A_s) SSCs which appear as single cell SSCs at the basement membrane of the seminiferous tubules. The A_s

undergo mitotic division to form another population of SSCs, A_{paired} (A_p) spermatogonia. Complete cell division of A_p give rise to two new A_s or they are sometimes joined together in chains connected by intracytoplasmic bridges, hence forming populations of cells: $A_{aligned-4}$, $A_{aligned-8}$ and $A_{aligned-16}$ (A_{al}). A_s, A_p and A_{al} make up the population of undifferentiated spermatogonia, which comprise about 0.3% of the entire germ cell population and their role is mainly to continually replenish the SSCs (Claire Huckins, 1971). To begin spermatogenesis, A_{al} differentiate into Type A1 spermatogonia (Figure 4). Type A1 spermatogonia further undergo successive mitotic divisions into A2, A3, A4, intermediate and Type B spermatogonia which differentiate into primary spermatocytes (Fig. 4).

SSCs reteain their properties in cell culture, and SSC cultures have been widely used to study their properties *in vitro*. SSC cultures have also been used to study, for examples, the effects of environmental toxicants which causes infertility in men on SSC function (McAbee et al., 2018).

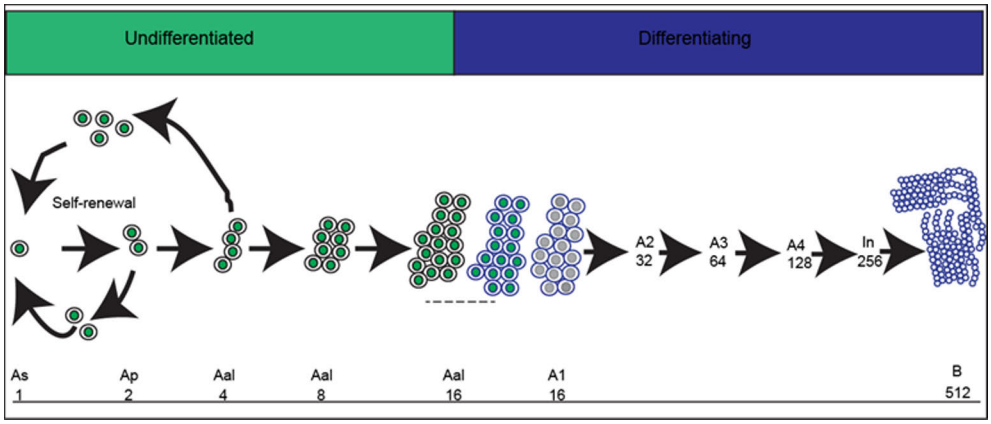

Figure 4. Schematic drawing to illustrate the clonal expansion of spermatogonial stem cells from A_s through to B type spermatogonium. The A_s to A_{al} types have an undifferentiated population which basically engage in self-renewing. Once differentiated into an A1 population, the cells continue with clonal expansion and differentiation into type B which differentiates into primary spermatocytes. Modified from the article, "Spermatogonial stem cells and spermatogenesis in mice, monkey and men" (Fayomi & Orwig, 2018).

2.1.3.3 Meiosis

A diploid set of chromosomes need to be transformed into haploid cells for gametogenesis to take place and this marks the beginning of life cycle of sexually reproducing organisms (Y. Huang & Roig, 2023). The process by which diploid cells (spermatocytes) undergo reduction division to form haploid round spermatids is called meiosis (Y. Huang & Roig, 2023). This occurs at different time points in the development of male and female gametes. In female mice, meiotic division of

oogonia is initiated under the influence of the retinoic acid gene 8 gene (*Stra8*) which stimulates the cells to differentiate into oocytes and this occurs before birth (Baltus et al., 2006; Koubova et al., 2006). In the male mice, meiotic division does not occur during the embryonic development because of the degradation of retinoic acid by Sertoli cell-producing enzyme CYP26B1. The meiotic division in males occur during the postnatal life when the expression of CYP26B1 is supressed and the activity of the retinoic acid resumes (E. L. Anderson et al., 2008; Koubova et al., 2006). This section will focus on meiotic division in male adult mice testes during spermatogenesis.

Mammalian meiosis is a tightly regulated process and it includes two subsequent divisions during spermatogenesis, Meiosis I and Meiosis II. The first part of meiotic division (especially, the Prophase of Meiosis I) is a slower process which takes about two weeks (Oakberg, 1956). The prophase of Meiosis I is divided into leptotene, zygotene, pachytene and diplotene phases, during which the primary spermatocytes in the leptotene phase undergo pairing of homologous chromosomes accompanied by the formation of a dense fibril called synaptonemal complex; this occurs between the homologous chromosomes to bind them together during the zygotene phase (Fawcett, 1956; Moses, 1956; Parra et al., 2003). Crossing over of non-sister chromatids takes place at the pachytene phase (Carpenter, 1987), leading to the formation of chiasmata (late Prophase I) that are prominent in the diplotene cells (Zickler & Kleckner, 1999). The paired homologous chromosomes are then aligned at the equatorial plane (metaphase), in the same region where the sex chromosomes are aligned. In males, a Y chromosome pairs with an X chromosome. In the last part of Meiosis I, the paired homologous chromosomes separate into two different daughter cells having half (haploid) the number of chromosomes. These two daughter cells are called the secondary spermatocytes. The second meiotic division occurs similar to the regular mitotic division as the two haploid daughter cells further divide into four haploid cells (round spermatids), each containing a single copy of each chromosome.

2.1.3.4 Haploid differentiation

During the haploid differentiation (also known as post-meiotic differentiation or spermiogenesis), after the completion of the reduction division of the spermatocytes, the formed haploid round spermatids undergo the process of morphological transformation into elongating spermatids. The elongating spermatids further develop into mature sperm that shed their cytoplasm and are released into the lumen of the seminiferous tubules, transiting to the epididymis for temporary storage and further development (V. G. Da Ros et al., 2015). Haploid differentiation is also described as spermiogenesis because it culminates in the production of sperm and

each of the steps involved are accompanied by morphological changes in the appearance of the developing cells. Some of the important features include: development of acrosome and nuclear shape, formation of axoneme structures and the flagella, accompanied by the development of cytoskeletal-based transport system required for motility, and finally, nuclear condensation (Pleuger et al., 2020).

The acrosome is a membrane-bound enzyme-secreting vesicle that is derived from the golgi apparatus and contains digestive enzymes (Berruti & Paiardi, 2011). The acrosome formation is initiated at the proximal part of the nucleus during early haploid differentiation of round spermatids (steps 3–5) and it continues throughout the development of haploid germ cells. The morphology of acrosome varies among different mammalian species, but in mice the acrosome is morphologically shaped in the form of a cap and consists of a large anterior part, which looks like a hook and the smaller part aligned to middle of the sperm head (Bedford, 2014). The acrosome consists of an outer membrane which is directly below the plasma membrane of the haploid round spermatids, and an inner acrosomal membrane, both of which fuse together with the oocyte at fertilization. Within the two acrosomal membranes are the hydrolytic and proteolytic enzymes and the main function of the acrosome is to release its enzymatic contents by exocytosis, facilitating the penetration of the zona pellucida and cumulus layers of the oocyte (Khawar et al., 2019).

During haploid differentiation there is formation of the motile cilia and flagella which together makes up the cytoskeletal axoneme structure of the sperm tail. The cilia and flagella begin with the formation of axoneme (a protrusion from centrosome of the nucleus) during steps 3–8 in the development of round spermatids (Lehti & Sironen, 2017). This phase of flagella formation in the round spermatids is termed intermediate haploid spermatids as it also initiates the elongation of the nuclei. The axoneme is composed of microtubules arranged in layers of cylindrical structures providing strength and support for spermatozoa maturation. Other important features formed during haploid differentiation include the manchette, fibrous sheath and mitochondrial sheath. The axoneme is surrounded by the mitochondrial sheath. Mitochondria is also formed in the mid-piece of the sperm tail, providing an energy supply to the sperm for transportation during fertilization (Lehti & Sironen, 2017).

During haploid differentiation, there is the condensation of the chromatin fibres which is the most dramatic changes during the elongation process. This process is facilitated by the gradual replacement of histone proteins first with transition proteins and then with protamines (Rathke et al., 2014).

2.1.4 RNA regulation during spermatogenesis

The progress of spermatogenesis is supported by specific, temporally controlled gene expression patterns in differentiating male germ cells. These dynamic changes in the

gene expression pose a high demand for different transcriptional and posttranscriptional RNA regulatory mechanisms in spermatogenesis.

In all mammalian tissues, genes are actively being transcribed in the nucleus by RNA polymerases (RNAPol I, II and III). While RNAPol I and III are involved with transcription of genes encoding ribosomal RNAs, transfer RNAs and the small non-coding RNAs (Turowski & Boguta, 2021), the RNAPol II is mainly involved with transcription of mRNAs (J. Huang & Ji, 2023). mRNAs are first transcribed as long pre-mRNAs containing introns interspersed among alternating exons. Introns are removed by splicing leading to the formation of mature mRNAs (Koch, 2017). Splice sites can be selectively used to skip a certain exon or a part of it, or retain a part of an intron or the whole intron in the mRNA. This alternative splicing is responsible for generation of multiple forms of mRNAs and protein products from a single gene (X. Yang et al., 2016), and it can be regulated by tissue- or cell type-specific manner. Differentiating germ cells exhibit an exceptionally diverse splicing landscapes compared to somatic cells, *i.e.* they produce a large number of different splice isoforms (Yeo et al., 2004).

In addition to splicing, pre-mRNA undergoes processing of its 5’ and 3’ ends, and the mature mRNA consists of a cap structure at the 5' end and a poly (A) tail at the 3' end. Mature mRNA has also cis-acting untranslated regulatory regions on both sides of its coding region (5' and 3' untranslated regions, UTRs). The cap structure, poly (A) tail and the UTR regions serve important regulatory functions and determines the stability, localizaton and fate of the mRNA. The mature mRNA is exported to the cytoplasm where they can be directed to ribosomes to be translated into proteins. In some situations, mRNAs are not translated immediately, but are destined to either be stored, repressed or turned over by decay processes.

2.1.4.1 Special features of germline transcriptome

Male germline differentiation, from embryonic germ cells to postnatal germ cells and finally to mature sperm, is characterized by drastic epigenetic alterations accompanied by dynamic transcriptome changes (Meikar et al., 2013). These processes must be carefully regulated to maintain sperm production and fertility. In addition to fertility, these changes in the epigenome and gene expression can affect the development and health of offspring through the process of epigenetic inheritance, i.e., the sperm epigenome-mediated transmission of the information on environmentally-induced conditions to the next generation (Rando, 2016).

The first major epigenetic remodelling that challenges transcriptome regulation takes place in embryonic germ cells that undergo epigenetic resetting to remove the majority of DNA methylation, followed by the re-establishment of epigenetic marks by male germ cell-specific *de novo* methylation (Kato et al., 2007). This allows the

activation of germline-specific genes and the establishment of male-specific patterns for the paternally imprinted genes (Kamimura et al., 2014). The global removal of methyl marks across the genome landscape of embryonic germ cells, if not properly regulated, will induce widespread activation of transposable elements capable of insertional mutations, which disrupt genomic integrity and results in spermatogenic defect and infertility (Hamidi et al., 2015). Therefore, the male germline has specific mechanisms, such as the piRNA pathway (discussed in section 2.1.4.3), to silence transposon expression and to preserve genome integrity of the germline (Kuramochi-Miyagawa et al., 2008b).

During postnatal spermatogenesis, meiotic and early postmeiotic cells are challenged by exceptional diverse transcriptomes (Soumillon et al., 2013). These cells show widespread gene expression, both in the protein-coding and intergenic non-coding parts of the genome. The exact reason for this transcriptome complexity is not known, but it is thought to be due to the dramatic epigenetic remodelling that occurs during meiotic chromosome synapsis and recombination (Soumillon et al., 2013). Other studies suggest that genome-wide transcription is required for transcription-coupled repair, which enables mutation scanning for genes expressed in germline and thus serves as an important quality control system and evolutionary driver for gene-specific modulation of mutation rates (Xia et al., 2020). Nevertheless, meiotic and early postmeiotic cells need specific posttranscriptional mechanisms to cope with the diverse transcriptomes.

In addition to widespread expression of the genome, germ cells also have highly complex splicing landscapes, and they express unusually high number of isoforms, many of them being testis-specific and they support the unique processes required for spermatogenesis (Q. Li et al., 2020). Interestingly, intron retention is also active in meiotic male germ cells, and intron-containing transcripts are retained in the nucleus for the temporally regulated translation (Naro et al., 2017).

After active widespread genome expression, transcription ceases during late spermatogenesis in elongating spermatids, when histones are replaced first with transition proteins and finally with protamines to allow tight packing of sperm chromatin (Rathke et al., 2014; T. Wang et al., 2019). This generates high demand for mRNA storage and translational regulation, because the majority of the transcripts encoding for proteins required in condensed spermatids are transcribed in earlier cell types and translationally repressed and stored in RNP complexes (H. Tanaka & Baba, 2005). Many mRNAs, for example, protamine mRNAs are even stored for several days before translation, and this kind of uncoupling translation from transcription is very common during spermatogenesis (Rathke et al., 2014).

A broad range of RNA regulatory mechanisms are used in male germ cells to respond to the dynamic transcriptome changes, one of these being the coordination

of RNA regulation in the cytoplasmic germ granules that will be discussed further in section 2.2.3.

2.1.4.2 RNA-binding proteins

Transcribed mRNAs are always associated with RNA-binding proteins (RBPs) through RNA-protein interactions in ribonucleoprotein (RNP) complexes, and the association of mRNAs with RBPs determine the fate of mRNAs at different stages from synthesis until translation or degradation (Corley et al., 2020; Harvey et al., 2018).

RBPs can be localized either in the nucleus or cytoplasm depending on their functions. Nuclear RBPs participate in transcriptional regulatory processes in the chromatin and they regulate pre-mRNA processing events (Ji et al., 2013). For example, the differentiating germ cells contain a large amount of different heterogeneous nuclear ribonucleoproteins (hnRNPs) in the nucleus where they contribute to different posttranscriptional regulation of RNA processing, such as RNA splicing, cleavage, polyadenylation, transportation to cytoplasm, localization, stability and translation of mRNAs (X.-L. Wang et al., 2023). These hnRNPs are characterized by different RNA-binding motifs and domains which enable their participation in these RNA processing events (Rajyaguru et al., 2012; Rajyaguru & Parker, 2012).

The differentiating male germ cells express a high number of RBPs, which facilitates posttranscriptional control that is required for the development and formation of fertile spermatozoa (Venables & Eperon, 1999). Disruption of RBPs can affect the progress of spermatogenesis (Paronetto & Sette, 2010). For example, the loss of RBPs such as RBM46, MSY2, DDX4, PIWIL1, PIWIL2, RBMXL2, STRA8 and C-KIT causes defects in the differentiation of spermatogonia and spermatocytes, thereby resulting in infertility (Bak et al., 2011; Busada et al., 2015; Ehrmann et al., 2019; Endo et al., 2015; Peart et al., 2022; Su et al., 2004; J. Yang et al., 2007). In humans, mutations in some of the RBPs, such as ADAD2 affects the differentiation of germ cells, thereby also causing azoospermia (B. Shi et al., 2023).

2.1.4.3 piRNA pathway

A large proportion of mammalian genome is known to be non-coding and a significant part (about 37% of mouse genome and 45% of human genome) is occupied by different species of repetitive mobile elements known as transposable elements (TE) (R. P. Alexander et al., 2010). They are called transposable elements because they have the ability to transpose (jump) from one part of the genome in order to be inserted into other parts and because of this, they affect the expression

and function of genes (Kapusta et al., 2013; van de Lagemaat et al., 2003). If not properly controlled, some species of activated TEs are capable of causing insertional mutations in germ cells leading to diseases or affecting the germline and embryonic development (Gagnier et al., 2019; Hancks & Kazazian, 2016). The germline has a self-activating, highly conserved germline specific RNA regulatory mechanism, the PIWI-interacting RNA (piRNA) pathway, which protects the genome against invading TEs (Brennecke et al., 2007; Saito et al., 2006; Vagin et al., 2006). These small non-coding RNAs protect the genome by activating the methylation of the transposons to suppress their activity or by posttranscriptional regulatory mechanisms.

The piRNAs were initially recognized as repeat associated small interfering RNAs (rasiRNAs) because of their emergence from repetitive genomic sequences (also known as transposons) in *Drosophila melanogaster* germ cells (A A Aravin, 2001, 2003). Aravin *et al* also revealed that rasiRNAs have sequences 24–29 nt, complementary to the transposon sequences and were highly enriched in the testes and early embryos. Though it is still intriguing how some specific clusters are selected for the production of rasiRNAs, Aravin suggested that these species of RNAs may be required for transcriptional repression of TEs activity and other chromatin modifications (A A Aravin, 2003). Apart from *Drosophila melanogaster,* rasiRNAs have been identified in other organisms (invertebrates and vertebrates) and later they were shown to associate with germline specific PIWI proteins, hence their change of name from rasiRNAs to piRNAs (Brennecke et al., 2007; Saito et al., 2006; Vagin et al., 2006). Currently, piRNAs are defined as small non-coding RNAs (24–35 nt) that are predominantly expressed in the germline and bind to PIWI proteins to form piRNA-protein complex required for preservation of genomic integrity and development of germ cells (D. N. Cox, 1998; Megosh et al., 2006; Szakmary et al., 2005).

The functions of piRNAs have being studied in different species of eukaryotic organisms. Across different species, piRNAs are believed to contribute to epigenetic and posttranscriptional silencing of transposons as well as posttranscriptional regulation of mRNAs and non-coding RNAs to support germ cell development (X. A. Huang et al., 2013; Yin & Lin, 2007). However, the detailed mechanism of piRNAs is still under investigation, and there also appears to be mechanistic differences between different organisms. Here, I will focus on mouse piRNAs as they are related to this study.

In mice, the piRNAs are associated with three PIWI proteins (MIWI2/PIWIL4, MILI/PIWIL2 and MIWI/PIWIL1) which are germline specific (Girard et al., 2006). These proteins have specific expression patterns at different point of germ cell differentiation. While the PIWIL4 is predominantly nuclear and expressed during embryonic development in pro-spermatogonia (Bao et al., 2014), PIWIL2 localizes

to the cytoplasm of pro-spermatogonia, spermatogonia, pachytene spermatocytes and early round spermatids (Kuramochi-Miyagawa et al., 2004b). The role of PIWIL4- and PIWIL2-associated piRNA have been linked to the regulation of methylation of transposable elements at the time of the global resetting of DNA methylation during embryonic germ cell development (Manakov et al., 2015). During this time of global demethylation, PIWI proteins and piRNAs function together with DNA methyltransferases (DNMT3A, DNMT3B and DNMT3L) to inactivate transposable elements, and to prevent deleterious effects in the genome (Hajkova et al., 2002; Trelogan & Martin, 1995). The third mouse PIWI protein, PIWIL1, is a cytoplasmic protein and it is expressed during postnatal spermatogenesis from late spermatocytes to early elongating spermatids (Wei Deng & Lin, 2002). The role of PIWIL2 and PIWIL1 in post-natal germ cells is further discussed in section 2.2.3.3.

In mice, there are three known categories of piRNAs defined on the basis of the dynamics of their expression and they include: embryonic (prenatal) piRNAs, pre-pachytene piRNAs and pachytene piRNAs. The prenatal piRNAs are also known as the fetal piRNAs (26–28 nt) and they form complex with PIWIL4 and PIWIL2 to silence transposable elements in the fetal testes (Manakov et al., 2015). PIWIL4-associated piRNAs are localized in the nucleus where they enhance epigenetic silencing of the transposable elements (Schöpp et al., 2020), and PIWIL2-associated piRNAs are localized in the cytoplasm where they mediate posttranscriptional silencing of their targets (Alexei A. Aravin et al., 2008; Alexei A Aravin et al., 2009; Z. Yang et al., 2016). The pre-pachytene piRNAs (26–27 nt) are expressed after birth and form complex with PIWIL2 during the early stages of spermatogenic cells until meiotic pachytene stage. They mainly originate from genic sequences, particularly 3' UTRs, and from TE sequences (Di Giacomo et al., 2013; Robine et al., 2009). The pre-pachytene piRNAs are important for posttranscriptional silencing of TEs during the mitotic and meiotic stages of spermatogenesis (Di Giacomo et al., 2013). The pachytene piRNAs (~ 30 nt) are the most abundant class of piRNAs, contributing more than 80% of small RNAs in adult testis and known to be produced from intergenic regions not overlapping with TE sequences (Özata et al., 2020). The expression of pachytene piRNAs is induced at the pachytene stage of meiosis and they form a complex with PIWIL2 and PIWIL1 (Ozata et al., 2019; P. H. Wu et al., 2020).

2.1.4.3.1 piRNA Biogenesis

The biogenesis of piRNAs in mice can be classified into primary and secondary biogenesis. The primary biogenesis begins with the transcription of long single stranded RNA precursors synthesized by RNA polymerase II from specific loci

known as piRNA clusters (X. Z. Li et al., 2013b). Transcription factor A-MYB has been recognized as a crucial regulator for pachytene piRNA precursor transcription in mice (X. Z. Li et al., 2013a). The long single stranded transcripts are further processed into piRNAs in a PIWI-dependent regulatory RNP complex involving multiple factors that are still not fully characterized (Alexei A. Aravin et al., 2008; X. Z. Li et al., 2013a). Some of the factors that function in the primary piRNA biogenesis include the helicase protein MOV10L1, which binds piRNA precursor transcripts and enhance further processing (Vourekas et al., 2015). The pre-piRNA is further processed by endonucleases such as PLD6 or mitoPLD (Watanabe et al., 2011) that generates the 5' end of piRNAs containing a monophosphate; the 3' end is then trimmed by the exonuclease activity and finally 2'-O-methyl group is added by HENMT1 (Kirino & Mourelatos, 2007). The 3' trimming requires the proteins PNLDC1, TDRKH and GASZ (Ma et al., 2009; Saxe et al., 2013). These factors are mainly associated with the mitochondria membrane (Ding et al., 2019).

The formation of the piRNA-PIWI complex is crucial for the secondary biogenesis of piRNAs as well as piRNA-mediated posttranscriptional RNA regulation of piRNA targets. The secondary biogenesis of piRNAs utilizes the primary piRNAs to amplify the production of piRNA pool in a process called ping-pong amplification that is known to facilitate repression of transposons (Czech & Hannon, 2016).

Interestingly, the piRNA production and piRNA-mediated RNA regulation has been linked to the function of cytoplasmic germ granules, the intermitochondrial cement (IMC) and the chromatoid body (CB) (Meikar et al., 2010b). This will be discussed in more details in the **section 2.2.3**.

