MATERNAL CONTROL OF EARLY MAMMALIAN EMBRYOGENESIS

MINJIE TAN

Maternal control of early mammalian embryogenesis

Minjie Tan

谭敏捷

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Minjie Tan

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Promotor:
Prof. dr. T.A.E. Stout

Copromotor:
Dr. B.A.J. Roelen

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Contents

Chapter 1

Introduction

In the gonads, germ cells undergo meiosis and the final maturation steps required for differentiation into mature gametes, either eggs (oocytes) or sperm. By fusing together during fertilization, the haploid male and female germ cells form the totipotent, diploid zygote, thereby giving rise to the next generation. The somatic cells of the gonads play an important role in supporting the differentiation towards competent oocytes or spermatozoa. In females, the germ cells that colonize the developing ovaries divide mitotically a number of times before initiating meiosis, soon after which they become arrested at the prophase of the first meiotic division. At this stage, the oocytes together with the surrounding ovarian somatic cells form follicles. Within these follicles, the majority of the oocytes will undergo apoptosis as early as the fetal stages of development. After birth, millions of primordial follicles are formed in the cortical region of the ovary; before puberty, primary follicles cannot mature, instead most of them undergo apoptosis and form atretic follicles. Following puberty, groups of oocytes develop in 'waves' and become sensitive to the stimulatory effects of the pituitary gonadotrophins, FSH and LH. During each ovarian cycle, one or more follicles (depending on species) will be selected for dominance and develop to the preovulatory stage. Shortly before ovulation of a follicle, the contained oocyte undergoes germinal vesical breakdown (GVBD) and resumes meiosis I (MI) leading to the extrusion of the first polar body containing one set of homologous chromosomes. The oocyte then arrests again, now at metaphase of the second meiotic division (MII), until fertilization.

Since transcription is halted between first and second meiosis resumption, and the activation of the embryonic genome will only happen after fertilization, it is apparent that maternal proteins and RNAs accumulated during early oocyte growth and maturation are important during the final stages of oocyte maturation and, following fertilization, during early embryo development. Together these proteins and RNA species regulate the stability and translation of stored maternal mRNA.

This thesis explores and discusses biochemical changes during mammalian oogenesis with the aim of providing novel information, at the transcriptomic and proteomic levels, about events and processes that help to support, direct and protect the genomic integrity of the early mammalian embryo.

Folliculogenesis and oogenesis: Prospects and challenges for the future

Based on the stage of folliculogenesis, follicles can be divided into 5 classes: primordial, primary, secondary, tertiary and Graafian. Oogenesis starts with the development of oogonia from primordial germ cells after their arrival in the female genital ridges. The oogonia first undergo mitosis to give rise to primary oocytes that become surrounded by somatic mesenchymal cells which then differentiate into flattened granulosa cells, thereby giving rise to the primordial follicle. Primordial follicles subsequently develop into primary follicles that are similar in appearance with the exception that the granulosa cells acquire a cuboidal shape. During follicle growth, the number of granulosa cell layer increases, as does the size of the oocyte. Eventually, a secondary follicle is formed with multiple layers of cuboidal granulosa cells, now called cumulus cells, that are now capable of being influenced by oocyte-secreted signals. Development to a tertiary follicle includes the formation of a small antral cavity. At this stage, the oocyte is surrounded by a zona pellucida, which is traversed by cumulus cell processes that terminate in the oolemma. The Graafian (advanced antral) follicle is the follicular stage present just before ovulation. It comprises a large fluid-filled antrum that makes up most of the volume of the follicle[1].

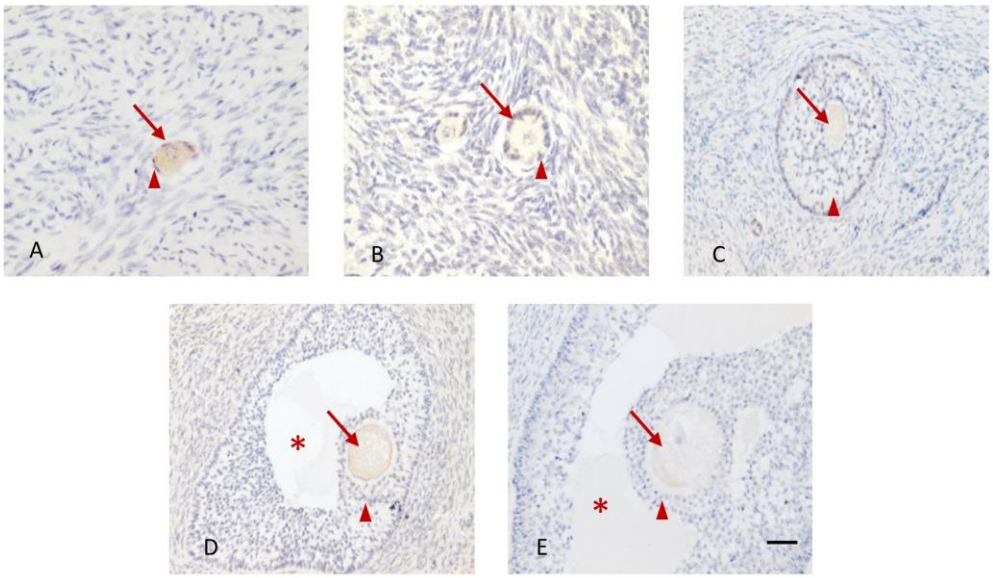

Figure 1: Representative hematoxylin stained images of oocytes at different stages of follicular development in bovine ovarian sections. (A) Primordial follicle. (B) Primary follicle. (C) Secondary follicle. (D) Tertiary follicle. (E) Graafian (advanced antral) follicle. Blue: Hematoxylin stained nuclei.

Arrows indicate oocytes; triangles indicate cumulus cells; asterisks denote the antrum. Scale bars, 50 µm.

PIWI/piRNA in mammalian germ cell development

Transposable elements in early development

Transposable elements (TEs) are DNA sequences with the capacity to "jump" from one genomic location to another[2]. It has been reported that almost two thirds of the human genome is comprised of TEs, indicating their potential for influencing human genome evolution[3]. Based on the transposition mechanism, TEs can be divided into two classes: Retrotransposons (class 1) and DNA transposons (class 2)[4]. Retrotransposons require an RNA intermediate with a reverse transcriptase enzyme to insert copy DNA elsewhere into the host DNA. Examples of retrotransposons include long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs) and long terminal repeat elements (LTRs). DNA transposons mainly excise and insert directly into a new genomic site using a "cut and paste" mechanism. In mammalian genomes, retrotransposons dominate the transposable element repertoire; for instance, around 42% of the human genome is made up of retrotransposons whereas only 2-3% is derived from DNA transposons[5]. Because of their mode of transposition, i.e. they are not excised from the donor site, retrotransposons can establish a high copy number in the cytoplasm of cells.

During mammalian embryogenesis, TEs have the potential to affect the genomic architecture, gene regulatory networks and protein function[6]. Even though transposons might benefit host genomes by enhancing genetic diversity and driving evolution, uncontrolled TE expression is harmful to genomic integrity and can disrupt developmental processes at both the transcriptional and post-transcriptional levels[7]. It has been reported that a dysregulated expression of TEs is associated with multiple developmental abnormalities in murine maternal and paternal germ cells. Increased LINE-1 expression can induce oocyte genome damage and meiotic defects, as well as reducing ovarian follicle reserves and predisposing to aneuploidy[8]. In addition retrotransposon activity leads to abnormal meiosis resulting in male sterility[9].

Retrotransposons are transcriptionally silenced by histone modification and DNA methylation[10]. During germ cell formation however, since several rounds of demethylation occur to restore cellular totipotency, an alternative control of TEs is required. In various animal species , TEs in germ cells are controlled by the PIWI/piRNA system[11–13].

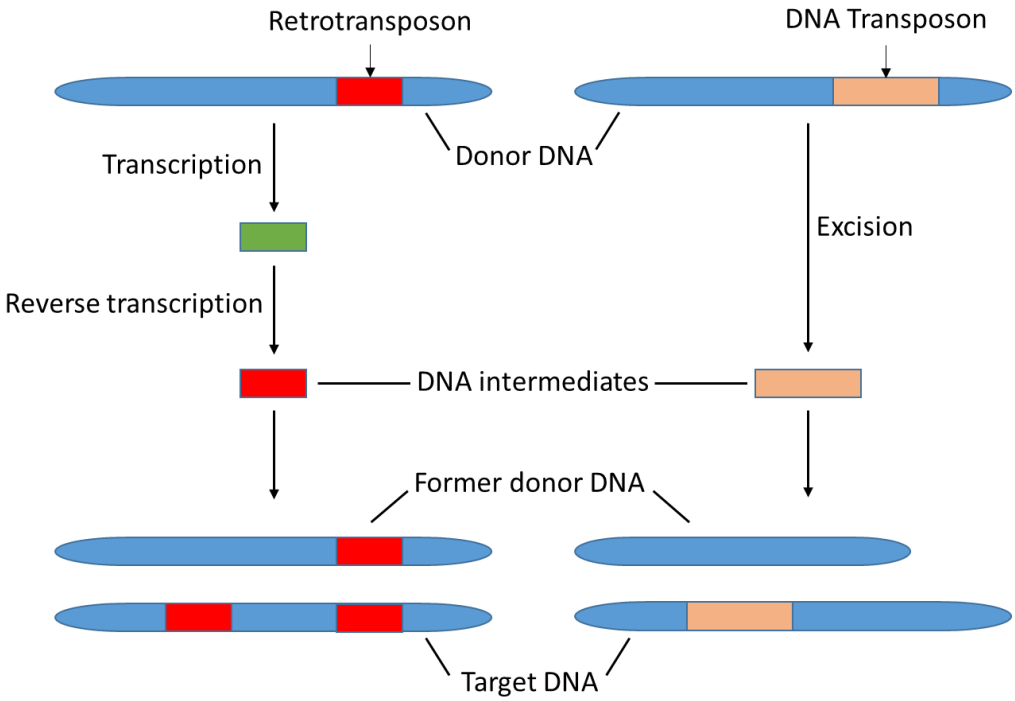

Figure 2: Two types of transposon and their mechanisms of mobilization. For retrotransposon mobilization, the DNA is first transcribed into RNA, the RNA copy is then reverse transcribed into cDNA and integrates into a new site in the genome. DNA transposons mobilize by a "cut and paste" mechanism. The transposon exits its genomic location and is incorporated at a new genomic location.

PIWI/piRNAs in mammalian germ cells

PIWI proteins belong to the Argonaute (AGO) protein subfamily and form specific RNA induced silencing complexes (RISCs) by interacting with a specific group of small non-coding RNAs known as PIWI-interacting RNAs (piRNAs)[14]. *Piwi* (P-element Induced wimpy testis) was originally discovered in *Drosophila*, where mutations abolish germline stem cell division leading to sterility in both males and females[15]. PIWI proteins contain four domains: the N-terminal, PAZ, middle (MID), and PIWI domains. The N-terminal region contains Arginine-rich

motifs that allow PIWI proteins to interact with Tudor family proteins to support piRNA biogenesis. The PAZ domain recognizes the 3'-end of piRNAs[16] while the MID domain specifically recognizes the 5' uridine (1U-bias) of piRNAs[17]. The PIWI domain contains catalytic residues, responsible for the cleavage of target mRNAs[18].

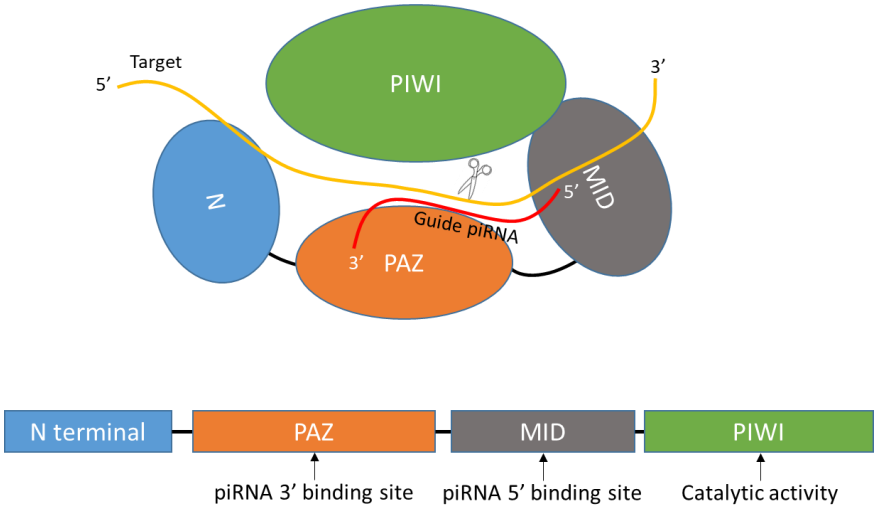

Figure 3: Schematic representation of a PIWI protein. N terminal: N terminal domain; PAZ: PIWI-Argonaute domain; MID: middle domain; PIWI: PIWI domain. The piRNA (the red strand) of the PIWI/piRNA complex guides the complex to a complementary target mRNA.

In most mammals, four different PIWI proteins exist, and have been called PIWIL1, PIWIL2, PIWIL3 and PIWIL4. However, studies on the functions of PIWIs in mammals have mainly focused on mice in which only 3 PIWI genes are present: *Piwil1* (*Mili*), *Piwil2* (*Miwi*) and *Piwil4* (*Miwi2*). Unusually, PIWI proteins in mice are predominantly expressed in male germ cells. MIWI and MILI are localized in cytoplasmic granules either expressed from the late pachytene stage to the round spermatid stage (MIWI) or from the gonocyte stage to the round spermatid stage (MILI) while MIWI2 is expressed in the nuclei of gonocytes[19,20]. Disruption of *Mili* or *Miwi2* leads to the activation of retrotransposons, resulting in meiotic arrest. *Miwi* deficiency also dysregulates the expression of retrotransposons which results in spermatogenesis arresting at the early spermatid stage[19,21,22]. Remarkably, knockout of any of the *P*iwi genes in mice leads to sterility of males but does not affect fertility in females. The absence of a female phenotype in mice after deletion of the *Piwi* genes (*Piwil1*, *Piwil2*, *Piwil4*) has led to the dogma

that the PIWI/piRNA complex is dispensable in mammalian oocytes. However, the genome of most other mammals including man and cattle, contains a fourth Piwi gene, PIWIL3. In man, cattle and rhesus monkeys this gene is exclusively expressed in oocytes of antral stage follicles[23].

piRNAs are small, 21-31 nucleotide (nt),RNAs characterized by a uridine at the 5' end and 2'-O-methylation on the 3' end[24]. Based on their biogenesis, piRNAs can be divided into primary and secondary piRNAs. Primary piRNAs are processed from long single-stranded transcripts encoded by piRNA clusters in the genome. The long piRNA precursors are first trimmed into shorter piRNA intermediates by the mitochondrion-anchored endonuclease Zucchini/mitoPLD with the preference for a uridine at the 5' end of pre-piRNAs[25,26]. Once bound to a PIWI protein, pre-piRNAs are trimmed at their 3' end by poly(A)-specific ribonuclease (PARN)-like domain containing 1 (PNLDC1) which physically interacts with a Tudor-type protein on the mitochondrion[27–30]. However, in Drosophila PNLDC1 is absent and Nibbler (Nbr) is instead used as a piRNA trimmer[31]. Finally, Hen1/HENMT1 2'-O methylates the 3' end of mature piRNAs[32,33].

The secondary piRNA generation pathway is referred to as the "ping-pong" cycle. In this process, primary piRNAs guide PIWI proteins to complementary RNA, for instance retrotransposon elements or mRNAs. As a consequence, the target RNAs are cleaved by PIWI proteins between the 10th and 11thnucleotides, which generates the 5' ends of the secondary piRNAs with an adenosine at the 10th nucleotide. The secondary pre-piRNAs can then be loaded onto other PIWI proteins and trimmed at their 3' end to form the mature piRNAs which in turn guide the PIWI proteins to RNA targets for slicing[34,35].

In the mouse, according to the expression stage in spermatocytes, piRNAs can be divided into two types: pre-pachytene and pachytene piRNAs. Pre-pachytene piRNAs are mainly derived from transposon elements and are present prenatally in pro-spermatogonia and postnatally in spermatogonia and are associated with MILI and MIWI2. Pachytene piRNAs are bound by MILI and MIWI and are present postnatally in pachytene spermatocytes and round spermatids and are generated from long precursor transcripts that predominantly originate from unannotated regions of the genome [21,22,36,37].

PIWI/piRNAs silence transposons in germ cells

piRNAs bind to PIWI proteins to form piRNA-induced silencing complexes (piRISC). Functional studies of mammalian PIWI/piRNA systems have focused on mice since, as a result of the possibility of generating embryonic stem cells, gene knockouts are relatively easy to establish when compared with other mammals.

Genome demethylation during oocyte and early embryo development can lead to the upregulation of several TEs including LINE-1 elements[38]. To minimize the risk of genome mutation, piRNA induced degradation is one of the strategies used widely in TE silencing. In various species, TE can be controlled by piRNAs in both the nucleus and the cytoplasm[39]. In the nucleus, piRNAs are implicated in the regulation of TE transcription while, in the cytoplasm, TE RNAs can be cleaved by the piRNA pathway through ping-pong amplification, a process that is unique because it combines piRNA biogenesis with target silencing[34]. In mice, various strategies of TE regulation in germ cells by piRNAs have been suggested. For instance, the piRNA pathway can act upstream of de novo methyltransferases (DNMT3L, DNMT3A and DNMT3B) to drive TE methylation in the genome. Most of the pre-pachytene piRNAs are derived from TE transcripts and MIWI's slicer activity is necessary for the accurate silencing of LINE1 elements[12,22,35]. A study in cattle revealed that almost 50 % of piRNAs in oocytes also map to TEs which indicates a role for piRNAs in TE silencing in maternal germ cells and early stage embryos of mammals[23,40].

TUDOR domain containing proteins are crucial partners for PIWI

Indispensable components of the PIWI/piRNA pathway include the Tudor domain containing (TDRD) proteins that functional in mediate protein-protein interactions. In germ cells of various animal species TDRDs are highly expressed[41] and interact with PIWI proteins[41,42]. Tudor domain proteins bind mainly via methylated arginine residues present in PIWI proteins and regulate the formation, localization and function of PIWI/piRNA complexes[43,44].

Tdrd2 also known as Tudor and KH domain-containing (TDRKH) is a conserved Tudor domain protein localized on mitochondria, and which is critical for pre-piRNA 3' end trimming[29,30,45].

In the mouse, TDRKH associates with the poly(A)-specific ribonuclease (PARN)-like domain containing 1 (PNLDC1) that has been identified as a 3'-5' exonuclease (trimmer) responsible for piRNA 3' end maturation. As the interacting partner of mouse MIWI, MIWI2 and to a lesser extent MILI, genetic deletion of TDRKH results in reduced levels of mature piRNAs and improper spermatogenesis[30,45]. In diverse animal models, it has been proposed that TDRKH specifically recruits PIWI proteins bound to piRNA intermediates and PNLDC1 to trim piRNA intermediates to their final lengths[46].

Besides TDRKH, other TUDOR proteins like TDRD9 are also reported to be essential for transposon silencing and male fertility, and have been proposed as nuclear effector proteins in the mammalian piRNA pathway[47]. TDRD5 is an RNA-binding protein that directly associates with piRNA precursors and is essential for pachytene piRNA biogenesis in mice[48].

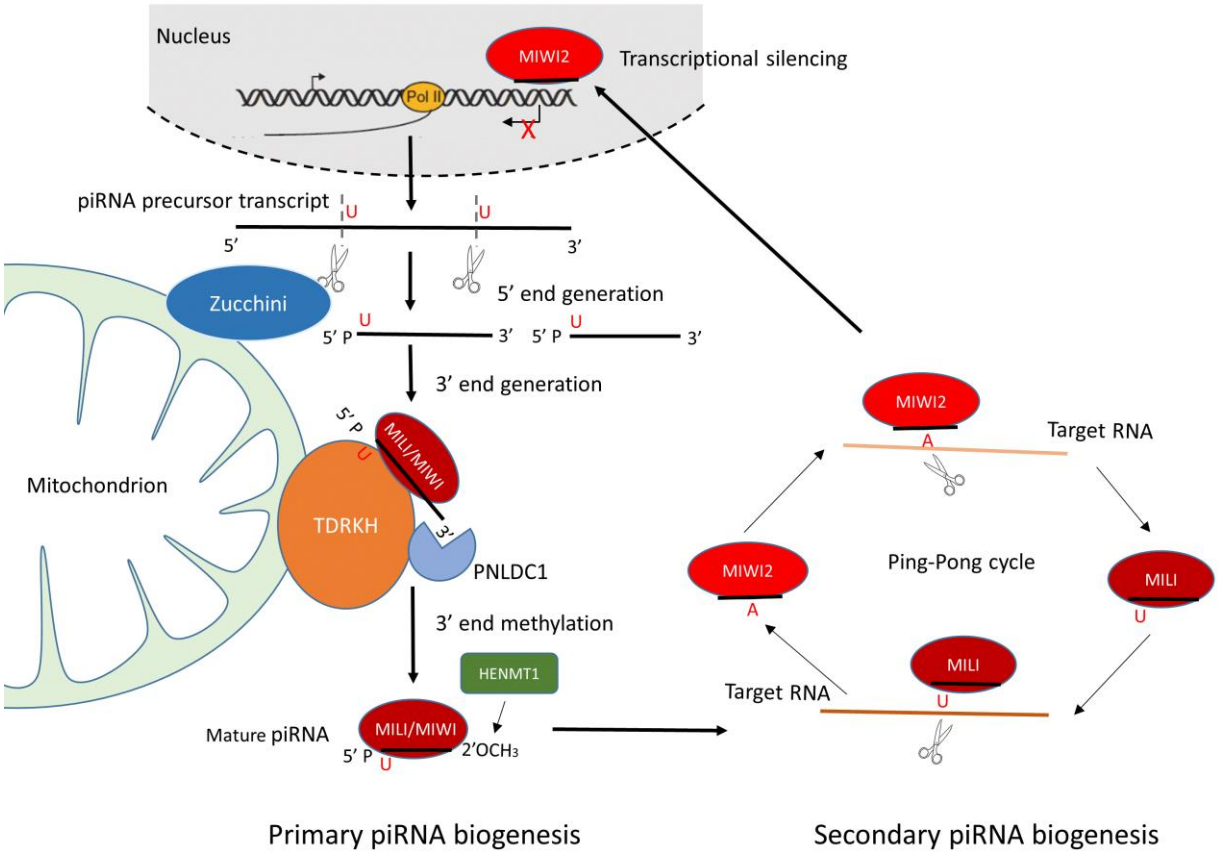

Figure 4: Schematic representation of piRNA biogenesis in the mouse male germline. piRNA precursors are generated from genomic loci by transcription and exported to the cytoplasm for primary piRNA biogenesis: The long piRNA precursors are first trimmed into short piRNA intermediates by the mitochondrion-anchored endonuclease Zucchini/mitoPLD, with the preference for a Uridine at the 5'

end of pre-piRNAs. Once pre-piRNAs are bound to a PIWI protein (MILI or MIWI), PIWI/piRNA complexes interact with TDRKH proteins on the mitochondrial surface and the 3' ends of pre-piRNAs are trimmed by poly(A)-specific ribonuclease (PARN)-like domain containing 1 (PNLDC1) which also physically interacts with TDRKH. After generating a piRNA 3' end with the mature size, Hen1/HENMT1 2'-O methylates the 3' end to complete primary piRNA maturation. These mature primary piRNA/PIWI complexes can then engage in the ping-pong cycle for secondary piRNA biogenesis. In this process, primary piRNAs guide MILI proteins to targets (TEs or RNA transcripts) that are complementary to the piRNAs; consequently, the target RNAs are cleaved by PIWI proteins, between the 10th and 11th nucleotides to generate the 5' end of the secondary piRNA, with an adenosine at the 10th nucleotide. The secondary pre-piRNAs are then loaded onto MIWI2 proteins and trimmed at the 3' end to form the mature piRNAs which in turn guide the PIWI proteins to RNA targets and slice them. The piRNA/MIWI2 complex would then translocate into the nucleus where it silences TEs at the transcriptional level by the recruitment of, for instance, the de novo DNA methylation machinery (DNMTs) as well as histone methyltransferases (HMTase), resulting in targeted DNA methylation and H3K9me3-mediated heterochromatinization of TE loci[39].

Overall, repression of TE damage to the genome in germ cells requires a range of proteins and mechanisms. Using PIWI/piRNA and TUDOR to control TEs seems to be a commonly used strategy in the germ cells of most model animals. However, most research has been conducted in lower organisms such as drosophila, zebrafish and the silkworm which may not be fully representative of human TE regulation. For this reason, further research needs to be performed focusing on mammalian species that provide a more accurate comparison to human germ cell development and, in particular, clarify how TE activity is regulated in oocytes.

The use of microinjection to study molecular function within individual oocytes

Microinjection is a direct method for the introduction of nucleotides, proteins or sperm cells into oocytes. It is an essential technique for research into the function of RNAs or proteins in oocytes and early embryos. For example, by injecting mRNA coding for a labeled protein into oocytes, the localization of the coded protein can be investigated without the need for an antibody. Particularly in species where specific antibodies are less readily available, this is extremely useful. Moreover, microinjection of specific siRNAs that target a transcript in

oocytes leading to its degradation can be an effective way to knock-down, and thereby study, gene function.

The microinjection set-up usually consists of a glass needle for injection, a holding pipette to fix the oocyte, a positioning device to control the movement of the micropipette, and a microinjector. Injection of fluid occurs using hydrostatic pressure. Injections are typically carried out under direct visual control, using a microscope (Figure 5)[49]. Even though microinjection is the simplest way to ensure effective delivery, it can be cumbersome because the oocytes need to be injected one by one.

Small interfering RNA (siRNA) applications

RNA interference (RNAi) is the biological mechanism by which small RNAs (20-30 nucleotides) induce gene silencing by targeting complementary mRNAs for degradation. RNAi was first discovered in plants and the worm *Caenorhabditis elegans*, and has subsequently been developed as a tool for research and a potential therapeutic strategy in various genetic diseases and types of cancer [50]. Synthetic small interfering RNAs (siRNA) specifically suppress expression of endogenous and heterologous genes in mammalian cell lines which enables its application in gene therapy[51]. In germ cells like oocytes, the siRNA technique is also widely used for fundamental research on gene function during in vitro maturation, fertilization and early embryonic development.

In vitro, siRNAs can be delivered to a cell in many ways, including electroporation, viral mediated delivery and microinjection. Microinjection is the most commonly used way to introduce siRNAs into oocytes. Even though siRNA microinjection is a successful and efficient way of investigating gene function, off-target effects i.e. the suppression of genes other than the desired target gene, are a serious concern[52]. In order to discriminate the specific effects, several combined siRNAs that target the same gene are usually injected to increase the efficiency of any knockdown effect. Moreover, a good negative control is an essential part of any siRNA experiment, this usually involves injection of a so-called ‘scrambled’ siRNA with similar nucleotide content but in a random (i.e. scrambled) order.

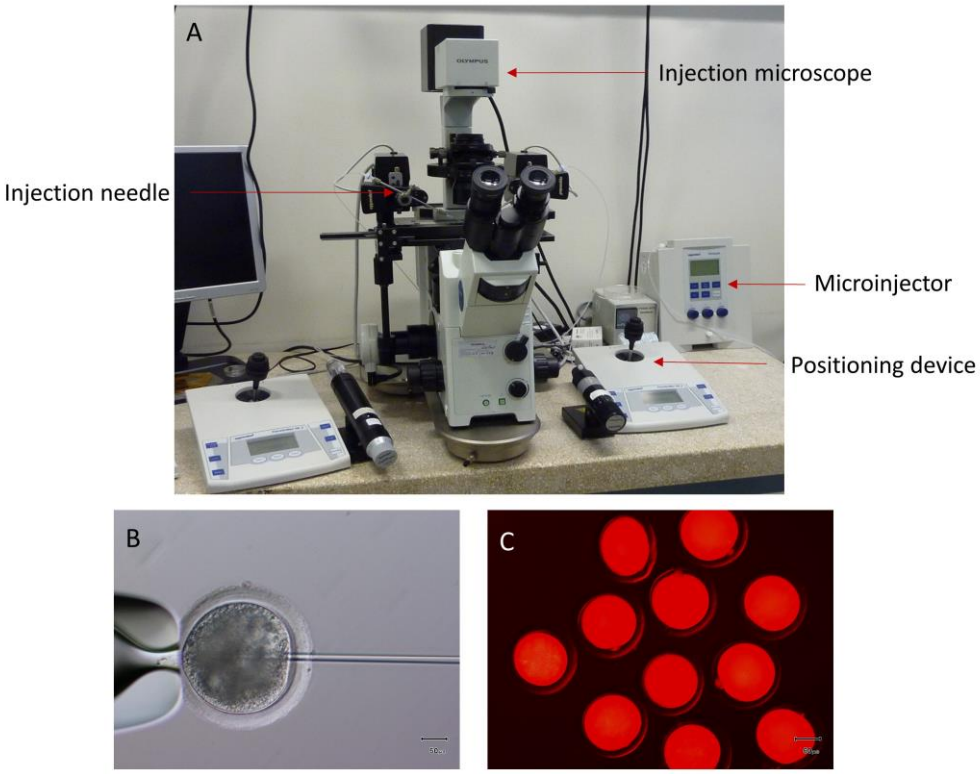

Figure 5: Microinjection manipulator set-up for an siRNA experiment. (A) Complete microinjection system. (B) Bovine oocyte during injection. (C) Dextran injected oocytes after maturation. Red fluorescence indicates successfully injected oocytes. Scale bars, 50 µm.

Membrane proteins in oocyte maturation and early embryo development

The plasma membrane provides a protective physical boundary between a cell and the environment. The cell membrane not only behaves as the barrier that controls which substances pass in and out of the cell but is also involved in diverse biological processes such as cell adhesion, invasion and cell-cell signaling. The plasma membrane is mainly composed of lipids and proteins. In fact, plasma membrane proteins constitute around 50% of the mass of the membrane. Based on their interactions and integration, membrane proteins can be divided into two categories: integral and peripheral proteins. Integral proteins have one or more segments that are embedded in the phospholipid bilayer and are permanently attached to the biological membrane. Peripheral proteins on the other hand are temporarily attached to the membrane via interactions with integral proteins or the lipid bilayer. Membrane

proteins are involved in multiple cellular functions including motility, adhesion, signaling and nutrient transport. In cancer biology, plasma proteins have been widely studied to aid disease biomarker discovery, and as potential targets for drug therapy[53]. In oocytes, plasma proteins have been studied to try to understand the mechanisms of maturation, sperm-egg binding and fusion[54,55].

Phosphorylation of oocyte membrane proteins

In oocytes, post-translational modification (PTM) plays a critical role in protein regulation involved in specific steps of embryonic development. Protein phosphorylation is a reversible PTM that is mediated by kinases and phosphatases that phosphorylate or dephosphorylate substrates, respectively. In eukaryotes, the amino acids commonly phosphorylated are serine, threonine and tyrosine. Protein phosphorylation is one of the most important post-translational modifications, and can act as a molecular switch for the coordination of cellular functions such as oocyte activation, recruitment of maternal mRNAs, DNA repair and resumption of the cell cycle[56].

During oocyte maturation, it has been reported that protein tyrosine phosphorylation is significantly higher in the cortical region, suggesting that distinct signaling pathways are used to mediate nuclear and cytoplasmic maturation[57]. In addition, multiple proteins localized in the cortical area that interact with plasma membrane proteins are reported to be critical to oocyte maturation. For example (Figure 6), members of the ezrin/radixin/moesin (ERM) protein family are expressed in an inactive form and translocate after activation by phosphorylation to the membrane, where they function as cross-linkers between plasma membrane proteins and the actin cytoskeleton[55]. In oocytes, consistent with a role in microvillus formation, phospho-ERMs are enriched within the oocyte cortex, and the activation of ERMs is important for maintaining cortical integrity and tension[55]. Actin-depolymerizing factor (ADF)/ cofilin are actin binding proteins essential for the cortical actin cytoskeleton. The binding abilities of ADF/cofilin are regulated by their phosphorylation and dephosphorylation. It has been reported that, in maturing oocytes of starfish, M-phase-promoting factor (MPF) is exported from the nucleus to the cytoplasm and acts as a kinase to activate the ADF/cofilin that is required for oocyte maturation[58]. In *Xenopus* embryos, the

protein phosphatase Slingshot is involved in Rho-dependent inactivation of ADF/cofilin, thereby promoting the rearrangement of the actin cytoskeleton essential for cytokinesis[59].

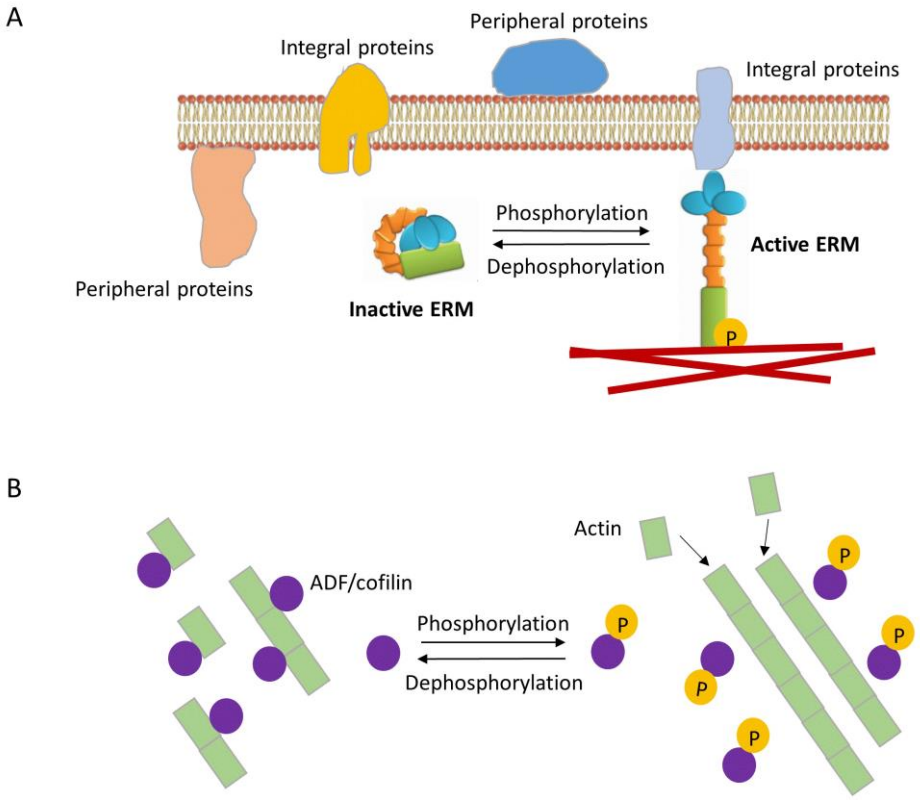

Figure 6: Schematic model for protein regulation based on phosphorylation. (A) Regulation of the ERM complex. The inactive ERM complex is folded, whereas after phosphorylation the complex is unfolded and acts as a crosslinker between plasma transmembrane receptors and the cytoskeleton. (B) Regulation of ADF/cofilin. ADF/cofilin protein enhances the depolymerization of actin filaments by inducing filament severing. Phosphorylation inhibits ADF/cofilin's protein binding activity to actin and promotes actin reassembly[60].

Because of the limitations of current plasma membrane extraction and detection methods, there is a large void in our understanding of plasma protein function in oocytes and early embryos. In this thesis, we investigate (phospho)proteome dynamics during meiosis of mammalian oocytes and fertilization.

Outstanding questions

To date, the PIWI/piRNA pathway has been studied mainly in a small number of animal models such as drosophila, the silkworm, zebrafish, C. elegans and the mouse. Although it is the only mammalian model used extensively for PIWI/piRNA investigation, there are a few issues that make the mouse questionable as the standard animal model for mammalian PIWI/piRNA studies. For example, experiments in which mouse Piwi genes were knocked out led to the assumption that piwi is dispensable in mammalian oocytes, because while disrupting piwi genes in the mouse causes male infertility, females retain normal fertility. Second, and as an exception among mammals, the PIWI paralog PIWIL3 is not present in the mouse genome. These murine peculiarities indicate that it is necessary to broaden our understanding of PIWI/piRNA biology in mammals by studying the function of PIWIL3. To date, PIWIL3 has been detected in oocytes from antral follicles in cattle, monkeys and women, but its subcellular distribution, mechanisms of action and function remain largely unknown.

Microinjection has been used to study the functions of genes in oocytes. Microinjection of siRNA into oocytes is a commonly used technique to downregulate gene activity in single cells. By delivering siRNA into an oocyte with a sharp needle however, mechanical injury can interrupt the integrity of the plasma membrane, damage the cytoskeletal structure and potentially influence cell signaling. In addition, the injected siRNA could also affect gene expression aspecifically, which could affect the accuracy of the results. To reduce the effect of untargeted gene regulation, injection of non-specific siRNA needs to be performed as a negative control. However, little is known about the actual impact of the microinjection technique *per* se on an oocyte's maturation and developmental capacity.

It is recognized that oocyte cortical and plasma membrane proteins play important roles during oocyte maturation and fertilization. However, little is known about the function of the plasma membrane phospho(proteome) in mammalian oocytes and embryos. Investigating oocyte plasma membrane function has, amongst other impediments, been hampered by the technological difficulty of efficient plasma membrane protein extraction. To better study plasma membrane protein function in oocytes and early embryos, it is critical to find an efficient way for plasma membrane protein purification and identification when limited cell numbers are available.

Thesis outline

The underlying theme of this thesis is to better understand biochemical events during, and the effects of commonly used technology which might influence, oocyte development and early embryogenesis in large mammals.

For example, little is known about the cellular localization and function of PIWIL3 in mammalian oocytes, primarily because of the absence of validated antibodies and the absence of PIWIL3 in the mouse genome. In **chapter 2**, using microinjection of PIWIL3-eGFP constructs, immunoprecipitation and small RNA sequencing, we clarified the localization, interaction complex and the small RNAs related with the PIWIL3/TDRKH complex in bovine oocytes.

In **chapter 2**, microinjection was used for the investigation of PIWIL3 localization and function. While siRNA introduction by microinjection can be useful to study the function of maternally expressed genes in oocytes, injection is a rather invasive procedure in that it damages the plasma membrane and disturbs the cell organelle distribution. Therefore, in **chapter 3** we examined the effect of introducing non-specific small RNAs into oocytes by microinjection, at the single cell transcriptome level.

Based on the results of **chapter 3**, a group of mRNAs with functions related to plasma membrane interaction are significantly altered by microinjection, which raises the question of the importance of plasma membrane proteins during oocyte maturation and fertilization. In **chapter 4**, the plasma membrane (phospho)proteome was examined in bovine GV and MII stage oocytes and the zygote to yield an inventory of the global protein profile, and to examine protein dynamics and phosphorylation during meiosis and fertilization. The results provide basic knowledge of candidate proteins and PTMs to enable further study of mechanisms that are critical for the development of healthy oocytes, and for normal fertilization.

In **chapter 5**, the findings of these studies are discussed, with recommendations for future areas of study.

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Chapter 2

PIWIL3 forms a complex with TDRKH in mammalian oocytes

Minjie Tan[1], Helena T.A. van Tol[1], David Rosenkranz[2], Elke F. Roovers[3], Mirjam J. Damen[4,5], Tom A.E. Stout[1,6], Wei Wu[4,5,*], Bernard A.J. Roelen[1,6,*]

[1]Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.

[2]Johannes Gutenberg-University Mainz, Institute of Organismic and Molecular Evolution, Anselm-Franz-von-Bentzel-Weg 7, 55128 Mainz, Germany.

[3]Biology of Non-coding RNA Group, Institute of Molecular Biology (IMB), Ackermannweg 4, 55128 Mainz, Germany.

[4]Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands.

[5]Netherlands Proteomics Centre, Utrecht, the Netherlands.

[6]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.

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Abstract

PIWIs are crucial guardians of genome integrity, particularly in germ cells. While mammalian PIWIs have been primarily studied in mouse and rat, a homologue for the human PIWIL3 gene is absent in the Muridae family, and hence the unique function of PIWIL3 in germ cells cannot be effectively modeled by mouse knockouts. Herein, we investigated the expression, distribution and interaction of PIWIL3 in bovine oocytes. We localized PIWIL3 to mitochondria, and demonstrated that PIWIL3 expression is stringently controlled both spatially and temporally before and after fertilization. Moreover, we identified PIWIL3 in a mitochondrial-recruited three-membered complex with TDRKH and PNLDC1, and demonstrated by mutagenesis that PIWIL3 N-terminal arginine modifications are required for complex assembly. Finally, we sequenced the piRNAs bound to PIWIL3-TDRKH-PNLDC1 and report here that about 50% of these piRNAs map to transposable elements, recapitulating the important role of PIWIL3 in maintaining genome integrity in mammalian oocytes.

Introduction

Genomic integrity is critical for faithful propagation within the species. Ensuring genome integrity entails controlling transposable elements (TEs), which are mobile DNA sequences that can migrate within the genome[1]. Amongst various TEs, retrotransposons are especially damaging as these can be transcribed, reverse transcribed, and reinserted at multiple locations in the genome. While retrotransposon activity may be minimized by repressive DNA (cytosine) methylation, germ cells and early embryos are particularly vulnerable to retrotransposon reinsertion because in these developmental stages, cells undergo genome-wide demethylation[2]. Maintenance of genome integrity in oocytes and early developing embryos against TEs is therefore extremely important, since any genetic change would be passed on to the next generation.

In germ cells, P-element induced wimpy testis (PIWI) proteins that interact with 21-31 nucleotides non-coding PIWI interacting (pi)RNAs form an efficient system to silence TE activity[3–6]. The PIWI-piRNA pathway has been shown to suppress transposon activity both post-transcriptionally and transcriptionally[3,7]. Interestingly, PIWI-piRNA pathway activation has been mainly reported in the contexts of gametogenesis and embryology, further highlighting probably a unique and critical role for PIWIs in controlling genome stability, germ cell maturation and early embryonic development[8,9].

Genetic mutation of different Piwi genes in mice (*Mili*, *Miwi*, and *Miwi2*) are associated with male sterility, but no abnormal phenotypes were observed in females[10–12]. This appears to imply that functions of mouse Piwi genes (homologues of human *PIWIL1*, *PIWIL2*, *PIWIL4*) are not critically needed during oogenesis, maturation and post-fertilization, at least in the murine system. We have previously established that human, macaque and bovine oocytes express large amounts of piRNAs, and 30-50% of these are enriched for transposon sequences, in particular, the expression of a fourth PIWI gene, *PIWIL3*, was detected in human and bovine species but absent in murine systems[13,14]. Due, in part, to the perennial shortage of PIWIL3-specific antibodies, and the lack of mammalian species expressing PIWIL3 that are amenable to genetic manipulations, little is known about the function and subcellular localization of PIWIL3 as well as biogenesis in PIWIL3 related piRNAs.

Here we investigated the expression, distribution, and interaction of PIWIL3 in bovine oocytes by combining microinjection and immune-labeling techniques with affinity purification, crosslinking and mass spectrometry, as well as small RNA sequencing. We demonstrate here a novel and developmental stage-specific PIWIL3/TDRKH/PNLDC1 interaction network, which sheds light on piRNA biogenesis and maintenance of genome integrity in mammalian oocytes prior to and shortly after fertilization.

Results

PIWIL3 is expressed in the cytoplasm during oocyte maturation and early embryo development

Since the subcellular localization of a protein often dictates the mode of action and accessibility to substrates and cofactors, we sought to trace the intracellular distribution of PIWIL3 over developmental stages, to further elucidate the functional role of PIWIL3. While the intracellular distributions of PIWIL1 , PIWIL2 and PIWIL4 have been elucidated in mouse testis[10,15] and human fetal oocytes[14], the subcellular compartment(s) in which PIWIL3 exerts its function remains uninvestigated to date. Herein, we follow the distribution and expression pattern of PIWIL3 in bovine oocytes and preimplantation embryos, by transiently expressing EGFP-PIWIL3 fusion proteins.

We generated EGFP-PIWIL3 fusion constructs (both N- and C-terminal tagging), performed *in vitro* transcription and directly injected *EGFP-PIWIL3* mRNA into bovine oocytes at the germinal vesicle (GV) stage and 2 pro-nuclei (2PN) stage zygotes after *in vitro* fertilization. Zygotes were cultured further *in vitro* for up to 8 days (blastocyst stage), during which embryos at various developmental stages post fertilization were harvested.

First we confirmed that the entire constructs were translated in the oocytes. Microinjection of the *EGFP-PIWIL3* and *PIWIL3-EGFP* fusion construct into oocytes resulted in proteins of 130 kDa, corresponding to the total length of EGFP (26 kDa) and PIWIL3 (100 kDa) (Figure 1a). Next, we followed the localization of EGFP-PIWIL3 in bovine oocytes. Starting from the GV stage, EGFP-PIWIL3 was localized largely to the oocyte cytoplasm in a punctate pattern but was excluded from the nucleus, whereas the EGFP control signal was distributed evenly throughout the cell. The expression of PIWIL3 remained cytoplasmic throughout preimplantation development but strongly reduced at the blastocyst stage (Figure 1b). The same localization patterns were observed using C-terminal EGFP tagging (Figure 1b). Collectively, these data support a largely cytoplasmic localization of PIWIL3 in bovine oocytes and preimplantation embryos.

Figure 1

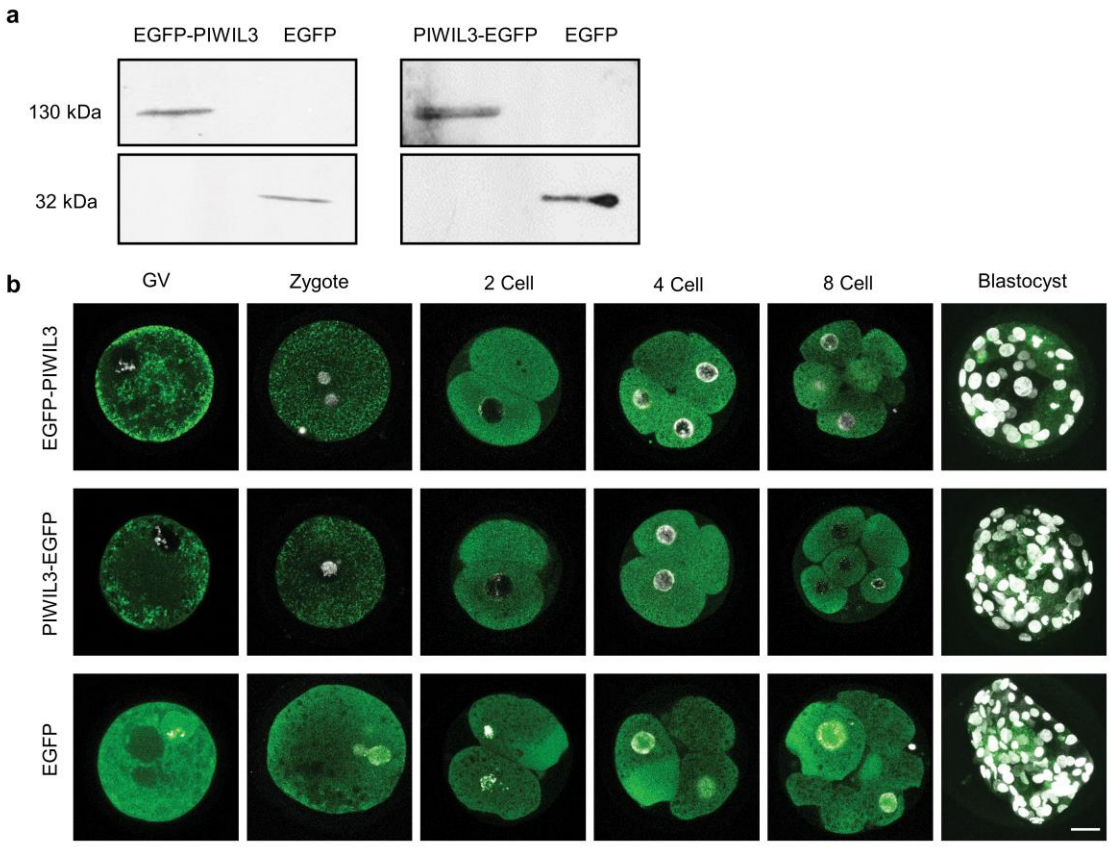

Figure 1: PIWIL3 is a cytoplasmic protein in oocytes and early stage embryos. a Detection of PIWIL3-EGFP, EGFP-PIWIL3 and EGFP in oocytes after mRNA injection by Western blotting. b Detection of EGFP and EGFP tagged PIWIL3 in oocytes and embryos by confocal laser scanning microscopy. Stages of oocyte and embryos are indicated at the top. DNA was stained with DAPI (white). Scale bar, 30 µm.

PIWIL3 co-localizes with TDRKH on mitochondria

In the absence of a working PIWIL3 antibody, the functional complexes involving PIWIL3 have not been characterized. Here, we utilize an alternative approach to investigate the promising PIWIL3 binding partner in bovine oocytes, as guided by existing knowledge of other PIWI interactions.

Tudor domain containing proteins have been shown to interact with other PIWI proteins[16–18], and we had established previously that Tudor and KH containing protein (TDRKH) is highly expressed in GV and Metaphase II (MII) oocytes[13]. We thus hypothesized that TDRKH might be a PIWIL3-associating protein in bovine oocytes. In further support of this possibility, we

examined the temporal expression pattern of TDRKH in bovine gonads, and TDRKH levels in different stages of bovine oocytes. Using immunoblotting, high levels of TDRKH were detected in oocytes and testis while lower levels were detected in complete ovaries, indicating that TDRKH was specifically expressed in the germ cells and not in the somatic tissue of the gonads (Figure 2a). This was further illustrated by immunohistochemical analyses of testis and ovary sections which showed again the expression of TDRKH in germ cells but not somatic cells. In bovine testis, TDRKH expression was localized to immature and mature sperm cells (Figure 2b). In bovine ovaries, TDRKH expression was detected in oocytes, but not in granulosa and theca cells, at all stages from primary to antral follicles (Figure 2c).

Using the same EGFP fusion constructs described above and injection of *EGFP-PIWIL3* mRNA into bovine oocytes at the GV stage, we detected the co-localization of TDRKH with EGFP-PIWIL3 (and PIWIL3-EGFP) *in vivo*, in cytoplasmic regions that co-stain with MitoTracker (Figure 2d, Supplementary Figure S1). Collectively these data indicate that PIWIL3 and TDRKH co-localize at mitochondria.

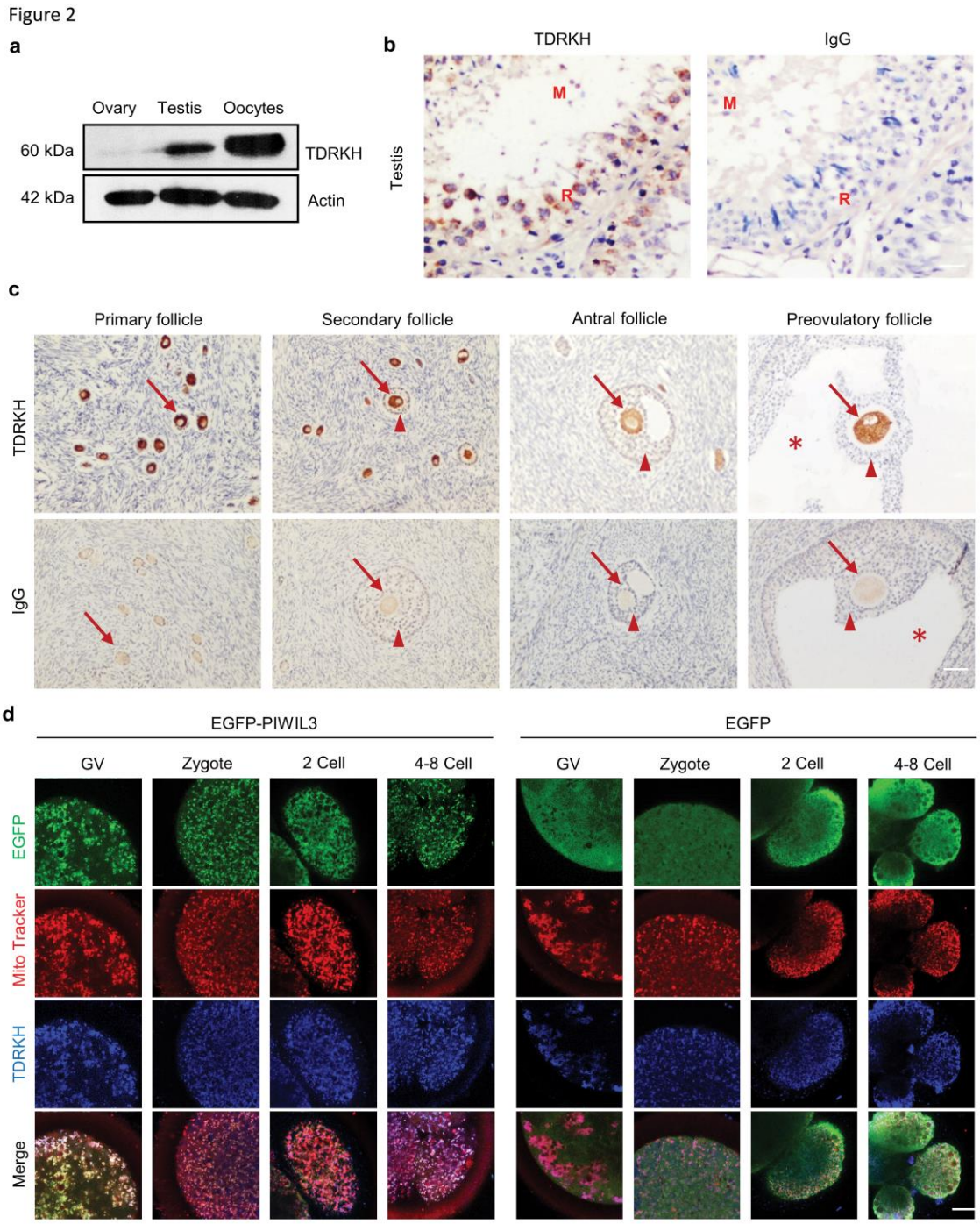

Figure 2: PIWIL3 colocalizes with TDRKH on mitochondria. **a** Western detection of TDRKH in bovine ovary, testis, as well as oocytes from antral follicles. **b** Immunostaining of TDRKH (brown staining) in paraffin sections of bovine testis. Blue: hematoxylin counterstaining. R: Round spermatid. M: Mature sperm. Scale bar, 100 µm. **c** Immunostaining of TDRKH (brown staining) in paraffin sections of bovine ovaries in different follicular stages. Blue: hematoxylin counterstaining. Arrows indicate oocytes; Triangles indicate cumulus cells; Asterisks indicate antrum. Scale bar, 100 µm. **d** Fluorescent detection

of EGFP-PIWIL3 (green), EGFP (green), MitoTracker (mitochondria, red) and TDRKH (blue) in microinjected oocytes and early stage embryos. Scale bar, 20 μm.

PIWIL3 is present in a complex with TDRKH and PNLDC1 in bovine oocytes

Since TDRKH and PIWIL3 fusion constructs co-localize in bovine oocytes, we were interested if these proteins also associate with each other.

We therefore proceeded to verify this interaction by reverse co-immunoprecipitation (co-IP) with a TDRKH antibody, followed by MS confirmation of the constituents in TDRKH complexes. To negate the possibility of non-specific proteins binding to GFP instead, we harvested bovine oocytes without mRNA injection for this experiment. By in-solution digestion of TDRKH co-IP eluates, the top three proteins detected were TDRKH, PNLDC1 and PIWIL3, making PIWIL3 and PNLDC1 highly confident interactors of TDRKH in bovine oocytes that were identified by many peptide spectra matches (Figure 3a, Supplementary Table S1). Other dominant proteins (OGDHL, IMMT, ARMC10 and PHB2) that showed enrichments in the immuno-purified TDRKH complexes were mostly mitochondrial, suggesting again that TDRKH connecting with PIWIL3 is docked on the mitochondria *in vivo*, in agreement with MitoTracker co-staining (Figure 2d and Supplementary Figure S1).

To further elucidate if PIWIL3 forms a complex with TDRKH and PNLDC1 simultaneously, or as two binary complexes (TDRKH-PIWIL3 and TDRKH-PNLDC1 respectively), we mildly crosslinked the proteins immuno-purified by TDRKH antibodies, eluted the complexes off Protein A/G-agarose beads, and detected by Western blotting a single complex species of >250kDa (Figure 3b). Since we did not observe two species of protein complexes containing TDRKH, and the theoretical combined size of PIWIL3-TDRKH-PNLDC1 alone is around 220kDa, we suspect that more proteins contribute to the complex. We report here that PIWIL3 is engaged in a TDRKH complex also including PNLDC1 in bovine oocytes.