2.2 Ribonucleoprotein (RNP) granules

RNP granules are non-membrane bound conspicuous structures that are formed by the coalition of different species of RNAs and proteins. The RNP granules have also been described as biomolecular condensates of liquid-liquid phase separation consisting of interactions among different species of RNAs and their associated RBPs (Banani et al., 2017). This means that within the RNP granules, both RNA-RNA, RNA-protein and protein-protein interactions, all contribute to the formation of the granule (Figure 5). Notably, the size of the formed RNP granule is dependent on the number of accumulated transcripts and their associated RBPs. In fact, smaller RNP granules can coalesce together to form a larger condensed RNP granule. Both internal and external stimuli that affect cellular homeostasis regulate RNP granule formation (Zarnack et al., 2020).

The role of RNP granules is widely studied in many different physiological processes (such as in growth, metabolism, development and in stress responses) as

well as in disease conditions (such as neurodegeneration, viral infections and cancer). There are different kinds of RNP granules existing in eukaryotic cells and each of them have their unique composition. They can be categorized on the basis of their localization or on the basis of their tissue specific occurrence (e.g in somatic cells versus germ cells). RNP granules mostly participate in the posttranscriptional regulation of RNAs.

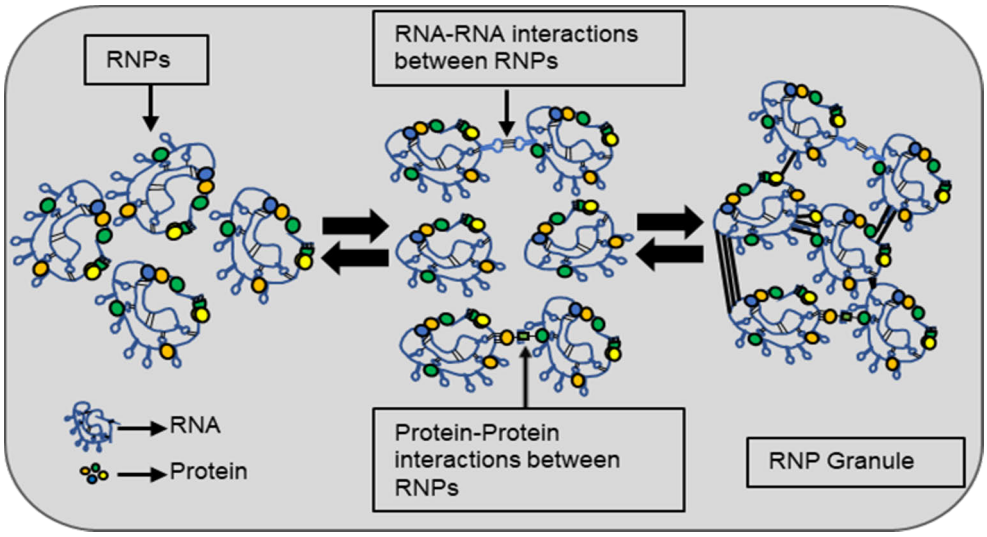

Figure 5. Assembly of Ribonucleoproteins (RNPs) to form larger Ribonucleoprotein granules by different RNA-RNA interactions and Protein-Protein interactions. They contain different species of RNAs and RNA-binding proteins.

The RNP granules can either be located in the nucleus or cytoplasm. Nuclear RNP granules include: nucleoli, polycomb, cajal bodies, and paraspeckles. Cytoplasmic RNP granules include: processing bodies, stress granules, the chromatoid body (CB) and the intermitochondrial cement (IMC). While the CB and IMC are germline specific granules, the other RNP granules also appear in somatic cells. In the following, I will discuss the cytoplasmic RNP granules.

2.2.1 Processing bodies

The processing bodies (PBs) are described as non-membranous organelles, appearing as droplets, in the cytoplasm of somatic cells in eukaryotic organisms and they serve as a platform for RNA metabolism (Standart & Weil, 2018). The PBs resemble other RNP granules due to their appearance and involvement in RNA-protein interactions, however they have unique molecular composition and functions which are currently unfolding. By the use of advanced immunofluorescence-

activated particle sorting (FAPS), the full molecular characterization of the PB composition have been analyzed and this has shown that they are highly enriched with many repressed mRNAs, microRNAs (miRNAs) and considerable amounts of RBPs functioning in different posttranscriptional RNA regulatory pathways (particularly in translational repression and RNA decay pathways and nonsense mediated decay) (Hubstenberger et al., 2017). The PBs also contain translation initiation factors, such as eiF4E and as opposed to earlier studies which suggested PBs as the potential site for mRNA degradation due to the accumulation of mRNA decaying enzymes (Parker & Sheth, 2007), there is still no clear evidence of the degradation of repressed mRNA targets (Hubstenberger et al., 2017). Thus, whether PBs function as a storage of untranslated mRNAs for later translation or for decay is not completely understood (Standart & Weil, 2018).

The PBs are very dynamic cytoplasmic structures which are assembled and disassembled during normal physiological processes. Physiological conditions, such as stress (for example glucose starvation) can induce the accumulation of translationally repressed mRNAs to PBs and the size is dependent on the magnitude of the exposure (Riggs et al., 2020). The formation of PBs is very crucial for cellular homeostasis and development. For example, PBs contribute to stem cell plasticity and cellular differentiation mechanisms (Riggs et al., 2020). In addition, conditions such as exposure to viral infection can promote PB disassembly (P. Anderson & Kedersha, 2008; L. Liu et al., 2019).

The molecular mechanism of PBs function is not well understood, some studies have suggested the involvement of RNA-interference-mediated (RNAi) RNA regulation (Rossi, 2005). The RNAi-mediated RNA regulation is crucial for gene regulation where small non coding-RNAs (micro-RNAs and siRNAs) form a complex with Argonaute proteins and associate with their targeted mRNAs through complementary base pairing. Interestingly, Argonaute proteins 1 and 2 (AGO1, AGO2 and AGO3) which are components of the RNAi pathways have been reported to localize to the PB in mammalian cells (J. Liu et al., 2005; Meister et al., 2004; Sen & Blau, 2005). These AGO proteins, especially, AGO2 has been shown to be involved in the cleavage of targets at the 3'-UTR to initiate mRNA silencing (Meister et al., 2004; Sen & Blau, 2005).

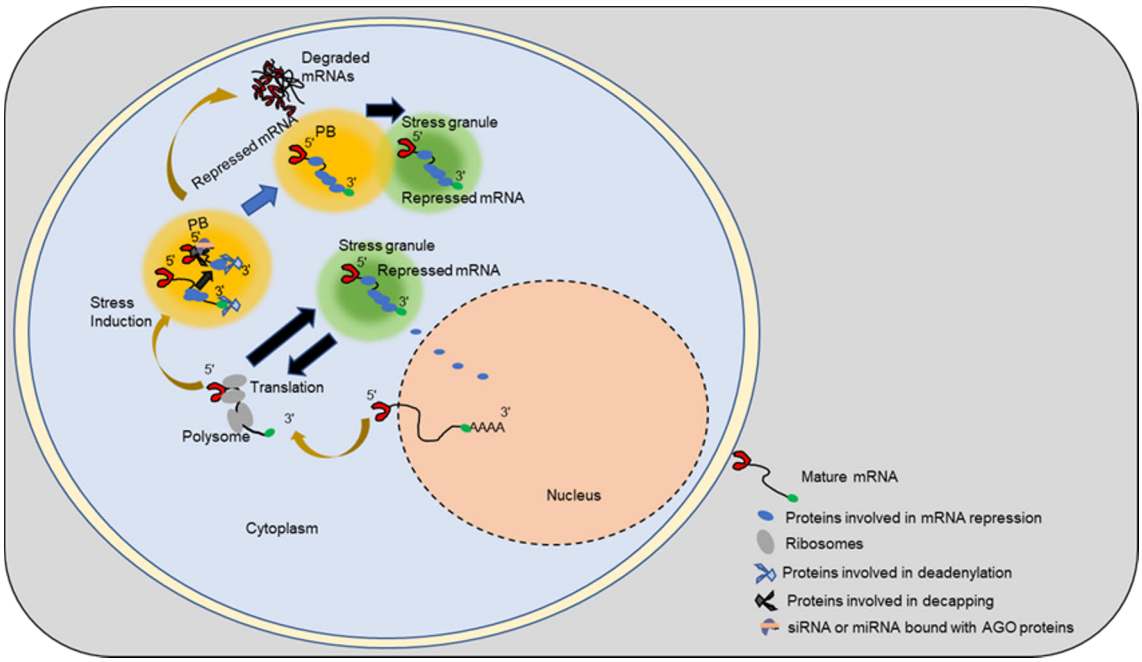

Figure 6. Assembly of PB and stress granules in the cytoplasmic compartment of a typical eukaryotic cell.

2.2.2 Stress granules

The stress granules (SGs) are membrane-less mRNP granules that are formed in response to stress condition (Protter & Parker, 2016). Similar to PBs, SGs are comprised of stalled untranslated mRNAs together with their associated RNA binding proteins that can be later translated into proteins. Under physiological condition, SGs formation is a protective mechanism of the cells in response to stress (Ivanov et al., 2019). SGs can be formed in the nucleus (nucleoli) or cytoplasm of eukaryotic cells (Mahboubi & Stochaj, 2014). While SG formation is always induced by stress, the PBs can be assembled in the absence of stress. For example, factors that promote mRNA deadenylation and dissociation from translational machinery can induce the assembly of PBs. Factors that inhibit translation affects polysome assembly, dissociating mRNAs from ribosomes and these untranslated mRNAs accumulate in the cytoplasmic SGs (Fig.6). SGs are similar to the PBs in that they both accumulate untranslated mRNAs, and sometimes they even fuse together under certain condition of stress and in this situation, they contain common proteins. Their functional relationship is not well understood.

The characterization of the molecular composition of the SGs showed that several RBPs are involved in the formation of SGs. The primary players involved in initiating the formation of cytoplasmic SGs include RBPs T-cell intracellular antigen

(TIA-1), TIA-1-related (TIAR) and the poly-A-binding protein 1 (PABP1) proteins (N. L. Kedersha et al., 1999). The stress condition triggers the phosphorylation of EIF2α which in turn facilitates the recruitment of RBPs (N. Kedersha et al., 2002). The ultimate goal of these RBPs is to enhance the recruitment of untranslated mRNAs from polysomes to the SGs. Therefore, any factor that inhibits the phosphorylation of EIF2α or these RBPs can directly affect the assembly of SGs. Another component of the SGs is a phosphorylated-dependent endoribonuclease Ras-GTPase-Activated protein, G3BP, which has been shown to contribute to the assembly of SGs (Tourrière et al., 2003). In addition, the assembly of SGs is closely associated with ATP-dependent protein-RNA complexes. For example, helicases (such as Dhh1) which are dependent on ATP and have been shown to enhance the targeting of mRNAs for decay during stress conditions (Carroll et al., 2011). Once the stress condition is over, the repressed untranslated mRNAs can be recycled back into polysomes where they are translated into functioning proteins. This reversible process is a cell adaptation mechanism to re-establish homeostasis under physiological condition. Therefore, unlike in PBs, untranslated mRNAs in SGs are not degraded but stored and recycled for later translation.

There are other emerging roles for SGs under chronic stress, such as in cancer, virus infections, ischemia and in neurodegenerative diseases. Given the circumstance that cancer cells are constantly being surrounded by low nutrient and a hypoxic microenvironment, there is high demand for oxygen and nutrients for their growth and survival. These conditions trigger the formation of SGs factors and SGs assembly and many of the proteins required for the formation of SGs have indeed been shown to be over expressed in different cancers. For example, the SG component G3BP1 which has a role in RNA stabilization has been shown to be expressed in human sarcomas upon activation by Y-box binding protein (YB-1), and it contributes to tumor progression and invasion in the lungs (Somasekharan et al., 2015). G3BP1 has also been shown to be widely expressed in primary renal cell carcinoma and its inhibition caused significant delay in cancer cell proliferation and metastasis (Y. Wang et al., 2018). Poly-A-binding protein 1 (PABP1) which is one of the main components of SGs functioning in the regulation of mRNA translation and stability has also been shown to be widely expressed in gastric cancer and suggested to be an oncogene (J. Zhu et al., 2015). The deletion of PABP1 causes the apoptosis of cancer cells (J. Zhu et al., 2015). The expression of these SGs-factors in different malignancies appears to contribute to the survival mechanism utilized by cancer cells and inhibition of the factors can promote cancer cell apoptosis. Interestingly, some of these SG-assembly factors are currently being studied as promising therapeutic targets in cancer drug development (Zhou et al., 2023).

The SGs have also been implicated in neurodegenerative disease. In this condition, there is sustained or prolonged formation of stress granules, which

prevents transportation of stalled mRNAs back for translation or degradation pathways. This may be caused by additive effect of exposures to different factors ranging from genetic mutations, epigenetic and environmental factors (Irwin et al., 2015).

2.2.3 Germline-specific RNP granules (germ granules)

The germline-specific RNP granules are membrane-less organelles that are highly enriched with different species of RNAs and RNA-binding proteins coalesced in the cytoplasm of differentiating germ cells. They are well conserved in many organisms and they contribute to germline specification and cell differentiation. In fact, in some organisms, such as Drosophila, *C. elegans*, Xenopus or Zebrafish the germ granules are transmitted from the oocyte to the embryo where they determine the germ cell fate during embryonic development (Evans et al., 2014; Hay et al., 1988; Tada et al., 2012; C Wang & Lehmann, 1991). In mammalian species, such as mice, the germ granules appear after germ cell specification and they contribute to the differentiation of germ cells (Meikar, 2013; Yokota, 2008).

Germ granules occur in the germline of male and female sexually reproducing animals. Unlike other known RNP granules whose formation is triggered by stress, the main reason for the formation of germ granules remains elusive. However, what is known is that they contains considerable amounts of RNAs and proteins, suggesting that they play an important role in different RNA regulatory processes in the germ cells (Voronina et al., 2011).

There are different names attributed to germ granules across different species of sexually active metazoans. Balbiani bodies occur in *Xenopus laevis,* P (posterior) granules in *Caenorhabditis elegans,* polar granules in *Drosophila melanogaster* (Strome & Wood, 1982). The two best characterized germ granules in mammalian species are the intermitochondrial cement (IMC) and the chromatoid body (CB) in differentiating male germ cells.

2.2.3.1 Intermitochondrial cement (IMC)

The IMC is a mitochondrial-associated germ granule (Fig. 7) in the cytoplasmic compartment of both fetal and postnatal germ cells, being particularly prominent in mid-to-late pachytene spermatocytes in the adult mouse testis (Eddy, 1974; Romrell et al., 1976). Under electron microscopy, the IMC appears as a dense material that glues mitochondrial clusters together, hence the name intermitochondrial cement (Eddy, 1974; Russell & Frank, 1978).

The germline-specific protein MVH/DDX4, is the best characterized marker of germ granules, and it also localizes to the IMC (Y. Fujiwara et al., 1994). DDX4

belongs to the DEAD box RNA helicase family of proteins which are very important in RNA metabolism (Bourgeois et al., 2016). The deletion of *Mvh* in mice caused arrest of germ cell differentiation at the premeiotic stage leading to sterility in males (Satomi S Tanaka et al., 2000). Another IMC-localized RNA-helicase is the MOV10L1, which localizes in the outer mitochondrial membrane in spermatocytes and is involved in piRNA biogenesis (Vourekas et al., 2015; Zheng et al., 2010). Tudor domain containing proteins such as Tudor domain containing 1 (TDRD1) are also found in the IMC as well as in other types of germ granules (Chuma et al., 2006). The deletion of *Tdrd1* results in a very similar phenotype as in the deletion of *Mvh*, where there is failure in IMC formation and progression of male germ cell differentiation (Chuma et al., 2006). The Tudor protein domains function as molecular adaptors or scaffolds as they bind other RNA-binding proteins responsible for RNA metabolism, RNA splicing as well as piRNA pathway (Pek et al., 2012; Ponting, 1997). Many of the proteins that forms complex with TDRD1 in the spermatocytes, such as mitoPLD, GASZ, TDRKH, GPAT2, TDRD6, MAEL, are mitochondrial proteins and many of their functions are associated with piRNA production (Akpınar et al., 2017; Saxe et al., 2013; Y Shiromoto et al., 2013; Yusuke Shiromoto et al., 2013; Watanabe et al., 2011; Xiol et al., 2012; J. Zhang et al., 2016). It has been suggested that the IMC serves as the main site for the production of piRNAs in pachytene spermatocytes, but a systematic characterization of the IMC molecular composition was still lacking prior the current study.

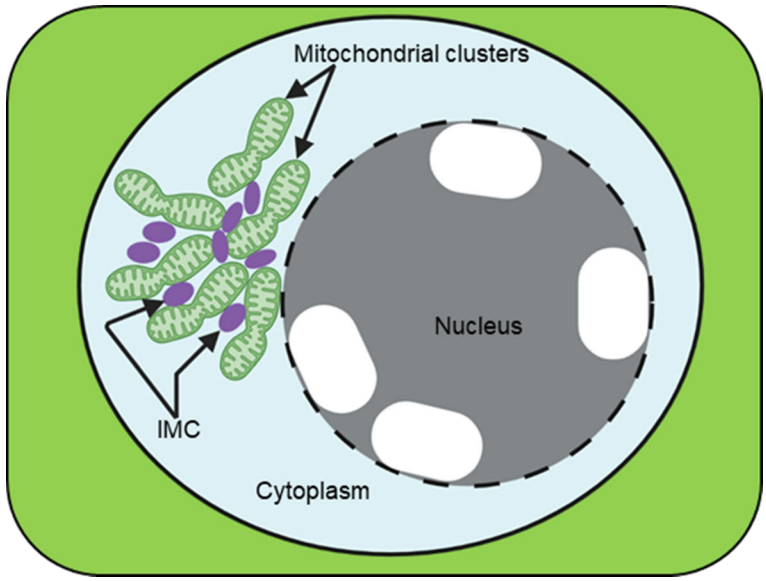

Figure 7. Intermitochondrial cement (IMC) (purple) is located among mitochondrial clusters (green) in the cytoplasm of germ cells, and appears particularly prominent in the early, mid- and late pachytene spermatocytes.

2.2.3.2 Chromatoid body (CB)

The chromatoid body (CB) is formed a little later than IMC during spermatogenesis, and it condenses into its final form in the cytoplasm of haploid round spermatids. Under electron microscopy, it appears as a single, unusually large and electron dense granule, about 0.5–1 μm in diameter (Meikar et al., 2010a; Parvinen, 2005).

The CB precursors (Fig. 8A) are formed in the late pachytene spermatocytes where they co-exist briefly with the IMC (Lehtiniemi & Kotaja, 2017; Olotu et al., 2023). After meiosis, the IMC disappears and the CB-precursors aggregate into a single, large granule in the perinuclear region of haploid round spermatids (one granule per nuclei) (Fig. 8B). The reason for prominence of CBs in haploid round spermatids is still not well understood, but it corresponds to the period of a highly active and widespread transcription of the genome during the transition of meiotic spermatocytes into haploid round spermatids. The CB is often found closely associated with nuclear pores, and it has been suggested to actively communicate with the nucleus via the nuclear pores to receive RNAs and RNA-binding proteins (M. Da Ros et al., 2017a; Matteo Da Ros et al., 2015). The CB exists throughout all the steps of round spermatid differentiation, but starts decreasing in size in late round spermatids (Onohara et al., 2010). During this time, the CB migrates to the base of the flagellum, and in early elongating spermatids it forms a ring like structure around the axoneme that migrates along the flagellum during the mitochondrial sheath formation, and finally disappears (Fawcett et al., 1970; Shang et al., 2010).

The CB has been suggested to have a central role in RNA regulation (Kotaja & Sassone-Corsi, 2007; Parvinen, 2005), which is supported by full molecular characterization of CBs isolated from mice testes (Meikar et al., 2014). The CB was shown to be highly enriched with different species of RNAs, including piRNAs, long non-coding RNAs, non-annotated intergenic transcripts and mRNAs. The CB was also packed with different RNA-binding proteins, the most abundant being the DEAD box helicases DDX4/MVH and DDX25/GRTH, the PIWI proteins PIWIL1/MIWI and PIWIL2/MILI, poly-A-binding protein PABP and Tudor domain containing proteins TDRD6 and TDRD7, HSP72 (Meikar et al., 2010a, 2011a, 2014). Many of the CB proteins have been implicated in the functioning of the piRNA pathway (Girard et al., 2006; Meikar et al., 2010b, 2014). Apart from the proteins involved in piRNA pathway, the CB also accumulates proteins involved in non-sense-mediated RNA decay (NMD) pathway, such as the NMD endonuclease SMG6 (Lehtiniemi et al., 2022a).

Another characteristic feature of the CB is its close association with cellular endomembrane system exchanging and transporting materials that are required for acrosome biogenesis in haploid round spermatids (Matteo Da Ros et al., 2015). Furthermore, one of the components of the CB is FYCO1, a motor protein has been

shown to interact with proteins functioning in autophagy-lysosomal pathway, hence the CB also function in FYCO1-dependent autophagy pathway (M. Da Ros et al., 2017b).

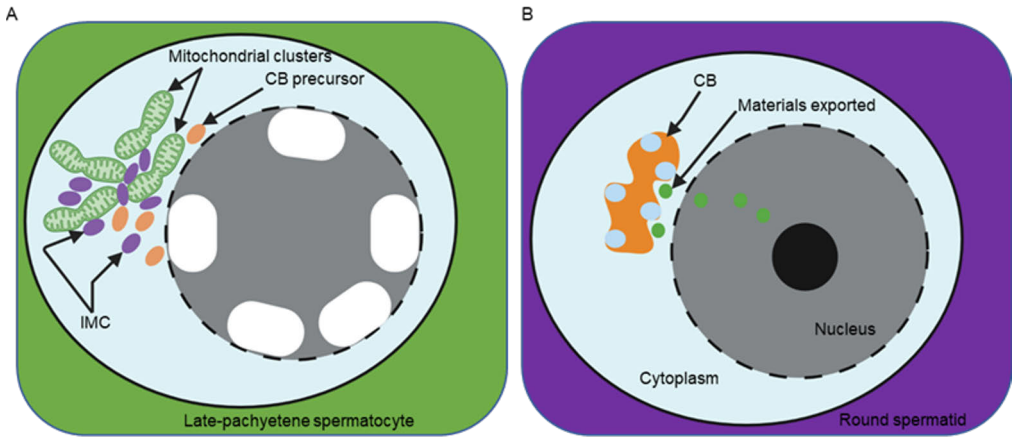

Figure 8. (**A**) Chromatoid body (CB) precursors (orange) co-exist with the IMC (purple) in the cytoplasm of late pachytene spermatocytes. (**B**) The CB appears as a large single granule (orange) in the cytoplasm of round spermatid. Materials (green) such as different RNA species are exported to the CB through the nuclear pore. The CB is adjacent to the nuclear pore.