Using qRT-PCR we examined the expression of *PIWIL3*, *TDRKH* and *PNLDC1* mRNA in oocytes and preimplantation embryos. The temporal expression pattern of these genes was similar, with expression in oocytes and embryos up to the 8-cell stage and decreased levels at morula

and blastocyst stages (Figure 3c). Taken together, these results suggest that PIWIL3, TDRKH and PNLDC1 are collectively engaged in a germ cell specific function *in vivo.*

Figure 3

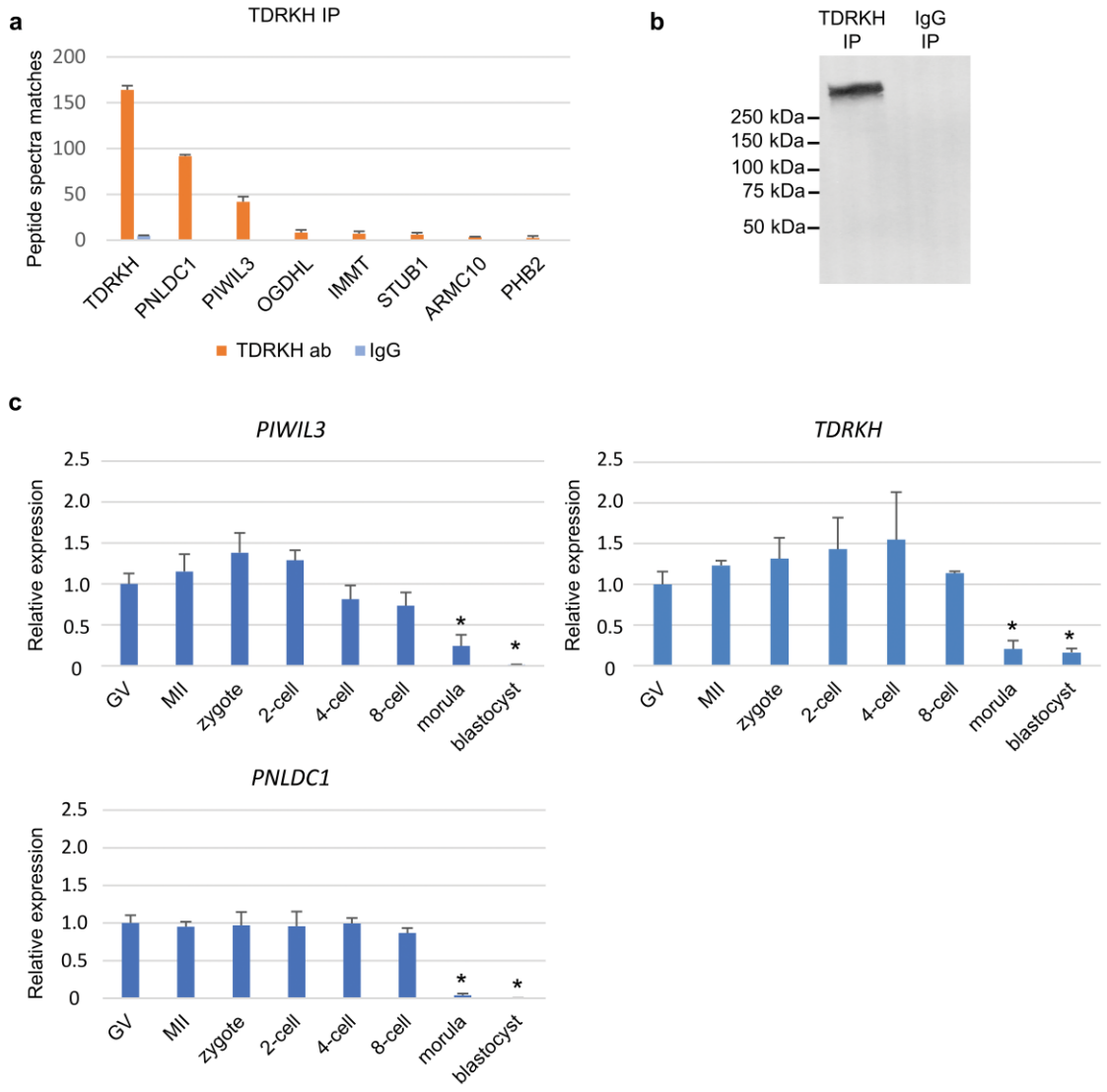

Figure 3: PIWIL3 forms a complex with TDRKH and PNLDC1 in oocytes. **a** Identification of TDRKH-associated proteins by mass spectrometry. Orange: TDRKH IP. Blue: IgG IP (negative control). **b** Crosslinked TDRKH complexes. Chemically crosslinked TDRKH complexes detected as a >250 kDa band by western blotting. **c** Bar graph showing *PIWIL3*, *TDRKH* and *PNLDC1* mRNA expression as detected by qRT-PCR in oocytes and early stage embryos. * $p<0.05$.

PIWIL3 interacts with TDRKH through N-terminal arginines

To further characterize the mechanism of PIWIL3-TDRKH interactions, we generated PIWIL3-EGFP N-terminal deletion and substitution mutants (Figure 4a) and injected in vitro synthesized mRNA into GV stage oocytes, to examine the regions critical for TDRKH interaction. In other animal model systems, PIWI family proteins have been suggested to interact with Tudor domain-containing proteins through symmetrically dimethylated arginines at the N-terminus[16,17,19]. We demonstrate here that N-terminal deletion of the GRARVHARG motif from PIWIL3 (G3-G11) abolished PIWIL3 interaction with TDRKH and recruitment to the mitochondrial surface. This is exemplified by the diffuse pattern of N-terminal truncated PIWIL3-EGFP cytoplasmic mislocalisation (Figure 4b, De1 panels), confirming that the N-terminal GRARVHARG motif in PIWIL3 is indeed essential for TDRKH binding in a higher mammalian model system.

We next sought extensively for spectral evidence of symmetric dimethylations that might exist on these arginine residues in the GRARVHARG motif, by affinity purification and mass spectrometry of peptides derived from TDRKH-bound PIWIL3. This was however hampered by general difficulties in N-terminal sequence coverage in mass spectrometry. Thus, we turned instead to site-directed mutagenesis for alternative evidence that indicate the importance of arginine modifications in this N-terminal stretch. PIWIL3 arginine residues in this GRARVHARG motif (R4, R10) are likely critical to mediate TDRKH docking, as conservative substitutions of both arginines to lysines in full-length PIWIL3-EGFP was already sufficient to prevent TDRKH binding (Figure 4b, Mu1 panel). In contrast, conservative substitutions of R58 and R63, two dimethylated arginines detected by mass spectrometry on TDRKH-bound PIWIL3, had no functional impact on TDRKH binding and mitochondrial recruitment, serving as very convincing negative controls for this experiment. Hence, by a combination of mass spectrometry and N-terminal truncation/conservative amino acid substitutions, we provide evidence here that PIWIL3 interaction with TDRKH likely requires arginine modifications in the N-terminal GRARVHARG motif, in strong agreement with previous postulates in other species[16,17,19].

Figure 4

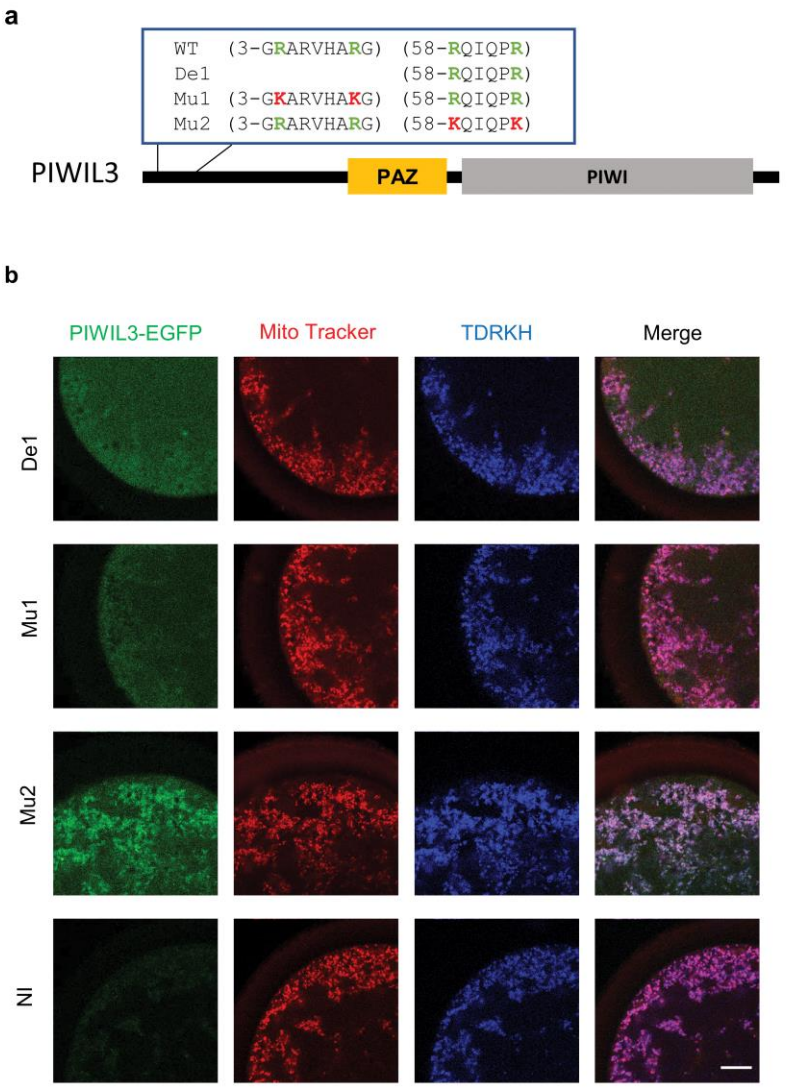

Figure 4: The N-terminal domain of PIWIL3 is critical for binding with TDRKH. **a** Domain map of PIWIL3 mutation construct. WT: wild type PIWIL3 sequence. De1: deletion construct lacking N-terminal GRARVHARG. Mu1/Mu2: mutation vectors. Green: original arginines; red: R→K substitutions. **b** Triple labeling of PIWIL3-EGFP mutants (green), mitochondria (red) and TDRKH (blue) in oocytes. Coding on the left indicates injected mRNAs. NI: Non-injected oocytes. Scale bar, 20 µm.

piRNAs are loaded onto PIWIL3/TDRKH/PNLDC1 complexes

With our experimental evidence for the interaction between PIWIL3, TDRKH and PNLDC1, a new avenue to study PIWIL3-bound piRNA species arose, even in the absence of PIWIL3-

specific antibodies. Instead of directly retrieving PIWIL3, the whole PIWIL3-TDRKH-PNLDC1 complex containing the bound piRNA could be immuno-precipitated with a TDRKH antibody.

Specifically, we used lysates of bovine oocytes as starting material to isolate endogenous PIWIL3-TDRKH-PNLDC1 complexes, and extracted the RNAs with Trizol to isolate small RNAs for sequencing. In parallel, we performed the same procedures on the input samples (oocytes lysate before IP), for comparison of piRNAs enrichment. The sequenced libraries contained about 30 million raw reads for input samples and IP samples. After trimming, length filtering (18-36 nt) and quality processing, approximately 13 million reads per replicate from TDRKH IP samples were mapped to the *Bos taurus* genome. In both duplicates of TDRKH immuno-precipitated piRNAs and total piRNAs (Input), high R^2 correlations of 0.97-0.98 were obtained between biological (independent IP and sequencing) replicates (Figure S2a). The raw data for RNA sequencing is appended in Supplementary information, Table S3.

Next, we examined the RNA species in the TDRKH IP samples to characterize the small RNA pool associated with the PIWIL3/TDRKH complex. While miRNAs, rRNAs, and tRNAs were underrepresented in the TDRKH IP samples compared to input samples, piRNAs had clearly higher affinity for PIWIL3-TDRKH-PNLDC1 complexes (Figure 5a). Both input (70 %) and TDRKH IP (80 %) samples predominantly comprised small RNAs that map to annotated piRNA producing loci[20] and exhibit a strong bias for uracil (U) at the 5’ position. The 5’ U bias was even stronger in the IP samples (Figure 5b; Supplementary information, Table S3). To further confirm the genuine presence of piRNAs, we assessed the RNA length distribution from the IP samples, which revealed a normal distribution about the peak of 24-27 nucleotides, strongly indicative of piRNA species. In addition, we also observed a strong enrichment of read pairs with 10 nt 5’ overlap, which is again highly indicative of the presence of primary and secondary piRNAs in the IP samples. These piRNA properties indicate that both primary and secondary piRNAs are bound by the PIWIL3-TDRKH complexes in bovine oocytes (Figure 5b-d, Figure S2b; S3).

Figure 5

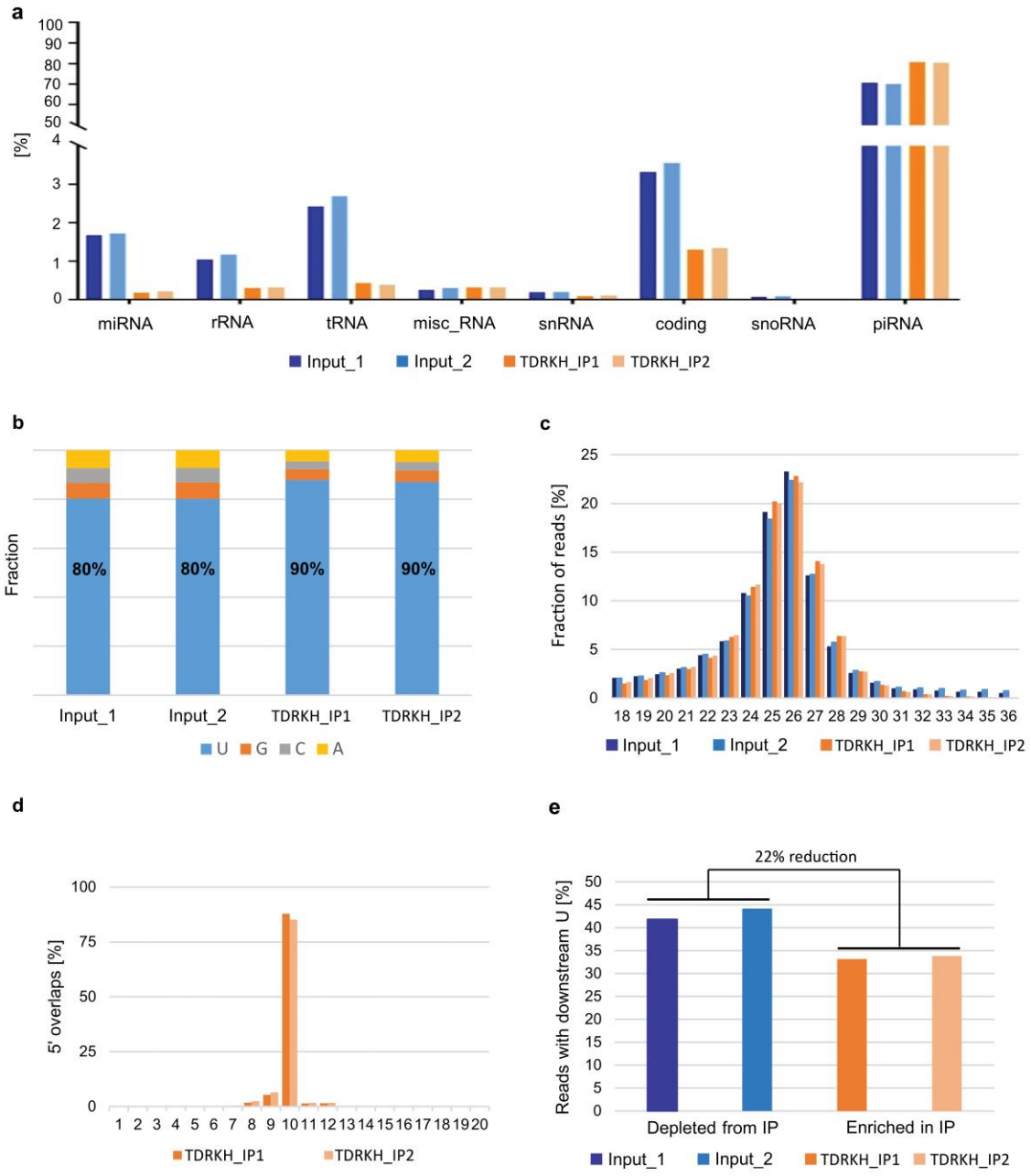

Figure 5: Analyses of piRNA sequences detected after TDRKH immunoprecipitation. Input 1, 2: duplicate total RNAs (blue). TDRKH_IP1, 2: duplicate immunoprecipitated RNAs (orange). **a** RNA categories detected in Input and TDRKH IP samples. **b** Nucleotide composition at the 1st position of the 5' end. **c** Length distribution of the small RNA libraries. **d** Overlaps of 5'ends of reads that are mapped to opposite strands of the same locus. **e** Frequency of downstream 1U in the small RNAs. Percentage reduction in reads with downstream 1U calculated with respect to Input samples.

According to the current model of piRNA biogenesis, the endonuclease Zucchini (phospholipase D family member 6: PLD6) functions to promote piRNA biogenesis by cleaving pre-pre-piRNAs to generate the 5' end of pre-piRNAs. A key characteristic of these intermediates is that they have a uridine directly downstream of the generated piRNA intermediate in the genome[21,22]. Once PIWI protein loaded with pre-piRNAs complexes with PNLDC1, 3' end trimming by PNLDC1, a PARN family 3'-5' exonuclease, is thought to start. Hence, piRNA intermediates on the TDRKH complex should lose their downstream U bias because of 3' end trimming. In contrast, piRNA intermediates that are not loaded on the trimmer complex may still keep the downstream U bias. We evaluated this hypothesis based on our data, and indeed found a 22% reduction in downstream 1U with respect to Input samples (Figure 5e), indicative of trimming activity by the PIWIL3-TDRKH-PNLDC1 complex.

Traditionally, piRNAs have been widely associated with TEs and repeat regions. To assess piRNAs in the mammalian PIWIL3/TDRKH complexes in relation to repetitive sequences, we compared the location of mapped small RNAs with the RepeatMasker annotation of the *Bos taurus* genome. We found that 45% of RNAs in TDRKH IP samples are mapped to transposon sequences, most notably LINE, SINE and LTR elements (Figure 6a). Within the LINE class, piRNAs map predominantly to L1 and BovB repeats, which is similar to the data previously reported for piRNAs extracted from regular oocyte samples (Figure S2c)[13,23]. Based on sequence homology we made a list of non-transposon putative target transcript genes (Table S4). These genes are not only targeted but also processed via the ping-pong cycle, indicating that they are piRNA targets.

We further addressed the chromosomal distribution of piRNA producing loci based on *de novo* piRNA cluster prediction with proTRAC (Figure 6b). We identified 674 distinct piRNA producing loci with a total size of 5 MB. In line with findings from other species, few piRNA clusters contribute to the majority of piRNA reads, as more than 80% of clustered piRNA reads were derived from only 12 predicted piRNA clusters up to 154 kb in length. Depending on the physical location of these piRNA clusters, the density of genomically encoded piRNAs per MB DNA can be diverse across different chromosomes. While the chromosomes 6, 17 and 23, which comprise the three largest piRNA clusters in terms of read counts, exhibit an overall density of >1600 aligned piRNA reads per MB, all other chromosomes exhibit on average of ~50 aligned piRNA reads per MB. We further noticed that the identified piRNA clusters

represent a main source of piRNAs antisense to transposon sequences, suggesting they are likely to be produced from piRNA clusters and regulate TEs. On average, 71% of transposon derived reads being reverse complementary to transposon sequences, while the same holds true for only 56% of transposon derived reads from outside of piRNA clusters (Figure 6c, Figure S2d).

Figure 6

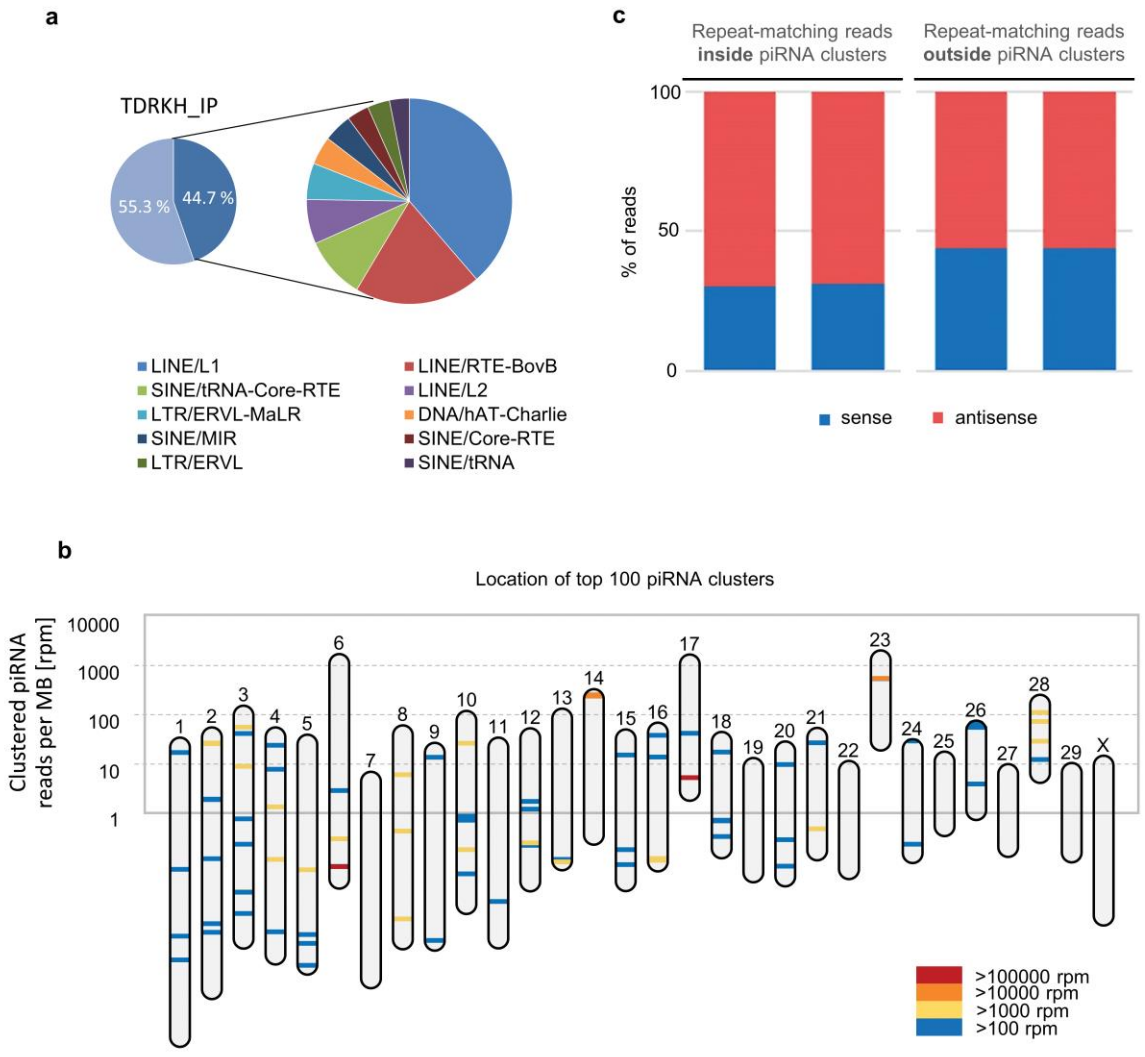

Figure 6: piRNAs mapping to transposons. **a** Pie chart depicting the transposon content (dark blue) of bovine piRNA populations from TDRKH IP samples, with further annotation in inset. **b** Mapping of piRNA clusters across chromosomes in the *Bos taurus* genome. Each piRNA cluster location is indicated by a bar colored according to its expression level. Y-axis refers to the number of clustered piRNAs per chromosome, normalized by chromosome size. **c** Repeat-matching reads inside and outside piRNA clusters in TDRKH IP samples.

Discussion

Since a *PIWIL3* ortholog is absent from the mouse genome, the function of PIWIL3 has not been studied by gene knockout and has remained largely unknown. Based on genetic deletion of the other 3 Piwi genes *Miwi*, *Mili* and *Miwi2* in the mouse, it was hypothesized that in mammals PIWI proteins are only essential for male germ cells. Here we reveal on the contrary that PIWIL3 is expressed in the cytoplasm of bovine maturing oocytes. Our data demonstrate that in oocytes from antral follicles, pre-piRNAs/PIWIL3 associated with TDRKH and PNLDC1 on mitochondria, most likely revealing the piRNA biogenesis mechanism in mammalian oocytes.

TDRKH is a conserved Tudor domain protein localized on mitochondrial that is critical for pre-piRNA 3' end trimming in diverse animal models. As interacting partner of mouse MIWI, MIWI2 and a lesser extent to MILI, knockout of TDRKH resulted in spermatogenesis arrested in meiosis and reduced levels of mature piRNAs[16,24,25]. Various PIWI proteins have been suggested to interact with Tudor proteins through multiple arginine-glycine and arginine-alanine (RG/RA) rich clusters at their N termini in either an arginine methylation dependent, or independent manner depending on the species[16,17,26–28]. For the first time in a mammalian system, we identified a symmetrical glycine-arginine-alanine motif in the N terminus of PIWIL3 as a binding site for TDRKH in oocytes, and demonstrated indirectly that post-translational modifications on the side chain of R4 and R10 might be critical for TDRKH docking. Whether TDRKH binding requires the arginines to be methylated in PIWIL3 remains to be demonstrated, and indeed it has been shown that TDRKH preferentially recognizes unmethylated arginines in the N-terminus of PIWIL1[28]. We also compared the N terminal domains of PIWIL3 in different mammalian species: cow, human, rabbit, rhesus macaque and golden hamster together with mouse PIWIL4 (MIWI2) (Figure S4). A conserved GRAR region is present in the PIWIL3 N terminal region of these species. R4 and R10 are conserved in most of the species except in rabbit and golden hamster where R10 is replaced by P and Q respectively. The N terminal part with repeated arginines seems to be the conserved part that interacts with TDRKH.

Interestingly, in bovine and human oocytes from antral follicles PIWIL3 was the only detected PIWI member[13,29], while it was absent from testis tissue. TDRKH on the other hand was also

strongly expressed in bovine testis and in mice is required for spermatogenesis indicating binding to a variety of Piwi molecules[16]. In mice, it has been reported that TDRKH acts as a key mitochondria-anchored scaffold protein that specifically recruits MIWI to the intermitochondrial cement and tethers PNLDC1 to couple MIWI recruitment and piRNA trimming during pachytene piRNA biogenesis. This TDRKH-mediated scaffolding function is essential for the production of MIWI-piRNAs, MIWI chromatoid body localization, transposon silencing and spermiogenesis[24]. We propose that in bovine oocytes, TDRKH is also important for PIWIL3 recruitment to mitochondria since PIWIL3 physically interacts with TDRKH and co-localizes with TDRKH on the mitochondria. This TDRKH binding brings the piRNA-bound PIWIL3 in close proximity to PNLDC1, a deadenylase responsible for the elimination of mRNA 3' poly(A) tails and trimming during pre-pachytene and pachytene pre-piRNA maturation[25,30,31].

In silkworms (*Bombyx mori*) it has been postulated that BmPapi (the silkworm homologue of TDRKH) recruits PIWI-pre-piRNA complexes on the surface of mitochondria to create an optimal platform for trimming. PNLDC1 binds with BmPapi and shortens the 3' end of pre-piRNAs to the mature length after which the 3' end is 2'-O-methylated by BmHen1[25]. In our data, sequences enriched in IP samples were slightly shorter (average size 25.2 nt of IP piRNAs versus 25.4 nt of input piRNAs). This was predominantly due to a smaller percentage of RNAs between 30 and 36 nt (Figure 5c). Moreover, with the support of reduced downstream U bias in the genome of IP samples, our results indicate that PNLDC1 is also responsible for the trimming of pre-piRNAs in mammalian oocytes. In our data (Figure S5), we also found around 18% of the piRNAs in IP samples are marked by an extension of 1-2 nucleotides. Among it, piRNAs display strong adenylation. Agree with the publication before[13], we think these piRNAs are kept relatively unstable and are likely to be a signal for degradation. Our qPCR data of expression of *PIWIL3*, *TDRKH* and *PNLDC1* mRNA indicate that the piRNA biogenesis complex in bovine embryos is mainly functional before the morula stage, also in agreement with a coordinated decrease in TDRKH and PNLDC1 levels during embryonic development detected previously[32]. While other PIWI proteins have been studied rigorously in lower model organisms, this study is the first to provide evidence in oocytes of mammals that piRNAs are present in a complex comprising both PIWIL3, TDRKH and PNLDC1.