2.3 RNA regulation in cancer

Gene mutations and epigenetic changes in the genes that control normal physiological processes have important roles in the etiology of cancer, and alterations in the function of tumor suppressors or proto-oncogenes can initiate the formation of cancer. In addition, cancer cells have an unstable genome which contributes to intra-tumor heterogeneity and the sustenance of their growth and development (S.-Y. Cho et al., 2019). In addition, alteration in RNA regulatory mechanism connected to RNA splicing, modification, transport, localization, stabilization, degradation and translation have also been implicated in cancer initiation and progression (Cadieux et al., 2006; N.-Y. Cho et al., 2007; A P Feinberg & Vogelstein, 1983; Andrew P Feinberg & Vogelstein, 1983; Iino et al., 2020; Lujan et al., 2018).

The RBPs are highly conserved and changes in their function can cause imbalance in cellular transcriptome that may support cancer progression (Neelamraju et al., 2015; Rappaport et al., 2013). Aberrant expression of different RBP proteins is widely studied in different somatic malignancies (Kechavarzi & Janga, 2014; Lilja, 1985; Pereira et al., 2017). Wei Li *et al,* revealed pronounced dysregulation of RBPs (about 300) in lung squamous cell carcinoma (W. Li et al.,

2020). Many of these aberrantly expressed RBPs were shown to be associated with different RNA regulatory mechanisms, and higher expression of some of the RBPs were shown to correlated with poor survival, therefore potentially useful as prognostic markers (W. Li et al., 2020). Some RBPs involved in RNA splicing, such as heterogenous nuclear ribonucleoproteins and serine/arginine-rich proteins, have been shown to be widely expressed in different cancers and to promote cancer cell proliferation and metastasis (Lu et al., 2022). Some other RBPs, such as poly-A-binding proteins which are involved in mRNA stability and translation have also been shown to be widely expressed in ovarian cancers promoting cancer metastasis (Feng et al., 2021).

2.3.1 Introduction to cancer

Cells in multicellular organisms are in constant interaction with one another for a common interest of growth, sustainability and development. They achieve this goal upon stimulations by different extracellular signals, and each of them has the responsibility of cooperating and performing their accepted roles (such as resting, growing, dividing, differentiating or dying). Therefore, any disturbance in the extracellular signals or irresponsible decisions of the cells will jeopardize the common goal of the multicellular society.

Cancer occurs when healthy cells acquire properties such as genetic and epigenetic mutation, leading to their ability to evade apoptosis, proliferate uncontrollably creating descendant cells, sustain their angiogenesis, ability to acquire self-sufficient growth signals, invade and create their own territories in the surrounding tissues and organs (Hanahan & Weinberg, 2011) (Fig. 9). Sometimes, the stimulus can induce cells to proliferate abnormally without becoming invasive, this kind of growth is neoplastic and the formed tumor is known as benign. Malignant cells proliferate out of control and they gain the propensity to invade surrounding tissues through a process of metastasis. Cancers are categorized depending on the kind of tissue or cell type they originated from. Cancer originating from the epithelial tissues are categorized as carcinomas and these are the most common cancers in the human population (Hinck & Näthke, 2014). Sarcomas are peculiar to the connective tissue or muscles, and leukemias and lymphomas are cancers related to the blood cells.

Nowadays, there are different kinds of therapies, such as chemotherapies of radiotherapies that are successful in the treatment of cancer especially at early detection of the disease and neoplastic cells or benign tissues can be surgically removed. However, when the tumor cells have become malignant in the late stages, the majority of cancer patients die due to metastasis into distant organs. Cancer remains one of the leading causes of death especially in advanced countries, such as the United States of America (Voronina et al., 2011).

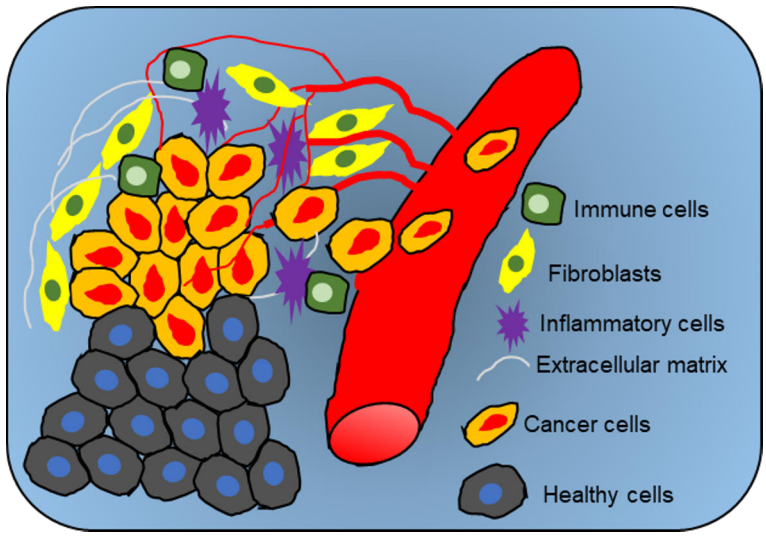

Figure 9. Healthy cells can be transformed to cancer cells, which in turn proliferate rapidly and progressively infiltrate into blood vessels to be transported in the blood to invade distant organs. The cancer cells, during their formation are able to create a niche where inflammatory cells, immune cells, fibroblasts, other growth factors and angiogenesis to further enhance their growth and survival.

2.3.1.1 Prostate cancer

Prostate cancer is the second leading cause of malignant tumor-related deaths after lung cancer in the United States (Siegel et al., 2022). The prostate is an exocrine gland whose secretion is slightly alkaline and contributes 30% to the semen and also functions to prevent urinary tract infections. The main cell-types in a normal prostate includes: basal cells that is at the prostate basement membrane, luminal cells and neuroendocrine cells which are secretory cells of the prostate gland (Fig. 10). The prostatic cells are surrounded by fibroblast, smooth muscles, macrophage and T-cells. Anatomically, the human prostate gland is located around the urethra proximal to the bladder and it is divided into four zones which are different on the basis of their embryologic origins and they include the anterior zone, central zone that originates from the wolffian duct, transition zone and the peripheral zone that originate from the urogenital sinus during embryogenesis (Ittmann, 2018; Mcneal, 1972; Selman, 2011). The majority of the cases of prostate cancer is believed to arise from the peripheral zone because it constitutes the largest portion (70%) of the prostate gland and this peripheral zone is similar to the dorsolateral lobe of the mouse prostate (Berquin et al., 2005). The transition zone is fibromuscular and constitute 5% of the entire prostate and has been notable for benign prostate hyperplasia (BPH) and cancer (25%) in older men (Ittmann, 2018; Mcneal, 1972).

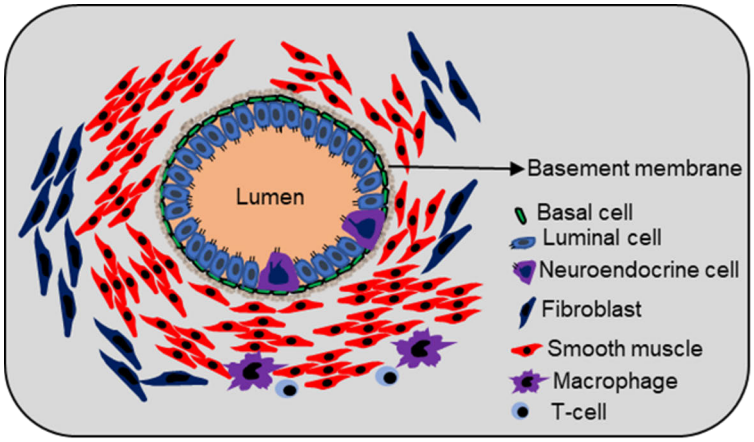

Figure 10. The main cell types in the exocrine prostate gland. The human prostate epithelium is lined by the acini consisting of basal cells, luminal cells and sparsely occuring neuroendocrine cells. All these cells secret their content into the prostate lumen. The prostate epithelium is surrounded by the stroma which consist of the smooth muscles, fibroblasts, macrophages and the T-cell.

Most of the prostate cancer cases are adenocarcinoma because they are derived from the prostate epithelial cells (Hartmann & Friess, 2017). Numerous factors such as DNA hypermethylation, which may result into silencing of several genes, such as tumor suppressor genes (PTEN, CDKN1B and TP53), and activation of other oncogenes (MYC and BCL2) are implicated in prostatic adenocarcinoma (Hartmann & Friess, 2017).

2.3.1.1.1 Prostate cancer initiation and progression

Concurrently, prostate cancer can be classified into categories on the basis of the initiation and progression of the disease: adenocarcinoma androgen-dependent, adenocarcinoma androgen-independent or castration-resistant prostate cancer (Bostwick & Brawer, 1987; Brawer, 2005; H. L. Kim & Yang, 2002; McNeal & Bostwick, 1986; Taplin et al., 1995).

Prostatic intraepithelial neoplasia (PIN) lesion is a precursor state to cancer, where some prostate cells have begun to look and behave abnormally. Pin lesion occur due

to abnormal proliferation of the prostate glandular epithelial cells, mostly attributed to the cells of the peripheral zone (peculiar to the acinar and ductal lining of the epithelium) (Bostwick & Brawer, 1987; Brawer, 2005; H. L. Kim & Yang, 2002; McNeal & Bostwick, 1986; Moch et al., 2016). Not all PIN lesions lead to cancer, the hyperproliferation of these glandular cells in advanced form of PIN is referred to as high-grade PIN (HGPIN) and it has been described as the main

precursor to prostate adenocarcinoma (Bostwick & Brawer, 1987; De Marzo et al., 2007, 2016; Moch et al., 2016). Prostate cancer is a disease of the elderly men; however, the PIN lesion can already be detected in the third decade of life in adult males either by biopsy and observation of the prostate histology or monitoring the PSA level (value of 4 ng/mL was considered normal) in the serum sample (Brawer, 1992; Diamandis, 1998; H. L. Kim & Yang, 2002; Sardana et al., 2008).

Over the years, the level of PSA detection has been a gold standard, used as a biomarker of prostate cancer (Merriel et al., 2022; Thompson & Ankerst, 2007), there are controversial arguments surrounding the relationship between emergence of PIN lesion, prostatic adenocarcinoma and the serum concentration of the PSA. Stamey *et al,* observed that though PSA is a very sensitive marker as it is detected in different stages of prostate cancer and could be used as a biomarker but a high level is also detected in benign prostatic hyperplastic cells suggesting that it may not necessarily be specific to cancer (Stamey et al., 1987). Brawer *et al,* further showed that the level of the PSA is not sensitive enough to classify the prostate cancer into stages (Brawer et al., 1989). Alexander *et al,* also reported that HGPIN-associated cancer did not contribute to the serum concentration of PSA (E. E. Alexander et al., 1996), and at some point, even patients with lower PSA value than 4 ng/mL were shown to have prostate cancer (Thompson et al., 2004). Given all these arguments, the continued use of serum PSA concentration as a biomarker for prostate cancer is controversial due to its lack of specificity, and several studies are now focusing on investigating novel biomarkers that are more specific for prostate cancer detection (Jethwani et al., 2022).

The androgen-dependent adenocarcinoma is another category of prostate cancer which requires androgen signaling for prostate cancer progression (Huggins & Hodges, 2002). Most of the initial stages of prostatic adenocarcinoma are androgen dependent so they are also called androgen-sensitive prostate cancer. The withdrawal of androgens could transiently affect the growth of prostate cancer. However, the main driver is the AR and not just the circulating androgens. The role of AR in the progression of prostate cancer is widely established. The AR is highly expressed by the secretory luminal cells, and expressed at a lower level by the basal cells as well as in the stroma cells. Under normal physiological condition, upon stimulation by androgens, AR receptors in the surrounding stroma effect the secretion of peptide hormones which are growth factors such as the keratinocytes growth factor, the insulin-like growth factor and the epidermal growth factors; thus initiating a downstream effect on the epithelial cells. These secreted growth factors in turn induce the production of other transcription factors that promote the differentiation of basal epithelial cells and columnar cells. The AR present in the epithelial cells contributes to the production of the secretory proteins and PSA (Cunha & Young, 1991; Murashima et al., 2015). The stroma AR receptor is very crucial to the

development of prostate gland (Cunha et al., 1992; Cunha & Chung, 1981; Lai et al., 2012; Marker et al., 2003). The AR has been one of the main targets in the development of drugs for cancer treatment (Gao, 2010). One of the current treatments for prostate cancer is surgical removal of the prostate gland (radical prostatectomy) (Kesch et al., 2021) or androgen deprivation therapy which generally leads to a decrease in PSA levels, complete abrogation of androgenic effect and apoptosis of prostate cancer cells (Denmeade & Isaacs, 1996; Kyprianou & Isaacs, 1988). In fact, both surgical removals of the prostate (bilateral orchiectomy) and drug interventions (hormone therapy) are considered to be androgen deprivation therapy (Choi et al., 2022).

The androgen-independent prostate cancers (AIPC) are the category of prostate cancer cells that re-occur and become resistant after androgen deprivation therapy (ADT) or surgery (Feldman & Feldman, 2001). It is also known as androgen-refractory prostate cancer which is no longer dependent on androgens for development (Debes & Tindall, 2004; Feldman & Feldman, 2001). The molecular mechanism of the formation of AIPC is very complex and not well understood but several studies are in agreement that these AIPC-cells continue to respond to AR-signaling despite androgen withdrawal in ADT. Factors that can enhance AR signaling include AR gene amplification which is one of the most common AR alteration in classically treated PCa, mutation of the AR gene such that there is continuous stimulation by other steroid hormones, over expression of the AR to amplify its function, increased secretion of androgens from other mechanism which in turn enhance AR responses, and activation of other AR-co factors enhancing its signaling as well as influence of cytokines and growth factors. Other investigations also reveal that some of these AIPC-cells show the phenotypic features of neuroendocrine cells of the prostate, hence have been given the name neuroendocrine-like cells (NE-like). Even though, NE cells in the normal prostate are not actively proliferating as they sparsely occur, a high proliferation of the NE cells has been observed in prostatic adenocarcinoma than in a normal prostate. Moreover, NE cells of the normal prostate gland do not express an androgen receptor and they do not secret PSA, but NE-like cells in AIPC are resistant to ADT. Some *in vitro* studies have also demonstrated that prostate tumor cells (LNCaP) could differentiate reversibly into NE-like cells upon treatment with cAMP-inducing agents (M. E. Cox et al., 1999). NE-related prostate cancer can be categorized as small cell neuroendocrine prostate cancer and they are very aggressive in metastasis causing death among prostate cancer patients (Aggarwal et al., 2018; Beltran et al., 2012).

To critically study the molecular mechanism of initiation, progression and metastasis of prostate cancer, researchers have initiated the use of different prostate cancer cell lines for both *in vitro* and *in vivo* cancer models. These studies have

reshaped the insight into understanding the complexity of the disease and identification of potential targets for drug development. Different kinds of prostate cancer cell lines have been used, and they have different features. To mimic the tumor environment, these cancer cell lines are being xenografted into the mouse subcutaneous skin or orthotopic and monitored over a period of weeks. In recent years, there have been proposals to reduce the use of laboratory animals in cancer studies. Another alternative model for tumor studies is the use of 3D-cancer model *in vitro*, an environment that promotes cancer cell differentiation and metastasis (Kimlin et al., 2013).

Three of the most commonly used prostate cancer cell lines are: PC3, LNCaP and VCaP cells. The LNCaP cells are characterized by expression of AR and they respond to androgen stimulation. The LNCaP cells also express PSA and cytokeratins, which are characteristics of adenocarcinoma (Horoszewicz et al., 1980, 1983). The VCaP cells were derived from the lumbar vertebral metastasis of the prostate cancer. The VCaP cells widely expreses PSA, cytokeratin-18, P53, prostatic acid phosphatase and the AR, which are characteristic features of prostatic adenocarcinoma (Korenchuk et al., 2001). The PC3 cells were originally derived from bone metastasis and lymph nodes (Kaighn et al., 1979). The PC3 cell are AR-negative and they express cytokeratins with characteristic feature of the small cell neuroendocrine prostate cancer, being aggressive (van Bokhoven et al., 2003).

2.3.1.2 Head and Neck squamous cell carcinoma

Head and Neck squamous cell carcinoma (HNSCC) is a malignant cancer of squamous epithelial origin and there are different primary tumor sites of detection, which include: the oral cavity, oropharynx, hypopharynx, larynx and the nasal cavity (Chow, 2020; Routila et al., 2021). Epidemiological studies have shown that the predominant risk factors are alcohol abuse and smoking (Chaturvedi et al., 2011; Denissoff et al., 2022; Gillison et al., 2008; Herrero et al., 2003). Other risk factors are infections caused by human papillomavirus (such as HPV-16) (Chaturvedi et al., 2011; Gillison et al., 2008; Herrero et al., 2003; Syrjänen et al., 1983).

The genetic heterogeneity of cancer cells and the different location of the categorized primary site of HNSCC make it difficult to understand the nature of the disease and even more challenging to find appropriate treatment measures for the disease (Alsahafi et al., 2019; Ha et al., 2009). Moreover, depending on the cause, some HNSCC (especially HPV-associated oropharyngeal cancer) have been successfully treated with chemoradiotherapy while some other treatment measures have not been successful as the patients eventually died (O'Shea et al., 2015). It is important to note that there are currently no clinically useful biomarkers for the early detection of HNSCC (Chow, 2020; K. Y. Kim et al., 2014).

Unlike the prostate cancer cells, the step-by-step initiation, progression of HNSCC and the characterization of the cells has not been established. The molecular mechanism of HNSCC is beginning to emerge. Many of the affected genes in HNSCC have been shown to be mainly associated with tumor suppressor regulatory pathways which work in concert with other pathways, cell cycle regulation, DNA damage pathways, WNT-β-catenin signaling and immune related pathways (Alsahafi et al., 2019; Ha et al., 2009; Lawrence et al., 2015; Leemans et al., 2018). Loss-of-function mutation of tumor suppressor genes, *TP53, NOTCH1-3* are widely detected in HNSCC (especially those associated with smoking) (Lawrence et al., 2015). In addition, transforming growth factor-beta (TGF-beta), when activated, serves as a tumor suppressor at the initial stages of the HNSCC has been shown to promote tumor progression and metastasis when repressed (Prime et al., 2004). Programmed death protein 1 (PD-1) which normally prevent autoimmune reactions upon binding to its ligand PD-L1 and PD-L2, has been reported to be widely expressed in HNSCC (especially the one caused by HPV-1). It has been regarded as an immune check point-inhibitor in cancer as it prevent T-cells from destroying cancer cells, thereby promoting cancer progression (Mcdermott & Atkins, 2013; Pardoll, 2012).

In order to better understand the molecular mechanism of HNSCC initiation, progression and classification, numerous cancer cell lines have been established from primary tumors of patients samples to be used for both *in vitro* and *in vivo* models (Lin et al., 2007). UT-SCC-14 cells, which was originally derived from the oral cancer is HPV-negative and it has been used for xenograft in athymic nude mice to characterize the global gene expression changes at different stages of tumor growth (Wilson et al., 2016).

2.3.2 Cancer germline antigens

Cancer germline antigens can be described as germline-specific proteins that are aberrantly expressed in different somatic malignancies. Many years ago, Van der *et al,* detected the expression of a specific antigen MZ2-E recognized by cytolytic T lymphocytes in human melanoma cell line (MZ2-MEL) (P van der Bruggen et al., 1991). The antigen was renamed as melanoma antigen-1 (MAGE-1) and was also detected in other melanoma cell lines but not in normal human tissues (P van der Bruggen et al., 1991). Other families of MAGE were identified and shown to be expressed by the T lymphocytes in all cancer tissue samples and in the testis but not expressed in other healthy tissue samples (Pierre Van Der Bruggen et al., 1994; C De Smet et al., 1994). It was intriguing that these tumor-associated antigens were specifically expressed by cancer specific immune cells and the testis. Notably, the

testes, just like the cancer cells, also have their own specialized immune response which contributes to testicular stem cell homeostasis (Kitadate et al., 2019).

The group of tumor-associated antigens that are also expressed in the testes were named cancer-testis antigens (CTAs) (Y. T. Chen et al., 1997; Old & Chen, 1998). Some of these CTAs are also detected in the placenta (Jungbluth et al., 2007; W. A. J. Silva et al., 2007). Some CTAs are also referred to as cancer germline antigens (CGAs), because they are specifically expressed in the germline, not in somatic tissue of the testis (Vodolazhsky et al., 2018). In the somatic cells, the germline genes are repressed due to DNA methylation of the CpG dinucleotides (Al Adhami et al., 2023; Gibbs & Whitehurst, 2018). Any factor that causes hypomethylation can activate intergenic transcription, including the repetitive elements, which can cause mutations and also activates the germline genes.

Systematic analysis of the transcriptome data identified around 1000 CGAs which shows that they are more highly expressed in different human cancers than in normal tissues (Cheng Wang et al., 2016). The expression level of these CGAs have been correlated with tumor progression and several studies have suggested that they should be considered as targets for cancer treatment (Goydos et al., 2001; Kurashige et al., 2001; Patard et al., 1995; Shukla et al., 2018; van Baren et al., 1999). However, the molecular mechanism of their role in tumor formation and progression is not well understood.

In the germ cells, many of the CGAs are associated with meiosis, and many RNA binding proteins, such as PIWIL1 and TDRD3 have been reported as CGAs shown to promote tumor formation (Morettin et al., 2017; S. Shi et al., 2020). PIWIL1 and TDRD3 are important germ granule components where they contribute to piRNA-mediated RNA regulatory mechanisms, enhancing germ cell differentiation. Interestingly, a DEAD-box family member DDX4, which is the best characterized germ granule component, is also among the CGAs reported to be expressed in different cancers (Y. Chen et al., 2018a; K. H. Kim et al., 2014; Lee et al., 2018; Schudrowitz et al., 2017). Recently, DDX4 was suggested to promote the survival of small cell lung cancer cells by activating proteins related to DNA repair and inflammatory response. However, the molecular mechanisms are not clear (Noyes et al., 2023).

2.3.2.1 Comparison of cancer cells and germ cells

It is currently unclear why the expression of germline genes is often reactivated in cancer cells (Karpf, 2009; Kortleve et al., 2022; Shukla et al., 2018; Simpson et al., 2005; Van Tongelen et al., 2017). The understanding of this relationship between germ cells and cancer cells could provide a better understanding of cancer formation,

progression and classification. In this section, I will dissect the features that are similar between germ cells and cancer cells.

One of the key features of the germ cells, which distinguish them from somatic cells, is their immortality (Medvedev, 1981). To preserve their population, the germ cells are known for transmission of genetic information from one generation to the next and they also have in-built mechanisms to prevent any alteration such as invasion by transposable elements (Watanabe et al., 2015). Spermatogonial stem cells (SSCs) self-renew themselves throughout the whole adult age to provide a continuous source of sperm production. Propensity for immortality is one of the hallmarks of cancer. One main cause of for this is the ability of cancer cells to reactivate their telomerase activity which enable them sustain their proliferative signaling pathways (Hrdlicková et al., 2012).