PIWI proteins and piRNAs are regulators of both transposon and mRNA expression[33]. About 45% of the piRNA sequences detected in IP samples map to retrotransposon elements, while

only fewer than 20% of piRNAs from adult bovine testis do so[20], strongly indicating that PIWIL3 is involved in the control of retrotransposon elements in oocytes. piRNAs from oocytes and Tdrkh-IPs map uniquely to intergenic regions of the genome, forming large piRNA clusters. While roughly half of the clustered piRNAs derive from transposon copies and are thus presumably involved in transposon control, the function of non-repeat derived piRNAs remain enigmatic. In agreement with Cuthbert et al[34], PIWIL3-bound piRNAs clusters were predominantly detected on chromosomes 6, 17, 14 and 23. In contrast however, we observed enhanced piRNA mapping to chromosome 28, but not to chromosomes 8 and X. Since no other PIWI proteins were detected in bovine oocytes it seems unlikely that the differences are caused by oocyte piRNAs bound by other proteins, but more likely due to analysis settings.

During mouse spermatogenesis, meiosis induces a period of transcriptional quiescence which leads to the post-transcriptional silencing of transposon mRNAs by multiple epigenetic mechanisms that are in conjunction with the piRNA pathway[35]. However, since PIWIL3 is absent from mouse oocytes, piRNAs may play a more important role in oogenesis of non-murine mammals. We conducted a *PIWIL3* siRNA microinjection experiment with GV oocytes and oocytes matured to the MII stage. Despite huge efforts and efficient reduction in *PIWIL3* mRNA levels, the PIWIL3 protein level did not significantly change (data not show), which indicates that siRNA mediated knock down is not an efficient way to address the function of PIWIL3 in oocytes due to putatively long half-life of PIWIL3 proteins and limited culture time during oocyte maturation.

Some Argonaute family members including MIWI and human Agos are small RNA-guided nucleases (slicers) that catalyse cleavage of target nucleic acids depending on the presence of a conserved catalytic motif Asp-Glu-Asp-His inside the PIWI domain[36,37]. We also found the conserved catalytic motif DEDH in PIWIL3, leading us to speculate that PIWIL3 may act as a slicer at some point during oocyte meiosis and embryo development. This assumption is supported by the presence of a ping-pong signature, which indicates the cleavage of piRNA target transcripts, in simultaneous absence of other PIWI proteins. Further biochemical experiments should be performed to confirm this activity.

Depletion of *Piwi* genes lead to male-specify sterility because of defects in sperm formation. However, the functions of PIWI proteins in mammalian females are still largely unknown. For

a long time, the function and piRNA-binding properties of PIWIL3 was not investigated, primarily due to the lack of animal systems expressing this important piRNA processing regulator, and the perennial shortage of PIWIL3-specific antibodies. Here we provide a first glimpse into the PIWIL3 piRNA repertoire in bovine oocytes. In future studies, mechanism and function of PIWIL3/TDRKH/PNLDC1 complexes in mammalian oocytes should be further investigated using knockout animal models, although the mRNA injection workflow we present here could also be useful for experiments with relatively shorter time durations.

Methods

Oocytes collection, maturation and fertilization

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in a polystyrene box at room temperature (RT) within 2 h after slaughter. After washing and removal of extraneous connective tissue, the ovaries were transferred to a flask containing 0.9% NaCl supplemented with 10 U/ml penicillin/streptomycin (Gibco BRL, Paisley, UK) and maintained in a water bath at 30 °C. Cumulus oocyte complexes (COCs) were collected, matured and fertilized *in vitro* as described previously[38].

RNA isolation, cDNA synthesis

An RNeasy Micro Kit (Qiagen, Valencia, CA, USA) was used to extract total RNA as described before[39]. The columns were eluted with 18 µl RNAse-free water. For cDNA synthesis, 10 µl RNA was mixed with 4 µl 5 x RT buffer (Invitrogen, Breda, The Netherlands), 0.2 µl RNAsin (Promega, Leiden, The Netherlands), 0.75 µl Superscript III reverse transcriptase (Invitrogen), 0.4 µl random primers (Invitrogen), 2 µl DTT (Invitrogen) and 1 µl dNTP (Promega). Minus RT blanks were prepared from 10 µl of the same RNA sample under the same conditions without the addition of reverse transcriptase. The mixture was incubated for 1 h at 55 °C, followed by 5 min at 80 °C before storage at –20 °C.

PIWIL3 plasmids construction

PIWIL3 cDNA was amplified from oocyte cDNA using PCR. PIWIL3 plasmid construction primers are shown in supplementary table S2a. The DNA was cloned into pcDNA3-EGFP, a gift from Doug Golenbock (Addgene plasmid # 13031). A Q5® site-directed mutagenesis kit (New England BioLabs, Ipswich, MA, USA) with forward primer 5'- CTACAGAAGAAAGCACAATG -3' and reverse primer 5'- AAGGTAAAAGAGAGATTTTGAC -3' was used to delete the stop codon in *PIWIL3*. The HiScribe™ T7 ARCA mRNA kit (New England BioLabs) was used to transcribe *PIWIL3-EGFP, EGFP-PIWIL3* and *EGFP* mRNA *in vitro*. The mRNA integrity was checked by electrophoresis on 1% agarose gels with MOPS buffer prior to microinjection.

Injection of mRNA into oocytes

Before injection, *EGPF-PIWIL3, PIWIL3-EGFP* and *EGFP* mRNA were diluted with RNase-free water to a final concentration of 100 nM. Subsequently, 6 denuded germinal vesicle (GV) stage oocytes were transferred to a 5 µl drop of HEPES buffered M199 with 10% FCS in a 60 mm dish overlaid by mineral oil at 37 °C on an IX71 inverted microscope (Olympus, Leiderdorp, the Netherlands) equipped with a heated stage at 38.5 °C . In total, 5 µl of the mRNA was loaded into a microinjection needle with a 30° angle and 4.3-4.9 µm inner diameter of the tip (Origio, Vreeland, The Netherlands). Injection was performed at 100 hpa for 0.2 seconds. After injection, oocytes were cultured in maturation medium with 25 µm Roscovitine for 8 h to collect GV stage oocytes; only oocytes with green fluorescence were washed in PBS and fixed in 4% paraformaldehyde (PFA) 30 min at room temperature (RT). Zygotes used for microinjection were collected 8 h after in vitro fertilization (IVF).

Immunoblotting

Groups of 60-100 injected oocytes or 100 normal oocytes, ovary and testis tissues were lysed in RIPA buffer (Pierce Biotechnology, Rockford, IL, USA) supplemented with 1% protease/phosphatase inhibitor (ThermoFisher, Waltham, MA, USA). Lysates were separated by electrophoresis on 8% SDS-PAGE gels and subsequently transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Blots were blocked with 5% milk in TBST (TBS + 0.1% Tween 20) for 1 h at RT and incubated with GFP antibody (1:1000, sc-9996, Santa Cruz Biotechnology, Dallas, TX, USA) or TDRKH antibody (1:1000, 13528-1-AP, Proteintech) overnight at 4 °C. Blots was washed three times (10 min each) in PBST followed by 1 h incubation with secondary antibody: HRP-conjugated goat anti mouse IgG (1:5000, sc-2005, Santa Cruz Biotechnology) or HRP-conjugated goat anti rabbit IgG (1:5000, 31460, Pierce Biotechnology) at RT. Antibody binding was detected using ECL Super Signal West Dura Extended Duration Substrate (ThermoFisher) and exposure to maxAgfa CL-XPosurelight films (ThermoFisher).

Mitochondrial staining and immunofluorescence of bovine oocytes

For mitochondrial staining, oocytes and early stage embryos were incubated in M199 with 500nM MitoTracker™ Red CMXRos (M7512, ThermoFisher) for 1 h in a humidified incubator at 38.5 °C and 5% CO_2. Oocytes and embryos were subsequently washed 3 times in PBS and fixed in 4% PFA for 30 min at RT.

Immunofluorescence was conducted largely as described before[39]. After fixation, oocytes were washed three times in PBST (PBS+10% FBS+0.1% Triton-100), permeabilized for 30 min using 0.5% Triton-X100 in PBS with 10% FBS and blocked in PBST for 1 h at RT. Incubation with TDRKH antibody (13528-1-AP, Proteintech ThermoFisher) 1:100 was at 4°C overnight. Oocytes were then washed three times in PBST for 15 min each and incubated with secondary goat anti-rabbit 1:100 (AlexaFluor 488, Life Technologies, Bleiswijk, the Netherlands) for 1 h in the dark at RT. After washing, oocytes were incubated with 4',6-diamidino-2-phenylindole (DAPI) for 20 min and mounted onto glass slides using Vectashield (Vector Laboratories, Burlingame, CA, USA). Fluorescence was examined by confocal laser scanning microscopy (TCS SPE II, Leica, Wetzlar, Germany).

Immunohistochemistry

Tissues were fixed in 4% PFA overnight at 4 °C and embedded in paraffin. Sections (5 µm) were deparaffinised, washed in water and citrate buffer (pH 6.0) for antigen retrieval followed by blocking with BSA. The sections were treated with the primary anti-TDRKH antibody (1:200, 13528-1-AP, Proteintech ThermoFisher) overnight at 4 °C. After incubation with HRP-conjugated goat anti rabbit secondary antibody (1:5000, #31460, Pierce Biotechnology), the slides were washed and developed with Diaminobenzidine (DAB) solution, K3468, Dako) and counterstained with hematoxylin. As a negative control, sections were incubated with rabbit Immunoglobulin (X0903, Dako Agilent, Santa Clara, CA, USA).

Immunoprecipitation and MS identification

Cells were lysed in IP lysis buffer (Pierce™ Crosslink IP Kit, 26147, ThermoFisher) and 10 µg TDRKH antibody (13528-1-AP, Proteintech) or rabbit Immunoglobulin (X0903, Dako Agilent) was coupled to Protein A/G-coated agarose beads, and crosslinked with 2.5 mM DSS to prevent co-elution. The antibody conjugated beads were mixed with bovine oocyte lysate incubated for 60 min to retrieve TDRKH complexes. The IP eluates were reduced with 4 mM DTT, alkylated with 8mM Iodoacetamide, and then digested with 1:75 trypsin at 37 °C overnight. The resulting peptides were desalted by c18, dried by vacuum centrifugation and reconstituted in 10% formic acid for analysis on an Orbitrap Q Exactive HF spectrometer (ThermoFisher) connected to a UHPLC 1290 LC system (Agilent). Raw files were processed using Proteome Discoverer 1.4.1.14 and searched using Mascot against the bovine database

(downloaded on 2017.11.29, containing 32206 entries). Cysteine carbamidomethylation was used as a static modification and methionine oxidation was set as a possible dynamic modification. Up to 2 missed cleavages were allowed. False discovery rates of 1% was set for both protein and peptide identification. For IP samples prepared for Western blot, 1 mM DSSO was used to crosslink protein complex before elution.

Quantitative RT-PCR

RNA isolation and cDNA synthesis were described as before in the methods. 20-30 oocytes or embryos were collected for each sample. Minus RT blanks were prepared from 5 µl of the same RNA sample under the same conditions, but without addition of reverse transcriptase. 1 µl of the resulting 20 µl cDNA was used for qRT-PCR analyses with the primers listed in Supplementary Table S2b. The qRT-PCR reactions were performed using a real-time PCR detection system (MyiQ Single-color Real-Time PCR Detection System; Bio-Rad Laboratories, Hercules, CA, USA) with IQ Sybr Green Supermix (Bio-Rad Laboratories). Three biological repeats were analyzed. The relative starting quantity for each experimental sample was calculated based on the standard curve made for each primer pair. Data normalization was performed using *GAPDH* and *SDHA* as reference genes with the same set of samples.

IP for piRNA sequencing

Bovine GV stage oocytes were isolated and snap frozen in liquid nitrogen. Upon collection, 500 oocytes per IP sample were taken up in 500 µL cold lysis/IP buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1% Triton-X100, 1 mM DTT, Protease Inhibitor (04693159001, Roche)) and sonicated 3 x 30 sec. The samples were centrifuged at 12 000 g at 4 ^{o}C and the supernatant was used for IP. Samples were incubated with anti-TDRKH 1:100 (13528-1-AP, Proteintech ThermoFisher) for 2 h at 4 ^{o}C, rotating. Next, samples were incubated for another 45 min at 4 ^{o}C with 30µL pre-washed Dynabeads protein G. Afterwards, the beads were washed 3 times with cold wash buffer (25 mM Tris pH 7.5, 300 mM NaCl, 1.5 mM MgCL2, 1 mM DTT) and taken up in Trizol (15596018, ThermoFisher), followed by RNA and protein isolation according to the manufacturer. The RNA samples were used for library preparation.

Library preparation

NGS library prep was performed with NEXTflex Small RNA-Seq Kit V3 following Step A to Step G of Bioo Scientific's standard protocol (V16.06). Libraries were prepared with a starting amount of 5ng and amplified in 25 PCR cycles. Amplified libraries were size selected on a 8% TBE gel for the 18 – 40 nt insert size fraction. Libraries were profiled in High Sensitivity DNA Assay on 2100 Bioanalyzer (Agilent) and quantified using the Qubit dsDNA HS Assay Kit, in a Qubit 2.0 Fluorometer (Life Technologies). All 4 samples were pooled in equiAL molar ratio and sequenced on 1 NextSeq 500 Flowcell, Highoutput 75-cycle-kit, SR for 1x 84 cycles plus 7 cycles for the index read.

Sequencing data processing

Trimming of adapter sequences comprising flanking 4 nucleotide random tags with subsequent filtering of putative PCR duplicates was performed as previously described[13,40]. Trimmed sequence reads <18 or >36 nt in length as well as low complexity reads were filtered and the remaining reads were collapsed to non-identical sequences using Perl scripts from the NGS TOOLBOX[13]. Mapping to the genome of *Bos taurus* (bosTau7) and subsequent filtering of alignments was performed as described[41]. The obtained map files were used as input for unitas small RNA annotation pipeline (v.1.5.3, additional options: -pp –skip_dust)[42]. Reference sequences were obtained from Ensembl Database (release 93)[43], GtRNAdb[44], piRNA cluster database[20], SILVA rRNA database (release 132) and miRBase (release 22)[45,46]. The amount of sequences that correspond to repetitive DNA was determined based on RepeatMasker annotation (RepeatMasker open-4.0.5, Repeat Library 20140131) using the custom Perl script RMvsMAP.pl. *De novo* piRNA cluster prediction was performed with proTRAC 2.4.2 for each dataset separately using default settings. The predicted cluster coordinates were merged as described[41]. piRNA target genes were predicted as described before without allowing any mismatches when mapping piRNAs to coding sequences[47].

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Author contributions

M.T, W.W and B.R designed the study and interpreted the data. M.T collected primary materials and performed plasmid construction, microinjection, and immunostaining. M.T and M.J.D performed IP experiments and MS analyses. H.T performed qRT-PCR experiments. E.F.R performed RNA sequencing. D.R performed the small RNA sequencing analysis. M.T, B.R, W.W and T.S. wrote the manuscript. All authors contributed and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Material and Correspondence

All data and material that support the findings of this study are available from the corresponding authors Wei Wu or Bernard A.J. Roelen upon reasonable request.

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Supplementary Figures

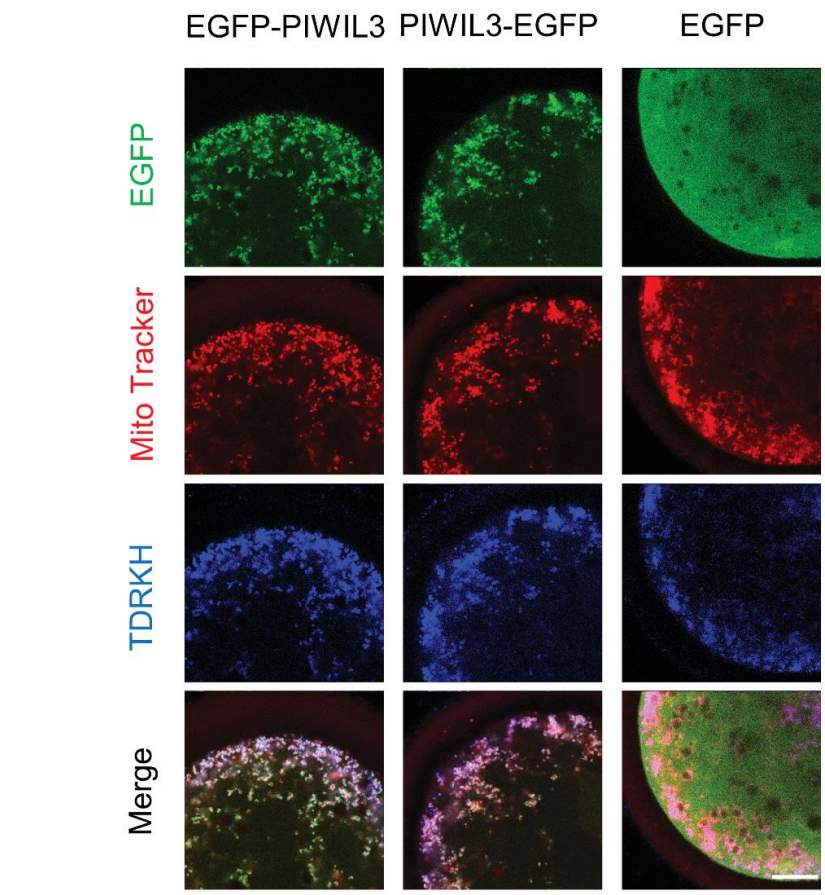

Fig. S1: Fluorescent localisation after microinjection of PIWIL3-EGFP or EGFP-PIWIL3 mRNA into GV stage bovine oocytes. Distribution of EGFP-PIWIL3 and PIWIL3-EGFP (green), MitoTracker (mitochondria, red) and TDRKH (blue) in oocytes. Scale bar, 20 µm.

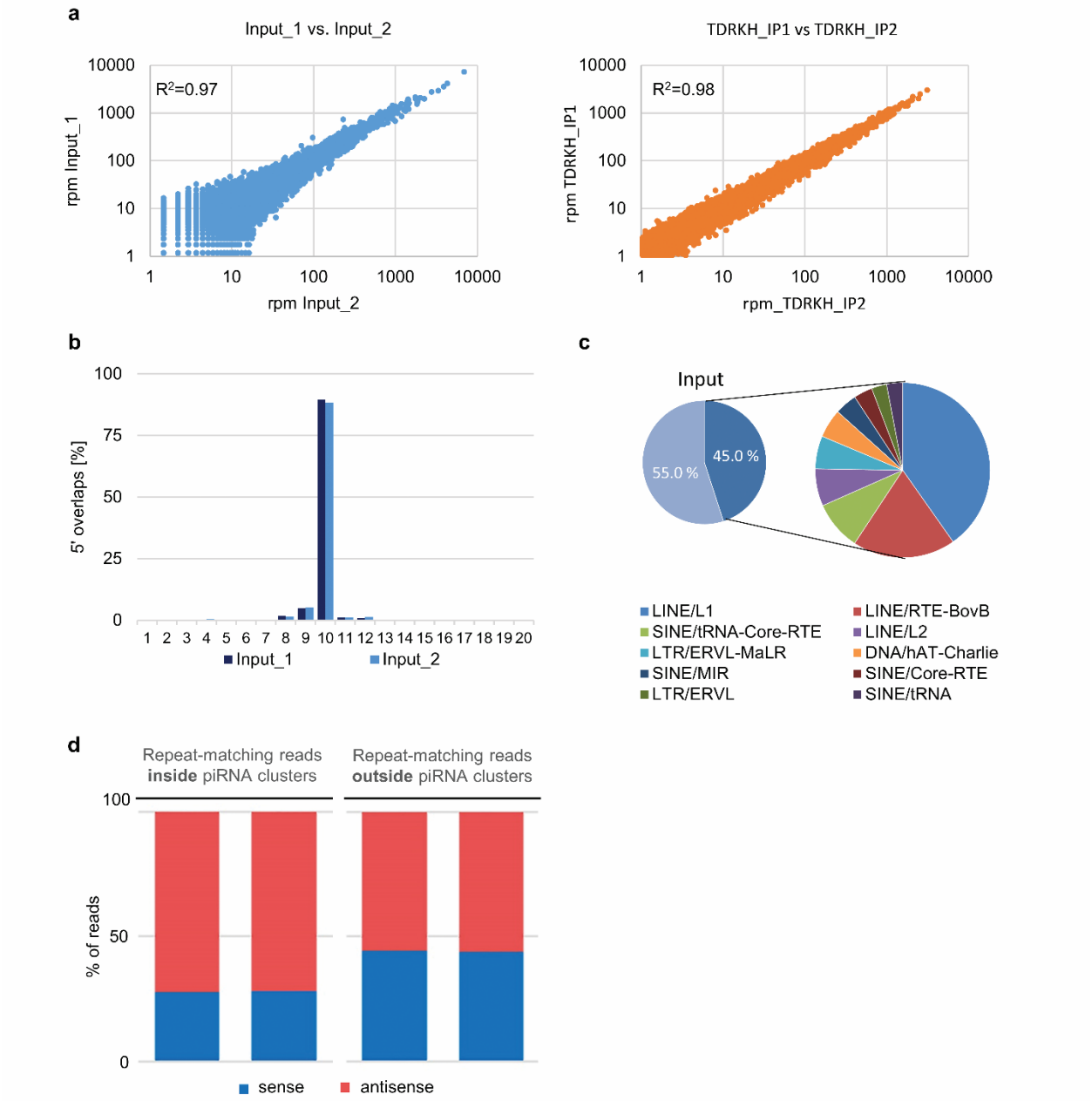

Figure S2: piRNA sequencing summary statistics. **a** Technical reproducibility. Pearson correlation between sequencing replicates varied in the range of $0.97<r^2<0.98$. rpm, reads per million. **b** Overlaps of 5' ends of reads that are mapped to opposite strands of the same locus. **c** Pie chart depicting the transposon content of bovine piRNA populations from Input samples. **d** Repeat-matching reads inside and outside piRNA clusters for Input samples.

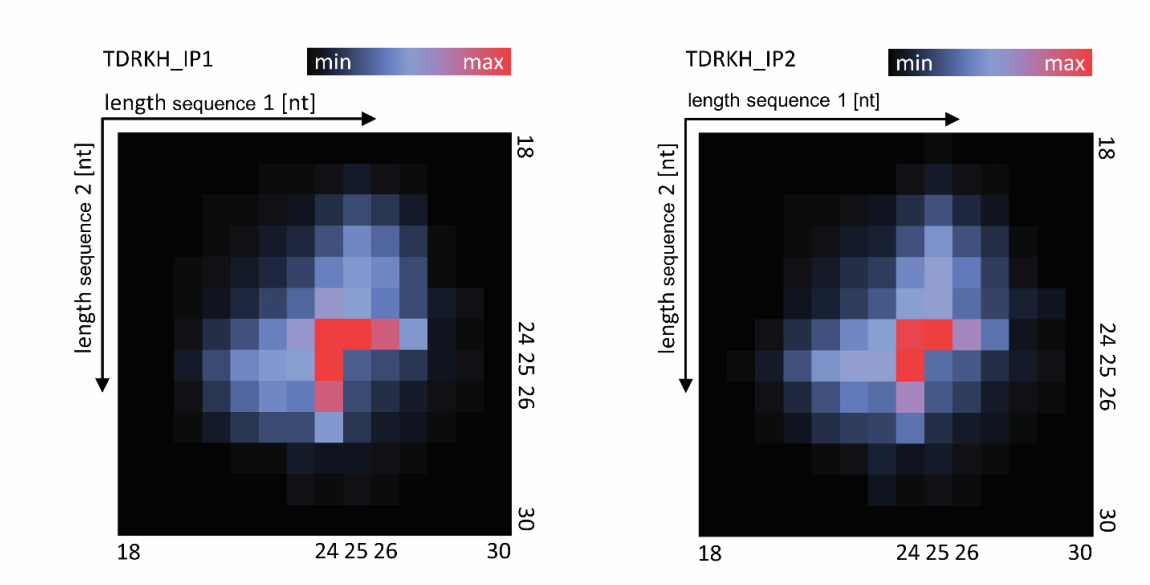

Figure S3: Sequence length analysis of piRNAs that participate in the ping-pong amplification loop. Ping-pong matrices illustrate frequent length-combinations of ping-pong pairs (sequences with 10 bp 5' overlap), indicated in red. X-axis and Y-axis refer to sequence read length of the two sequences of a ping-pong pair.

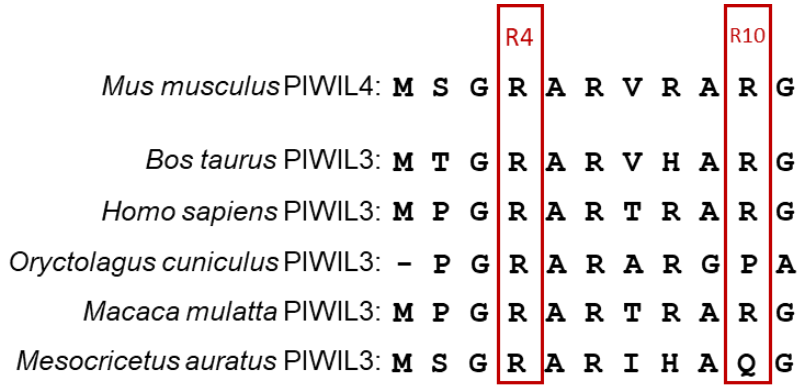

Figure S4: Sequence alignment of mammalian PIWI N terminal domain. R4 and R10 illustrate the arginines in *Bos taurus* PIWIL3 mutated in our experiment.

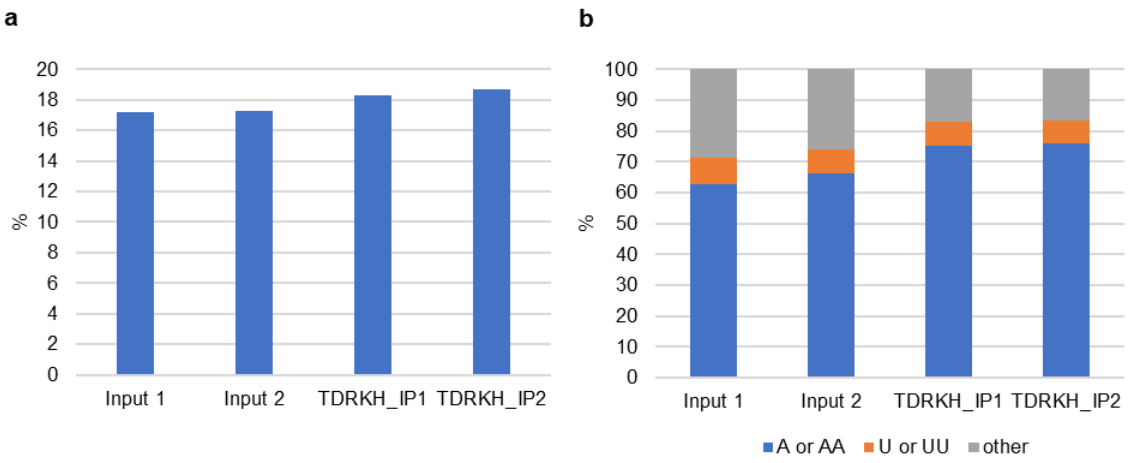

Figure S5: Non-templated nucleotide analysis. a Frequencies of non-template nucleotide at the 3' end from the indicated libraries. **b** Frequency of the identified non-template nucleotides into "A", "U" or "other" tails.

Table S1 TDRKH IP MS data (Excel file).

Please refer to Excel document "SI_Table S1".

Table S2: Primers for PIWIL3 (a) plasmid construction and (b) qRT-PCR. "f" and "r" indicate forward and reverse respectively.

a

Gene	Accession number	Sequence (5'-3')	Direction	Annealing temperature (°C)
PIWIL3	XM_010814123.1	CGGGGTACCAGAGGAGGAAAGACGGAA G GGCGATATCCCCATTGTGCTTTCTTCTG	f r	66
PIWIL3 De1	XM_010814123.1	AGACGGCGGGATACACCA AGTCATTGCTGTCCTGAATCCG	f r	68
PIWIL3 Mu1	XM_010814123.1	TCACGCCAAAGGCAGACGGCGGGATACA ACTCTGGCCTTGCCAGTCATTGCTGTCCTG	f r	67
PIWIL3 Mu2	XM_010814123.1	CAGCCTAAACTCCAGTCATTGACAGGTGT TAG GATTTGTTTAGGTTGCTGAGCCGCTGT	f r	66

b

Gene	Accession number	Sequence (5'-3')	Direction	Annealing temperature (°C)
PIWIL3	XM_010814123.1	AGAAGGAGCTTCGAGACTGG GATTCTGCTGCAAGGTCAGG	f r	61
TDRKH	NM_001105375.1	GCAAAGCGCGCGAAGGCTAAC CCCACGGATCTCTGGGGGAC	f r	58
PNLDC1	XM_015464886.1	TGCAAGGGGCTTTTCTGTGT CCAGAGTTCTTGGTGGGGTT	f r	60
GAPDH	NM_001034034.2	AGGCCATCACCATCTTCCAG GGCGTGGACAGTGGTCATAA	f r	61
SDHA	NM_174178	GCAGAACCTGATGCTTTGTG CGTAGGAGAGCGTGTGCTT	f r	64

Table S3: Small RNA sequencing data (Excel file).