Another important similarity between the germ cells and cancer cells is their ability to undergo epithelial mesenchymal transition (EMT) during their development. EMT is one of the essential biological processes crucial for embryonic development and is accompanied by phenotypic changes such as loss of adhesion between cells, loss of cellular polarity, then the loosened cells gain migratory and invasive potentials (D. H. Kim et al., 2017; Oda et al., 1998; Thiery et al., 2009). For the establishment of the germ cells in their niche, the primordial germ cells need to migrate from the wall of the yolk sac to the genital ridge where they become gonocytes. It has been established that the PGCs migrate in clusters such that the cells maintain cell-cell contact. While E-cadherins enhance their cell-cell contact, the other Cadherins (P- and N-cadherins) promotes their migration (Bendel-Stenzel et al., 2000; Packer et al., 1997; J.-C. Wu et al., 1993). The *C-kit* also contributes to the migration of the PGCs to the genital ridges (Matsui et al., 1990). Furthermore, the gonocytes develop into pro-spermatogonia, which is further differentiated into spermatocytes, round spermatids and elongated spermatids. The migration continues when spermatozoa are released from the epithelium of the seminiferous tubules to the lumen where mature sperm migrate to the epididymis and after ejaculation, through female reproductive track.

Another important feature peculiar to both germ cells and cancer cells is the formation of stable intercytoplasmic or intercellular bridges between sister cells after division. In the germ cells, formation of cytoplasmic bridges occurs at different times in their differentiation during development. They occur during the differentiation of A_{single}-type spermatogonia to A_{pair}-type and type B spermatogonia (C Huckins, 1978). Also, cytoplasmic bridges are formed during differentiation into haploid round spermatids. These cytoplasmic bridges enhance the sharing of materials among the germ cells in the syncytium (Ventelä et al., 2003). In the case of the cancer cells, there are emerging reports that well differentiated cancer cells, such as squamous cell carcinoma consist of cytoplasmic bridges and that poorly differentiated

squamous carcinoma cells do not have cytoplasmic bridges (Carlile & Edwards, 1986; Nguyen et al., 2016). However, the role of cytoplasmic bridges in differentiated cancer cells have not been investigated.

Both the male germ cells and cancer cells have their immunological micro-environment. The testicular germ cells are heavily protected by the blood-testis barrier (BTB) which prevents foreign toxic agents from penetrating into the luminal compartment of the seminiferous epithelium where the final differentiation of sperm takes place (Qu et al., 2020). The germ cells are surrounded by their own immune cells such as macrophages testicular dendritic cells, mast cells and T-lymphocytes which contributes to inflammatory response within the testis (Bhushan et al., 2015; DeFalco et al., 2015; Duan et al., 2011; Frungieri et al., 2002; Qu et al., 2020; Rival et al., 2006; Rosa et al., 2020; Winnall et al., 2011). Therefore, the differentiating male germ cells are among the immune-privileged cells (DeFalco et al., 2015; O'Bryan et al., 2000; S. Zhao et al., 2014).

The cancer cells like the germ cells, have the ability to build their own immune machinery around themselves, this enhances their growth and survival within their micro-environment. At the initial stages, there is production of innate immune cells, such as natural killer cells and $CD8^+$ T cells, which provides cytolytic activity against the abnormally proliferating cells (Teng et al., 2015). However, as tumor formation progresses, these tumor cells become resistant and bypass the immune checkpoint pathway and therefore utilize the property of the immune cells to enhance their advancement. The role of programmed cell death protein (PD-1) has been implicated in this resistance. For example, in HNSCC, PD-1 has been shown to bind PD-L1 ligand which is located on the immune T cell, causing inhibition of the immune cells and further destroying the cancer cells (Chow et al., 2016; Clarke et al., 2021b; Teng et al., 2015). Tumor-associated inflammation has been shown to contribute to cancer progression by creating an immunosuppressive tumor microenvironment (TME). Accumulation of released immune factors, such as cytokines, chemokines and growth factors, stimulates the activity of the PD-1, thereby enhancing the metastatic potential of the cancer cells (Clarke et al., 2021a).

2.3.2.2 piRNA pathway in cancer

Although piRNAs and PIWI proteins are predominantly expressed in the germline, there are increasing number of reports on the low-level expression of piRNA-like small non-coding RNAs in the somatic cells such as in ovarian follicular cells (Goriaux et al., 2014). However, experimental data showing whether they are functional associated with PIWI proteins is still lacking (Théron et al., 2014). Furthermore, many studies suggest the possible involvement of piRNAs in cancer

epigenetics but their molecular mechanisms is not known (Esteller, 2011; Siddiqi & Matushansky, 2012).

The PIWI proteins are also aberrantly expressed in many human tumors where they function as oncogenes. For example, PIWIL1 was shown to promote gastric cancer progression (S. Shi et al., 2020). Interestingly, the abrogation of piRNA-binding activity of the PIWIL1 did not affect the oncogenic function of PIWIL1 (S. Shi et al., 2020), suggesting that the function of PIWIL1 in cancer is independent of piRNAs. The widespread expression of PIWI proteins in other cancers, such as pancreatic adenocarcinoma, oesophageal squamous cell carcinoma, and genitourinary cancers have been reported (Grochola et al., 2008; Hanusek et al., 2022; He et al., 2009; J. J. Liu et al., 2010), but the involvement of piRNAs in the function of PIWI proteins in these cancers has remained unclear. Overall, these studies suggest that both PIWI proteins and piRNAs contributes to cancer progression, but PIWI proteins can also function independently of piRNAs in cancer.

The expression of PIWI proteins and piRNAs in cancer makes them potential cancer biomarkers (Al-Janabi et al., 2014; Busch et al., 2015; Hanusek et al., 2022; Y. Li et al., 2015; Zuo et al., 2019). For example, PIWIL2 is expressed at different stages of breast cancer and has been suggested as a novel biomarker in breast cancer progression (J. J. Liu et al., 2010).

3 Aims and Objectives

The aim of this study was to clarify germline-specific RNA regulatory mechanisms in order to gain novel information about the factors required for normal spermatogenesis and male fertility. Furthermore, the aim was to understand the functional similarities of germ cells and cancer cells and to study the involvement of germline-specific RNA regulatory mechanisms in cancer progression.

The specific objectives included:

1. To reveal the protein composition of the IMC to be able to understand the functional differences between IMC and the CB
2. To characterize the expression of the two novel germ granule components, EXD1 and EXD2, during spermatogenesis, and to study their involvement in piRNA biogenesis
3. To investigate the expression of the germ granule protein DDX4 in cancer cells, and to study its role in cancer progression.

4 Materials and Methods

4.1 Cell culture (Paper I & III)

Cell lines used in this project include: HeLa, PC3 and UT-SCC-14 cells (American Type Culture Collection). All cell lines were maintained using Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco Life Technology) without phenol red and supplemented with 10% Fetal Bovine Serum (FBS; PromoCell), penicillin/streptomycin (Cat code: 15140-122, Gibco Life Technology) 37 °C in a humidified 5% CO_2 incubator.

4.1.1 Generation of *DDX4*-null cells (Paper III)

To study the function of *DDX4* in cancer cells, *DDX4* was deleted in the prostate cancer cell line, PC3, and head and neck squamous cell carcinoma cell line UT-SCC-14 cells using CRISPR/Cas9 technology. We targeted different loci of exon 11 of *DDX4* by designing two guide-RNAs (gRNAs) (**see construct in Table 1 of paper III**) with a freeware package CRISPOR (Concordet & Haeussler, 2018) (http://crispor.tefor.net). Both gRNAs were assembled *in silico* into a single functional unit and then purchased as a double-stranded DNA gene block (IDT, USA). The gene block was ligated into pSpCas9(BB)-2A-Puro plasmid (Ran et al., 2013) and PX45_V2.0 (Cat. 62988, Adgene repository) with *T7*ligase and *BbsI* (from New England Labs) (Cong & Zhang, 2015). The ligation products were transformed into chemically-competent 10β Escherichia coli (NEB), plated on LB-agar plates treated with 100 µg/ml of ampicillin and incubated overnight at +37ºC. Bacterial colonies formed on the LB-agar plate were selected and screened by genomic PCR (Paramonov et al., 2018) using primers #1–2 (**see construct in Table 2 of paper III**). Selected bacteria clones, exhibiting PCR products of the expected size were run on agarose gels, were then validated with Sanger sequencing (Macrogen Europe, the Netherlands) and used for targeting plasmid isolation (NucleoBond Xtra Maxi Plus; Macherey-Nagel). *DDX4*-targeting plasmid was transfected into PC3 cells and UT-SCC-14 cells (see below).

4.1.2 Transfection (Paper I & III)

To study the role of EXD2 and EXD1 *in vitro* (Paper I), HeLa cells (~ 300,000 cells) were seeded in each well of 6-well culture dish (Catalogue code: 657160, greinerbio-one) overnight. Seeded cells were transfected with 2 µg EXD2 (Myc-DDK-tagged, Catalogue code: MR207967) and 2 µg EXD1 (Myc-DDK-tagged, Catalogue code: MR219928) and 2 µg PIWIL2-FLAG overexpressing plasmids diluted in OPTI-MEM®1 (1×) (Catalogue code: 31985-062, Gibco Life Technology). Transfection reagent, Fugene® HD (Catalogue code: E2311/2, Promega, USA) was used in ratio 2:1 of the plasmid DNA. After 24 hours of transfection, cells were washed in ice-cold PBS then lysed with lysis buffer (5 mM Tris-HCl, 25 mM NaCl, 0.1% Triton-X-100, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF) containing protease inhibitor cocktail (Catalogue code: 11873580001, Roche). Cell samples were allowed to lyse on ice for 30 minutes, then centrifuged 16,000 × g for 20 minutes at +4 °C. Supernatant samples were used for immunoprecipitation and western blotting.

To study the role of *DDX4* in cancer cells (Paper III), we transfected PC3 cells and UT-SCC-14 cells with *DDX4*-targeting plasmid. Briefly, on the first day, PC3 or UT-SCC-14 cells (~ 300,000 cells) were seeded per well, using 6-well culture plate (657160, Greinerbio-one) in 2 mL of DMEM/F12 supplemented with 10% Fetal Bovine Serum (FBS; S1810, Biowest) and 1% penicillin/streptomycin (15140-122, Gibco Life Technology). The next day, the attached PC3 cells were transfected with the *DDX4*-targeting plasmid (2.5 µg plasmid DNA per well), using Xfect reagent (Cat. -No. 631317 & 631318 Clontech Laboratories). After a recovery phase of 72 hours, the cells were subjected to puromycin selection (A1113803, ThermoFisher; 1.25 µg/mL in the complete medium), which lasted approximately 7 days. To achieve monoclonal population of mutant cells, we used the method of limiting dilution, first by allowing cells that were resistant to puromycin treatment expand for 2 weeks, then transferring them into 96-well plates (Corning, 3367), aiming to have less than 1 cell per well. The resultant monoclonal populations were further expanded for about 2 weeks and then screened for *DDX4* mutations by PCR.

4.1.3 Proliferation assay (Paper III)

Following the deletion of *DDX4* in cancer cells, proliferation of *DDX4*- null PC3 cells and *DDX4*-null UTSCC14 cells were monitored as follows: *DDX4*-null PC3 cells and WT PC3 cells or *DDX4*-null UT-SCC-14 cells and WT UT-SCC-14 cells were seeded (~ 4000 cells/well) in a 96-well plate (Corning, 3367). These were placed in the incucyte S3 live-cell imaging analysis system to monitor the profile of growth over 110 hours for UT-SCC-14 cells and 70 hours for PC3 cells. The growth profile of the *DDX4*-null cells was acquired and compared with their counterpart WT cells at the termination of the experiment.

4.1.4 Apoptosis assay (Paper III)

To monitor and compare the cellular events associated with program cell death in *DDX4*-null PC3 cells and WT PC3 cells, phosphatidylserine-based detection by Annexin V-FITC fluorescence probe binding assay was utilized. ~250,000 cells (*DDX4*-null PC3 cells and WT PC3 cells) were seeded in 6-well plate (Catalogue code: 657160, Greinerbio-one). Growing cells were detached from the plate by adding Trypsin EDTA. After trypsinization, cells were resuspended in ice-cold PBS, centrifuged 300 × g for 5 minutes at 4 °C. Cells were resuspended in Annexin V binding buffer, incubated in Annexin V-FITC and Propidium iodide (PI) solution for 10 minutes on ice in the dark moisty incubation chamber (according to the manufacturer's description). Stained live cell suspension were diluted to a final volume of 250 µl/assay with ice-cold IX Annexin V binding buffer, then analyzed by flow cytometry (ACEA NovoCyte® Flow Cytometer). The cells that have lost membrane integrity (dead cells), were stained with PI while Annexin V-FITC conjugated stained cells were with intact plasma membrane (living cells).

4.1.5 3D-spheroids culture (Paper III)

To study the behavior of *DDX4*- null cancer cells (PC3 and UT-SCC-14 cells) in 3D *in vitro* tumor environment, both WT cancer cells and *DDX4*-null cancer cells were seeded between two layers of Matrigel (BD Biosciences) on uncoated µ-plate 96-well angiogenesis (Mediq 1700414). Wells were filled with 10 µl of Matrigel/DMEM/F12 culture medium (1:1; 50%) without phenol red and kept in the incubator at 37 °C for 30–60 minutes to polymerize. Both PC3 cells (WT and *DDX4*-null cells) and UT-SCC-14 cells (WT and *DDX4*-null cells) were seeded gently on top of the Matrigel/medium layer and allowed to form a monolayer (~ 700–800 cells/well). The cells were allowed to attach for 1–2 hours at 37 °C in the incubator. The cells were covered with a second layer of Matrigel containing 20 µl (Matrigel/culture medium; ratio 1:4, 25%), placed in 37 °C incubator overnight. The culture medium was changed every second day.

To monitor and evaluate the viability and morphology of 3D-cancer cell spheroids, both spheroids formed by *DDX4*-null and WT cancer cells were incubated in calcein AM dye (Catalogue code: ab270788) diluted in cell culture medium without phenol red for 1 hour, then images of the viable spheroids were acquired by spinning disk confocal microscope (objective 63 × oil). The morphology of the spheroid images was analyzed using AMIDA software.

4.2 Animals (Paper I & III)

Experiments involving animals were carried out in agreement with the guidelines in the Finnish legislation and good laboratory practice that has been approved by the ethical committee of animal experimentation, University of Turku animal care center. For the use of *in vivo* cancer models in research and drug development, project license number: 21485/2020. 4–5 weeks old male Hsd: Athymic Nude-Foxn1nu mice from Envigo France were used for the xenograft experiments (Paper III). Mice (15 mice per group) were housed in ventilated cages under controlled 12 hours light and 12 hours dark conditions, humidity (55%±15%), and temperature of 21±3 °C. Also, mice were fed with Teklad 2920 soy-free, irradiated, with 20% protein (RM3; Special Diet Services, Witham, UK) and autoclaved or UV-treated water *ad libitum.* For the mouse spermatogenesis experiments, using Licence:2009-1206-Kotaja, Wild-type C5BL/6NHsd and FVB mice were used for tissue collections in the Animal facility of the University of Turku (Paper I). The mice were grown under pathogen-free conditions.
Exd2 mutant mice were generated at the mouse transgenic facility of the EMBL Monterotondo, Rome, Italy (Paper I). Animal experiments in Grenoble, France, were covered by an authorization (no. 381007) from the Direction Departmentale de la Protection des Populations, Prefecture de 1' Isere. All mice were euthanized by carbon dioxide followed by cervical dislocation before tissue collections.

4.2.1 Xenograft experiments (Paper III)

To prepare cell samples for subcutaneous and orthotopic injections, PC3 cells and UT-SCC-14 cells were maintained as described earlier (section 4.1). Cells were harvested on the day of inoculation as follows: Cells were washed in PBS, and then treated with EDTA trypsin solution to detach the cells from the plate. Trypsinization was quenched by adding growth medium, collected and then centrifuged at 300 × g for 5 minutes in +4 °C. The supernatant was removed and cell pellets resuspended in PBS solution. The number of cells were counted (cells/mL) and the viability determined with trypan blue staining (at least 80% of the cells should be alive). For subcutaneous injections, the cells were mixed with a cell culture medium (without antibiotics) and cold Corning Matrigel™ in a ratio of 1:1. It was noted that all equipment (tubes, needles, syringes, pipettes) must be cold when using the Matrigel™, which must be placed on ice to avoid coagulation. The cell/Matrigel suspension was mixed carefully and taken to the cold syringe just before injection. Approximately, 1 million cells were gently injected into each of the right and left flank of the subcutaneous skin of the Athymic nude mice. The size of the needle used for subcutaneous and orthotopic injection of cancer cells was 25G. In the case of orthotopic injection, animals were given analgesics: Buprenorphine Temgesic 0.3

mg/ml (Schering-Plough Nv, Brussels, Belgium) via the intraperitoneal route); animals were also anaesthetized with isoflurane (induction 3–4%, upkeep 2–3%) before the commencement of orthotopic injections. The injection volume for orthotopic was 20 µl per prostate and the amount of cancer cells injected was 1 million cells/prostate and was kept on ice. The weight gain of mice was monitored at least once per week over the duration of the experiment. The tumor growth was monitored weekly by using either the Vernier callipers or ultrasound machine.

4.2.2 Histology (Paper I, II & III)

To process tissue samples for histological analysis, mice were euthanized in carbon IV oxide chamber followed by cervical dislocation. Testes tissues (Paper I & II) were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline, left in gentle rotation overnight at room temperature. PC3 derived tumor samples (Paper III) (*DDX4*-null PC3 tumor and WT PC3 tumors) were dissected and fixed in 10% formalin solution for about 48–75 hours, left in gentle rotation at room temperature. The fixed tissue samples were dehydrated in series of ethanol concentrations: 2 × 50% ethanol, 2 × 70% ethanol, then embedded in paraffin. Tissue sections (4 µm) were cut and placed onto polylysine-coated slides. For normal morphological examinations, samples were stained with haematoxylin and eosin (HE) or periodic acid-Schiff (PAS) staining according to standard protocols. Other samples were used for immunostaining (as described in the sections below).

4.2.2.1 Immunohistochemistry analysis (Paper III)

To investigate localization of proteins in tissue samples, using immunohistochemically-staining approach, FFPE samples (4 µm in thickness) were rehydrated as follows: 3 × 5 minutes in xylene, 2 × 5 minutes in 100% ethanol, 2 × 5 minutes in 96% ethanol, 2 × 5 minutes in 70% ethanol, 1 × 5 minutes in distilled water. Hydrated tissue samples were permeabilized in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and boiled in the pressure cooker at 120 °C for 20 minutes. Samples were allowed to cool down to room temperature for 1 hour 30 minutes. Permeabilized samples were washed 2 × 5 minutes in 0.05% Tween in PBS solution.

To prevent non-specific binding, samples were incubated in 10% bovine serum albumin, 0.05% Tween in PBS (blocking solution) at room temperature. After the blocking step, samples were placed in a humidified chamber and incubated in the primary antibody (diluted in blocking solution) over night at +4 °C. Samples were washed 2 × 5 minutes in 0.05% Tween in a PBS solution. To block endogenous peroxidase activity, samples were incubated in 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) diluted in 0.05% Tween in PBS for 20 minutes at room

temperature. Samples were washed 2 × 5 minutes in 0.05% Tween in PBS solution. Samples were incubated in Dako EnVision®+System-HRP labelled polymer against rabbit IgG (secondary antibody) solution for 30 minutes at room temperature. Samples were washed 2 × 5 minutes in 0.05% Tween in PBS. Samples were incubated with 3,3'-Diaminobenzidine (DAB) substrate chromogen system (Agilent Corp., CA, USA) solution for 10 minutes, and then washed 3 × 3 minutes immediately with distilled water.

For nuclear background staining, DAB-stained samples were counter stained with Mayer's Haematoxylin solution for 45 seconds then washed in distilled water for 5 minutes at room temperature. Samples were dehydrated as follows: 2 × 5 minutes in 70% ethanol, 2 × 5 minutes in 96% ethanol, 2 × 5 minutes in 100% ethanol, and, 2 × 5 minutes in xylene solution. Samples were dried and mounted with a Pertex mounting medium and a 60 mm glass cover slip. Mounted samples were allowed to solidify for 2–4 hours then images were acquired by scanning using the Panoramic® 250 flash series digital slide scanner from 3DHISTECH Ltd (Budapest, Hungary).

4.2.2.2 Immunofluorescence analysis (Paper I, II & III)

To investigate protein localization in tissues samples, using an immunofluorescence staining method, formalin-fixed paraformaldehyde embedded (FFPE) tissue samples, 4 µm sections were deparaffinized as follows: 3 × 5 minutes in xylene, 2 × 10 minutes 100% ethanol, 2 × 5 minutes in 96% ethanol, 2 × 10 minutes 70% ethanol, and 2 × 2 minutes milliQ water (Catalogue code: MPGP02001, Millipore). To open up the epitopes in the cells of the tissues, samples were permeabilized in citrate solution (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) or Tris-EDTA buffer (10 mM Tris-EDTA 0.05% Tween 20, pH 9.0), boiled at 120 °C for 20 minutes. Samples were allowed to cool down to room temperature while in the pressure cooker. Samples were washed 4 × 3 minutes in milliQ water and once in PBS for 5 minutes. For cells cultured in Millicell® EZ slide, 4-well (Catalogue code: PEZGS0416, Millipore), samples were fixed in 4% paraformaldehyde for 10 minutes at room temperature, then washed 3 × 5 minutes in 1×PBS, permeabilized in 0.2% Triton X-100 for 5 minutes at room temperature.

To prevent unspecific binding, samples (both FFPE tissues and fixed cells) were incubated in the blocking solution (10% bovine serum albumin, 1XPBS containing 0.1% Triton X-100) at room temperature in a moist incubation chamber for 1 hour. Samples were then incubated in specific primary antibodies (diluted in the blocking solution) (Table 1) at +4 °C overnight. Samples were washed 3 × 5 minutes in PBS. Samples were incubated 1 hour at room temperature with Alexa 488/594/647-conjugated secondary antibodies (Life Technologies) (diluted in blocking solution)

that correspond to the primary antibody host. Samples were washed 3 × 5 minutes in PBS, then stained with 4', 6-diamidino-2-phenylindole (DAPI) (D9542, Sigma-Aldrich) diluted 1/20000 in PBS. Samples were mounted with ProLong Diamond Antifade Mountant (P36970, Life Technologies) and allowed to solidify at room temperature overnight. Images were acquired with a 3i spinning disk confocal microscope (objective 100 × 1.4 oil, Intelligent Imaging Innovations).

For immunofluorescence analysis of the 3D-spheroids at 15th day time point (Paper III), cell culture medium was suctioned out and the spheroids carefully washed in PBS in a manner that did not disturb the structure of the spheroids. Samples were fixed in 4% PFA for 15–20 minutes at room temperature, then washed 3 × 5 minutes in PBS. Samples were permeabilized in 0.7% triton-X in 20% horse serum, 0.5% BSA in PBS for 1 hour at room temperature. Samples were incubated in primary antibodies in a humidified chamber, overnight at +4 °C. Samples were washed 3 × 5 minutes in PBS, incubated in secondary antibody (diluted in 20% horse serum and PBS) for 2 hours at room temperature, then washed 2 × 5 minutes in DAPI (1:20000 diluted in PBS). The samples were finally washed and left in PBS. Immunofluorescence images were acquired by the spinning disk confocal microscope and images processed with slide book readers and Fiji image J using the maximum intensity projection of the z-stack (Paper I, II & III).

4.2.3 Immunoprecipitation (Paper I & III)

To isolate IMC by crosslinking, using the same conditions as described earlier for the CB isolation (Meikar et al., 2010b), six 15-day old male mouse testes were collected in PBS, then dissected and seminiferous tubules digested in 0.5 mg/mL collagenase type 1 (LSS004196, Worthington Biochemical) in PBS-containing 0.1% glucose and 1 µg/mL DNase I (Catalogue code: LS006353, Lakewood New Jersey) in rotation at room temperature for 50 minutes. Digested seminiferous tubules were filtered (100 µm) (Catalogue code: 352360; BD biosciences), washed in 0.1% glucose in PBS, and cross-linked with 0.1% (v/v) paraformaldehyde (Catalogue code: 50-980-487, Thermo Fisher Scientific) at room temperature for 20 minutes. Cross-linked cells were centrifuged at 300 × g at 4 °C for 5 minutes, then the supernatant was discarded and cells lysed was sonicated, using sonicator (UCD-200, Diagenode) in 1.5 mL Radio Immunoprecipitation Assay buffer RIPA (50 mM Tris-HCl at pH 7.5, 1% NP-40, 0.5% w/v sodium deoxycholate, 0.05% w/v sodium dodecyl sulphate, 1 mM EDTA, 150 mM NaCl, IX complete™ Mini Protease Inhibitor Cocktail (11836153001; Roche), 0.2 mM PMSF and 1 mM DTT) using medium settings for 6 × 30 seconds intervals. Cells lysates were centrifuged at 300 × g for 10 minutes at +4 °C. Pellet was discarded and supernatant fraction precleared with 10 µl of washed Dynabeads Protein G (10003D, Invitrogen), and then the

precleared lysate was subjected to immunoprecipitation with beads coupled with 2 µg of anti-PIWIL2 antibody or negative control IgG overnight on a rotator at +4 °C. The beads-antibody-antigen complexes were washed three times in 1 mL RIPA buffer.

To investigate protein-protein interaction in testes (Paper I) or tumor samples (Paper III) without cross-linking, tissues (4 adult mice testes or two PC3 derived xenograft tumors) were dissected and homogenized with 5 mm stainless steel beads (Catalogue code: 69989, QIAGEN-Hilden Germany) and tissue-lyser LT (ETI-09019957-A, Qiagen Hilden Germany) in isotonic lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, IX complete™ Mini Protease Inhibitor Cocktail, 0.2 mM PMSF and 1 mM DDT. Samples were allowed to lyse on ice for 30 mins, then centrifuged at 300 × g for 10 minutes at +4 °C. Pellet was discarded and supernatant fraction precleared with 10 µl of washed Dynabeads Protein G (10003D, Invitrogen). Lysates were precleared and subjected to immunoprecipitation with beads coupled with 2 µg of primary antibody (anti-EXD2 for testes, anti-DDX4 for PC3 xenograft tumors) or negative control IgG overnight on a rotator at +4 °C. The beads-antibody-antigen complexes were washed three times in 1 mL lysis buffer.

To study the interactions of ectopically expressed proteins (Paper I), the transfected HeLa cells were washed in ice-cold PBS after 24 hours of transfection, and then lysed with the isotonic lysis buffer for 30 minutes on ice. Both tissue and lysed cells were centrifuged 17000 × g at +4 °C for 20 minutes. Supernatant lysed samples were used for immunoprecipitation.

4.2.4 Mass spectrometry (Paper I & III)

Samples for mass spectrometry analysis (antibody-antigen-beads complexes) were washed 3 × 1 mL of 50 mM Tris pH 8.0, digested and analysed at the Turku Proteomics Facility of the Turku Bioscience Center using LC-ESI-MS/MS (2X Q Exactive HF Mass Spectrometer) according to standard protocol. Database searches for samples were performed by Mascot search engine against a Swissprot database. Briefly, the LC-ESI-MS/MS analyses were performed on a nanoflow HPLC system (Easy-nLC1200, Thermo Fisher Scientific) coupled to the Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ionization source. Peptides were first loaded on a trapping column and subsequently separated inline on a 15 cm C18 column (75 µm × 15 cm, ReproSilpur 5 µm 200 Å C18-AQ, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany).

The mobile phase consisted of water with 0.1% formic acid (solvent A) or acenotrile/water (80:20 (v/v)) with 0.1% formic acid (solvent B). A 30 minutes gradient from 8 to 43% B was used to elute peptides. MS data was acquired

automatically by using Thermo Xcalibur 3.1 software (Thermo Fisher Scientific). An information dependent acquisition method consisted of an Orbitrap MS survey scan of mass range 300–2000 m/z followed by HCD fragmentation for 10 most intense peptide ions. Data files were searched for protein identification using Proteome Discoverer 2.2 software (Thermo Fisher scientific) connected to an in-house server running the Mascot 2.6.1 software (Matrix Science). Data was matched against SwissProt database (version 2018_04) with a taxonomy filter '*mus'*. The following database search parameters were used. Enzyme: Trypsin, Variable modifications: Oxidation (M), Fixed modifications: Carbamidomethyl (C) ** peptides were alkylated with iodoacetamide, Peptide Mass Tolerance: ± 5 ppm, Fragment Mass Tolerance: ± 0.02 Da, Max Missed Cleavages: 1, Instrument type: ESI-TRAP. Results discovered from Proteome were exported and saved as Excel files. Proteins with high and medium confidence and minimum 2 peptides per protein are enlisted.

4.2.5 Western blotting (Paper I & III)

To perform protein expression analysis in tissue samples, protein concentrations were measured with a Pierce BCA protein assay kit (Catalogue code: 23227, Life Technologies) and absorbance taken with Victor™ X4 plate reader (Catalogue code: 2030-0050, PerkinElmer). Protein lysate concentrations were normalized and diluted with 2 × Laemmli buffer (containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.04% bromophenol blue, 0.125 M Tris HCl, pH 6.8). Samples were heated to 95 °C before loading on the gel. Proteins were separated by 4–20% Poly Acrylamide Gel Electrophoresis (PAGE) MiniPROTEAN® TGX (456–1093) in running buffer (containing: 250 mM Tris base, 1.92 M glycine and 10% SDS) at 100 Volts.

Proteins were transferred onto the PVD membrane (10600023, RPN303F, Amersham™ Hybond™) at 90 Volts, 400 mA for 1 hour in +4 °C or on ice. To prevent unspecific binding of proteins, the membrane was either incubated in 100% methanol, air dried at room temperature overnight or the membrane was incubated in 5% skimmed milk in PBS containing 0.1% Tween®-20 (Catalogue: P2287, USA) (PBST) for 1 hour at room temperature. Membranes were incubated in a primary antibody, diluted in PBST, overnight at +4 °C. The antibodies used are listed in Table 1. The membranes were then washed in PBST 3 × 10 minutes, then incubated in Horseradish Peroxide (HRP)-linked anti-rabbit (Catalogue code: 7074S, Cell signaling) or HRP-linked anti-mouse antibody (Catalogue code: 7076S, Cell signaling). The membranes were washed for 3 × 10 minutes in PBST and the proteins detected by incubating in western lightening ECL pro (Nell200IEA, Perkin Elmer) for 1 minute. Band intensity signals were observed with LAS4000 (Fujifilm) or

Azure Sapphire Bio-molecular Imager (Azure Biosystems) and images were saved as 16-bit TIFF files. Adobe Photoshop creative Suite 6 was used for image processing of the detected proteins.

4.2.6 RNA extraction (Paper I & III)

The total RNA was extracted from the testes with TRIzol™ reagent (Invitrogen) and from PC3-derived tumors with TRIsure (Bio-38033, Meridian Bioscience) according to the manufacturer's instructions. DNA contamination was removed by treating the samples with DNase1 (AMPD1, Sigma-Aldrich). RNA pellets were resuspended in 100 µL RNAse free water (Catalogue code: P119C, USA) and concentration measured by NanoDrop (Thermo Fisher Scientific, USA) or 2100 Bioanalyzer (Agilent Technologies) using Agilent RNA 6000 Nano Kit (5067-1511, Agilent Technologies) and samples sent for mRNA sequencing analysis. For small RNA isolation (Paper I), 10 µg of total RNA was run through a 15% denaturing Urea- PAGE gel and the region of gel containing small RNAs was cut out and extracted through incubation in 400 µL of 0.4 M NaCl at 25 °C for 4 hours on a shaker. The incubated sample was centrifuged 17000 × g for 2 minutes at +4 °C and incubated in 40 µL of 7.5 M ammonium acetate, pH 5.5, 1 µL Glycogen RNA grade (20 ng/mL) and 1 mL of 100% EtOH for 5 hours at -20 °C to precipitate small RNA. The sample was centrifuged 17000 × g for 2 minutes, the supernatant discarded and the RNA pellets washed in 80% ethanol. The sample was centrifuged again and resuspended in RNAse free water then sent for illumina library preparation (Paper I). RNA samples from PC3 derived tumors were sent for library preparation at the Finnish Functional Genomics Centre (FFGC), Turku Centre for Biotechnology, Finland (Paper III).

4.2.6.1 mRNA sequencing (Paper I & III)

For the preparation of RNA-seq libraries (Paper I), ribosomal RNA was depleted from 1 µg of total RNA using NEBNext® rRNA depletion Kit v2 and libraries prepared using NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®. The mRNA-seq libraries were sequenced at the GENOMICS platform of the University of Geneva using Illumina HiSeq 4000 platform with single-end run with a read length of 50 nucleotides (nt) (Paper I). The reads were sorted into individual libraries based on the barcodes then mapped to the mouse genome (GRCm38: Ensembl release 95) using STAR (parameters: runThreadN 10-outFilterTypeBySJout—limitOutSJcollapsed 50000000—limitIObufferSize 1500000000). EdgeR was used to compare the read counts of each pachytene piRNA cluster of *Exd2*$^{\Delta}$ to *Exd2*$^{+/+}$. Only the longest ones of the overlapping clusters were

retained. To construct the volcano plot, obtained log2fold changes with statistical significance were used. Deletion of *Exd2*$^{\Delta}$ was confirmed by the presence of TTGGAATTGACTGTGAATGG sequence (the 3' end of exon2) in individual libraries. The mean read coverage (rpm) was normalized and plotted along the *Exd2* locus to show expression in *Exd2*$^{\Delta}$ and *Exd2*$^{+/+}$ mice. To see how the deletion in *Exd2*$^{\Delta}$ mice affects the final transcript, the position of the exon2-exon3 splice junctions obtained from STAR SJ.out.tab files were plotted. Uniquely mapped reads were considered with at least 5 reads abundance across all samples. Whereas in *Exd2*$^{+/+}$ mice, only canonically spliced reads were detected, the deletion in *Exd2*$^{\Delta}$ mice results in aberrant splicing of exon 2, thereby resulting in the absence of 3' end of exon 2 or whole exon 2 skipping. The most common splicing pattern in *Exd2*$^{\Delta}$ produces intron at chr12:80476090-80480454 resulting in the in-frame deletion of 33 amino acids, including the DCE motif.

For RNA isolated from PC3-derived tumors and PC3 cells (Paper III), library preparation was done according to Illumina TruSeq® Stranded mRNA preparation guide (part#15031047). The quality of library was confirmed with advanced Fragment Analyzer and the concentrations of the libraries were quantified with Qubit® Fluorometric Quantitation, Life Technologies. Only good quality libraries were sequenced. Typically RNAseq library fragments are in the range of 200–700 bp and the average size of the fragments is 250–350 bp. The samples were normalized and pooled for the automated cluster preparation which was carried out with Illumina cBot station. The 16 libraries were pooled in one pool and run in one lane. The samples were sequenced with illumine HiSeq 3000 instrument. Paired-end sequencing with 2 × 75 bp read length was used with 8 + 8 bp dual index run.

4.2.6.2 Small RNA sequencing (Paper I & Paper III)

In paper I, Small RNA-sequence libraries were prepared using NEBNext® Multiplex small RNA library preparation set for illumina®, and the quality of libraries validated in 2% agarose gel. Libraries were run into a 3.6% Agarose (MetaPhorTM) gel for size selection, desired size region was cut out and lysed in QIAGEN MinElute Gel extraction kit to extract the DNA. The small RNA-seq libraries were sequenced at the GENOMICS platform of the University of Geneva using illumine HiSeq 4000 platform with single-end run with a read length of 50 nucleotides (nt) (Paper I).

In Paper III, small RNA libraries were prepared according to Qiagen QIAseq miRNA library Kit Handbook (11/16) at the Finnish Functional Genomic Centre, University of Turku and Åbo Akademi and Biocenter Finland. Briefly, the first two steps of the library preparation workflow 3' and 5' adapters were added to each end of the RNA molecules. After the adapter ligations reverse transcription (RT) reaction with a Unique Molecular Index (UMI) containing RT-primer was carried out to

create a single stranded cDNA. After RT, a clean-up of the cDNA was performed using a magnetic bead-based method with QIAseq miRNA NGS beads (QMN beads). The cDNA was then PCR amplified (16 cycles) using a common forward primer and a unique indexing primer containing six-base indices for labeling each sample with a unique barcode. Integrated UMIs enable unbiased and accurate miRNome-wide quantification of mature miRNAs by NGS.

After amplification, a size fractionation and clean-up of miRNA libraries were carried out using QMN beads. The quality of the libraries was confirmed with an Advanced Analytical Fragment Analyzer and Agilent Bioanalyzer 2100 and the concentrations of the libraries were quantified with Qubit® Fluorometric Quantification, Life Technologies. The samples were normalized and pooled for the automated cluster preparation which was carried out with Illumina HiSeq sequencer. The samples were sequenced with an Illumina HiSeq 2500 instrument using Truseq v2 Rapid sequencing chemistry. Single-read sequencing with 1 × 75 bp read length was used, followed by 6 bp index run.

4.2.6.3 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Paper III)

The RNA sequencing analysis of differentially expressed genes was validated by qRT-PCR. To achieve this, the total RNA was isolated from PC3 derived tumors and also from cell samples as described above (in section 4.2.6). 1 µg of the total RNA was used to synthesize complementary DNA (cDNA) using SensiFast cDNA synthesis kit (BIO-65054, Meridian Bioscience) and qPCR performed DyNamo Flash SYBR Green qPCR kit (F415L, Thermo Scientific) according to the manufacture's protocol. The qPCR samples were run on CFX384 Real-Time PCR system (Bio-Rad) with the following settings: 95 °C for 7 mins in initial denaturation, and subsequently for 40 cycles, 95 °C for 10 seconds, 60 °C for 15 seconds, 72 °C for 10 seconds, followed by final elongation at 72 °C for 10 minutes. The PCR products were run on 1.5% agarose gel and the amplified products visualized by staining the gel with Midori Green Advance DNA stain (MG04, Nippon Genetics). The primers used for the qPCR were ordered from Integrated DNA Technologies (IDT) and are listed in Table 2. Fold gene expression was calculated using *hRPL19* as a reference gene.

Table 1. List of antibodies, where they were used in the original publications (I, II & II), applications, dilutions, catalogue codes and the manufacturer.

ANTIBODY NAME	HOST	1°/2°	APPLICATION	DILUTION	CAT. CODE	MANU-FACTURER
DDX4 (I & III)	Rabbit	primary	IF	1/1000	ab13840	Abcam
EXD1 (I & II)	Rabbit	primary	IF/WB/IP	1/1000	17313-1-AP	ProteinTech
EXD2 (I)	Rabbit	primary	IF/WB/IP	1/1000	20138-1-AP	ProteinTech
VIMENTIN (III)	Mouse	primary	IF/WB	1/500	GTX40346	Gentex
ɣH2AX (I & II)	Mouse	primary	IF	1/500	05-636	Millipore
DDX4 (III)	Rabbit	primary	WB/IP	1/1000	510421-1-AP	ProteinTech
CDH6 (III)	Rabbit	primary	WB	1/1000	48111S	Cell Signaling
CDH1 (III)	Rabbit	primary	WB/IF	1/1000	14472	Cell Signaling
CDH2 (III)	Rabbit	primary	WB	1/1000	ab1934	Abcam
CDH7 (III)	Rabbit	primary	WB	1/1000	6505	Cell Signaling
DDX25 (I)	Goat	primary	IF	1/200	sc-51269	Santa Cruz
ASZ1 (I)	Rabbit	primary	IF/WB	1/500	21550-1-AP	ProteinTech
GAPDH (I)	mouse	primary	WB	1/5000	5G4	HyTest
ACTIN (III)	mouse	primary	WB	1/5000	A1978	Sigma
DDX4 (I)	Goat	Primary	IF	1/1000	AF2030	R&D systems
TDRD1 (I)	Rat	Primary	IF	1/100	MAB6296	R&D systems
PIWIL2 (I & II)	Mouse	Primary	IF/WB	1/1000	MABE363	Millipore
CYTOCHROME C (I)	Mouse	Primary	IF	1/1000	556433	BD Pharmingen
IgG (I & III)	Rabbit	Negative control	IP		NC100-P1	Neomarkers
IgG (I & III)	Mouse	Negative control	IP		Sc-2025	Santa Cruz

Table 3. Primers used for qRT-PCR in validation of RNA sequencing results (Paper III).

Gene	Sense primer (5'→3')	Reverse Primer (5'→3')
ADAM12	*GAGGAGCTGGTCTTAGAGAGG*	*CGGTCACGACACTGAAGGTG*
CCND2	*ACCTTCCGCAGTGCTCCTA*	*CCCAGCCAAGAAACGGTCC*
CDH6	*AGAACTTACCGCTACTTCTTGC*	*TGCCCACATACTGATAATCGGA*
CDH7	*TCAAATACATCTTGTCAGGCGAA*	*TGGCATGAATATCCCCAGTGT*
CYP27C1	*AGAAACCGTGACCAATGTCAAT*	*CAGCCCAAACGACTCTCATAAA*
MYLK	*CCCGAGGTTGTCTGGTTCAAA*	*GCAGGTGTACTTGGCATCGT*
CEACAM1	*TGCTCTGATAGCAGTAGCCCT*	*TGCCGGTCTTCCCGAAATG*
SFRP1	*ACGTGGGCTACAAGAAGATGG*	*CAGCGACACGGGTAGATGG*
CLEC2B	*GTTCCACTCAACATGCCGAC*	*TGCCATCTTCAGTCCAATCCA*
CSPG4	*CTTTGACCCTGACTATGTTGGC*	*TGCAGGCGTCCAGAGTAGA*
HSD17B2	*TCTTCTCGGTGTCATGCTTCC*	*CAAAACTCCGGCAAATACCGT*
IGSF9B	AGAGGAGCCCGAGTTTGTGA	CACTCTACGACATAGGGTGGG
MUC2	GAGGGCAGAACCCGAAACC	GGCGAAGTTGTAGTCGCAGAG
MGP	TCCGAGAACGCTCTAAGCCT	GCAAAGTCTGTAGTCATCACAGG
RPL19	AGGCACATGGGCATAGGTAA	CCATGAGAATCCGCTTGTTT
SFRP1	ACGTGGGCTACAAGAAGATGG	CAGCGACACGGGTAGATGG
SMOC1	AGGTCCTACGAGTCCATGTGT	CACTGCACCTGGGTAAAGG

5 Results and Discussion

5.1 IMC accumulates piRNA biogenesis factors (I)

We investigated the piRNA machineries in IMC-mediated RNA regulation by characterizing the protein composition of the IMC. To achieve this, we established a protocol for isolating IMC from mouse testicular cells that are mildly crosslinked with 0.1% paraformaldehyde, and harnessing the knowledge from our previously established CB isolation protocol (Meikar et al., 2010b). The crosslinking helps to preserve the integrity of the IMC. Therefore, we isolated the IMC by using an antibody against one of the mouse PIWI proteins, PIWIL2, which is known to accumulate in the IMC, and then identified proteins co-immunoprecipitating with PIWIL2 by mass spectrometry.

The PIWIL2 has been shown to be expressed exclusively in the cytoplasm, particularly prominent in the pachytene spermatocytes and is also found in the early CB of round spermatids. Therefore to avoid any CB contamination, we used testes from 15-day old (P15) mice as the starting material. The CB precursors are formed in the late pachytene spermatocytes that appear in P18 testes (Lehtiniemi & Kotaja, 2018). Immunofluorescence staining of P15 testes with PIWIL2 antibody confirmed the prominent localization of PIWIL2 in the cytoplasm of pachytene spermatocytes (I: Figure 1A). To confirm the absence of CB precursors in P15 testes, an antibody against DDX25, a well-established component of the CB (Anbazhagan et al., 2022), was used.

The result of the mass spec analysis from two replicate samples of PIWIL2-associated IMC identified 64 common proteins (I: Figure 1C, Table S1), which were referred to as the IMC components. To further understand the function of these IMC-localized PIWIL2 protein complexes, GO term analysis was performed, using gProfiler tool (https://biit.cs.ut.ee/gprofiler/gost). Results showed that GO terms connected to different RNA regulatory mechanisms were enriched among the proteins forming complex with PIWIL2 in the IMC. Moreover, and 37 out of 64 IMC proteins were found under the GO term “RNA metabolic process” (I: Table S2). Interestingly, 14 proteins were listed under the GO term connected to “piRNA metabolic process” (I: Figure 1D, Table S2). Indeed, many core proteins were found in the piRNA biogenesis machinery, including ASZ1, PLD6, MOV10L1, GPAT2,

TDRKH, and FKBP6 (Ozata et al., 2019); they were identified as IMC components. Many of these proteins have been shown to be associated with mitochondrial membranes (X. Wang et al., 2020). Therefore, the involvement of mitochondria in piRNA biogenesis is probably the reason for the typical morphological structure of IMC as a cement between mitochondrial clusters. We also observed an enrichment of one of the mitochondrial proteins, cytochrome C, in PIWIL2-associated IMC suggesting mitochondrial functions of IMC. Cytochrome c is localized exclusively in the cytoplasmic compartment of mitochondria, and our results showed that it colocalized with PIWIL2 in the IMC of pachytene spermatocytes.

In this study, the method used for the isolation of IMC is similar to the one we previously used for the CB isolation (Meikar et al., 2010b). In the CB isolation, we took advantage of the large size of the CB, which formed a pellet during low-speed centrifugation. The pellet fraction was highly enriched in CB-associated DDX4 (resulting in high immunopurified CB), whearas the supernatant fraction contained other cytoplasmic components excluded from the CB. However, in this study, we were unable to obtain an IMC pellet with the low-speed centrifugation. Therefore, we had to use the whole-cell lysate as the staritng material for the IMC immunoaffinity purificaiton. Due to this limitation, we cannot exclude the possibility that some PIWIL2-interacting proteins interact with PIWIL2 outside the IMC in the cytoplasm. However, the prominent localization of PIWIL2 in the IMC as detected by immunofluorescnece suggests that most PIWIL2-interactions occur in the IMC.