Please refer to Excel document “SI_Table S3”.

Table S4: Non-transposon putative target transcript genes (Excel file).

Please refer to Excel document “SI_Table S4”.

Chapter 3

Microinjection induces changes in the transcriptome of bovine oocytes

Minjie Tan[1], Helena T.A. van Tol[1], Michal Mokry[2], Tom A.E. Stout[1,3], Bernard A.J. Roelen[1,3]*

[1]Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.

[2]Epigenomics Facility, University Medical Center Utrecht, Utrecht, the Netherlands

[3]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.

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Abstract

Gene knockdown techniques are widely used to examine the function of specific genes or proteins. While a variety of techniques are available, a technique commonly used on mammalian oocytes is mRNA knockdown by microinjection of small interfering RNA (siRNA), with non-specific siRNA injection used as a technical control. Here, we investigate whether and how the microinjection procedure itself affects the transcriptome of bovine oocytes. Injection of non-specific siRNA resulted in differential expression of 119 transcripts, of which 76 were down-regulated. Gene ontology analysis revealed that the differentially regulated genes were enriched in the biological processes of ATP synthesis, molecular transport and regulation of protein polyubiquitination. This study establishes a background effect of the microinjection procedure that should be borne in mind by those using microinjection to manipulate gene expression in oocytes.

Introduction

The generation of gene knockout models based around embryonic stem (ES)cells and chimeric animals has significantly enhanced our knowledge of the function in specific genes. A variety of techniques are available to manipulate or alter the genome including homologous recombination, transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9)[1–3].

In oocytes and early stage embryos, gene transcription is arrested in the period between maternal meiotic resumption and embryonic genome activation, in addition, most maternal mRNAs are eliminated soon after the time of embryonic genome activation[4]. In the period of transcriptional arrest, the oocyte and then the blastomeres are dependent on transcripts produced in the oocyte prior to germinal vesicle (GV) breakdown. For this reason, gene knockout techniques are less practical for genes critical to oocyte maturation or embryonic development before embryonic genome activation. Particularly where homozygous gene knockout leads to embryonic lethality, gene function during oocyte development cannot be studied because functional transcripts will be present in heterozygous mothers.

As an alternative, mRNA translation can be downregulated by the introduction of small interfering RNA sequences (siRNA) into oocytes or early embryos[5,6]. *In vivo*, mRNA expression is regulated by small RNAs with sequences homologous to the target mRNA that use proteins such as DICER to form a multicomponent nuclease, RNA-induced silencing complex (RISC), to eliminate the target mRNA[7]. For species where ES cells are not available, but also for maternally expressed genes, RNA interference (RNAi) can be a useful tool for generating information about gene function. Small RNAs directed against mRNA sequences can be artificially synthesized and introduced into oocytes using electroporation or by direct injection to downregulate mRNA expression[8–10].

However, genome-wide analysis has revealed that siRNA sequences can also regulate expression of unintended targets[11]. So-called off-target effects can complicate the interpretation of phenotypic effects during gene-silencing experiments [12,13]. Off-target effects can be minimized, but not eliminated, by strategies such as careful selection of the siRNA sequence and the comparison of several different siRNA sequences that target the same mRNA[12,14].

When mRNA expression is downregulated by siRNA sequences, nonspecific RNA sequences, preferably of similar size and containing the same nucleotides but in a 'scrambled' order to the gene specific siRNA sequence, are used as a negative control. In oocytes and single-cell zygotes the preferred delivery method for siRNA is microinjection. During microinjection the plasma membrane is damaged, cytoskeletal structures may be disrupted, positions of organelles can be changed, and foreign components are introduced into the cell. Although injection of scrambled siRNA is an important control for validating the specificity of siRNA and its targeting for mRNA downregulation, the damaging effects of microinjection and introduction of short RNA sequences per se on the cell have not been analyzed in any depth.

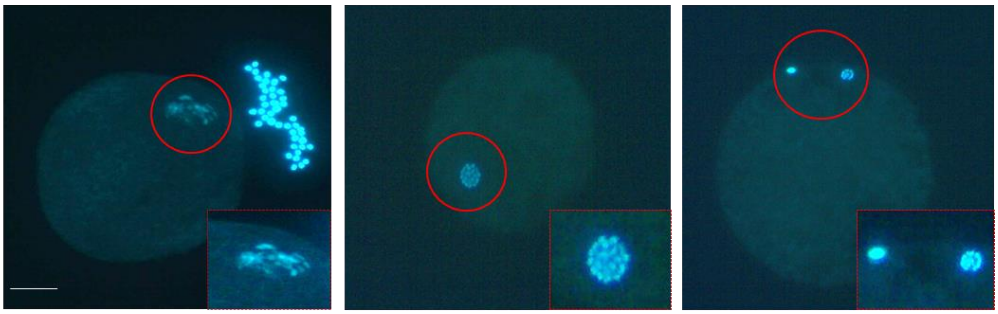
Here we evaluate the effect of injecting small RNA sequences into oocytes, in particular with respect to changes in the transcriptome. Denuded oocytes were injected at the GV stage and cultured first for 16 hours in the presence of roscovitine to delay germinal vesicle breakdown and allow more time for the siRNA to exert its influence[15,16]. Single oocyte RNA sequencing (RNA-Seq) was performed to examine changes in RNA expression as a result of the injection procedure.

Results

Microinjection does not significantly influence the ability of oocytes to mature *in vitro*

Denuded GV stage bovine oocytes were microinjected with non-specific siRNA containing Dextran-TRITC, as an indicator of successful injection. Only oocytes that showed red fluorescence were cultured further. Regular *in vitro* maturation of bovine oocytes takes 24 h, which might be too short for the RNAi machinery or any other intervention to properly downregulate mRNA expression, in that case, researchers usually extend the reaction time with chemical inhibitors such as roscovitine to delay *in vitro* maturation. Therefore, oocytes were first cultured in the presence of the cyclin-dependent kinase (CDK) inhibitor roscovitine for 16 h to delay meiotic resumption, followed by culture in conventional maturation medium for 22 h. After 16 h of culture in roscovitine, most oocytes had reached the MI stage (Figure 1 A). At the end of IVM, injected MII stage oocytes still contained red fluorescence, indicating that the injection procedure had not caused significant leakage (Figure 1B). The maturation rate (based on the numbers of oocytes with a first polar body) of the siRNA injected group (80.4%) was not different to that of the control group (86.8%, Table 1); this was expected given that an injected siRNA was chosen with little homology to known mRNA sequences. These results demonstrate that the injection procedure *per se* did not affect the oocyte's capacity to resume meiosis.

A



B

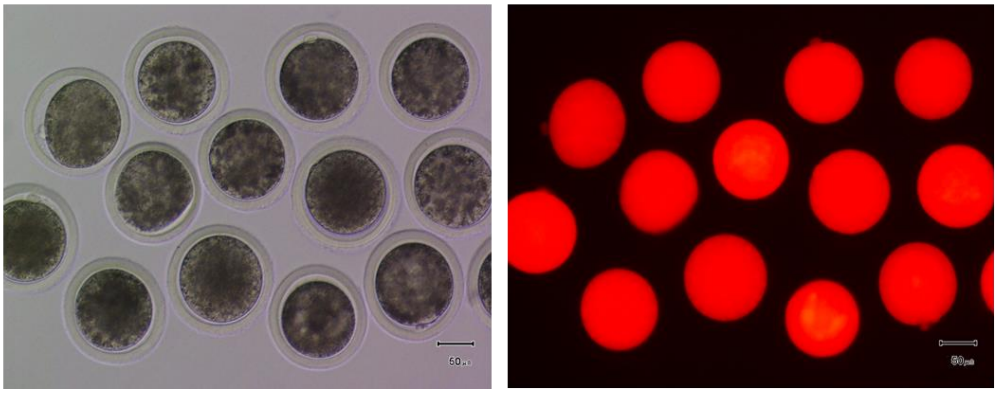


Figure 1. Microinjection of bovine oocytes. Oocytes injected with non-specific siRNA mixed with Dextran-TRITC at the germinal vesicle stage and cultured in roscovitine for 16 h before maturation *in vitro*. (A) Oocytes at germinal vesicle (left), Metaphase I (middle) and Metaphase II (right) stages, based on chromatin distribution (DAPI staining in blue. Insets show magnified view of red encircled areas. Scale bars: 30 µm. (B) Bright field (left) and fluorescence (right) microscopic images of matured Metaphase II stage oocytes after siRNA injection. Red fluorescence indicates successful injection. Scale bars: 50 µm.

	Non-specific siRNA injection	**Non-injected**
Oocytes	97	68
Matured	78	59
Percentage matured (NS)	80.4%	86.8%

* Pooled data from 3 replicates. Maturation rate: % of oocytes in metaphase II. NS: Non significant by with *p* value of 0.29.

Table 1. Non-specific siRNA microinjection does not influence the maturation rates.

Single oocyte RNA sequencing

To improve the accuracy of RNA sequencing result and reduce random transcriptome difference based on single oocytes, 12 single oocytes per group (microinjected and non-injected group) were chosen for Cell-seq2[17]analyze. The expression of each transcript in each sample was normalized with reads per million (RPM). Analyses of RPM values for each replicate showed that the overall ranges and distribution of the RPM values were consistent among the samples (Figure 2). These results indicate that our RNA-seq data are reliable, reproducible and of high quality, and meet the conditions for differential expression analysis.

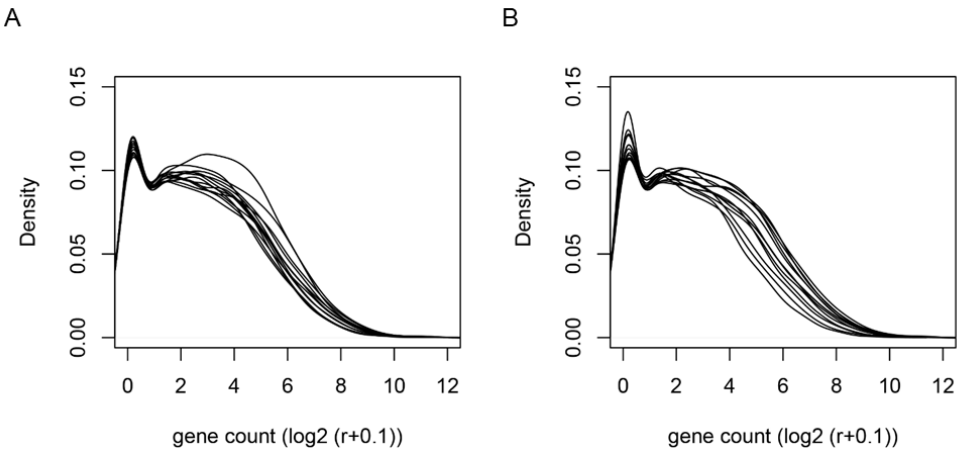


Figure 2. Distribution of expression levels of each single cell transcriptome in (A) non-specific siRNA injected and (B) non-injected oocytes. Individual lines represent individual oocytes, 12 oocytes per group.

Microinjection alters gene expression

To analyze the effect of microinjection in oocytes, we compared the gene expression profiles between the injected and non-injected groups. The total numbers of detected genes ranged from 9153 to 12094 over the two groups. On average, 10577 different mRNAs were detected in the non-injected group whereas 10696 expressed genes were detected in the siRNA injected group. Among these, 10086 mRNAs were detected in both groups, while 491 and 610 were detected exclusively in non-injected and siRNA injected oocytes, respectively (Figure3A). Principal component analysis (Figure 3B) revealed only a minor difference between oocytes from non-injected and siRNA injected groups. Nevertheless, 119 transcripts were differently expressed between the two groups based on an adjusted p value <0.05 with a fold change >

1.2 (Figure 3C, supplementary table 1). Of the differently expressed mRNAs, 76 transcripts were down-regulated and 43 transcripts were up-regulated in the injected oocytes (Figure 3D).

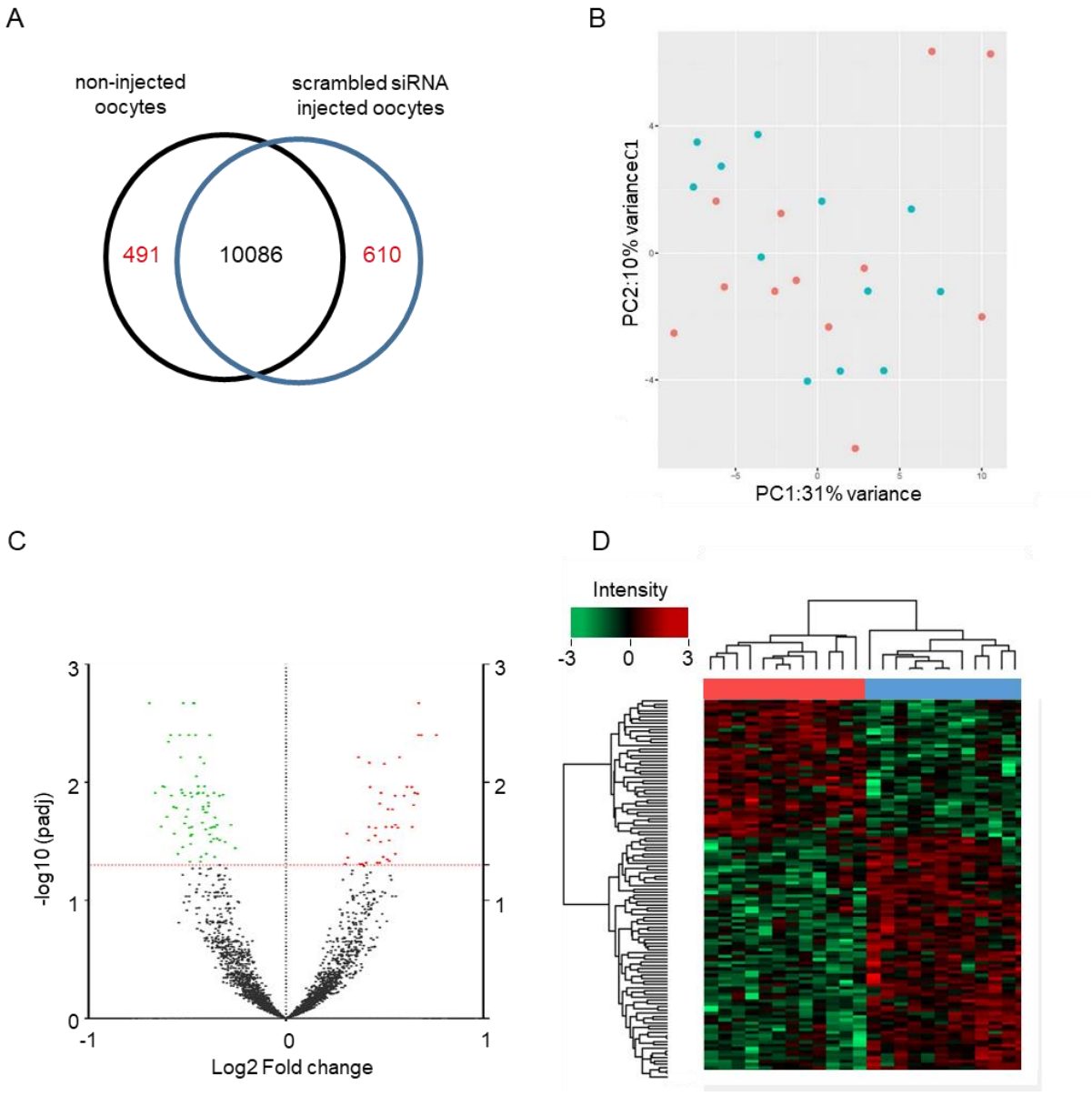


Figure 3. Gene expression differences between non-injected and siRNA injected oocytes. (A) Venn diagram showing overlapping and specific transcripts in the two groups. (B) Principal component analysis (PCA) of the transcriptomes; PC1 and PC2 represent the top two dimensions of the differentially expressed genes among the groups. Blue: non-injected oocytes; red: injected oocytes. (C) Volcano plot showing the estimated fold change (X-axis) versus the –log10 values (Y-axis) for non-injected and siRNA injected oocytes. Red dots represent genes significantly up-regulated in the injected group, green dots represent genes significantly down-regulated in the injected group (adjusted P-value<0.05). Black dots represent transcripts for which expression did not differ

significantly. (D) Heatmap showing the results of cluster analysis for differentially expressed genes between the two groups; n=119 (red: upregulated; green: downregulated). Red bar: injected oocytes. Blue bar: non-injected oocytes.

Microinjection alters the expression of mitochondrial and transmembrane related genes

To gain better insight into alteration of gene expression after siRNA injection, Gene Ontology (GO) term enrichment analysis was performed using ClueGO software (Figure 4, Table 2). We identified 'biological process, 'cellular component' and 'molecular function' as being enriched among these differently expressed mRNAs. For up-regulated transcripts, 8 GO terms within 'biological process' were significantly enriched and these incorporated 8 genes: *VCP, ATP5H, SNAPIN, COX1, UBE2I, SLC6A8, ABI1 and HMGB2.* Among them, *VCP* was included in 4 different 'biological process' lists, namely "positive regulation of Lys63-specific deubiquitinase activity", "ATP biosynthetic process", "flavin adenine dinucleotide catabolic process" and "endosome to lysosome transport". Two cellular component GO terms were significantly enriched, "mitochondrial proton-transporting ATP synthase, stator stalk" which includes *ATP5H*, and "heterotrimeric G-protein complex" which includes *GNA11* and *GNB2*. Six genes (*COX1, UBE2C, UBE2I, SLC6A8, CDKAL1* and *RPIA*) contributed to 5 significantly enriched GO terms for 'molecular function' relating to activity of multiple enzymes. For the down-regulated transcripts, 3 GO terms were enriched within 'biological process', where "negative regulation of protein polyubiquitination" was the most significant GO term, and related to two genes (*PLAA* and *TRIP12*), while "palmitoyltransferase complex" was the only GO term enriched within 'cellular component'.

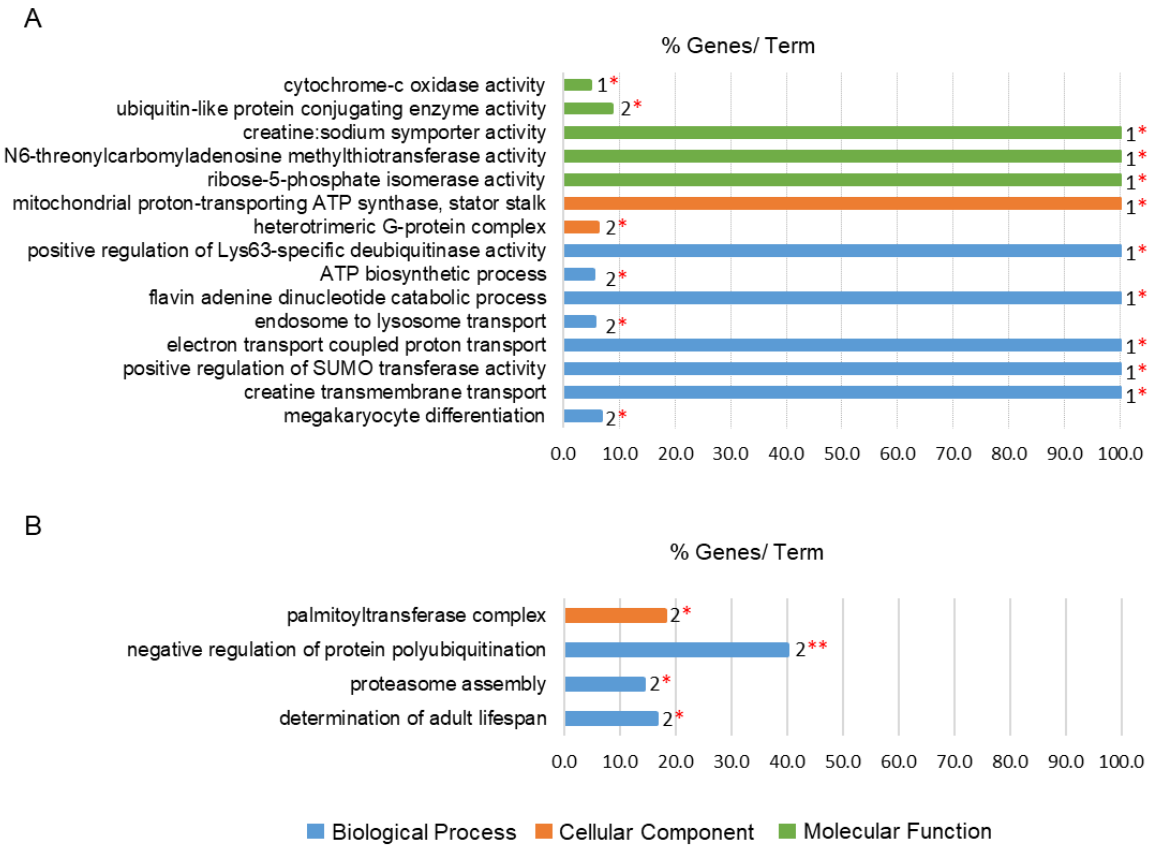


Figure 4. Functionally grouped gene ontology (GO) terms for genes significantly up-regulated / down-regulated in non-specific siRNA injected oocytes compared to non-injected oocytes. A: Up-regulated GO terms in siRNA injected oocytes. B: Down-regulated GO terms in siRNA injected oocytes. Number of genes (number behind bar chart) and the percentage of mapped genes from the total number of genes of the term is shown. GO Term is labeled on the left hand side. The level of significance of the terms is shown as ** (p value < 0.001), * (0.001 < p value < 0.05). Same bar color indicates the Go term as being in the same ontology.

GO_ID	GO_Term	Genes
GO:0004129	cytochrome-c oxidase activity	[COX1]
GO:0061650	ubiquitin-like protein conjugating enzyme activity	[UBE2C, UBE2I]
GO:0005309	creatine: sodium symporter activity	[SLC6A8]
GO:0035598	N6-threonylcarbomyladenosine methylthiotransferase activity	[CDKAL1]
GO:0004751	ribose-5-phosphate isomerase activity	[RPIA]
GO:0000274	mitochondrial proton-transporting ATP synthase, stator stalk	[ATP5H]

GO:0005834	heterotrimeric G-protein complex	[GNA11, GNB2]
GO:1903007	positive regulation of Lys63-specific deubiquitinase activity	[VCP]
GO:0006754	ATP biosynthetic process	[ATP5H, VCP]
GO:0072389	flavin adenine dinucleotide catabolic process	[VCP]
GO:0008333	endosome to lysosome transport	[SNAPIN, VCP]
GO:0015990	electron transport coupled proton transport	[COX1]
GO:1903755	positive regulation of SUMO transferase activity	[UBE2I]
GO:1902598	creatine transmembrane transport	[SLC6A8]
GO:0030219	megakaryocyte differentiation	[ABI1, HMGB2]

GO_ID	**GO_Term**	**Genes**
GO:0002178	palmitoyltransferase complex	[GOLGA7, SPTLC1]
GO:1902915	negative regulation of protein polyubiquitination	[PLAA, TRIP12]
GO:0043248	proteasome assembly	[POMP, PSMG1]
GO:0008340	determination of adult lifespan	[IDE, MSH2]

Table 2. Lists of functionally grouped gene ontology (GO) terms significantly up-regulated / down-regulated in siRNA injected oocytes compared to non-injected oocytes. Upper table: Up-regulated GO terms and gene names in siRNA injected oocytes. Lower table: Down-regulated GO terms and gene names in siRNA injected oocytes.

Discussion

RNAi has been widely used as a method for suppressing gene expression[18]. In oocytes and single-cell zygotes, microinjection is the preferred method of delivering siRNA, because the timing and amount of introduced RNA can be controlled[19,20]. A disadvantage of this technique, compared with for instance transfection, is the physical stress that the cells are subjected to. Independent of the delivery method, the RNAi machinery needed for the degradation of mRNA, and natural turnover for downregulation of protein levels, requires a period of time longer than the 22h during which cattle oocytes normally reach the MII stage when matured *in vitro*. Oocytes were therefore cultured in the presence of the CDK inhibitor roscovitine for 16 h before being cultured in maturation medium for 22h[15,21]. To distinguish non-specific from specific effects, scrambled siRNAs are usually injected and compared with oocytes/zygotes injected with specific siRNAs. A possible downside of this approach is that the effects of the injection procedure itself might be masked, but influence the final results.

Oocyte integrity after microinjection has been studied, but primarily to assess the impact of intracytoplasmic sperm injection (ICSI). For ICSI, injected oocytes are usually already at the MII stage; otherwise, the injection procedure is very similar to that required for siRNA injection. After ICSI, ultra-structural changes within oocytes have been reported, and include membrane-bound vacuoles and plasma membrane inclusions[22]. Here we analyzed the potential damage of the siRNA microinjection procedure *per se* by comparing non-injected oocytes with oocytes injected with siRNAs that did not target a specific mRNA. After injection, similar percentages of oocytes matured to the MII stage, indicating that the initial developmental capacity of the oocyte was not altered by the microinjection procedure. We then examined the transcriptome in individual injected oocytes and compared it to that from non-injected oocytes[17]. Transcriptome analysis of single oocytes has been reported in various species including mouse, cow and man[23–25]. We detected approximately 10600 genes as being expressed in bovine oocytes, which is similar to what has been reportedpreviously[24,26,27].

To understand transcriptome and biological pathway changes after siRNA microinjection and to define a background reference effect for conducting future siRNA microinjection experiments, we performed GO analysis based on genes with significantly altered expression levels. Our study identified 11 biological processes, 3 cellular components and 5 molecular

functions that were significantly affected by microinjection, based on up and down-regulated genes.

The oocyte membrane and cytoskeleton play important roles in maintaining cell shape, and enabling cytokinesis during cell divisions. During microinjection, the injection needle disrupts the integrity of the plasma membrane and it is therefore not surprising that expression of genes related to transmembrane proteins, cytoskeleton and actin filaments were significantly altered by siRNA microinjection. For instance, *GNA11* and *GNB2* that belong to the heterotrimeric G-protein complex alpha and beta subunits respectively are involved as modulators or transducers in various transmembrane signaling systems. G-alpha subunits are tethered to the plasma membrane and activate the enzyme adenylyl cyclase. ACTR2*,* also known as ARP2 Actin Related Protein 2 Homolog, is part of the ARP2/3 complex that mediates the formation of branched actin networks in the cytoplasm and is important for asymmetric division, spindle migration as well as the formation and completion of oocyte cytokinesis during meiotic maturation[28,29]. The upregulation of *ACTR2 expression* suggests that actin remodeling is required during oocyte maturation after injection. *GOLGA7*, which was down-regulated by siRNA injection codes for the palmitoyltransferase complex and may also be involved in vesicular protein transport from the Golgi apparatus to the cell surface[30].The downregulation of *GOLGA7* transcripts in our data suggests that injection interrupts signaling between cytoplasm, plasma membrane and organelles.

Mitochondria are essential for multiple biological processes via ATP synthesis in maturing oocytes[31]. Indeed, oocyte quality can be interrupted by mitochondrial dys-function and inadequate ATP production[32]. *ATP5H* and *VCP* are related to ATP synthesis and were up-regulated in oocytes injected with siRNA, indicating that microinjected oocytes require extra ATP to reach the MII stage.

Ubiquitin is involved in post-translational modification of target proteins. During the maternal to zygotic gene transition, maternal proteins are degraded by the ubiquitin-proteasome system and new proteins are synthesized from the embryonic genome. Reduced proteasome activity leads to accumulation of maternally derived oocyte proteins which can result in early embryonic developmental arrest[33]. Various types of ubiquitin-mediated modifications are thought to have specific functions. As an ubiquitin ligase, activity of TRIP12 is indispensable

for mouse embryogenesis. Gene inactivation of *Trip12* results in embryonic lethality during midgestation[34]. PLAA is required for the Ubiquitin-mediated sorting of membrane proteins from the early to late endosome, targeting them for lysosomal degradation[35]. However, to fully understand its role in oocyte maturation requires further investigation. In conclusion, these results suggest that microinjection affects the degradation of maternal proteins and compromises the oocyte's developmental competence.

The cellular response to mechanical injury could result in the dysregulated expression of certain genes. We used RNA-seq to identify changes in the transcriptome of single oocytes after non-specific siRNA injection. Our results show that, after microinjection, cattle oocytes are just as capable of maturing to the MII stage during *in vitro* culture as non-injected oocytes. On the other hand, we detected 43 upregulated and 76 downregulated genes in the injected oocytes, mainly involved in the cytoskeleton, proteasome and ATP synthesis processes. This result demonstrates that there is a 'background effect' of microinjecting oocytes and indicates that one should be careful when interpreting data from microinjected oocytes.

Materials and Methods

Oocyte collection and in vitro maturation

Cattle ovaries were collected from a slaughterhouse in the Netherlands and transported to the laboratory in a polystyrene box at room temperature, within 2 h after slaughter. After washing, the ovaries were maintained in 0.9% NaCl supplemented with 0.1% penicillin/streptomycin (10,000 U/ml: Gibco BRL, Paisley, UK) at 30°C. Cumulus oocyte complexes (COCs) were aspirated from 2-8 mm follicles[36]. Only oocytes with a multi-layered, compact cumulus complex were selected for microinjection.

Injection of siRNA into oocytes and *in vitro* maturation

Microinjection of a commercial, non-specific siRNA: UGGUUUACAUGUCGACUAA (D-001810-01, ON-TARGETplus Non-targeting siRNA #1, Dharmacon, US) was as described previously[37]. In short, siRNA with a final concentration of 25 µM was mixed with 0.1 mg/ml tetramethylrhodamine (TRITC) labeled 3kDa dextran (Dextran-TRITC; Molecular Probes, Eugene, OR, USA) in water. For microinjection, 6 denuded GV oocytes were transferred to a 5 µl drop of HEPES buffered M199+ 10% FCS overlaid by mineral oil in a 60 mm dish at 37°C on an IX71 inverted microscope (Olympus, Leiderdorp, the Netherlands) equipped with a heated stage. 5 µl siRNA mix was loaded into a microinjection needle with a 30° angle and a tip with a 4.3-4.9 µm inner diameter (Eppendorf, Hamburg, Germany). A Femtojet pressure injection system (Eppendorf) was used to inject for 0.2 sec at a pressure of 120 hpa. After injection, only oocytes with red fluorescence were selected for culture in 25 µm roscovitine (Sigma, R7772-1MG) in maturation medium without FSH for 16 h in a humidified atmosphere of 5% CO2-in-air at 38.5°C, followed by maturation medium for 22 h (M199 supplemented with 0.02 IU/mL follicle-stimulating hormone [Sioux Biochemical Inc., Sioux Center, IA, USA], 10% FCS, 100 U/ml penicillin and 100 µg/mL streptomycin). Non-injected control oocytes were cultured in the same conditions. After maturation, oocytes with a first polar body were washed in PBS and individually collected into RNase free tubes containing 200 µl RNA lysis buffer and stored at -80°C until RNA isolation.

Single oocyte RNA isolation and Cel-Seq

A total of 24 oocytes (12 injected and 12 non-injected) were collected for total RNA isolation using an RNeasy Micro Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions.

Sequencing library preparation was performed using the CEL-seq2 protocol as described previously[17] and sequencing was performed on an Illumina NextSeq500 using 75-basepairpaired-end sequencing (Utrecht Sequencing Facility).

Sequencing data were processed as described previously[38]. In brief, after demultiplexing, sequencing reads were mapped to a cDNA library, reads mapping to the same gene with identical UMI were counted as a single read.

Identification of differentially expressed gene

In order to identify genes with altered expression levels, R package DESeq2 was used. A *P* value was computed based on Poissonian statistics and multiple testing corrected by the Benjamini-Hochberg method[39]. Genes with an adjusted p-value<0.05 as calculated by DESeq2 were considered to have undergone significant gene enrichment. For identification of up and down-regulated genes in injected oocytes, a Cytoscape App ClueGO was used based on the terms " biological processes " , "cellular components" and "molecular function" in the *Bos taurus* database[40]. Statistical significance was set for a K score of 0.4. The function "GO Term fusion" was selected and GO term restriction levels were set at1 – 20, with a minimum of one genes or 4% genes in each GO term。

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Author contributions

MT, BAJR, TAES designed the study. MT and HTAvT collected primary materials and performed the experiment.MM performed RNA-seq data analysis. MT and BAJR wrote the manuscript. All authors contributed and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Material and Correspondence

All data and material that support the findings of this study are available upon reasonable request.

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Chapter 4

Plasma membrane (phospho)proteome dynamics during mammalian oocyte meiosis and fertilization

Minjie Tan[1], Mirjam J. Damen[2,3], Tom A.E. Stout[1,4], Bernard A.J. Roelen[1,4], Wei Wu[2,3]*

[1]Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.

[2]Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands.

[3]Netherlands Proteomics Centre, Utrecht, the Netherlands.

[4]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.

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Abstract

Due to the difficulty in extracting plasma membrane proteins and the limited amount of material in an oocyte, little is known about plasma membrane phospho(proteome) dynamics in mammalian oocytes. Here, we used an optimized plasma membrane protein purification method followed by phospho enrichment and mass spectrometry to investigate the oolemma phospho(proteome) in cattle GV and MII stage oocytes and zygotes. We identified a number of protein complexes that connect with the plasma membrane, including the Arp2/3, ERM and SCMC complexes. Moreover, we detected multiple new phospho-sites that were not previously reported. In addition, we found that PALM3, PB41L2 and TACC3 increased significantly at both the protein and phospho-protein levels during oocyte maturation. Our research validated an effective method for plasma membrane protein identification from samples of limited protein content, and also provided general knowledge on plasma membrane proteins activated during oocyte maturation and fertilization. These include promising candidates for more in-depth investigation of plasma membrane rearrangements in oocytes and early embryos.

Introduction

Plasma membrane proteins constitute about 50% of the plasma membrane by mass, and represent an important functional component of the membrane barrier. Plasma membrane proteins are responsible for a wide range of biological functions such as cell signal transduction, cell to cell interaction and adhesion.

In germ cells, the plasma membrane is the final barrier to fertilization and the creation of a zygote, and plasma membrane components are critically regulated to allow penetration of the fertilizing spermatozoon while preventing polyspermy. Potential differences or changes in plasma membrane proteins during oocyte maturation and fertilization have mostly been investigated at low resolution[1–3]. Nevertheless, these efforts have revealed that plasma membrane proteins associate with the actin cytoskeleton to strengthen the cell cortex and stabilize other structures essential for the success of meiosis and cell polarization, even though the spatial and temporal resolution of these associations is limited by the poor sensitivity and reproducibility of existing quantitative plasma membrane profiling techniques.

More importantly, upon fertilization a variety of oocyte-specific plasma membrane elements including surface receptors and extra and intracellular signaling molecules are dynamically regulated to prevent additional sperm fusion and polyspermy. The exact changes in membrane protein composition are still poorly characterized, although it is known that upon plasma membrane fusion between the oocyte and the fertilizing sperm, the contents of the cortical granules are released into the perivitelline space leading to modifications in the zona pellucida that prevent binding with additional spermatozoa. In-depth and focused analysis of the oocyte plasma membrane at each of these critical stages before and after fertilization will likely provide better understanding of these changes that help protect the genetic integrity of the zygote.

A rapid block to polyspermy is desirable but this is unlikely to be provided by the synthesis and insertion of newly translated proteins into the plasma membrane. It is therefore more plausible that the plasma membrane changes that take place involve or include post-translational modification (PTM) of proteins or assembly of complexes, which could take place rapidly after fertilization. During fertilization and early embryo development, phosphorylation

is implicated in a variety of physiological processes including meiotic spindle regulation, cortical granule translocation, and polar body formation and extrusion[4]. This stimulated us to better profile the phosphorylation dynamics of plasma membrane proteins and plasma membrane recruited phospho-proteins.

The overwhelming majority of existing methods to study plasma membrane presentation and complex assembly involve detergent solubilization. While surface active agents may provide an alternative solubilization environment to extract membrane proteins[5,6], detergents may also interfere with complexes involving highly or very poorly soluble proteins. In the presence of a high detergent concentration, denaturation of proteins and deactivation of protein interactions, structure and activity would occur[7].

Here, we tested a non-denaturing and detergent-free method to isolate plasma membrane proteins from cultured cells, and used this method to profile plasma membrane presence, phosphorylation and the assembly of plasma membrane complexes in bovine oocytes before and after fertilization. By using oocyte material from bovine germinal vesicle (GV), metaphase II (MII) stage oocytes and zygotes we hoped to reveal plasma membrane protein dynamics during oocyte maturation and fertilization.

Since the use of proteomics to study early development has mainly been performed on whole eggs[8–11], we aimed for the first time to apply proteomics to the plasma membrane of oocytes from a large mammal. Our results include global identification and quantification of plasma membrane proteins, phosphorylation dynamics during oocyte maturation and fertilization and describe methods for membrane protein purification in a native environment. The method should be widely applicable to mass spectrometric protein studies, especially in situations when the amount of material available is limited.

Results

Proteome analysis of Hela cell line

To validate the efficiency of a method for plasma membrane protein identification on small numbers of cells, we used a cultured cell line (Hela cells). After lysis, samples were separated into plasma membrane (PM), other membranes in the cytoplasm (OM) and cytoplasm (Cyto) components (Figure 1A). After fractionation and protein digestion, 10 µg of total peptides per sample was used for phosphopeptide enrichment followed by mass spectrometry.

After label free quantitative proteomic analysis, we identified 4247 proteins in the whole cell lysate whereas a total of 5107 proteins were found in the three fractions combined. We detected 3151 proteins in the plasma membrane fraction, 2627 proteins in the other membranes fraction and 3950 in the cytoplasm fraction (Supplementary table 1). Multi-scatter plots (Figure 1B) and principal component analysis (Figure 1C) indicate the distinct proteomes of the fractions, and their reproducibility. Pearson correlation values (within groups: 0.958-0.996; between groups: 0.44-0.56) further confirmed the repeatability.

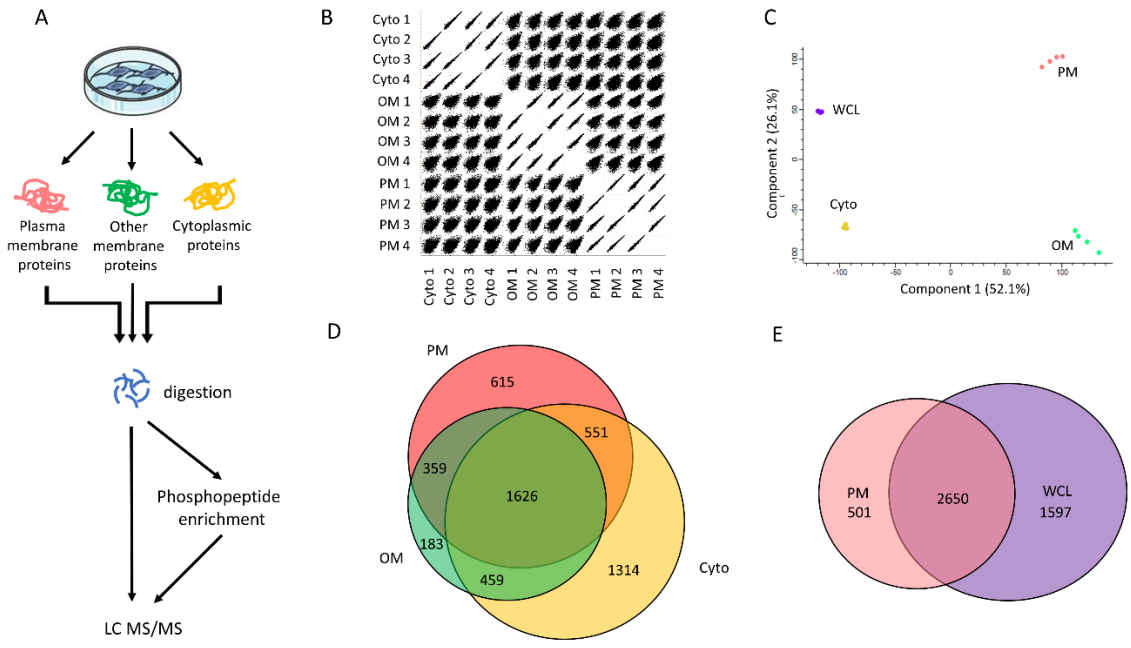


Figure 1: **Hela cell line proteome analysis**. WCL: Whole cell lysate; Cyto: cytoplasmic proteins; OM: other membrane proteins; PM: plasma membrane proteins. **A**: Workflow outlining protein extraction and phosphopeptide identification. **B**: Multi-scatter plot of protein intensities between fractions. **C**:

Principle component analysis of proteins in each group. **D**: Venn diagram depicting the overlap of proteins in fractions. **E**: Venn diagram depicting the overlap of proteins between the PM fraction and WCL.

Fractionation increases protein identification in Plasma membrane

To explore the similarity and difference of detected proteins, Venn diagrams were made to map overlaps between proteins in the different groups (Figure 1D). 1626 proteins were shared in all three fractions, while the numbers of unique proteins in each fraction were 1314 (Cyto), 183 (OM) and 615 (PM). A complete list of all identified proteins from each replicate along with the LFQ intensity, sequence coverage, number of unique peptides, and number of spectra is presented in Supplementary Table 1. When the WCL protein fraction was compared with that of the PM, 2650 proteins were shared and 1597 and 501 proteins were found exclusively in the WCL or PM, respectively (Figure 1E). Gene Ontology annotation verified enrichment for membrane proteins (Supplementary figure 1). For the 1597 proteins unique in the WCL (Supplementary figure 1A), GO annotation revealed enrichment in cytoplasm, cytosol and nuclear chromatin proteins. However, the unique proteins in the PM fraction were enriched for cellular localization of intrinsic, integral components of membrane, and plasma membrane components (Supplementary figure 1B). The results strongly indicate that our method is efficient for separating and identifying plasma membrane proteins from whole cell lysates without much contamination from other fractions.

Fractionation improved phosphoprotein identification in Plasma membrane

To investigate the number of phosphorylated peptides in the WCL and other fractions, we performed phosphorylated peptide enrichment as described previously[12]. A total of 12520 phospho-sites were identified (Figure 2, Supplementary table 2) of which 10954 were identified with high confidence (probability>0.75) (Figure 2A). When comparing the WCL samples with the plasma membrane, other membrane and cytoplasm fractions (Figure 2B), only 2339 phospho-sites were re-detected in the pWCL fraction (2164 with the localization probability > 0.75). Most phosphosites were identified in the other fractions, 7120 in the cytoplasmic fraction (>0.75: 6265) whereas OM and PM fractions contained similar number of phosphosites: 6318 (>0.75: 5518) and 6078 (>0.75: 5340) respectively.

The identified phosphosites were compared between groups to determine which were location specific. When comparing the WCL samples with the other three fractions, only 106 sites were unique for WCL samples (Figure 2C). We further compared the number of sites detected between all groups (Figure 2D). There were 1118 common sites in the four fractions while in OM, Cyto and PM samples, 2041, 2569 and 2145 sites were exclusive to the respective groups. The results indicate that, after fractionation, additional phospho-sites can be detected in various cellular components.

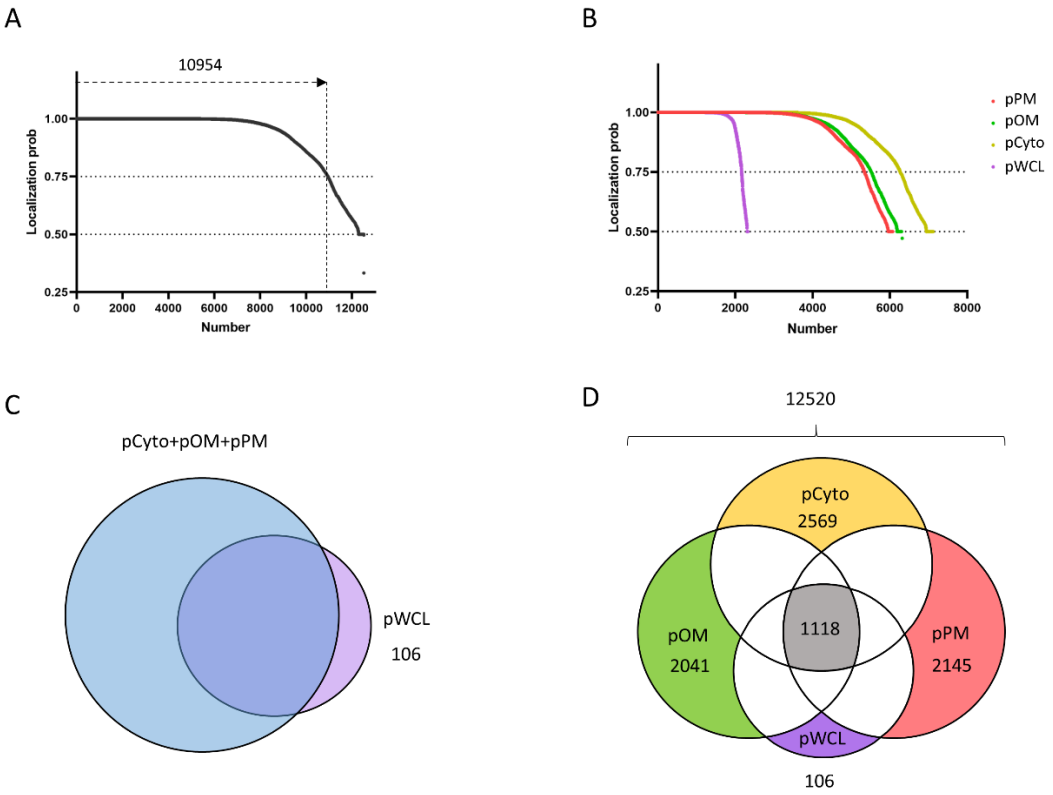


Figure 2: Hela cell line phosphoproteome. pWCL: phospho-sites in whole cell lysate proteins; pCyto: phosphosites in cytoplasm fraction proteins; pOM: phosphosites in other membrane proteins; pPM: phosphosites in plasma membrane proteins. **A**: Distribution of phosphosite localization probability scores for all identified sites. Cutoff scores for class I (⩾0.75 probability), class II (between 0.5 and 0.75 probability) and class III (<0.5 probability) are indicated by dashed lines. **B**: Distribution of phosphosite localization probability scores for identified sites in each groups. **C**: Venn diagram depicting the overlap of phosphosites between WCL and the other three fractions. **D**: Venn diagram depicting the unique and common phosphosites in the four groups.

A phosphosite is a serine, threonine or tyrosine with a distinct location in the protein sequence that can be phosphorylated. We analyzed the phospho Serine (S), Threonine (T) and Tyrosine (Y) residues in each group. This should give us information about the specific site that is modified. We expect that in the PM fraction, Tyrosine phosphosites will be enriched since Tyrosine kinase is essential for initiating and propagating downstream signaling via the plasma membrane[13]. In our results, most of the amino acids that were phosphorylated were S, followed by T and Y. While in the PM samples, Y sites are enriched more than other fractions, as was expected (Table 1A).

	S	T	Y	Total
PM	5265	757	56	6078
OM	5583	717	18	6318
Cyto	6250	841	30	7120
WCL	2127	200	12	2339

Table 1A: Phosphorylated Serine (S), Threonine (T), and Tyrosine (Y) sites in plasma membrane (PM), organelle membrane (OM), cytoplasm (Cyto) and whole cell lysate (WCL) fractions.

	S	T	Y	Total
PM	1750	357	38	2145
OM	1778	255	8	2041
Cyto	2185	372	12	2569

Table 1B: Unique Phosphorylated (Serine) S, (Threonine) T, (Tyrosine) Y sites in each fractions.

	New seq + p site	New p site	Share p sites	Total p site sequence	New P site (%)
pPM	70	502	1182	1754	33%
pOM	69	471	1100	1640	33%
pCyto	73	535	1536	2144	28%

Table 2: Concluding table of new phosphosites in Hela cell fractions. New seq+ p sites: Newly detected phosphosites in peptides with unknown phosphosites; New p sites: Newly detected phosphosites in known peptides with phosphosites; Share p sites: phosphosites that have been detected previously.

To further examine differences between fractions, we also performed S, T and Y site distribution based on unique sites in each groups (Figure 3A, Table 1B). We also found the enrichment of Y phosphosite in PM fraction that is four times higher than other fractions (Figure 3B). In order to check how many new phosphosites we detected based on our method, we compared the unique phosphosites in each group with the phosphosite database online (PhosphoSite Plus). On average, around 30% of the P sites found in each fraction were new, while the rest were previously described phosphosites (Table 2; Figure 3C).

Overall, the data reveals that after fractionation, we were able to increase the number of proteins that can be detected by mass spectrometry. In addition, the proteins in each fraction could be separates from each other. The methods can be used not only in cultured cell lines but also in a variety primary cells or germ cells with low abundance.

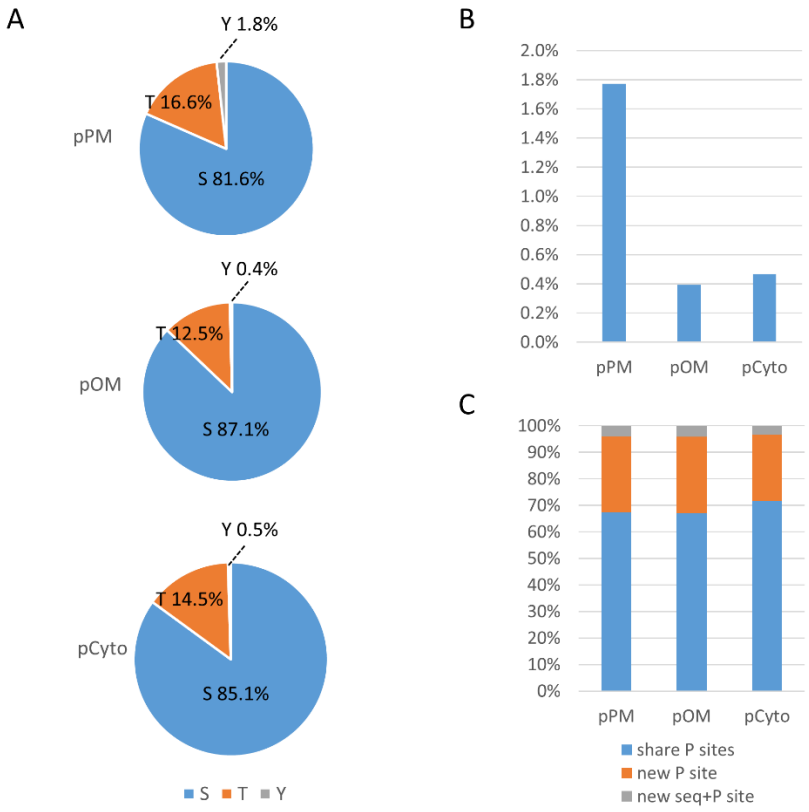


Figure 3: Characterization of phosphosites in Hela cells. **A**: Distribution of the proportion of amino acid residues on phosphopeptides with localization probability ⩾0.75. Phospho Serine (S), Threonine (T) and Tyrosine (Y) sites. **B**: Proportion of Tyrosine (Y) residue phosphopeptides in each fraction. **C**: Proportion of new phosphosites detected. Share p sites: phosphosites that have been detected previously; New p sites: Newly detected phosphosites in known peptides with phosphosites; New seq+ p sites: Newly detected phosphosites in peptides with unknown phosphosites.

Plasma membrane protein identification during bovine oocyte meiosis and fertilization

The technique of (phospho)protein identification was next applied to bovine oocytes and zygotes to provide insight into membrane biology in mammalian oocytes during maturation and fertilization.

Triplicate oocyte samples from GV, MII and zygotes (24 h after fertilization; Figure 4A) were collected for plasma membrane extraction followed by phosphopeptide enrichment and MS proteomic analysis, Principle component analysis revealed three distinct clusters based on cell stage (Figure 4B). A total of 327, 375 and 374 proteins were characterized in GV and MII oocytes and zygotes, respectively (Figure 4C; Supplementary table 3) of which the majority (272) were present at all stages.

According to the GO analysis results (Figure 4D), proteins related to the cellular component of membrane parts and, in particular, of the plasma membrane parts were significantly enriched in oocytes. In addition, we detected multiple proteins that contributed to protein complexes related to cortical actin dynamics, for instance the Arp2/3 complex, ERM complex, SCMC complex and the TACC3/ch-TOG/clathrin complex (Figure 6). The GO results and information on cellular complexes indicated the enrichment and purification of plasma membrane protein extraction.

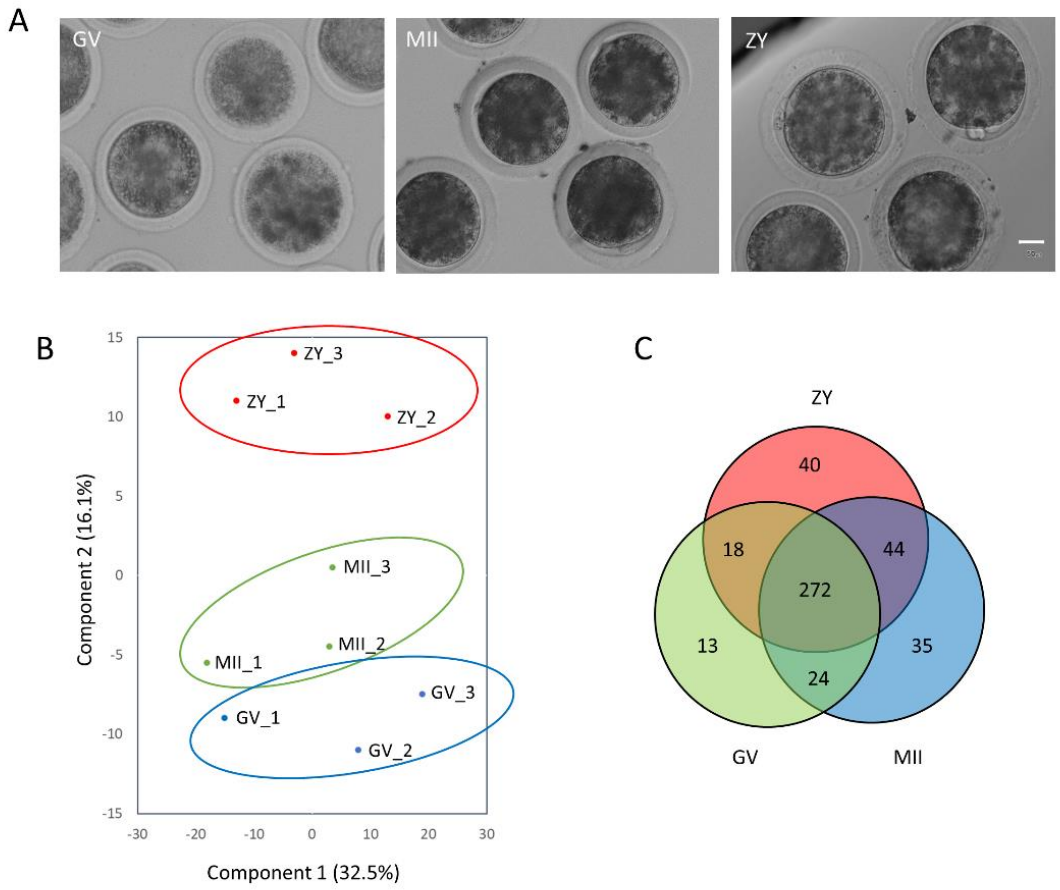
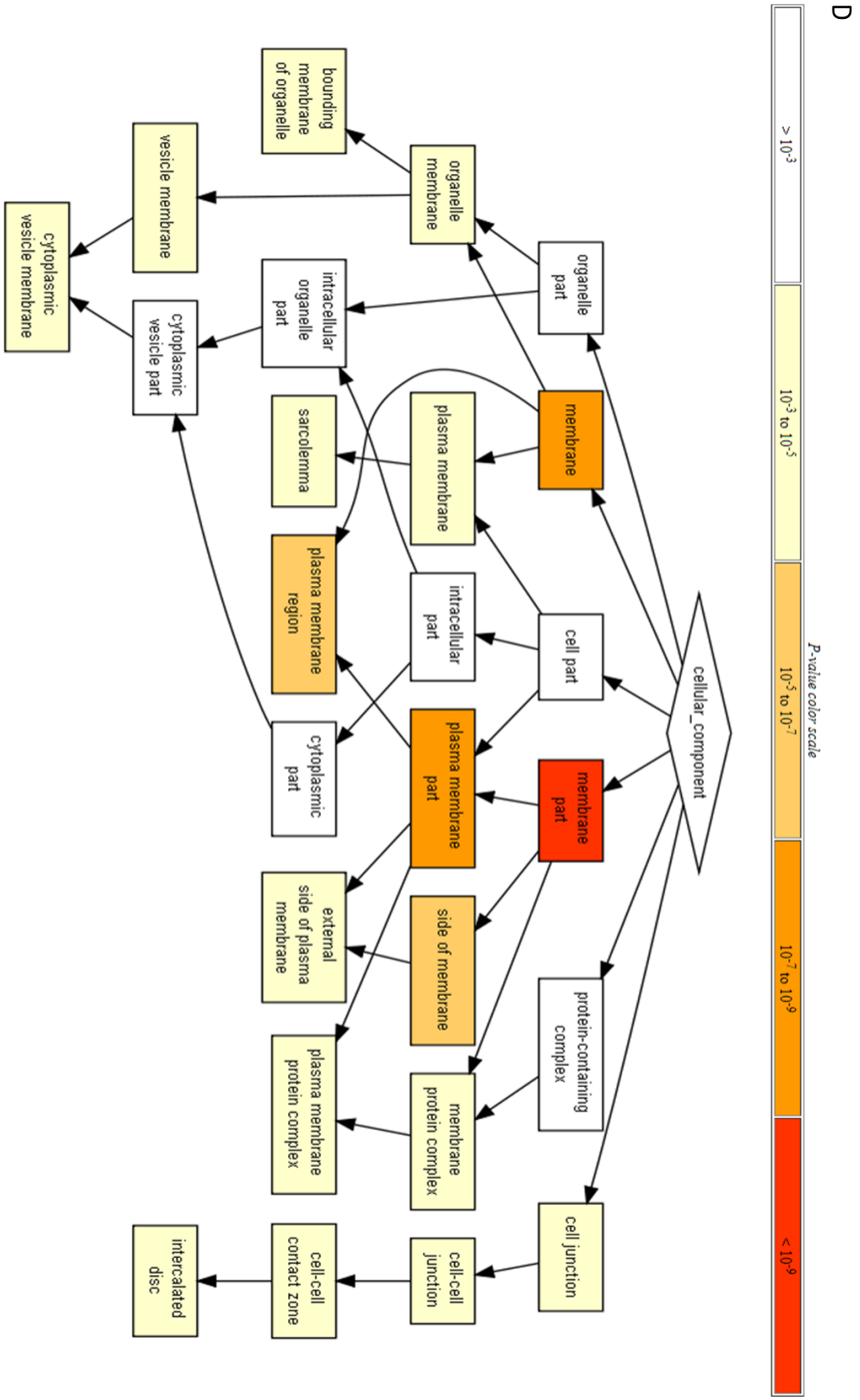


Figure 4: Oocyte proteome. **A**: Light microscopic images of germinal vesicle (GV) and metaphase II (MII) oocytes, and zygotes (ZY) **B**: Principle component analysis of plasma membrane proteins at the various stages. **C**: Venn diagram showing the overlap of proteins detected in each group. **D**: GO cellular component annotation of 272 common proteins detected at the three stages. GO clusters indicated the cellular components enriched among these proteins.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vesicle membrane
cytoplasmic vesicle membrane
cytoplasmic vesicle part
sarcolemma
external side of plasma membrane
side of membrane
plasma membrane protein complex
membrane protein complex
protein-containing complex
cell junction
cell-cell junction
cell-cell contact zone
intercalated disc

Dynamic changes of the (phospho)proteome during oocyte maturation and fertilization

Next, we examined differential expression of plasma membrane proteins during oocyte maturation and fertilization. In total, 63 proteins were differentially expressed (p<0.05) between the three groups. GO analysis revealed enrichment for biological processes involved in regulation of protein localization to the early endosome, homotypic cell-cell adhesion and mitotic cytokinesis with the enriched molecular function of cation-transporting ATPase activity (Supplementary Table 4).