5.1.1 EXD1 and EXD2 are novel germ granule proteins

Among the proteins that form complexes with PIWIL2 in the IMC in pachytene spermatocytes there were two novel exonuclease 3'–5' domains containing (EXD) proteins, EXD1 and EXD2. EXD1 has previously been shown to form a protein complex with one of the protein adaptors TDRD12 to promote piRNA production in embryonic testes (Pandey et al., 2018; Z. Yang et al., 2015), while the role of EXD2 in piRNA pathway has remained uncharacterized.

Both EXD1 and EXD2 contain 3'–5' exonuclease domain (amino acids 134–325 in mouse EXD1 and 105–291 in mouse EXD2), but have very little sequence similarity outside this domain (only 12.7% identity and 23.1% similarity between the two proteins in EMBOSS Needle pairwise alignment). In addition, mouse EXD1 has N-terminal like-Sm (Lsm) domain (amino acids 1–93), and EXD2 has a transmembrane domain in its N terminus (amino acids 6–25).

In order to understand their role in germ cells, we first characterized their expression in different mice tissues by western blotting to discover whether they were testis-specific proteins. This was not the case, but EXD1 and EXD2 were

shown to be ubiquitously expressed in different tissues (testis, epididymis, ovary, liver, heart, intestine, kidney, spleen, olfactory, lungs and brain) (I: Figure 2A,B). We next characterized the expression of EXD1 and EXD2 during the first wave of spermatogenesis using the testicular tissues collected from juvenile mice at different time points (P0, P7, P14 and P21 testes). Both EXD1 and EXD2 expression increased toward the later time points of spermatogenesis (I: Figure 2C,D).

By immunofluorescence staining, we showed that both proteins localized in the prominent cytoplasmic granules in pachytene spermatocytes. In addition to the expression in the spermatocytes, EXD1 appeared prominent in the cytoplasm of haploid round spermatids while EXD2 was not detected in haploid cells. (I: Figure S1B,C,D). In summary, our immunofluorescence analysis revealed that both EXD1 and EXD2 have prominent expression in the pachytene spermatocytes, and in addition, EXD1 continues to be expressed in haploid spermatids.

5.1.2 EXD2 localizes to IMC, while EXD1 localizes to both IMC and CB

The granular cytoplasmic localization suggested that both EXD1 and EXD2 localized in the IMC while only EXD1 localize in the CB. In order to confirm this, we conducted co-immunostaining of EXD proteins and proteins that were known markers of IMC and CB. The result showed that EXD2 colocalized with IMC proteins (TDRD1, PIWIL2 and DDX4) in pachytene spermatocytes and also associated with mitochondrial protein Cytochrome c in the IMC (I: Figure 3B,C). EXD2 did not localize to CB-precursors in late pachytene spermatocytes nor to CBs in round spermatids, as shown by using DDX25 and PIWIL2 as CB markers (I: Figure 3D,E). The close association of EXD2 with germ granules was confirmed by co-immunoprecipitation of germ granule components PIWIL2, DDX4, and ASZ1 with EXD2 from adult testicular cells without any crosslinking (I: Figure 5A). Although the role of EXD1 in embryonic germ cells have been demonstrated (Z. Yang et al., 2015), its expression during postnatal spermatogenesis was not known. In this study, we showed that EXD1 colocalize with PIWIL2 and TDRD1 to IMC, and also with DDX25 to CB-precursors in late pachytene spermatocytes and CB in round spermatids (I: Figure 4A,B,C). These results validated that both EXD1 and EXD2 are IMC components, and revealed the localization of EXD1 also to CB.

Although the localizations of both EXD1 and EXD2 are highly associated with germ granules, their differential localization in the IMC vs. CB suggests some functional differences even though they contain a similar 3'–5' exonuclease domain. EXD1 and EXD2 have very little sequence similarity outside the 3'–5' exonuclease domain that is located in the amino acids 134–325 in mouse EXD1 and at 105–291 in mouse EXD2. In addition, the mouse EXD1 has N-terminal like-Sm (Lsm)

domain (amino acids 1–93), and EXD2 has a transmembrane domain in its N terminus (amino acids 1–93). In fact, EXD2 has been shown to function in mitochondrial membranes in somatic cells (Hensen et al., 2018; J. Silva et al., 2018), which explains its localization in the IMC but not in the CB that has lost the association with mitochondria.

5.1.3 Functional differences between IMC and CB

The IMC and CB appear during different phases of spermatogenesis, but co-exist briefly in late pachytene spermatocytes. They are known to share some protein components (Lehtiniemi & Kotaja, 2018), but their interrelationship has remained elusive. Morphologically, the main difference between the two germ granules is that IMC appear as a glue between mitochondrial clusters, while the CB has lost the connection with mitochondria.

The comparison of the proteomes of the IMC (this study) and CB (Meikar et al., 2014) by mass spec analysis showed that 25 of the IMC proteins were shared with CB, suggesting that IMC and CB have a functional relationship. Among the common proteins were PIWIL2, PIWIL1, DDX4, MAEL and Tudor domain-containing proteins, all with piRNA pathway-associated functions. The shared components between the IMC and CB suggest that these granules communicate during their short co-existence in late pachytene spermatocytes. This supposed communication may include transferring of materials from IMC to CB for further processing or storage.

Despite the presence of PIWI proteins and other proteins implicated in the piRNA pathway in the CB, the major core proteins that are crucial for piRNA biogenesis are absent from the CB, but exclusively in the PIWIL2-associated IMC. This confirms the earlier hypothesis that piRNA biogenesis takes place in the IMC of pachytene spermatocytes. The role of mitochondria-associated IMC but not CB in piRNA biogenesis is further supported by earlier studies showing the involvement of mitochondrial membrane-localized proteins in piRNA biogenesis (H. Huang et al., 2011; X. Wang et al., 2020; Watanabe et al., 2011; J. Zhang et al., 2016). The accumulation of piRNAs, PIWI proteins and a diverse set of mRNAs and lncRNAs that could serve as targets for piRNAs in the CB (Meikar et al., 2010a) suggests that the CB could function as a platform for piRNA-mediated RNA regulatory mechanisms.

Our results show that both CB and IMC have a role in piRNA pathway, and further supports other studies both in mammals, Drosophila and *Caenorhabditis elegans* that have suggested a role for the germ granules as platforms for the piRNA pathway (Chuma et al., 2006; W Deng & Lin, 2002; Kuramochi-Miyagawa et al., 2004a; Meikar & Kotaja, 2014; S S Tanaka et al., 2000). However, our results suggest that the functions of IMC and CB differ, IMC being involved in the

biogenesis of piRNAs, while CB probably mediating downstream regulatory functions of piRNAs.

5.2 *Exd2* mutation affects the expression of specific piRNA clusters but does not affect fertility in mice

To understand the role of EXD2 in piRNA pathway, we first clarified whether the interaction between PIWIL2 and EXD2 is dependent on the germ granule environment since EXD2 is not a germ cell specific protein like PIWIL2. To achieve this, we overexpressed EXD2 and PIWIL2 by transfecting HeLa cells with the plasmids that are encoding EXD2 and PIWIL2, followed by immunoprecipitation and western blotting with antibodies against EXD2 and PIWIL2. Indeed, there was interaction between EXD2 and PIWIL2 (I: Figure 5B), suggesting that the germ granule environment is not required for their interaction.

EXD2 contains metal-binding sites Asp137 and Glu139 in its DCEW motif sequence in exon 2, which is required for the 3'–5' exonuclease activity on both DNA and RNA (Broderick et al., 2016; J. Silva et al., 2018). Therefore, to dissect the physiological role of EXD2 in the testes, we engineered the deletion of the 3' end of exon 2 of the mouse *Exd2* gene locus and part of the downstream intron, using guide RNA/Cas9 endonuclease complex. This mutation resulted in an in-frame deletion of 33 amino acids including the removal of sequences encoding for catalytic residues DCE within the DCEW motif. The deletion of *Exd2* did not affect the size of the testes (I: Figure 5C) and the histology showed normal organization of the seminiferous epithelium. In addition, there was no effect on the progression of germ cell differentiation and $Exd2^{\Delta}$ mice were fertile, indicating that the catalytic activity of EXD2 is not required for spermatogenesis.

We then decided to explore the effect of *Exd2* deletion on piRNA expression since our mass spec result showed that EXD2 is an IMC component and therefore, potentially involved in the piRNA pathway. To investigate the piRNA status, we analysed small RNAs in the $Exd2^{\Delta}$ testes and compared the result with control testes. There was no difference in the relative abundance or size distribution of piRNAs in the $Exd2^{\Delta}$ and control testes (I: Figure 5D), suggesting that generally piRNA biogenesis was not affected and this would explain why spermatogenesis was not affected. These results showed that the DCE motif of EXD2 which is responsible for 3'–5' exonuclease activity is not required for the production of piRNAs.

During postnatal spermatogenesis, piRNAs are produced from genomic piRNA clusters (A. Aravin et al., 2006). Therefore, we further analysed the levels of piRNAs from individual piRNA clusters. We showed that twenty clusters were downregulated ($\log2FC < -1$, $padj \leq 0.05$) and only one cluster was upregulated

(log2FC > 1, padj ≤ 0.05) (I: Figure 5G). This imbalance of piRNAs suggests that deletion of the DCEW motif indeed affected the piRNA processing from specific clusters although there was no effect on overall piRNA production and no effect on male fertility. The reduced number of piRNAs produced from these specific clusters of the *Exd2* mutant does not correlate with the levels of their precursor transcripts, therefore the reduced amount of the precursors cannot explain the redueced levels of piRNAs produced. Our preliminary analysis failed to identify any speicifc features in these clusters that would target them for EXD2-dependent regulation (e.g. epigenetic features etc.), Further investigation is required to understand the specific role of EXD2 in the expression of piRNAs from specific clusters during the male germ cell differentiation.

Unfortunately, our study on the role of EXD2 *in vivo* remained quite superficial due to the very limited amount of material for the analysis of *Exd2*$^{\Delta}$ mice. The mouse line had been discontinued earlier, and we only had some frozen testes available for the validation of the mutation and for the RNA-seq analysis. Therefore, we only confirmed the deletion by genomic PCR and also by RNA sequencing analysis. Due to the limited material we were not able to verify the effect of deletion at the protein level by western blotting or immunostaining. Therefore, we do not know if the frame shift mutation caused total absence of the protein, or production of a truncated protein. Owing to these limitations, we can conclude that the absence of DCEW motif in EXD2 did not affect for the overall production of piRNAs, eventhough there is slight imbalanced in piRNA population from specific clusters. This is a novel finding that is important for the field. It is clear that further investigation is required to clarify the role of EXD2 in spermatogenesis, and the detailed analysis of spermatogenesis and the germ granules in *Exd2*$^{\Delta}$ mice should be carried out in the future.

Furthermore, although we showed that the DCEW motif of EXD2 is not required for piRNA biogenesis and spermatogenesis, we cannot rule out the possibility that other functional domains are sufficient to carry out the critical functions of EXD2. Therefore, a full knockout mouse model that fully abolishes the expression of EXD2 will enable us study the role of EXD2 during spermatogenesis. Given that EXD2 is not a germline-specific protein, if the full knockout is embryonic lethal, then a germ cell-specific knockout mouse model would be required in order to gain insight into the germline-specific role of EXD2. In addition, further biochemical analysis should be employed to study different functional domains of EXD2 and this will provide more mechanistic insight into the specific role of EXD2 in different cells, including germ cells.

5.3 EXD1 functions with TDRD12 to promote piRNA biogenesis in embryonic mouse testes

From our mass spec analysis of PIWIL2-associated IMC, we identified EXD1 as one of the novel proteins interacting with piRNA machineries in the IMC of spermatocytes. We also characterized EXD1 localization in the IMC and CB during postnatal spermatogenesis. Immunofluorescence staining of the earlier testes time point (P0) showed a strong signal of EXD1 in the cytoplasm of developing germ cells (1: Figure S1C), suggesting that EXD1 might already have a notable function during embryonic development of the testes before birth. In fact, piRNA functions, including its biogenesis and prevention of invasion by transposable elements, are already initiated during the pro-spermatogonium development (Watanabe et al., 2018).

Apart from PIWIL4 and PIWIL2 proteins which are widely known as the main PIWI proteins functioning in the embryonic testes, Pandey *et al* investigated one of the Tudor domain containing proteins, TDRD12, as an essential factor for piRNA biogenesis, silencing of transposable elements and spermatogenesis (Pandey et al., 2013). Homozygous *Tdrd12* mutant mice were infertile with atrophied testes, and this phenotype already originated during the embryonic development. The heterozygous *Tdrd12* mice were completely fertile and their testes contained all the developing germ cells (Pandey et al., 2018). The Tudor family of proteins are recognized as adaptors having dimethyl arginine at their N-terminus which forms complexes with PIWI proteins for posttranscriptional control of RNAs (Siomi et al., 2010). Yang *et* al further demonstrated by *in vitro* studies that TDRD12 requires an RNA-binding protein EXD1 to form a PET (PIWI-EXD1-TDRD12) complex with PIWIL4 to promote nuclear production of piRNAs and silencing of transposons during the development of embryonic testes (Z. Yang et al., 2016). EXD1 has Lsm domain, which forms complex with the helicase domain of TDRD12. Yang *et al* further showed that, although, the deletion of *Exd1* did not affect male fertility, there was a notable reduction in the PIWIL4-associated piRNA production (Z. Yang et al., 2016).

Therefore, in order to understand the specific function of EXD1 and how it functions together with TDRD12 to promote piRNA biogenesis in embryonic testes, we carried out deletion of *Exd1* in *Tdrd12* heterozygous background to generate sensitized *Exd1* mutant ($Exd1^{-/-}; Tdrd12^{+/-}$) (II: Figure 1A) and studied the effect on germ cell development and postnatal spermatogenesis.

5.3.1 *Exd1* deletion in *Tdrd12* heterozygous background disrupts postnatal spermatogenesis

While $Exd1^{-/-}$ and $Tdrd12^{+/-}$ male mice have normal testis histology and fertility, the testicular size of the sensitized Exd1 ($Exd1^{-/-}; Tdrd12^{+/-}$) adult mice was relatively smaller than the control testes (II: Figure 1B), suggesting that there is a functional

connection between *Exd1* and *Tdrd12*. To begin to understand the effect of *Exd1* deletion in *Tdrd12* heterozygous background, we first analyzed the histology of the adult testes. The result showed disruption of spermatogenesis. The arrest was not uniform, but different cross-sections of the seminiferous tubules appeared to be arrested at different phases of differentiation. Many seminiferous tubules showed the presence of mainly Sertoli cells and pre-meiotic or early meiotic cells (II: Figure 1C), while some other sections contains a few pachytene spermatocytes or round spermatids and elongating spermatids (II: Figure S1E,F). We also analyzed the histology of the cauda epididymis where mature spermatozoa are temporarily stored before ejaculation. Indeed, the cauda epididymis of the sensitized *Exd1* mutant testes showed an absence of matured sperm (II: Figure 1D).

In order to further characterize the spermatogenic defects, we performed immunofluorescence analysis of the sensitized *Exd1* mutant testes with various molecular markers. Sertoli cells (immunostained with anti-SOX9) and undifferentiated spermatogonia stem cells (immunostained with anti-LIN-28) were present in both sensitized *Exd1* mutant and control testes (II: Figure 1E). Detailed examination of the pachytene spermatocytes using antibodies against the synaptonemal complex (anti-SCP3) and sex body (anti-γH2AX) showed lack of a synaptonemal complex and a sex body-containing pachytene spermatocytes in about 27% of the seminiferous tubules in the sensitized *Exd1* testes (II: Figure 1F,G and H), suggesting an arrest at early meiosis during spermatogenesis. About 30% of the seminiferous tubules were positively stained by SCP3 antibody but not positive for anti-γH2AX, suggesting that they were arrested at the leptotene and zygotene spermatocyte stages after the induction of SCP3 expression but before the sex body formation (II: Figure 1H). In some tubules spermatogenesis had progressed further, and staining with anti-DDX4 antibody and PNA lectin showed that approximately 23% of the tubules had round spermatids and 7% had elongating spermatids (II: Figure 1F,I). The arrest at the early spermatocyte stage is non-uniform in the seminiferous tubules of sensitized *Exd1* mutant testes, an observation which is not the case in homozygous *Tdrd12* mutant testes as they have a uniform arrest at an early meiotic phase in all the seminiferous tubules (Pandey et al., 2018).

To study the localization of piRNA-binding PIWI proteins in sensitized *Exd1* mutant testes, we performed immunostaining using antibodies against DDX4, PIWIL2 and PIWIL1. Both DDX4 and PIWIL1 were localized to the CB and were unaffected in sensitized *Exd1* mutant compared to control testes (II: Figures S2A, S2B, S2C and S2D). However, localization of PIWIL2 was affected by the deletion of *Exd1* in *Tdrd12* heterozygous background. PIWIL2, which normally localized to the germ granules, appeared diffused in the cytoplasm of the pachytene spermatocytes of sensitized *Exd1* mutant testes, suggesting a possible defect in the

piRNA processing pathway, particularly in the IMC, which affects the fertility of the mice.

We further examined the juvenile testes at P24 to understand if the arrest we observed in the early meiotic spermatocytes occur already during the first wave of spermatogenesis. Acrosome staining with PNA lectin indicated that at P24, the spermatogenic cells had progressed until the round spermatid development in the *Exd1* mutant and control testes, but not in sensitized *Exd1* mutant and homozygous *Tdrd12* mutant testes (II: Figure 2A,S1G). Quantification showed that about one third of the seminiferous tubules in sensitized *Exd1* mutant P24 testes did not reach the pachytene spermatocyte phase as indicated by the absence of SCP3-staining in the seminiferous tubules (II: Figure 2B). In the homozygous *Tdrd12* mutant testes, both SCP3- and sex body-positive pachytene spermatocytes were completely absent (II: Figure 2B) and 23% of the tubules contained only spermatogonia and Sertoli cells (II: Figure 2B).

Given the already reported function of TDRD12 and EXD1 in forming a PET complex to promote piRNA biogenesis and repress transposable elements (Pandey et al., 2013; Z. Yang et al., 2016), we wanted to understand whether the infertility in sensitized *Exd1* mutant is connected to de-repression of transposable elements. To achieve this, we performed immunofluorescence staining and western blotting analysis of adult sensitized *Exd1* mutant, homozygous *Tdrd12* mutant and control testes with antibody against L1ORF1p, which is protein expressed from Line1 (L1) elements. Immunofluorescence analysis revealed high expression of L1 elements in 75% seminiferous tubules of sensitized *Exd1* mutant testes, and 24% of the tubules in homozygous *Tdrd12* mutant were positive for L1. The lower percentage of L1 expressing cells in homozygous *Tdrd12* mutant cells is most probably due to the dramatically reduced number of meiotic cells that usually show transposon expression. This high expression of L1 in sensitized *Exd1* mutant was confirmed by immunofluorescence staining (II: Figure 2C,D,E,F). These results showed that, even though the deletion of *Exd1* has a mild effect on the L1 elements which did not affect spermatogenesis, the deletion of the *Exd1* in *Tdrd12* heterozygous background caused concomitant de-repression of L1, resulting in the arrest of spermatogenesis and male infertility.

5.3.2 *Exd1*$^{-/-}$ deletion in *Tdrd12* heterozygous background affects the production of PIWIL4-associated secondary piRNAs

TDRD12, PIWIL4 and PIWIL2 all contribute to the production of embryonic piRNAs (Kuramochi-Miyagawa et al., 2008b; Pandey et al., 2013; Z. Yang et al., 2016). Due to the massive transposon de-repression and the affected PIWIL2

localization, we wanted to clarify whether PIWIL4-associated piRNAs were affected in the neonatal sensitized *Exd1* mutant testes. To examine this, we first analyzed the total small RNAs (20–40 nt) from neonatal (P0) testes of the sensitized *Exd1* mutant (*Exd*$^{-/-}$; *Tdrd12*$^{+/-}$) compare to the control testes (*Exd1*$^{+/-}$;*Tdrd12*$^{+/-}$) (II: Figure S3A). The piRNA-sized reads at 26–28 nt were found in both the sensitized *Exd1* mutant and the control (II: Figure 3A,3B). Interestingly, an additional peak of 30–34 nt was observed in the sensitized *Exd1* mutant (II: Figure 3A,3B) that was absent in the control as well as in *Exd1* mutant without deleted *Tdrd12* allele (Z. Yang et al., 2016). This peak corresponded to the tRNA-derived small RNAs (tsRNAs) produced from the 5' end of mature tRNAs (II: Figure S3I). Previous studies have found that the expression of tsRNAs can be induced under unfavorable conditions such as stress (Pan et al., 2021; Sanadgol et al., 2022), and the expression of tsRNAs have been implicated in different diseases (Chujo & Tomizawa, 2021). It is currently unknown why tsRNA expression is induced in the sentisized *Exd1* mutant. It is, for example, possible that the disruption of the piRNA pathway, which leads to de-repression of transposons, cause an increase in tsRNA expression due to the cellular stress. Further investigation is needed to understand the connection between deletion of piRNA components and expression of tsRNAs as well as their role in infertility.

Further analysis of the piRNAs originating from repeat sequences showed piRNAs mapping to repeats in both a sense and an antisense orientation were reduced, with antisense piRNAs showing the most dramatic reduction (2.5-fold decrease) in sensitized *Exd1* mutant compared to the control (II: Figures S3C, S3D). Antisense piRNAs mapping to both non-LTR (L1 and SINE) and LTR transposable elements were shown to be affected (II: Figure 3D–G, S3D). These results suggest that loss of *Exd1* in *Tdrd12* heterozygous background caused an impairment in the piRNA production.

In the embryonic testes, antisense piRNA production is dependent on a ping-pong amplification mediated by PIWIL2 slicing and PIWIL4 (Z. Yang et al., 2015). To show that *Exd1* is functioning in this secondary biogenesis of piRNAs in embryonic testes, we performed PIWIL2- and PIWIL4-immunoprecipitation, using biological replicate samples of P0 testes (infertile sensitized *Exd1* mutant and fertile *Exd1*$^{-/-}$), followed by a 5' labelling to visualize any associated small RNAs and a deep sequencing for sequence-level analysis. Indeed, PIWIL4-piRNAs were reduced in the sensitized *Exd1* mutant, while PIWIL2-piRNAs were not affected (II: Figure 4A). Sequencing showed that the antisense repeat piRNAs that mapped to L1 elements were most dramatically affected (II: Figure 4C). The reduced production of PIWIL4-associated antisense piRNAs is most probably sufficient to activate de-repression of transposable elements.