The differentially expressed proteins could be classified into four different expression clusters (Figure 5A). Group A consists of proteins that were highly expressed in zygotes. Group B contains proteins with low expression at the GV stage. Proteins in group C are highly expressed at the GV stage, whereas group D is made up of proteins that are highly expressed at the MII stage.

As one of the most widely studied post-transcriptional modifications, phosphorylation is an important cellular regulatory mechanism during germ cell development. Therefore, the plasma membrane phosphoproteome of oocytes at the GV and MII stages and of zygotes were examined. After, plasma membrane protein extraction and digestion, titanium oxide columns were used for phosphopeptide enrichment, followed by MS identification of the flow-through. In GV stage oocytes, 35 phosphopeptides were detected, of which 33 had a localization probability >0.75. In the MII stage oocytes, the number of phosphosites (47; 43 with probability >0.75) was similar to the amount of peptides in zygotes (44; 39 with probability>0.75) (Supplementary Table 5). The dominant phosphorylated residue in the three groups was Serine, followed by Threonine, while no phosphorylated Tyrosine residues were detected. The data were compared with the human database using PhosphoSitePlus. Overall, we identified 36 new phosphosites located on 24 proteins and 13 phosphosites that are were described previously. Among them, we found 7 different new phosphosites in KHDC3L, followed by JPT2 and EPB41L2 that each contained 3 previously unidentified phosphosites.

In total, 17 peptides were identified with significantly changed phosphorylation states (Figure 5B). There was an overall tendency to increased phosphorylation during maturation, and reduced phosphorylation at the zygote stage.

In short, new phosphosites have been detected indicating that phosphor modification is important for plasma membrane proteins during oocyte development and fertilization.

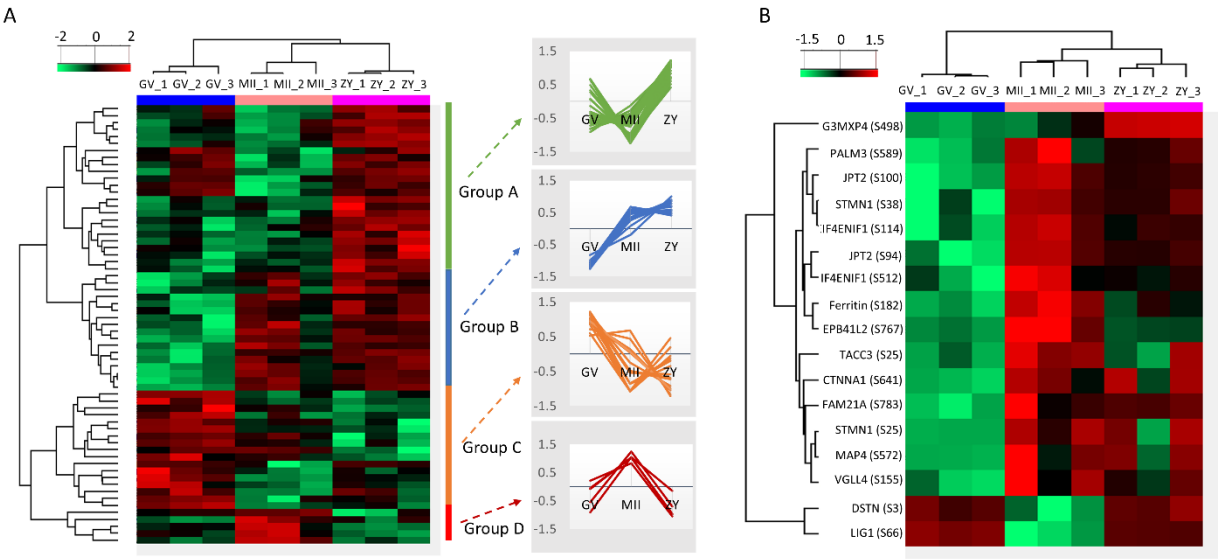

Figure 5: Hierarchical clustering based on oocyte plasma membrane proteome quantification. A: Heatmap of differentially expressed proteins over three developmental stages ($p < 0.05$). Plot on the right side indicating tendency of quantitative changes of proteins clustered in 4 groups. **B:** Heatmap of significantly changed protein phosphorylation over three developmental stages ($p < 0.05$).

Discussion

Plasma membrane proteins are critical for a wide range of cellular functions and are promising drug targets[14]. The challenges to plasma membrane studies centers on the difficulty of plasma protein extraction from the cellular phospholipid bilayer, their low solubility and low abundance[15]. Moreover, most current extraction methods result in the loss of interactions, which makes it difficult to appreciate the collective function of PM proteins in the context of their signaling environment. Here, we used an optimized detergent free plasma membrane protein extraction technique followed by fractionation, phosphosite enrichment and mass spectrometry to identify membrane proteins from Hela cells and compared the results with normal whole cell lysate proteomic data obtained under the same conditions. Although different methods exist for plasma membrane protein extraction, the method described was

able to extract plasma proteins from the phospholipid bilayer in a native condition that preserved their physical structures and interactions. This generated novel information about protein complexes that related to membrane dynamics[16]. The scalability of this method makes it amenable to low input samples, such as early embryos, as well as larger tissues. Since membrane proteins frequently interact with other molecules based on post-translation modifications such as phosphorylation, understanding the phosphorylation associated with plasma proteins is essential. For protein phosphorylation analysis, an enrichment step is critical for downstream identification, especially when only limited amounts of material are available[12].

As show in the results, based on 10 ug input of peptides from Hela cells, we identified additional proteins in three fractions: plasma membrane, cytoplasm and organelle membranes. A total of 12520 phosphopeptides were detected, which is roughly three times the number of peptides found in Hela cell digests previously[12]. The increased percentage of tyrosine residues detected in the plasma membrane fraction accompanied by the GO annotation strongly indicates that the plasma membrane fraction contains proteins from the plasma membrane with high enrichment. In this respect, ligand-induced tyrosine phosphorylation is important for the transmission of signals across the plasma membrane[17]. Overall the method we describe here has a number of advantages: ease of extraction, quantitative, membrane complex preservation and phosphorylation detection within the plasma membrane environment.

Plasma membrane (phospho)proteome during meiosis and fertilization

The proteome of oocytes has been investigated in a variety of species, to the best of our knowledge most studies of the phosphoproteome have been performed in *Xenopus*[8,9]. The identification of plasma membrane proteins has mainly been used to investigate cell-cell interactions, molecular transport and to identify therapeutic targets in tissues or cell lines. However, little research has focused on mammalian oocyte plasma membrane dynamics, partly because of the relatively large amount of material needed for proper analysis using poorly sensitivity existing methods.

Here, by applying the strategy described above, we investigated the plasma membrane proteome and phosphoproteome over the developmental period extending across

mammalian oocyte maturation and into zygote formation. Oocytes from the GV and MII stages and zygotes collected 24 hours after fertilization were used; the study was performed with three biological replicates in each group, and around 300 bovine oocytes per sample.

A total of 446 proteins and 65% new phosphosites which had not been annotated previously were found to be enriched in the cellular component of the plasma membrane. We also found a small group of enriched proteins to be related to cytoplasmic vesicle membranes, most likely because of plasma membrane protein-protein connections. 63 of the 446 proteins exhibited significant changes in abundance during development. Many of these proteins are involved in cellular transportation, cell communication and cell deviation, illustrating these plasma proteins can be an active element in these biological events during early development.

Actin binding proteins are recruited beneath the plasma membrane during oocyte maturation

The cortex of the oocyte represents a significant assemblage of the plasma membrane, extracellular coat, and subsurface skeleton to which a characteristic set of cytoskeletal elements, organelles and macromolecules adhere. It behaves as a functional unit during the reorganizations that precede and follow fertilization[18]. In our data, multiple protein complexes that concentrated in the cortex region were detected, for instance the Arp2/3 complex and the ERM complex.

The Arp2/3 complex primarily localizes in the cortex of oocytes close to the plasma membrane as a major component of the actin cytoskeleton and has been found to be an important regulator of actin remodeling, when chromosomes move to the oocyte cortex they trigger Arp2/3 complex activation and induce cortical polarization, thickening and spindle positioning during oocyte maturation and preimplantation embryo development[19–22]. Here, we detected 5 proteins from the Arp2/3 complex (ARP2, ARP3, ARPC2, ARPC3 and ARPC1B) which represent 70% of the subunits in the complex[23]. In addition, the IQGAP2 protein which has been reported to interact with Arp 2/3 to regulate actin assembly[24,25] was also detected. Both ARPC3 and IQGAP2 are highly expressed in zygotes, the coordination of their change in expression indicating their activation in bovine oocytes after fertilization. This suggests a role in formation and completion of cytokinesis and actin cap formation which is essential for the

regulation of asymmetric division, polar body extrusion and cell division during maturation and fertilization.

Another interesting membrane protein complex found is the ERM complex consisting of three proteins EZR, RDX and MSN. Phosphorylation of the ERM complex leads to their localization under the plasma membrane where they function as a general bridge between plasma membrane proteins and the actin cytoskeleton. Active ERM adopts an open conformation that binds F-actin and the plasma membrane. ERM proteins are involved in the functional expression of membrane proteins at the cell surface[26,27]. In mammalian oocytes, the ERM proteins have been reported to contribute to cell polarity during the completion of meiosis[28]. However, research into these complexes has been mainly in mouse oocytes, and their dynamic in higher mammalian species are largely unknown. Here we found the three proteins of the ERM complex to be expressed at low levels in GV oocytes but more highly in MII oocytes and zygotes. Even though we did not find any phosphosites in the ERM complex, the elevated expression of ERM complex proteins during bovine oocyte maturation suggests a function in spindle positioning, polar body formation and completion of meiosis II in bovine oocytes[3].

Thus, we show enrichment of two protein complexes that are important for actin cytoskeleton dynamics in mammalian oocytes by extracting plasma membrane proteins. The enrichment indicates the localization of these complexes just beneath the plasma membrane in cattle oocytes which is similar to reports in oocytes of other species. Further biological research needs to be done to reveal the mechanism for their differential expression in oocytes at different stages, as well as to investigate any interaction between the Arp2/3 and ERM complexes and how do investigate how they contribute to actin filament dynamics.

Multiple new phosphosites are found in a single protein

An interesting finding in our data is protein KHDC3L, a maternal effect protein. In human oocytes, immunofluorescence studies revealed that KHDC3L localizes to the cytoskeleton predominantly at the cortical region in growing oocytes and translocates to the nucleus from the morula stage of embryo development, which suggests that it has a function in the integrity and organization of the cortical region during oocyte development[29]. Further study of this protein reveals that it is one of the members of the subcortical maternal complex (SCMC) which is uniquely expressed in mammalian oocytes and early embryos, with a critical function

during early embryogenesis. The SCMC has a specific localization in the subcortex of mammalian oocytes and preimplantation embryos[30]. SCMC is composed of at least five proteins: oocyte expressed protein (OOEP), NLR family, pyrin domain containing 5 (NLRP5), transducin-like enhancer of split 6 (TLE6), peptidyl arginine deiminase 6 (PADI6) and KH domain containing protein 3 (KHDC3; also known as FILIA). Based on our data, we find KHDC3L not only but also other membranes of the complex in the oocyte plasma membrane. This is the first report of their identification in cattle, especially when based on plasma membrane protein extraction. Our results indicate that they are probably localized under the plasma membrane in bovine oocytes, where they have been proposed to be important regulators of their distribution of mitochondria, spindle positioning, cytoskeleton interaction, oocyte-specific translation and epigenetic reprogramming during oocyte maturation and fertilization[31]. However, expression of KHDC3L in bovine oocytes has only previously been investigated at the transcript level; RT-PCR showed KHDC3L gene expression in oocytes[32]. Even though the protein has been reported in multiple species, its phosphosites were still unknown. Here, we found 7 phosphosites and therefore helped to fill the gap of knowledge in terms of possible KHDC3L post-translational modification. KHDC3L is significantly more highly expressed in GV stage oocytes which agree with its protein expression in human oocytes, while its phosphorylation level seems to be stable during the three stages studies[33]. The new phosphosites we found further validate the efficiency of membrane protein investigation after plasma membrane extraction, and its role in generating new information about the status of the proteins after enrichment. Moreover, our date indicates that expression and phosphorylation of KHDC3L may play a role in membrane dynamic of oocytes.

Coordinated dynamic changes in proteins during meiosis and fertilization

ANOVA analysis suggested that 17 phosphosites underwent significant differential expression ($p< 0.05$). The major notable features of these changes were from GV to the MII stage, when the number of phosphorylation increased, followed by a decline from MII to the zygote stage; this pattern is similar to previous reports of changes in global phosphorylation[8]. We found three proteins (PALM3, EPB41L2 and TACC3) that had similar abundance tendencies in the proteome and phosphoproteome. All three exhibited increased phosphorylation during oocyte maturation but a decrease at the zygote stage.

PALM3 (Paralemmin-3) is a plasma membrane protein that belongs to the paralemmin protein family implicated in plasma membrane dynamics such as filopodia and microspikes, membrane signaling, and induction of cell expansion, process formation and cell shape control through membrane flow or membrane-cytoskeleton interaction[34–36]. EPB41L2 (4.1 G protein) is a membrane skeletal protein that serves as an adapter between transmembrane proteins and the underlying membrane skeleton and is required for cell adhesion, spreading, migration and equal cell division during mitosis[37,38]. So far, little is known about the distribution, expression and function of PALM3 and EPB41L2 protein in oocytes. Our study demonstrates the expression of these proteins in GV and MII stage bovine oocytes as well as zygote; expression was significantly increased at the MII stage and maintained in zygotes. Furthermore, we found a phosphosite at the position of S (589) in the PALM3 protein and three phosphosites at positions 586, 682 and 767 in EPB41L2; none of which had been reported previously. In addition, the phospho level at site 767 was significantly increased at the MII stage. In short, we revealed a novel expression tendency and phosphosites for these proteins which provide a basis for further study of their function in oocytes.

TACC3 is a microtubule associated protein involved in mitotic spindle assembly and chromosome alignment following phosphorylation[39]. In oocytes, expression of TACC3 increasing during meiosis and it is mainly located around the metaphase spindle. Knock-down and Aurora-A kinase inhibition experiments led to disruption of spindle formation indicating a function for phosphorylated TACC3 in maintaining meiotic spindle stability[40]. It is known that TACC3 coupled with clathrin helps assist mitotic spindle organization.

In our data, TACC3 showed significantly increased protein and phosphoprotein expression during oocyte maturation, in accordance with previous Western blot results[40]. In addition, we detected the other three proteins involved in the TACC3/ch-TOG/clathrin complex in our data (TACC3, CKAP5 and clathrin); this complex is formed to stabilize kinetochore fibers by crosslinking adjacent microtubules to regulate spindle assembly and chromosome congression[41]. Since the TACC3/ch-TOG/clathrin complex was reported to act on the spindle and centrosome, it is therefore not entirely clear why we also found these proteins in our plasma membrane fraction. One possibility is that a TACC3 coupled complex also functions in

spindle interaction with the plasma membrane late in MII to help regulate first polar body formation[42,43].

Aurora A phosphorylates TACC3, recruiting and stabilizing it on the microtubules that help to ensure proper cell division. In man, Aurora A phosphorylates TACC3 on several residues (S25, S34, S552, S402 and S588)[44,45]; in bovine oocytes, Aurora A localizes at the contractile ring, suggesting a function in cytokinesis during polar body formation. Both inactivity and over-activity of Aurora A results in aberrant cytokinesis, underlining its importance in the completion of cell division[46]. Here, we found a phosphosite at position S25 the expression of which significantly increased during meiosis indicating that it may contribute to maturation.

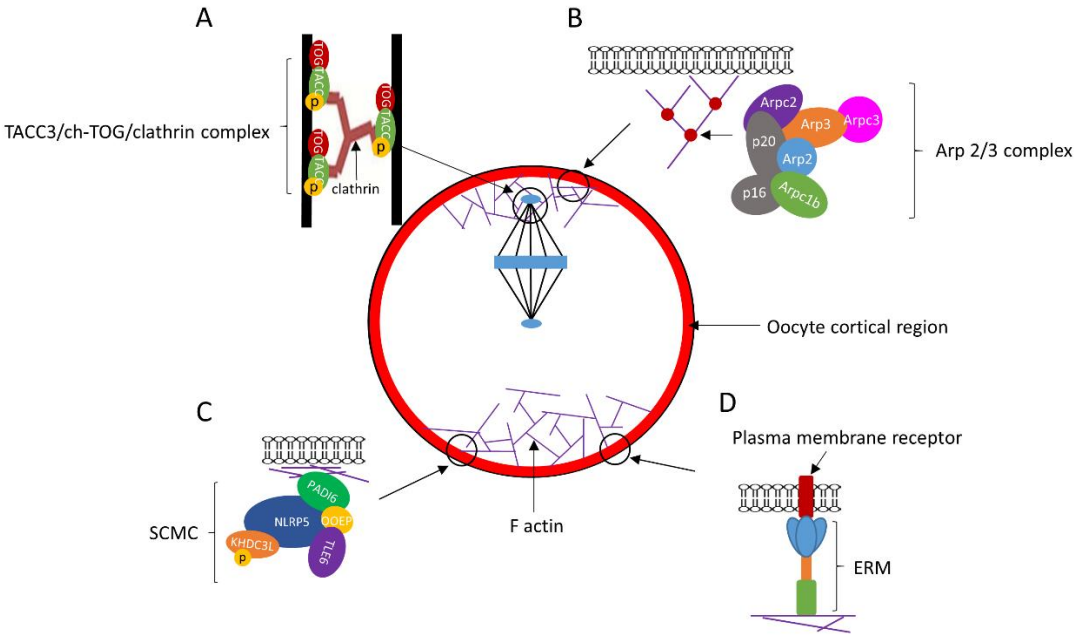

Figure 6: Various molecular complexes in maturing oocytes. **A:** Model of the TACC3/ch-TOG/clathrin complex components during oocyte maturation. Phosphorylated TACC3- ch TOG complex interacts with clathrin to medicate microtube growth and stability in the regulation of microtubule dynamics at the meiotic spindle. **B:** ARP2/3-mediated actin polymerization is responsible for the formation of the cortical actin cap, and cortex thickening during oocyte maturation; the grey color indicates proteins not detected in this study. **C**: SCMC complex localizes adjacent to the oocyte plasma membrane and is responsible for mitochondria redistribution, spindle positioning, cytoskeleton interaction, oocyte-specific translation and epigenetic reprogramming during oocyte maturation and fertilization. **D**: ERM complex functions to crosslink transmembrane receptors to the cytoskeleton. The complex is involved in maintaining cortical tension.

Overall, in this paper, the optimized plasma membrane extraction and identification method enabled us to boost the detection of proteins and phosphosite with poorly abundant material. The detection of large functional complexes (Figure 6) that relate to plasma membrane dynamics for example: the Arp2/3 complex and ERM complex indicates that a method without detergents for plasma membrane protein extraction helps protect the native structure of protein complexes and therefore aids detection of functional interactions among proteins. The multiple new phosphosites detected further illustrate the enrichment of plasma membrane proteins and their phosphopeptides, and how this helps discovery of proteins that would be difficult to detect in whole cell lysates. Besides highlighting the technological advance, the data reveals a number of biological insights into plasma membrane dynamics during mammalian oocyte meiosis and fertilization. GO analysis yielded expected enrichments in biological processes related to regulation of protein localization to early endosome, homotypic cell-cell adhesion and mitotic cytokinesis that are all involved in trafficking related to the plasma membrane and cytoplasm. Examination of the (phospho)proteome suggested that proteins such as PALM3, PB41L2 and TACC3 work in tandem, with increased proteome and phospho expression at the MII stage suggesting a function in regulating oocyte maturation following post-translational modifications. This study therefore identifies molecular targets that could be used to improve the maturation and development of bovine oocytes and embryos in assisted reproductive technologies.

Material and Methods

Hela Cell Culture

Hela cells were cultured in DMEM (Lonza) supplemented with 10 % FBS (Gibco) and 1 mM Glutamine (Lonza) (all from Invitrogen, Carlsbad, CA), and harvested at 80 % confluency. Cells were washed in PBS and stored as frozen pellets at -80 ^{o}C before plasma membrane extraction.

Oocyte collection, maturation and fertilization

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in a polystyrene box at room temperature (RT) within 2 h after slaughter. After washing and removal of extraneous connective tissue, the ovaries were transferred to a flask containing 0.9 % NaCl supplemented with 0.1 % penicillin/streptomycin (10.000 U/ml: Gibco BRL, Paisley, UK) and maintained in a water bath at 30 °C. Cumulus oocyte complexes (COCs) were collected, matured and fertilized in vitro as described previously[47]. Oocytes at germinal vesicle and metaphase II stages and zygotes (24 h after fertilization) were collected and stored at -80 °C. Around 300 oocytes were collected in each sample and triplicates were used as experimental groups.

Membrane Protein Preparation

Plasma membrane protein extraction was performed using a Plasma Membrane Protein Extraction Kit (ab65400, abcam, USA) with modifications and adaptations to suit the oocyte material. Briefly, oocyte samples were homogenized on ice with 200 µl ice-cold homogenizing buffer supplemented with protease inhibitor (Roche Complete EDTA Free), PhosSTOP phosphatase inhibitor mixture (Roche Applied Science, Meylan, France). The homogenates were centrifuged at 700 × g for 10 min at 4 °C and the pellet was discarded. The supernatant was subjected to a second centrifugation step at 10.000 × g for 30 min at 4 °C. The pellet contained total cellular membranes in “sheets”. Plasma membranes were further isolated by their differential partitioning properties into the upper phase. In the final step, the upper phase containing the plasma membranes was precipitated by dilution in MQ water, and pelleted at 12.000 × rpm for 30 min.

MS sample preparation and identification

The plasma membrane pellets were dissolved in 8 M Urea, 50mM AMBIC protease and phosphatase inhibitors. Denatured plasma membrane proteins were reduced with 4 mM DTT for 1 hr at room temperature, alkylated with 8mM Iodoacetamide at room temperature for 30min in the dark, and then digested sequentially with 1:75 LysC and 1:75 trypsin at 37 °C for 4hrs and overnight, respectively. The resulting peptides were acidified and purified by home-made SCX stage tips, dried by vacuum centrifugation and reconstituted in 10 % formic acid for analysis on an Orbitrap Q Exactive HF spectrometer (ThermoFisher Scientific) connected to a UHPLC 1290 LC system (Agilent).

Phosphopeptide enrichment

Phospho-peptide enrichment was performed as described previously[12], using 10 µg equivalent of triptic peptide digests.

LC-MS/MS analysis

Peptides were trapped (Dr Maisch Reprosil C18, 3 µM, 2 cm × 100 µM) for 5 min in solvent A (0.1 % formic acid in water) before being separated on an analytical column (Agilent Poroshell, EC-C18, 2.7 µm, 50 cm × 75 µm). Trapping was performed for 5 min in 0.1 % formic acid (Solvent A) and elution with 80 % ACN in 0.1 %formic acid (Solvent B) in 90 min gradient time, at a passively split flow rate of 300 nl/min. MS data were obtained in data-dependent acquisition mode. Full scans were acquired in the m/z range of 375–1600 at a resolution of 60,000 (*m*/*z* 400) with AGC target 3E6 in the maximum filling time of 20 ms. The top 15 most intense multiply charged precursor ions were selected for HCD fragmentation performed at a resolution of 30,000 using NCE 27 %, after accumulation to target value of 1E5 in the maximum filling time of 50 ms. Dynamic exclusion was set to 16 s.

Database search

The raw data were processed using Maxquant 1.6.5.0 and searched against the Uniprot human database containing 171063 entries (downloaded in June 2019) or the Uniprot bovine database containing 32206 entries (downloaded in Nov 2017). Enzyme specificity was set to Trypsin, and up to 2 missed cleavages were allowed. Cysteine carbamidomethylation was set to fixed modification, whereas variable modifications of methionine oxidation and protein N-

terminal acetylation were allowed, together with phosphorylation of Ser, Thr, Tyr (STY) residues (phosphoproteomics).

Data visualization and statistical analyses

Protein filtering by significance was performed with Perseus 1.6.2.2. One-way ANOVA analyses were performed with a p-value < 0.05. Heatmaps, hierarchical clustering and trend plots were also visualized by Perseus 1.6.2.2. Gene ontology annotations were extracted using Gorilla. Venn diagrams, piecharts and bar charts were made with Venny and Microsoft Excel respectively. Peptide hydrophobicity was computed using SSRCalc.