What is interesting is that the reduction of antisense piRNAs observed in the infertile sensitized *Exd1* mutant is similar to that of fertile *Exd1* mutant mice.

Therefore, this overall reduction of antisense piRNAs cannot explain the infertile phenotype of sensitized *Exd1* mutant mice. The reduction of PIWIL4-associated secondary piRNAs, particularly the antisense L1 piRNAs, was somewhat more pronounced in the infertile sensitized *Exd1* mutant than in the fertile *Exd1* mutant (II: Figure 4A). It is possible that this difference in the loading of antisense L1 piRNAs to PIWIL4 in the absence of other *Tdrd12* allele causes more dramatic de-repression of LINE elements in the sensitized *Exd1* mutant compared to fertile *Exd1* mutant, leading to infertility. However, the exact reason for the spermatogenic defects in the sensitized *Exd1* mutant still remains to be elucidated.

5.3.3 Function of EXD1 during postnatal spermatogenesis

We have already shown that EXD1 cooperates with TDRD12 to promote the production of PIWIL4-associated piRNAs in the embryonic testes, and is reflected as de-repression of L1 retrotransposons. The defects in the transposon silencing in embryonic germ cells during the epigenetic resetting usually results in male infertility with the arrest of postnatal spermatogenesis at the early meiotic phase (Zamudio et al., 2015). The spermatogenesis phenotype of the sensitized *Exd1* mutant resembles the phenotype of these mice, and therefore, it is likely that the phenotype originates from the embryonic function of EXD1 and TDRD12 in the production of PIWIL4-associated secondary piRNAs.

We have shown that EXD1 is also expressed during postnatal spermatogenesis, where it localizes to the IMC together with PIWIL2 and TDRD12 (I: Figure 1C). Therefore, it is likely that EXD1 continues functioning in the postnatal testes after it participates in the production of PIWIL4-associated piRNAs in embryonic germ cells. PIWIL4 is expressed only in embryonic germ cells, and the relative amount of transposon-targeting secondary piRNAs is much lower in postnatal germ cells than in embryonic germ cells (Kuramochi-Miyagawa et al., 2008a). However, transposons are known to be expressed in meiotic spermatocytes that have an open chromatin and are broadly expressing their genome (Soumillon et al., 2013), and it is possible that EXD1 still participates in piRNA production and transposon silencing in meiotic and early postmeiotic cells. Both the transposable element transcripts and the piRNA machinery, including EXD1, are found in the CB in round spermatids (Meikar et al., 2010b, 2011b, 2014; Meikar & Kotaja, 2014), raising a possibility that the CB could also be involved in the transposon silencing.

Unfortunately, the existing mouse models do not allow us to address the postnatal function of EXD1/TDRD12 due to potential secondary effects caused by the defective embryonic germ cell regulation that interferes with the evaluation of the direct effects on postnatal spermatogenesis. Therefore, mouse models with different conditions would be required to dissect the function of EXD1/TDRD12

complexes in postnatal spermatogenesis., e.g. by using a Cre-loxP system and promoters that induce CRE expression and gene deletion specifically in the germline after birth.

5.4 The germ granule protein DDX4 form cytoplasmic granules in many different types of cancer

Germline-specific genes are often found to be expressed in cancer cells, and due to their tissue-restricted expression pattern and immunogenic property, these cancer-germline antigens (CGAs) are considered potential therapeutic targets in the treatment of cancer (Fratta et al., 2015; Mcdermott & Atkins, 2013; Nin & Deng, 2023). Germline-specific genes are repressed in somatic tissues by epigenetic mechanisms such as DNA methylation (Loriot et al., 2009). Cancer cells are shown to undergo general hypomethylation of their genome, which could be responsible for activation of these germline genes in cancer cells (Charles De Smet & Loriot, 2013). However, the exact mechanisms and reasons for the expression of germline-specific genes in cancer is not understood.

DDX4, which is one of the major components of germ granules, is an RNA-binding protein belonging to the super family of DEAD-box helicases (Linder, 2006). It has previously been shown to be expressed in different human cancers (Y. Chen et al., 2018b; K. H. Kim et al., 2014; Lee et al., 2018, 2018; Noyes et al., 2023; Schudrowitz et al., 2017), hence classified among the CGAs (Cheng Wang et al., 2016). However, the cytoplasmic localization of DDX4 in cancer cells has not been investigated, and its functional role in cancer progression has remained unclear. In this study, we wanted to investigate the role of DDX4 expression in cancer cells, utilizing our knowledge and expertise on germline and germ granules. Interestingly, we were able to show that 22 of the published CB components (Meikar et al., 2014), including DDX4, were also identified as CGAs (III: Figure 1A).

We then investigated a panel of different somatic cancer samples, both epithelial cancers (breast, colon and lung adenocarcinoma) and connective tissue cancers (fibrosarcoma and leiomyosarcoma) by immunostaining, and showed that DDX4 is widely expressed as prominent cytoplasmic granules in different human cancer samples and not in normal somatic tissues (III: Figure 1B). We also found DDX4 granules in xenograft tumors originated from human head and neck squamous cell carcinoma cell line UT-SCC-14 and prostate cancer cell line PC3, which enabled us to proceed to functional studies using animal models (III: Figure 2A,B,C). Interestingly, DDX4 granules did not appear in the cytoplasm of cancer cells when they were cultured in 2D-culture plate. We carried out western blotting of xenograft tumor samples and showed that the intensity signal of DDX4 protein expression was

significantly lower in the PC3 cells (cultured on 2D-plate) compared to PC3-derived xenograft tumor (III: Figure 2D).

These results suggest that DDX4 only form cytoplasmic germ granule-like granules in cancer cells when they are grown in an environment that mimics their natural microenvironment but not when cultured as a monolayer. Cancer cells have a special capability to interact with their surrounding cells and this creates a microenvironment that promotes extracellular matrix formation, immune cell infiltration, communication with stromal cells, blood vessels formation by angiogenesis, and other processes that support their survival and cancer progression (N. M. Anderson & Simon, 2020). Cancer cells grown on a 2D petri dish-based culture environment only form cell monolayers, and this environment does not mimic the tumor microenvironment (Pampaloni et al., 2007). In many cases, to study the effects of drugs in cancer treatments or for effective investigation of cancer cell properties, they are either grown in 3D-cultures of *in vivo* xenograft models (Barbosa et al., 2021). In this study, the presence of DDX4 granules in tumors but not in 2D cultured cancer cells suggests that the 3D or tumor environment is required for the localization of DDX4 in cancer cells. Since the presence of DDX4 granules is indicated in tumor formation, further studies are needed to clarify whether the 3D or tumor environment is directly required for cytoplasmic DDX4 localization in cancer cells.

In this study, we observed the heterogeneous localization of DDX4 in the cytoplasm of tumor cells. We also observed that not all cancer cells (neither in the immunostained patient samples nor in the tumor xenograft samples) have conspicuous DDX4-positive granules, as always seen in differentiating germ cells. During their clonal expansion, cancer cells are highly heterogeneous as many of them are in different stages of progression. Consequently, the inconsistent DDX4 staining in cancer cells may be a possible indication of the status of cancer cell growth, especially in a tumor environment. Heterogeneity in cancer is one of the major causes of therapy resistance in cancer cells. Further investigation needs to be conducted to understand the role of cytoplasmic expression of DDX4 in individual cancer cells.

5.5 *DDX4* deletion delay tumor growth

Many DEAD box helicases have been shown to have an oncogenic function in promoting cancer formation (Cargill et al., 2021; Fuller-Pace, 2013). To investigate the physiological role of *DDX4* in cancer cells, we performed a CRISPR/Cas9-mediated deletion in PC3 cells, specifically targeting exon 11 of *DDX4*, which resulted in the loss of 103 nucleotides inducing frame-shift mutation (III: Figure 3A,B,S1A). Two *DDX4* mutant cell clones were generated with the same deletion,

and they were referred to as *DDX4*-null PC3 cells. The deletion of *DDX4* did not cause any significant change in the proliferation rates of *DDX4*-null compared to WT PC3 cells, (III: Figure 3C) and there was no difference in the number of apoptotic cells (III: Figure 3D).

Based on our observation that there was no significant difference in the proliferative capability of *DDX4*-null cells compared to WT PC3 cells, we decided to grow the cancer cells in an environment that mimics the tumor microenvironment and check whether the deletion of *DDX4* would affect the tumor formation or growth. Firstly, PC3 cells were grown in a miniaturized 3D culture environment with matrigel which is enriched with extracellular matrix proteins, and the progress of spheroid formation was monitored at two different time points (day 5 and day 10). The result showed that the deletion of *DDX4* compromised the spheroid (tumor) formation in *DDX4*-null PC3 cells. The area of spheroids formed at day 5 and was significantly smaller in *DDX4*-null PC3 cells compared to the WT and these differences were more pronounced at day 10 (III: Figure 3E,F). Previous 3D *in vitro* studies have shown that at day 9, spheroids formed by PC3 cells start to possess invasive metastatic potentials, such as formation of protrusions described as stellates at day 14 (Härmä et al., 2010). Using a similar approach, we examined whether deletion of *DDX4* affects the invasive properties of PC3 cells and indeed the length of invasive structures of spheroids formed by *DDX4*-null PC3 cells was significantly reduced when compared to WT PC3 spheroids ($P<0.0001$) (III: Figure 3G). We then performed immunofluorescence staining of the *DDX4*-null spheroids with antibody against Vimentin, an established marker for cancer metastasis (Gebhard et al., 2016; Usman et al., 2021) and showed that Vimentin signal in *DDX4*-null PC3 spheroids was disorganized (III: Figure 3H). This suggests that the invasive potential was affected upon *DDX4* deletion. Importantly, we repeated the same experiment in another cancer cell line (UT-SCC-14 cells) (III: Figure S1B,C), and showed that the deletion of *DDX4* also affected spheroid formation and the invasive potential of UT-SCC-14 cells in the 3D culture (III: Figure S2A,B).

We confirmed the results observed in the *in vitro* 3D culture condition by performing *in vivo* subcutaneous inoculation of WT and *DDX4*-null PC3 cells in male athymic nude mice. Upon monitoring the growth of subcutaneous tumors over a period of four weeks, we showed that the deletion of *DDX4* caused significant reduction in the size of the *DDX4*-null PC3 tumors compared with WT PC3 tumors throughout the entire period ($P<0.0001$ on week 1, week 2 and week 3; $P=0.0151$ on week 4) (Fig. 3I). We validated *DDX4* deletion in a PC3 tumor by performing immunofluorescence staining with an antibody against DDX4 and showed that the DDX4-positive granules are absent in *DDX4*-null PC3 tumor cells (III: Figure 3K). When we repeated the same xenograft experiment with UT-SCC-14 cells, we observed the same result, confirming that deletion of *DDX4* in cancer cells causes

delay in tumor growth (III: Figure S2D–G). This result suggests that DDX4 promotes tumor growth, and the germline-specific functions of DDX4 that are normally required for germ cell differentiation are utilized in cancer cells to promote malignant properties.

5.6 *DDX4* deletion affects cancer transcriptome

To gain more insight into the role of *DDX4* in cancer progression, we isolated total RNA from monolayer-grown PC3 cells WT and *DDX4*-null PC3 cells and xenograft-derived WT and *DDX4*-null PC3 tumors, then performed RNA-sequencing analysis to study the effects of *DDX4* deletion on cancer cell transcriptome. The result revealed a dramatic transcriptome imbalance in monolayer-grown *DDX4*-null PC3 cells compared with WT PC3 cells (III: Figure 4A,S3A, Table S1A). Out of the over 3000 dysregulated genes, 2196 genes were upregulated ($|Log2FC| \geq 1$, p-adjust value ≤ 0.05) and 1328 genes downregulated ($|Log2FC| \leq -1$, p-adjust value ≤ 0.05). In the case of *DDX4*-null PC3 xenograft tumors, 88 genes were upregulated and 54 downregulated compared to WT PC3 tumors (Figure 4A, Table S1B from paper III). About 95% of the differentially expressed genes in tumors were also differentially expressed in cultured cells. We found much fewer differentially expressed genes in tumors compared to cells. One reason for this could be the heterogeneity of tumors; when the tumor grows in *in vivo* environment, the tumor mass consist of heterogenous population of cells and different tumors may have differences in the balance between the cell populations. Increasing the number of tumor samples in the RNA-seq analysis could have revealed more differentially expressed genes. Due to tumor heterogeneity, it would be interesting to analyze the cancer cells by single-cell RNA sequencing to better understand the role of DDX4-mediated RNA regulation in tumor progression.

GO term enrichment analysis of the misregulated genes revealed enrichment of genes from biological processes connected to cell-cell adhesion, cell junctions and cell migration, which are processes that are known to promote cancer progression (III: Figure S3B–E). This suggests that cancer cells utilize DDX4-mediated transcriptome regulation to support malignant properties.

It was interesting to discover that cultured PC3 cells had such massive transcriptome imbalance even though we did not observe any significant change in the proliferation capacity of *DDX4*-null PC3 cells compared with the WT PC3 cells, or any significant change in the apoptosis. According to the RNA-seq analysis, many genes involved in cell migration and cell-cell adhesion were upregulated in the absence of *DDX4* in PC3 cells. However, cell migration assay did not show any significant difference in migration of the *DDX4*-null cells compared to the WT cells (data not shown). The effects of *DDX4* deletion only becomes apparent when the

cells were grown either in a 3D culture or as xenografts. This suggests that DDX4 also regulates gene expression in cultured cancer cells, but the misregulation of these genes did not disrupt the proliferation or migration of PC3 cells. Instead, the misregulation interferes with the capacity of cancer cells to form tumors. It is also possible that the imbalance in the transcriptome triggered signalling pathways that compensated for the loss of *DDX4* in cancer cells cultured under 2D conditions. Future studies should be conducted to determine the precise role of *DDX4* in 2D-cultured cancer cells, for example, in regulatingcancer cell properties such as stemness.

5.6.1 Expression of tumor suppressors and genes involved in epithelial-mesenchymal transition is mis-regulated in *DDX4*-null cancer cells

To further understand the role of *DDX4* in tumor progression, we carried out literature search on different pathways of some of the commonly affected genes in cancer progression and metastasis. Genes that are notable for tumor suppressive functions were mostly effected. Firstly, we compared the misregulated genes in *DDX4*-null PC3 tumors with the genes that have been shown to function as tumor suppressors as enlisted in the database (https://bioinfo.uth.edu/TSGene/). Our result revealed 15 known tumor suppressors among differentially expressed genes in *DDX4*-null PC3 tumors. Interestingly, 12 of these (*OPCML, NDN, PPP1R1B, DCC, CTDSPL, CAMK2N1, DIRAS1, PROX1, GJB2, LOX, GJA1, and CEACAM1*) were upregulated (III: Figure 4B,C), suggesting that DDX4 negatively regulates these tumor suppressors, and their upregulation upon *DDX4* deletion protects the cells from cancerous behaviour. Seven of these genes (*CTDSPL, CAMK2N1, PPP1R1B, PROX1, LOX, DIRAS1 and GJB2*) were also upregulated in *DDX4*-null PC3 cells grown in a monolayer (III: Table S1).

Supporting our results that deletion of *DDX4* affects the invasiveness of DDX4-null PC3 spheroids, many genes shown to promote epithelial-mesenchymal transition (EMT) in cancer metastasis were also misregulated in *DDX4*-null cells. For example, the disintegrin and metalloprotease 12 (ADAM12) involved in extracellular matrix organization has been shown to promote gastric cancer metastasis (H. Zhu et al., 2022), was among the downregulated genes in *DDX4*-null PC3 tumor (III: Figure 4C,D). In addition, genes belonging to cadherin family, such as *CDH6* and *CDH7* that are involved in the EMT pathway and cancer metastasis (Machado et al., 2019; Z. Zhao et al., 2021) were downregulated in *DDX4*-null PC3 tumors (III: Figure 4C) as well as in *DDX4*-null PC3 cells (III: Table S1). The results of mRNA dysregulation of these genes (*ADAM12, CDH6* and *CDH7*) and other genes (*CEACAM1, SFRP1* and *CLEC2B*) that have supportive functions in tumor

formation and progression, were validated by RT-qPCR (III: Figure 4D). When expressed during spermatogenesis, DDX4 functions to support germ cell differentiation and development of spermatozoa. Cancer cells have the capacity to differentiate when in their natural environment and this enhances their metastatic potential such as expression of EMT pathway components. Therefore, it is possible that cancer cells also harness DDX4-mediated differentiation function to regulate the expression of EMT proteins, and this is compromised in *DDX4*-null PC3 tumors.

Overall, our transcriptome analysis provides a starting point for mechanistic studies. Further studies are needed to investigate the direct effects of *DDX4* deletion on various signaling pathways involved in carcinogenesis and metastasis. This will elucidate the molecular mechanism of the role of DDX4 in cancer, thus supporting our previous findings from 3D culture and xenograft experiments showing that tumor formation and growth are impaired in the absence of DDX4.

5.6.2 Role of DDX4 in the regulation of splicing landscape

DDX4 belongs to the DEAD-box family of helicase proteins that are widely implicated in RNA metabolism (Linder & Jankowsky, 2011). To elucidate the mechanisms by which DDX4 may regulate cancer transcriptome, we first characterized the proteins that are interacting with DDX4 in the cytoplasm of cancer cells. In order to achieve this, we used an antibody against DDX4 to perform immunoprecipitation of DDX4 from PC3 subcutaneous xenograft tumors, followed by mass spectrometry analysis to identify DDX4-interacting proteins. We were unable to use the previously established CB-isolation protocol (Meikar et al., 2010b) because of the smaller size of cancer-associated DDX4-granules, which prevented them from forming pellet during the centrifuation step of the isolation protocol. Therefore, we used the native co-immunoprecipitation protocol without the cross-linking approach. With this protocol, we could not ensure the purity of the isolated DDX4 granules because there may be other pulled interaction partners that are widely distributed in the cytoplasm. Therefore, the localizaton of interaction partners to DDX4 granules should be verified by co-immunofluorescence in the future.

We identified 214 proteins that formed a complex with DDX4 in two biological replicate immunoprecipitation samples, but were not found in the IgG control samples (III: Table S2A). We conducted GO term analysis and showed that enrichment of biological processes connected mostly to different posttranscriptional RNA regulatory processes, such as translation, regulation of splicing, mRNA processing, RNA transport and RNA localization (III: Figure 5B, Table S2B). Importantly, the DDX4-complexes included proteins earlier identified as germ granule components (Meikar et al., 2014), indicating that germ granule-related functions may operate in cancer cells.

Interestingly, proteins involved in the regulation of splicing were enriched among the interacting partners of DDX4 (III: Figure 5B, Table S2B). Alternative splicing is one of the RNA regulatory mechanism driving cancer progression (Bonnal et al., 2020; Martinez-Montiel et al., 2018; Y. Zhang et al., 2021). We did not observe any of the proteins associated with core spliceosome as DDX4-interacting proteins. This is in line with the spliceosome-mediated splicing taking palce in the nucleus and DDX4 granules being present in the cytoplasm. Therefore, we speculate that DDX4 could be involved in the regulation of spliced transcripts that are exported to the cytoplasm during cancer formation and progression. We therefore analyzed five different splicing events in *DDX4*-null versus WT PC3 cells using the RNA sequencing data: skipped exon (SE), alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), mutually exclusive exons (MXE) and retained intron (RI) (III: Figure 5D). The result showed significant changes in the SE, MXE, A3SS, A5SS and RI, confirming our hypothesis that DDX4 contributes to the regulation of the balance between the differentially spliced transcripts in cancer cells.

Interestingly, while SE, MXE, A3SS and A5SS events were equally divided between a decrease or an increase, the majority of RI events were decreased (25 increased, 160 decreased) (III: Figure 5DE). There are studies showing that the genomic instability in cancer cells cause accumulation of intron-containing transcripts, which contributes to cancer metastasis and prognosis (Dong et al., 2022; Dvinge & Bradley, 2015; Shah et al., 2022), and it is tempting to speculate that DDX4 could regulate the intron-containing transcripts. However, how the regulation takes place is still unclear. Spliceosome-mediated splicing takes place in the nucleus, and the mature mRNAs are then exported to the cytoplasm for translation or storage. Although there are some studies showing that splicing can also occur in the cytoplasm after nuclear export of intron-containing transcripts (Buckley et al., 2014); it is not very likely that cytoplasmic DDX4 is involved in the alternative splicing process, but rather in the regulation of spliced transcripts. Our results suggest that spliced transcripts that are still bound with specific splicing regulators are exported to the cytoplasm where they are associated with DDX4 granules for futher regulation. Cancer cells are known to have impaired splicing landscape (Aktas Samur et al., 2022; Bessa et al., 2020) and increased intron retention (D. Zhang et al., 2020). It is possible that improperly spliced gene products are transported into the cytoplasmic DDX4 granules for the regulation of their fate. For example, DDX4 could stabilize them to enhance cancer progression and metastasis. In this way, *DDX4* deletion could lead to the altered levels of intron retention in cancer cells. Further studies are needed to test this hypothesis.

5.7 DDX4 is a prognostic marker in human Head and Neck squamous cell carcinoma and prostate cancer

Having shown that DDX4 is widely expressed in different somatic malignancies, we wanted to investigate the prognostic property of DDX4-positive granules in cancer. Due to our functional studies on UT-SCC-14 and PC3 cell lines, we focused on HNSCC which is a non-hormonal cancer and prostate cancer (PC) which is a hormonal cancer. We performed immunostaining of the cancer samples with antibody against DDX4 and scored the presence of DDX4-positive granules in 46 HNSCC and 166 PC patient samples. In HNSCC, out of 46 patients' samples, 34 samples of HNSCC showed cytoplasmic DDX4-positive granules, and the expression was significantly associated with the size of the HNSCC tumor in HNSCC patients ($p<0.08$). We then correlated the result of the DDX4-positive granules scores with the patient's survival, and interestingly, the expression of DDX4 in HNSCC samples was significantly associated with poor survival (5 years) ($p<0.0075$) (III: Figure 6A).

In the case of PC patients, 122 of the 166 samples showed cytoplasmic DDX4-positive granules. Correlation of the expression of DDX4-positive granules in PC epithelial cells with the Gleason score pattern showed that the expression of DDX4 in the prostate cancer epithelial cells was significantly associated with the higher Gleason pattern in the core ($p=0.0234$). Furthermore, in the lower Gleason grade, there is a trend towards a worse progression of free survival in patients with DDX4-positive granules compared with patients without DDX4-positive granules ($p=0.0833$) (III: Figure 6B).