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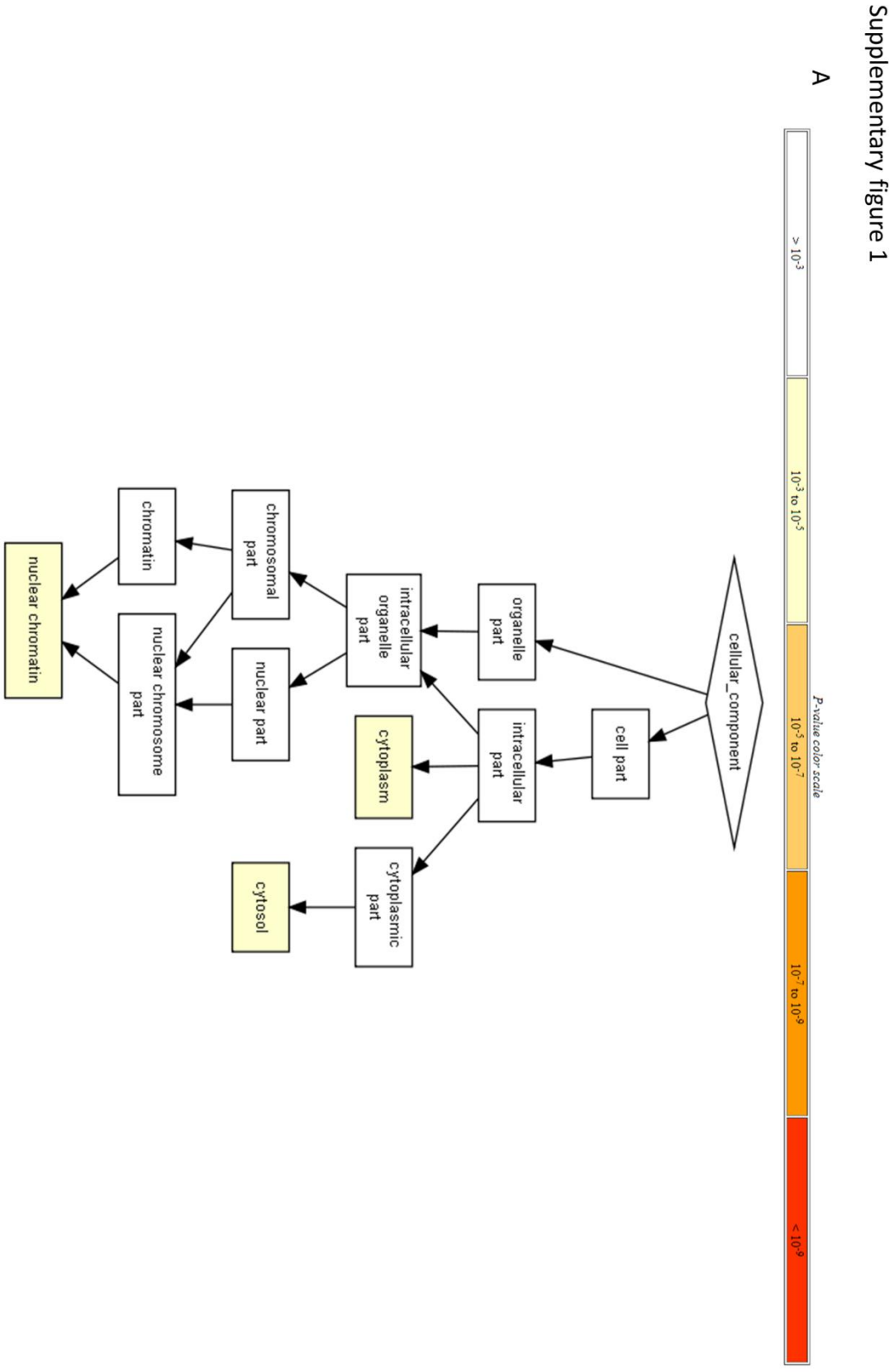
Supplementary Figures



Supplementary figure 1

B

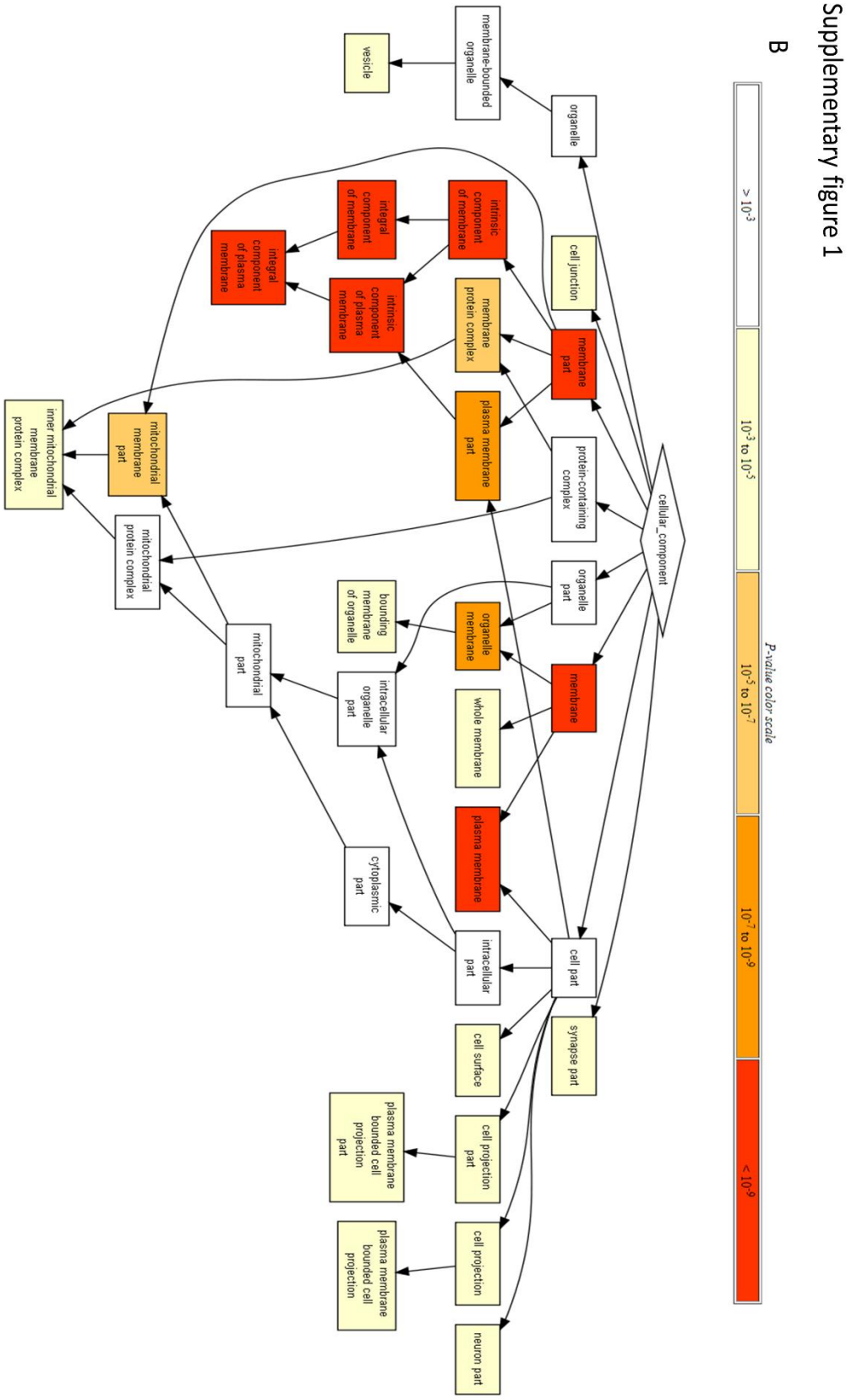


Fig. S1: GO cellular component annotation of proteins. A: GO clusters indicated the cellular components enriched among proteins unique in the WCL of Hela cell line. B: GO clusters indicated the cellular components enriched among proteins unique in the PM fraction of Hela cell line.

Chapter 5

Discussion

Discussion

This thesis explores biochemical pathways and processes that are important for oogenesis and early embryo development, and includes the validation of techniques to facilitate future research. We explored the PIWIL3/piRNA system in bovine oocytes and examined transcriptome changes caused by microinjection of oocytes prior to maturation. Lastly, we investigated plasma membrane protein dynamics during oocyte maturation and zygote formation. In this chapter, I will discuss the significance of these findings and identify directions to further explore important aspects of oocyte maturation, fertilization and genetic integrity of the zygote and early embryo.

PIWI/piRNA pathway - from less complex organisms to large mammals

PIWI proteins belong to the Argonaute protein family and are essential for protecting the integrity of the germline during early development. In 2006, various research groups identified a type of small RNA (piRNA) that interacts with PIWI proteins in a variety of species, including fruit flies, zebrafish and rodents. This signaled the start of studies examining the functions of the PIWI/piRNA system in different organisms[1–4]. During the last decade, the PIWI/piRNA system has been studied in depth in animals including *Drosophila* and mice. In the *Drosophila* genome, three Piwi protein encoding genes are present (*aub*, *ago3* and *piwi*), while the mouse genome also contains three genes coding for PIWI proteins (*Miwi*, *Miwi2* and *Mili*). PIWI mutations in *Drosophila* have been linked to failure of transposon repression, and it is interesting to note that in *Drosophila* whereas *Piwi* and *Aub* are required for both male and female fertility, *Ago3* is only essential for female fertility; this underlines the importance of transposon repressing argonaute proteins for ensuring the integrity of the female germline. In mice, however, PIWIs are predominantly expressed in the testes at various stage of spermatogenesis, and deficiency of PIWIs leads to male infertility. On the other hand, female mice with genetic deletion of Piwi proteins exhibit normal fertility, suggesting that PIWIs are not involved in the formation and function of mouse oocytes. Indeed, regulation of transposon activity in the murine female germline has been reported to be performed via Dicer and Dicer-dependent endogenous small interfering RNAs (endosiRNAs)[5,6]. In contrast, the genome of other mammals including the cow, monkey and man, encodes an additional PIWI protein,

PIWIL3, for which the mouse has no homologue. PIWIL3 is expressed in bovine oocytes and is the only PIWI protein specifically present in GV and MII stage oocytes, suggesting that it may play an important role in mammalian oocyte development[7].

In chapter two, we investigated the localization of PIWIL3 in bovine oocytes and early stage embryos and, to try to improve our understanding of the function of PIWIL3 in mammalian oocytes, we examined the proteins and piRNAs that interact with PIWIL3. In the absence of a specific antibody against bovine PIWIL3, we identified the cellular location of PIWIL3 by microinjection of mRNA coding for eGFP-PIWIL3 and PIWIL3-eGFP. We found that PIWIL3 was predominantly expressed near mitochondria and that it co-localized with TDRKH. The distribution and sub-cellular location of PIWI proteins have previously been reported in the female germ cells of silkworms and Drosophila[8–10]. However, this is the first time that PIWIL3 has been localized in oocytes in a mammalian species. The localization of PIWIL3 in mammalian oocytes was an important first step to establishing and understanding the function of PIWI during oocyte maturation. Using a TDRKH antibody and immunoprecipitation, we identified that a functional PIWIL3/TDRKH/PNLDC1 complex forms in bovine oocytes. Association of TDRKH with a Piwi protein and PNLDC1 has only previously been demonstrated in mammalian testis or the ovary of lower organisms[11,12]. In the silkworm, PNLDC1 and its homologue Trimmer are thought to be important for production of primary piRNAs[13–15]. In male germ cells in the mouse, the membrane-anchored TDRKH recruits MIWI, but not MILI, and PNLDC1 for 3'end trimming of pre-piRNAs to the appropriate size[12]. Interestingly, MILI also localizes to mitochondria, and trimming of MILI-bound piRNA requires PNLDC1, indicating that PIWI proteins are specifically recruited to mitochondria for piRNA biogenesis in different species. On the basis of its localization, complex formation and piRNA abundance, we propose that the PIWIL3/TDRKH/PNLDC1 complex in bovine oocytes is important for piRNA biogenesis. We additionally demonstrated that the repertoire of piRNAs bound to the TDRKH/PIWIL3 complex is very similar to the piRNA sequence repertoire found in bovine GV oocytes previously, indicating that most of the piRNAs in GV oocytes are associated with PIWIL3[7].

During preimplantation development, genome wide DNA demethylation occurs, removing targeted repression of DNA transcription and thereby increasing the risk of transposon activity. During this demethylation process, piRNAs expressed in GV oocytes presumably play a critical role in transposon suppression given that almost half of the piRNA species we detected map

to transposon sequences. It has been reported that piRNAs are highly expressed in bovine oocytes and blastocyst stage embryos but that significantly fewer piRNAs are present in 8 cell stage embryos, indicating that production of transcripts against transposable elements is important during the maternal to zygote transition[16].

The absence of *Piwil3* in the mouse genome has inhibited studies of the function of this gene using embryonic stem cell driven gene knockout technology. Theoretically, the CRISPR-Cas9 system could be used to inactivate *PIWIL3* in cattle, but the long generation interval of these animals renders these experiments impractical. Other animal models are therefore needed to study the role of PIWIL3 in mammalian oocytes, for instance the rabbit or hamster. An in-depth study of the function of PIWIL3 in mammals would give us a better understanding of the role and importance of this protein and the associated non-coding small RNAs during oocyte formation and early embryo development.

Microinjection during oocyte maturation alters the transcriptome

A method described for the efficient downregulation of mRNA expression to assist studies of gene function is RNA interference using siRNAs. In the oocyte, the technique used most commonly to introduce siRNAs is microinjection, because it allows relatively precise control of the amount of siRNA introduced. One of the downsides of this method however, is that it requires every oocyte to be handled and injected individually, thereby imposing a practical limit on the number of oocytes that can be subjected to gene silencing. In addition, the oocytes are subjected to physical stresses during microinjection. As a negative control during siRNA injection experiments, siRNA sequences with a similar GC content to that of the specific siRNA sequences but in random order (scrambled siRNA) are injected and the responses compared to the oocytes injected with the functional sequence. However, whether the microinjection procedure itself causes changes to oocyte function is something that has not been addressed in any depth. In order to determine how microinjection with siRNA affects oocyte function, in chapter three we performed nonspecific siRNA microinjection into oocytes, followed by single cell RNA-sequencing. To increase the reliability and reproducibility of the experiment, 12 individual oocytes were used in each comparison group. We identified 119 transcripts for which the expression levels were significantly altered by non-specific siRNA injection, out of

an average of 10696 genes that were expressed in oocytes. After gene ontology (GO) term enrichment analysis, we found that the microinjection procedure mainly alters the expression of mitochondrial and transmembrane related genes, which is logical given that microinjection interrupts the membrane structure. Most importantly, these findings demonstrate the potential effects on oocyte function of microinjection *per se*, which should be taken into consideration when investigating gene function after siRNA injection.

Even though siRNA injection is a useful method for investigating gene function in many situations, oocytes may be an important exception. Oocytes retrieved from slaughterhouse ovaries are at the germinal vesicle stage of development but resume meiosis immediately after isolation from the follicle and loss of contact with the follicle wall, this means that the time available for the effect of siRNAs aimed to silence function of a gene/protein involved in oocyte maturation is limited to the time span of maturation, in bovine oocytes ~24 hrs. In an attempt to investigate the function of PIWIL3, we downregulated *PIWIL3* expression by injecting a PIWIL3 siRNA into oocytes. Quantitative RT-PCR indicated that while *PIWIL3* gene expression was decreased by approximately 10-fold, no change in PIWIL3 protein expression was observed (data not shown). Most likely, the time course of PIWIL3 turnover is such that proteins formed or present prior to oocyte collection (e.g. during follicular growth) remain functional for at least 23hrs after resumption of meiosis. Recently, a method was described to rapidly downregulate endogenous protein expression, this so-called Trim-Away method utilizes antibodies to remove native proteins within minutes of application[17]. Although specific bovine PIWIL3 antibodies are not available, it is possible that targeting TDRKH using this technique may help to improve our understanding of the function of the PIWIL3/piRNA pathway in mammalian oocytes.

Role of plasma membrane (phospho)proteins in the mammalian oocyte and zygote

Over the last decades, it has been widely recognized that plasma membrane proteins are critical to cell dynamics. The study that examined transcriptome changes after microinjection underlines the importance of plasma membrane proteins for proper cell function. In chapter 4, we used a plasma membrane protein extraction method optimized for samples of limited quantity to identify the plasma membrane phospho(proteome) in bovine oocytes and zygotes.

By using a detergent free method for protein extraction, we were able to not only identify plasma membrane proteins but also some of their natural interactions. This method may have a variety of applications both in diverse cell lines, but also in samples for which only a limited amount of material is available.

Phosphorylation is a common post-transcriptional modification used to regulate protein activity, localization and stability. During oocyte meiosis, kinase signaling pathways are active in, for example, chromosome condensation and segregation, the cell cycle, spindle dynamics and chromatin modifications[18,19]. During mouse oocyte maturation, protein phosphorylation increases particularly in the cortical region, including the oocyte plasma membrane[20]. To determine the phospho-dynamics of plasma membrane proteins during meiosis and fertilization, we performed phospho enrichment and mass spectrometry on maturing bovine oocytes. We detected around 40 different phosphosites. Among these, the status of 17 phosphosites changed during maturation and development, with a tendency to increase during meiosis but decrease after fertilization. For this study, we used batches of approximately 300 bovine oocytes for plasma membrane purification, phosphoenrichment and protein identification, yielding less than 1μg of protein per sample for the measurements. By increasing the amount of input proteins, we assume that additional proteins and phosphosites could be detected. The results underline the importance of protein phosphorylation and associated kinase and phosphatase pathways during oocyte maturation and early embryo development[21].

Chapter 4 addressed both technical and biological goals. Firstly, by using the optimized detergent free method for cell fractionation combined with phosphoenrichment and a sensitive Mass Spectrometric technique, proteins and new phosphosites could be identified from samples of low abundance. This conclusion is supported by the identification after fractionation of novel proteins and a large number of phosphosites, which included around 30% of novel phosphosites. Secondly, the detergent free method enabled us to study the native information regarding protein interactions within the plasma membrane. We detected multiple protein complexes that relate to plasma membrane function in oocyte samples such as ERM (Ezrin, Radixin, Moesin), SCMC (subcortical maternal complex), Arp2/3 (actin-related protein) and the TACC3/ch-TOG/clathrin complexes, as described in chapter 4. These complexes are mainly localized in the cortical region near the plasma membrane and their

isolation with plasma membrane proteins indicates the complex network involved in maintaining oocyte structure. The findings also demonstrated the sensitivity of the isolation technique, which could be applied in a variety of fields other than oocyte plasma membrane investigation.

The quantification of protein and phosphosite expression in bovine oocytes at the GV and MII stages and in zygotes provides important basic information for future studies of plasma membrane dynamics during oocyte maturation and fertilization in large mammals. For example, the ERM complex is formed from the Ezrin, Radixin and Moesin proteins and can function as a crosslinker between plasma membrane receptors and the actin cytoskeleton, after activation by phosphorylation. Indeed, the ERM complex is known to be involved in signaling pathways during cell migration, adhesion and cytoskeleton reorganization[22]. In oocytes, ERM proteins contribute to the successful completion of meiosis II by modulating cell polarity[23]. It has been reported that, in mouse oocytes, the levels of ERM proteins and phosphorylation are reduced[23]. Here, we did not detect active phosphorylation sites within ERM proteins during oocyte maturation, but we did determine a significant increase in all ERM proteins during oocyte maturation from the germinal vesicle to meiosis II stage, something that is contrary to previous findings in mouse oocytes. Possibly, localization specificity accounts for these differences. Functional experiments with ERM proteins would be helpful to uncover their roles during mammalian germ cell development.

The SCMC is a protein complex consisting of numerous maternal effect proteins, and is required for normal mouse pre-implantation development. Accumulating evidence suggests that SCMC can be used to identify new maternal-effect genes, to clarify the molecular mechanism of other maternal-effect genes, and to explore the regulation of early embryonic development in mammals. Additionally, SCMC may be conserved in human oocytes and early embryos. Importantly, mutations of human SCMC genes are associated with several reproductive disorders. Collectively, these findings indicate that the SCMC may be an important complex orchestrating the maternal-to-zygotic transition in mammalian early development. Further investigations of the SCMC will greatly promote the understanding of molecular regulation in mammalian early embryo development, significantly contributing to human reproductive medicine. Overall, our findings in terms of plasma membrane proteins and their phosphorylation sites can lay the foundations for future research. By examining

protein complexes related to the plasma membrane, such as ERM and SCMC, we identified worthwhile candidates for future in-depth studies aimed at investigating oocyte function.

Overall, in this thesis, we addressed a number of important gaps in our understanding of oocyte development and embryogenesis. Future research still needs to be done to extend our findings on how the PIWL3/piRNA system ensures genetic integrity of oocytes and zygotes, the influence of microinjection on the transcriptome of oocytes is something that should be borne in mind when designing intervention studies in mammalian oocytes , while the dynamic changes that take place in the plasma membrane (phospho)proteome are an exciting starting point to understand the important roles the plasma membrane and associated proteins play in the various stages of oocyte maturation, fertilization and early embryo development.

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Addendum

Nederlandse Samenvatting

De productie van gezonde gameten (eicellen en spermacellen) is essentieel voor de vorming van nakomelingen. Een scala aan moleculen en mechanismen is nodig voor de juiste differentiatie van gameten gedurende de embryonale ontwikkeling maar ook in de reproductieve periode. In dit proefschrift staan de resultaten beschreven en bediscussieer ik verschillende experimenten die onze kennis omtrent de ontwikkeling van gameten, in het bijzonder eicellen vergroten. Ook bediscussieer ik technieken om eicellen verder te bestuderen om onze kennis over deze cellen te vergroten

Hoofdstuk 1 geeft een algemene introductie over de ontwikkeling van eicellen in zoogdieren, in het bijzonder in relatie tot PIW/piRNA mechanismen, microinjectie, de zuivering van celmembraaneiwitten en posttranscriptionele modificatie zoals fosforylering, als inleiding voor de experimentele hoofdstukken 2, 3 en 4.

Hoofdstuk 2 is gericht op de karakterisering van PIWIL3 in eicellen van het rund als een model voor zoogdieren. De functies van PIWI/piRNA in zoogdieren zijn voornamelijk bestudeerd in de muis. In dit modeldier zijn PIWI eiwitten (MIWI, MILI en MIWI2) aanwezig in de testes, maar niet of nauwelijks in vrouwelijke dieren. Inactivatie van alle genen in de muis die coderen voor Piwi leiden tot infertiliteit bij de mannen maar niet bij de vrouwen, waaruit geconcludeerd is dat in zoogdieren het PIWI/piRNA system geen rol speelt bij vrouwelijke dieren. Een gen dat codeert voor PIWIL3, dat aanwezig is in het genoom van de meeste zoogdieren inclusief de mens, ontbreekt echter in het genoom van de muis, waardoor dit dier minder geschikt is als model voor de functie van PIWIL3 in zoogdieren. Eerder is aangetoond, gebruik makende van het rund als modeldier, dat PIWIL3 voornamelijk tot expressie komt in eicellen van antrale follikels. Door afwezigheid van een geschikt antilichaam tegen PIWIL3 was niets bekend over de intracellulaire lokalisatie van PIWIL3 in eicellen. In Hoofdstuk 2 worden de resultaten beschreven van injectie van expressie-plasmiden coderend voor een N-terminaal en C-terminaal-eGFP gelabelled PIWIL3 in rundereicellen. Immuunfluorescentie met een antilichaam tegen GFP op deze eicellen liet zien dat PIWIL3 voornamelijk aanwezig was in het cytoplasma en dan vooral op mitochondriën, maar niet in celkern. Door een dubbelkleuring te doen met een antilichaam tegen TDRKH, dat specifiek gebonden is aan mitochondriën, werd de aanwezigheid van PIWIL3 op mitochondriën bevestigd. Deze data suggereren tevens dat PIWIL3 en TDRKH aan elkaar binden. Immuunprecipitatie met een TDRKH-antilichaam gevolgd door massaspectrometrie toonde aan dat PIWIL3 inderdaad een complex vormt met

TDRKH. In dit complex werd bovendien PNLDC1 gevonden. Tevens konden we door kleine RNAs te sequencen na immuunprecipitatie met een TDRKH-antilichaam, een groot scala aan piRNAs identificeren die hoogstwaarschijnlijk gebonden zijn aan PIWIL3 in het TDRKH-PIWIL3-PNLDC1 complex. Mogelijk wordt PIWL3 door TDRKH naar mitochondria gerekruteerd en worden piRNAs hier door PNLDC1 op de juiste grootte gebracht. Deze data laten zien dat PIWIL3 belangrijk is voor de vorming van ontwikkelingscompetente eicellen in zoogdieren.

In hoofdstuk 3 is onderzocht in hoeverre microinjectie van eicellen verschillen in RNA expressie kan veroorzaken. Microinjectie wordt heden ten dage routinematig gebruikt om exogene componenten zoals DNA, RNA of eiwitten te introduceren in eicellen. Het is duidelijk dat de plasmembraan en het cytoskelet door injectie worden beschadigd, maar in hoeverre dit een gevolg heeft voor het functioneren van de eicel en expressie van genen is niet bekend. Door middel van microinjectie zijn niet-specifieke siRNAs ingebracht in ongematureerde rundereicellen waarna onderzocht werd in hoeverre de eicel in staat was om te rijpen tot het metafase II stadium. Tevens werd door middel van single-cell sequencing het RNA expressiepatroon onderzocht en vergeleken met dat van niet-geïnjecteerde eicellen. Er is gekozen voor injectie van niet-specifiek siRNA omdat bij experimenten waarbij de expressie van een gen wordt geremd vaak gebruikt gemaakt wordt van specifieke siRNAs, waarbij een niet-specifieke siRNA als negatieve controle wordt gebruikt.

Een niet-specifieke siRNA werd geïnjecteerd in rundereicellen van het germinal vesicle stadium. Bij experimenten waarbij met behulp van siRNA gen-expressie wordt geremd worden eicellen vaak in Roscovitine gehouden om meiose uit te stellen en het siRNA tijd te geven om effectief te zijn. Daarom werden in deze experimenten de eicellen ook 16 uur gekweekt in aanwezigheid van Roscovitine, alvorens maturatie in vitro te stimuleren in specifiek medium. Het percentage eicellen dat matureerde tot het metafase II (MII) stadium verschilde niet tussen controle eicellen en geïnjecteerde eicellen. Single cell sequencing van 12 MII stadium eicellen liet zien dat de expressieniveau's van 119 transcripten, uit 12000 gedetecteerde transcripten, significant verschilde na microinjectie. Door middel van gen-ontologie werden deze genen geclassificeerd onder de biologische processen ATP synthese, molecuultransport en eiwit polyubiquitinatie.

Uit hoofdstuk 3 kan geconcludeerd worden dat wanneer siRNA microinjectie wordt gebruikt in eicellen dit veranderingen in het genexpressie patroon kan geven die niet veroorzaakt worden door specifieke mRNA downregulatie, maar aspecifiek door de injectieprocedure.

Omdat eicellen niet zoals cellen van een cellijn in zeer grote hoeveelheden gekweekt kunnen worden is de extractie van voldoende hoeveelheden plasmamembraan voor analyse beperkt. Daarom is in hoofdstuk 4 een effectieve en gevoelige methode ontwikkeld om plasmamembraaneiwitten te isoleren en identificeren. Deze techniek is gebruikt voor isolatie van eiwitten uit de plasmamembraan van rundereicellen, waarna het (fosfo)proteoom van gedurende rijping en bevruchting is onderzocht. Allereest is de efficiëntie van de techniek getoetst met behulp van HeLa cellen, waarbij massaspectrometrie werd gebruikt om eiwitten en fosfopeptiden te identificeren. Daarna werd dezelfde techniek toegepast op germinal vesicle en MI stadium eicellen en zygoten. Hierbij werden verschillende eiwitcomplexen geïdentificeerd zoals het Arp2/3 complex, ERM en SCMC, als ook nieuwe fosfopeptide-gebieden in verschillende eiwitten. Deze data geven nieuwe mogelijkheden om de functie van de plasmamembraan in rijping en bevruchting te onderzoeken met als doel de efficiëntie van eicelrijping en bevruchting bij reageerbuisbevruchting te maximaliseren.

Curriculum Vitae

Minjie Tan, was born on the 29th of December, 1988 in Hunan province, China. In 2012, she obtained her bachelor degree in the college of Animal Science, Guangxi University, China. After that, she started her master study in the laboratory of Animal Reproduction, State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Guangxi University, China. During the master program, she did a one year internship in the laboratory of the Engineering Research Center for Healthy Livestock and Poultry, Institution of Subtropical Agriculture, Changsha, China. After graduate from Guangxi University, she applied the Chinese Government scholarship which supports her PhD study in the Department of Veterinary Medicine, Utrecht University, Netherlands. The achievements during her PhD study are described in this thesis.

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2020 seems to be a difficult year. The COVID-19 is spreading rapidly in the world. The situation accidentally changed many people’s life. In this case, I would like to speak to the world, be strong, human beings would finally win the battle that fight against the virus. I would like to speak to my friends and family, be positive, no matter what life brings to you, keep going. I would also like to speak to myself, be prepared, to start the next step in your life.


3'
Piwi
5'
N
PAZ