These are promising results since they demonstrated that the expression of DDX4 in cancer samples could be used as an indicator for patients' poor survival, therefore highlighting DDX4 as a potential prognostic marker. This study only addressed the granular expression of DDX4 in cancer, so further investigation should be carried out on the serum, blood or urine samples of the cancer patient to show the level of DDX4 compared to samples from healthy subjects. Many of the tumor antigens end up in body fluids, such as blood serum and urine; and are therefore used as cancer biomarkers (Duquesne et al., 2017; Tang et al., 2017). Our result showed that DDX4 is not just a germline-specific protein but also expressed in different tumors of diverse origins (both epithelial and connective tissue-tumors). There is the likelihood that DDX4, like other tumor antigens leaks into the surrounding tumor and can be circulated in the blood serum or urine. Therefore, it would be interesting to detect and measure the level of DDX4 in the blood serum and urine samples of cancer patients, in comparision to the results from non-cancer patients' samples.

DDX4, being a member of the DEAD-box family of helicases with ATP-binding activity, is also a potential drug target for cancer treatment. However, the high

similarity in the domain structure of the DEAD-box helicases may cause some challenges in the drug design because of the difficulty to only target the specific helicases. The germline-restricted expression of DDX4 increases its value as a potential drug target, since the only expected side effect of targeting DDX4 would be infertility in males but not in females. The majority of cancer cases are often diagnosed in older patients, most of whom have already passed the reproductive age, so in these cases infertility would not be considered as a serious side effect.

5.8 DDX4 in transcriptome regulation in cancer vs. germ cells

Cancer cells share a considerable number of similarities with germ cells, one of these is the massive epigenetic remodeling and widespread expression of the genome, resulting in diverse transcriptome (Soumillon et al., 2013; Zatzman et al., 2022). Germ cells have specific RNA regulatory mechanisms, including germ granules, to cope with the diverse transcriptome. Our results show that the activation of these germline-specific RNA regulatory mechanism may be used in cancer cells to regulate the transcriptome and to support cancer progression and metastasis.

In differentiating male germ cells, the transcriptome diversity peaks in meiotic spermatocytes and round spermatids, and these cells also have an unusually complex splicing landscape. Interestingly, just like germ cells, cancer cells also have a very high level of alternative splicing events (Qi et al., 2020; Song et al., 2020), which increases transcriptome diversity. During spermatogenesis, the high demand of RNA regulation is accompanied by the appearance of cytoplasmic germ granules that participate in the transcriptome control (Lehtiniemi & Kotaja, 2018). For example, the components of the translation-coupled RNA quality control mechanism, the NMD, is accumulated in the haploid cell-specific CB (Lehtiniemi et al., 2022b), and it also contains several proteins involved in the regulation of splicing (Meikar et al., 2014). This suggests that the cytoplasmic appearance of the germ granules might be a direct consequence of widespread genome transcription. The appearance of germ granule-like DDX4 granules in many types of cancer suggest that this could also be used as an RNA regulatory mechanism in cancer cells.

Interestingly, the germ granules appear one per round spermatid nuclei in mouse, and two to three granules in human. However, in cancer cells, these germ granule-like DDX4 granules are unevenly distributed between different cancer cells (0–5 granules per nuclei). This uneven localization in cancer cells suggests that, firstly, the cancer cells are highly heterogenous and are at different stages of differentiation, and secondly, the number of granules may reflect the requirements of transcriptome regulation in each cancer cell. Single cell RNA sequencing analysis can be used to address this hypothesis.

Alternative splicing is a physiological process of transcriptional and post-transcriptional regulatory mechanism that is responsible for multiple generations of mRNA and different protein isoforms from a single gene in eukaryotic organisms. Interestingly, the level of alternative splicing is dependent on the cellular activity and varies among different tissues and also depends on the condition. Brain, liver and testicular cells, especially the differentiating germ cells have broad genome transcription and therefore make high demands on the alternative splicing mechanism for their gene regulation and activation of tissue specific proteins. In a similar way, cancer cells have unstable transcriptome and many of their genome are spliced to enhance production of oncogenes and other cancer specific genes that are beneficial for their survival.

The piRNA pathways are the main molecular pathways that accumulate in germ granules (Meikar et al., 2010a), but they also gather together other RNA regulatory proteins involved in the RNA storage and quality control (Werner et al., 2015). In cancer cells, some species of piRNAs and PIWI proteins are aberrantly expressed, but many cancer-related functions of PIWI proteins seems to be piRNA-independent (F. Li et al., 2020; S. Shi et al., 2020). The results from this study did not show any indications of the collaboration of DDX4 with the piRNA pathway in cancer cells, despite the reported contribution of DDX4 in piRNA production in germ cells (Wenda et al., 2017; Xu et al., 2021)). In addition, we also did not observe any major defects in the piRNA production in the absence of *DDX4* in cancer cells. This suggest that although DDX4 forms cytoplasmic germ granule-like granules in cancer cells, the piRNA pathway related functions of these granules is not conserved in cancer cells. It appears that, instead of piRNA processing, DDX4 is involved in the posttranscriptional regulation of mRNAs, including the regulation of alternatively spliced transcripts and translation. While the exact mechanisms of DDX4-mediated RNA regulation in cancer still requires further investigation, our results show that DDX4-granules and DDX4-mediated RNA regulation is beneficial for cancer cells, and they start expressing this germ cell-specific protein with the capacity to regulate the transcriptome to support cancer progression.

6 Summary/Conclusions

The role of germ granules in the development of germ cells has been studied for decades. From the development of the embryonic male germ cell to the formation of fertile sperm, germ granules serve as a unique cytoplasmic platform that mediates various posttranscriptional gene regulatory mechanisms. Any alteration in the germ granule-mediated RNA regulatory pathways can impair germ cell maturation and lead to male infertility.

In this study, we characterized the two well-known germ granules in mammalian species, the IMC and CB. For the first time, we have isolated the IMC and identified its protein components. We also demonstrated the accumulation of mitochondrial-associated piRNA biogenesis factors in IMC. In addition, we identified two exonuclease 3'–5' domain containing proteins (EXD1 and EXD2) as novel IMC components and characterized their germ granule-associated expression and their role in piRNA biogenesis during spermatogenesis. Interestingly, the deletion of the active DCEW motif in EXD2 did not affect spermatogenesis or fertility in *Exd2* mutant testes. Further biochemical analysis is required to be able to establish other important functional domains in EXD2 that are required for spermatogenesis.

We also investigated the role of the secondary piRNA biogenesis factor EXD1 during spermatogenesis. Although deletion of *Exd1* resulted in a decrease in PIWIL4-associated piRNAs and had no observable effects on testicular histology and fertility (Z. Yang et al., 2016), deletion of *Exd1* in *Tdrd12*-heterozygous mice resulted in a significant decrease in PIWIL4-associated piRNA production, derepression of transposons, loss of meiotic germ cells, and consequently mouse infertility. Therefore, this study demonstrated the collaboration between EXD1 and TDRD12 for the production of PIWIL4-associated piRNA biogenesis and male fertility.

Importantly, the results of this study elucidate the functional relationship between the IMC and the CB, which has not yet been adequately explored. Based on our results, we know that they share many protein components and communicate with each other during the late phases of meiosis and co-exist for short period of time. Furthermore, our data shows that the function of the mitochondrial-associated IMC is associated with piRNA biogenesis, while the CB, which is not associated

with mitochondria, appears to serve as the site for piRNA-mediated downstream RNA regulatory activities.

Although, we are only beginning to understand the role of germ granules in spermatogenesis, this study has further broadened our horizon into their novel roles in cancer biology. We have demonstrated that the germline-specific DDX4 forms cytoplasmic granules in cancer cells when they are in a tumor environment, and that DDX4 has a functional role in tumor formation, progression and metastasis. The cancer-related function of the DEAD-box RNA helicase seems to be strongly linked to the posttranscriptional regulation of genes functioning as tumor suppressors and genes involved in cancer promoting functions such as EMT. Significantly, we provided novel insight into the DDX4-mediated mechanisms by revealing a connection of DDX4 function to the regulation of alternative splicing. The correlation of the presence of DDX4-granules in cancer samples with poor survival probability in HNSCC patients and higher Gleason scores in prostate cancer patients provides new insights into the development of tools for cancer classification and prognosis.

In conclusion, this interdisciplinary research elucidated the contribution of germ granules to the development of male germ cells and male fertility. It also utilized the knowledge from germ cell research in cancer research to characterize the role of germ granule-related mechanisms in the transcriptome regulation in cancer cells to enhance their malignancy.

Acknowledgements

This research work was carried out during the years 2016–2023 at the Research Centre for Integrative Physiology and Pharmacology Unit (IPP), Institute of Biomedicine, University of Turku, Finland.

My heartfelt gratitude goes to my excellent supervisor, amazing mentor, and exceptional boss, Professor Noora Kotaja. You have given me the opportunity and privilege to work on this novel interdisciplinary research project and to discuss all relevant technical and scientific issues in-depth. Boss your attitude to science and life in general, is always a motivation for me and I greatly appreciate the aura of excellence around you. You have helped me grown into what I love to be. Throughout all these years, you were always easily accessible creating time for discussing both project related-questions and other life-related discussions. You are a very selfless leader and I am indeed very grateful for the positive influence you have had on me. In addition, I really appreciate the freedom to design and execute different experiments, including many failed ones, you have shown me patience and flexibility. Thank you for giving me the opportunity to contribute to other projects (both within the group and the international collaborative projects), priviledge to teach younger students and the freedom to present my results at different conferences (both international and national). I have learnt a lot from you, including writing academic grant applications and scientific articles. Your financial support was immense and I never had to fret. All of these contributed to the success of my PhD.

I would also like to express my gratitude to Professor Emeritus Martti Parvinen, for being the trailblazer in Chromatoid body research in Finland. Thank you so much for your persistence in the field even when no one believed you. Your legacy lives on.

Next, I would like to express my deep appreciation to the members of my PhD follow-up committee: Professor Jukka Westermarck, your questions and suggestions during our annual meetings especially the discussions about my cancer project, were very helpful and contributed to this great achievement; Professor Jorma Toppari, MD, PhD, for your constant willingness to inquire about the progress of my projects; the stories you have told me about your experiences during your PhD have been empowering, motivating and I do not take it for granted.

I would like to thank my external reviewers, Associate Professor Minni Änkö and Leena Latonen, Ph.D., for their critical review of my thesis. Your comments and suggestions have significantly improved the quality of my work, and deepened my knowledge.

I would like to thank all the co-authors of my publications, Paper I: Mark Dowling, David Homolka, Magdalena N. Wotjas, Panyi Tran, Tiina Lehtiniemi, Matteo Da Ros, Ramesh S. Pillai and Noora Kotaja; Paper II: Radha Raman Pandey, David Homolka, Ravi Sachidanandam, Ramesh S. Pillai and Noora Kotaja; & Paper III: Anna-Riina Koskenniemi, Lin Ma, Valeriy Paramonov, Sini Laasanen, Elina Louramo, Matthieu Bourgery, Tiina Lehtiniemi, Samuli Laasanen, Adolfo Rivero-Müller, Eliisa Löyttyniemi, Cecilia Sahlgren, Jukka Westermarck, Sami Ventelä, Tapio Visakorpi, Matti Poutanen, Paula Vainio, Juho-Antti Mäkelä and Noora Kotaja. Your great contributions and collaborations made this thesis possible. I wish we can continue to collaborate in the future.

Next, my appreciation goes to Elina Lauramo, Elina Valkonen and Noora Kotaja for helping me in translating my thesis abstract from English into Finnish language. Thanks so much for your support.

Next, I would like to appreciate all the members of Kotaja research team, the alumni: Karin Sõstar and Oliver Meikar, even though you already left when I joined the team, your good legacy continued to speak even till now; Matteo Da Ros, for being a great teacher to me, I have learned so many molecular biology techniques from you, you are a great scientist and also a wonderful friend to me and my family, the wonderful movie nights we spent together are indelible in my heart; Hanna Hyssälä, for your kind words and always willing to assist; Mari Lehti, for being an efficient manager and a great scientist in Kotaja team. I learnt a lot from you, thank you for bringing good energy and happiness to the team; Ram Yadav Prakash, for bringing brightness and vigour to the team. Special thanks to Tiina Lehtiniemi (my partner in ‘lab crimes’), who brings great enthusiasm and progress to the team and is always willing to contribute to other projects. Tiina, you are a true friend that I will always have; Margareeta Mäkelä, thank you for your contribution to the Kotaja team. I also appreciate Juho Asteljoki, you are always ready to help when you are needed. Special thanks to Niina Smolander and Elina Tuomikoski for their help with the projects in summer 2018 and 2019. I really appreciate ever smiling Elina Louramo for bringing sunshine to the team, thank you for bring enthusiasm to the working environment. You worked extensively on the validation of the primers, also went with me to the animal facility to carry out ultrasound measurement of xenograft tumors, sometimes during the evening. You are always helping when needed and you were always smiling. My warm gratitude goes to the current members of Kotaja team: I am very grateful for meeting and working with Juho-Anti Mäkelä, thanks so much for bringing your expertise in “stem cells” to the team, I admire your scientific

mind and I appreciate your immense support for me all through my work. Thank you for organizing all the “hang-outs”, “hunting trips” and sauna weekends; your kindness is exceptional and I will never forget. All these gestures enhanced my motivation to have gone this far. I appreciate Matthieu Bougery and Lin Ma for bringing bioinformatics expertise to Kotaja team. I have benefited a lot from our discussions and thanks for your contributing to my success story. I sincerely appreciate Anna Eggert for your medical advice and contributions in during our weekly meetings. You are always willing to help in the best way you can. Special thanks to Ammar Ahmedani, for bringing strong enthusiasm and brightness to Kotaja team. I admire your inquisitive scientific mind and the passion you bring. You are an amazing friend. I will never forget Samuli Laasanen’s contribution. Thank you for always being willing to help; Sini Leskinen, thank you for your contribution to my cancer project and for making the team happy. I also appreciate Jasmin Lassila and Riikka Palimo for your good works and contributions to the team. The legacy of the Kotaja team will always be a part of me. The brainstorming and problem solving ideas in the regular “Kotaja Team Meetings” every Monday were the building blocks that contributed to my success today, and for that I am eternally grateful.

I am very grateful for the friendly and supportive atmosphere created by all the Proffessors, Docents and Senior Researchers that I have been privileged to interact with, those who have previously worked and those currently working at the C5/B6 floors of Medisiina/University of Turku: Jorma Toppari for your exemplary personality in science, you are worthy of emulation, I am grateful for meeting people like you on my scientific journey; Helena Virtanen, for your kind heart and always supportive; Ilpo huhtaniemi, thank you for being the pioneer and for initiating great principles in physiology department, you were the one who walked me into Noora’s office when I newly arrived physiology department; Manuel Tena-Sempere, thank you for your great contribution to science; Harri Niinikoski, thanks for your contribution to science; Matti Poutanen, I really appreciate your great initiative and ideas about science. You are always ready to give your support. Thank you so much for your contribution to my cancer project. Nafis Rahman for your contribution to scientific community in Turku and Finland; Jukka Kero, thank you for your kind heart and for loving sports; Leena Strauss, all your encouraging words helped me a lot; Petra Sipilä, I really appreciate your warm personality, your door was always open for discussions (both scientific and otherwise) and you are always willing to help. I am grateful that I met you.

An excellent lab atmosphere is needed to work and produce great results. These would not have been possible without the help of excellent staff at the IPP/Physiology. I especially want to thank the former Laboratory Manager, ever-kind Tuula Hämäläinen, Minna Lindroth and Johanna Järvi for helping with ordering the necessary reagents, chemicals and antibodies, Marko Tirri for setting up the

experimental equipment and making a functional working environment, Taina Kirjonen and Satu Tölkkö for your help in organizing the reagents. You were always willing to help, the whole department would be a mess without you.

I am thankful to all the people I have been privileged to interact with on scientific and social matters. I would like to appreciate Mirja Nurmio, Mari Vähä-Mäkilä, and Taija Heinosalo. I also appreciate Leena Karlsson, Niina Saarinen-Aaltonen, Kalle Rytkönen, Mikael Piha, Janne Hakkarainen, Heli Jokela, Jaako Koskenniemi, Sofia Pruikkonen, Christoffer Löf, Donata Ponikwicka-Tyszko, Matias Knuutila, Ashutosh Trehan, Mahesh Tambe, Sergey Sadov, Valeriy Paramonov, Milena Doroszko, Ida Björkgren, Emmi Rotgers, Satu Orasniemi, Suvi Ruohonen, Antti Perheentupa, Meeri Jännäri, Kristiina Makkonen, Nadeem Asghar, Laura Lewandowski, Pauliina Toivonen, Janne Sulku and Salli Kärnä. Your kind words, comments, suggestions, technical supports contribute to the success of my PhD.

I would like to appreciate the Physiology singing team, which I have been part of, Johanna Järvi, Noora Kotaja, Laura Marinoja, Tiina Lehtiniemi and Marko Tirri. Singing together with you gives me joy, and enhanced my devotion to my PhD projects. I will never forget this.

I would like to thank Tiina Lehtiniemi, Sheyla Cisneros-Montalvo, Gabriela Martinez Chacon, Michael Gabriel, Konrad Patyra, Wiwat Rodprasert, Hanna Heikelä, Riikka Huhtaniemi, Arttu Junnila and Laura Mairinoja. Thank you very much for your great friendship and support.

My heartfelt gratitude also goes to Madeleine Latvala, Clara-Theresia Kolehmainen, Ida Hyötyläinen, Elina Valkonen, and Jenni Airaksinen. I am grateful that our path crossed. Thanks for your great friendship and support.

I am very grateful for meeting these amazing scientists, Ammar Ahmedani, Guillermo Martinez Nieto, Lin Ma, Krisztina Kukoricza, Matthieu Bougery, Inka Raimoranta and Kamila Pulawska. Thank you for your great friendship and support.

I am very grateful to the physiology coffee team: Noora Kotaja, Petra Sipilä, Leena Strauss, Madeleine Latvala, Clara-Theresia Kolehmainen, Ida Hyötyläinen, Jenni Airaksinen, Marko Tirri, Arttu Junnila, Pauliina Toivonen, Taina Kirjonen. Thank you for sharing your knowledge about life in general outside science during coffee breaks.

I am thankful to the doctoral thesis committee members at the Faculty of Medicine, University of Turku for insightful comments on improving my PhD thesis manuscript. I am proud to be a part of the Turku Doctoral Programme of Molecular Medicine (TuDMM), where I have enjoyed great scientific events, travel grants for scientific conferences and social activity organized by TuDMM. My heartfelt appreciation also goes to Eeva Valve, PhD, my coordinator in TuDMM, former director, Kati Elima (M.D., Ph.D., Adjunct Professor), and current director Prof. Noora Kotaja, thank you so much for your guidance and support all through my

journey as a doctoral researcher. I also thank the entire TuDMM Student Council members for creating a friendly and supportive environment.

I appreciate Jasmin Kaivola, Jens Luoto, Jesse Mattsson and Mervi Toriseva for all the insightful discussions we had when I started working using 3D-spheroid culture techniques.

I am grateful to Mona Niiranen and Jonna Palmu. I really appreciate your guidance and willingness to help at every time.

I'm grateful to University of Turku center for Biosciences for providing advance imaging core facilities. Special thanks to members of imaging core facility, Ruth Fair-Mäkelä, Markus Peula, Jouko Sandholm and Markus for training me how to use different advanced microscopes. I learnt a lot from you all. Jouko Sandholm, you answered my call every time.

My sincere gratitude goes to Anna-Marja Säämänen (PhD; Adjunct Professor), thanks for bringing calmness to me in the catastrophic situation in the bacterial lab. Thank you for your support though my PhD journey.

My gratitude also goes to Joanna Pylvanainen. Thank you for your guidance and support in image analysis.

My heartfelt gratitude to the entire Team of Turku Centre for Disease Modeling (TCDM), especially the research manager Petra Sipilä (PhD; Adjunct Professor), and the team members: Heli Niittymäki, thank you for always willing to help me when needed; Heidi Liljenbäck, thanks so much for teaching me how to use the ultrasound machine to measure xenograft tumors; Katri Hovirinta and Janne Suikkila, thanks so much for your support and for helping out in xenograft experiments.

I am grateful to the personnel at Central Animal Laboratory, University of Turku, who have been taking care of mice and ear sampling for genotype that has lightened my workload tremendously. Every time I needed mice for experiment, usually my first go-to are: Niina Kulmala, Terhi Hiltula-Maisala, Nea Konttinen and Niina Messner of Central Animal Facility of the University of Turku and there was never a time you did not help me out in providing mice for experiments.

I really appreciate Pekka Haapaniemi, Anne Rokka and other staff members at the Turku Bioscience Centre for proteomics for always receiving our samples for mass spec and I appreciate our discussion with Anne Rokka, suggesting a change of protocols in sample preparation for mass spec.

Special thanks to the members of the Histocore facility, Erica Nyman and Marja-Riitta Kajaala. Thank you so much for your assistance. You were always willing to help even during the covid-19 situation.

I am very grateful to Prof. Jukka Westermarck, Dr. Marcin Chrusciel and Dr. Nafis Rahman, for your generosity in providing tumor samples for initial testing of antibodies, and for discussions.

I am very thankful to my parents Mr. Lawrence Omolola Olotu and Mrs. Stella Edileola Olotu. Thank you for your unconditional love and support given me over the years. Without your teachings and selfless love, I probably would never strive to become better in life. Thank you for contributing to my achievements. I want to thank my uncle, Pastor Favor Olayemi Olotu. Thank you for your prayers, love and support through my PhD journey. I am grateful to my elder sisters: Mrs. Olayinka Alaboru, Mrs. Ayoola Oniyinde and their husbands, Mr. Charles Alaboru and Mr. Olayemi Oniyinde. Thank you all for your love and care all through these years. You supported all through this journey. My heartfelt gratitude to my brother Olanrewaju Olotu, thank you for being my motivation to come to Finland and for supporting my progress all through this PhD journey.

I also appreciate my in-laws Bishop Ezekiel Dahunsi and Mrs. Alice Dahunsi. Thank you for your love and support through my PhD. I also thank my brother-in laws, Reverend Sam Dahunsi and Temitayo Dahunsi, and my sister-in-law Mrs. Temitope Sunday. Thank you so much for the love and support.

I appreciate all my friends from the African community in Turku, Pori, Vaasa, Helsinki, Eespoo and Kuopio Finland. Thank you all for your support which contributed to the fulfillment of my PhD.

Finally, I want to appreciate my dear wife, Toluwanise Olotu and my children, Oluwajomiloju Grace and Oluwabusayomi Praise Olotu. Thank you for all the love and support which has contributed to the successful completion of my PhD.

This PhD study was financially supported by research grants from Academy of Finland, Sigrid Jusélius Foundation, Novo Nordisk Foundation, Jalmari ja Rauha Ahokas Foundation, Finnish Cultural Foundation, Turku Doctoral Programme of Molecular Medicine (TuDMM), Turku University Foundation and Institute of Biomedicine, University of Turku.

Opeyemi Olotu

Opeyemi Olotu

22.11.2023

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TURUN
YLIOPISTO
UNIVERSITY
OF TURKU

Painosalama, Turku, Finland 2023

ISBN 978-951-29-9548-6 (PRINT)
ISBN 978-951-29-9549-3 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)