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Age-related differences in translation of mammalian oocytes

S věkem spojené rozdíly v translaci proteinů v savčích oocytech

Doctoral thesis

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I hereby declare that I wrote this thesis independently, using the cited literature. This work or a substantial part of it has not been submitted elsewhere to obtain any other academic degree.

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Podpis:

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Contents

Abstract

Female germ cells (also known as oocytes) from mammalian species are found in the ovaries in a state of meiotic arrest at prophase I. It is not until puberty that oocytes start to be selected to grow, overcome their meiotic arrest and ovulate, so they become capable of being fertilized and give rise to new individuals. Half of the genetic information from these new individuals comes directly from the oocyte itself, therefore, oocyte and meiosis quality are of great importance for the reproduction.

One of the factors, which can drastically reduce oocyte quality in several mammalian species is the advanced age of females. In both mice and humans age-related poor oocyte quality is reflected by a large increase of chromosomal aneuploidy rates. Having an incorrect number of chromosomes reduces embryo viability and may cause severe clinical outcomes. The work of this thesis was primarily directed towards a better understanding of the causes behind age-related aneuploidy in mice oocytes.

One of the most characteristic features of oocytes is the fact that they become transcriptionally silent after meiotic resumption, relying heavily on translational control for protein expression. Here we show that after nuclear envelope break down (NEBD), one of the main protein kinases regulating translational initiation, mTOR becomes highly active and as a response 4E-BP1, one of its substrates is inactivated. This documents the tight connection between NEBD and timely initiation of translation.

Our results show that oocytes from aged females (AF, 12 months old) have faster meiotic progression than oocytes from young females (YF, 2 months old). AF oocytes underwent NEBD and polar body (PB) extrusion 30 minutes earlier than YF oocytes, accompanied by a faster phosphorylation of the nuclear lamina component LAMIN A/C. Accordingly, the results of our further experiments revealed an increased activity of maturation promoting factor (MPF), the main regulator of meiotic maturation, in AF oocytes.

Furthermore, we adapted a polysomal profiling method, which allowed us to extract RNAs bound to polysomes (therefore more prone to be translated) from post-NEBD oocytes of both maternal age groups followed by next generation sequencing. Utilizing this technique we were able to reveal a considerable amount of transcripts, which were differentially translated between YF and AF. We validated these differences on protein level at the post-NEBD stage of choosing four most interesting proteins in both age groups: SGK1, CASTOR1, AIRE and EG5. All these four proteins were localized at the spindle region. Moreover, when the levels/activity of SGK1 and CASTOR1 were altered in YF oocytes, we detected significant defects in chromosome alignment and cytokinesis, which supports a possible role of these proteins in age-related aneuploidy.

Altogether, our results show that the post-NEBD stage is the fundamental starting point, in which oocytes from YF and AF start to manifest their differences basically through differential translation of specific RNAs. These differences in the translation of these specific RNAs could be then involved in the higher aneuploidy rates seen in AF oocytes, since our results show that the alteration of their expression or activity can lead to cytokinetic abnormalities.

Abstrakt (česky)

Samičí zárodečné buňky (známé též jako oocyty) se u savců nacházejí ve vaječnících, kde je jejich vývoj zastaven ve stádiu profáze 1. meiotického dělení (profáze I). Teprve až v průběhu puberty dochází k selekci oocytů, které následně rostou, uvolňují se z výše zmíněného profázního bloku, ovulují a stávají se pak schopnými být oplodněny a dát základ budoucímu embryu. Polovina genetické informace embrya tedy pochází přímo z oocytu a tudíž kvalita oocytu a jeho meiotického zrání hraje v reprodukci klíčovou úlohu.

Jedním z faktorů, který může dramaticky snížit kvalitu oocytů u několika druhů savců je pokročilý věk samic. Jak u myší, tak u člověka se snížená kvalita oocytů pocházejících od starších matek projevuje především zvýšením výskytu chromozomální aneuploidie. Důsledkem je přítomnost nesprávného počtu chromozomů v embryo, což snižuje jeho viabilitu a může se projevit i dalšími klinickými příznaky. Práce, která je obsahem této dizertace byla primárně zaměřena na pochopení příčin zvýšeného výskytu chromozomálních aneuploidií v myších oocytech pocházejících od starších samic.

Jedním z nejdůležitějších znaků, kterými se oocyty vyznačují, je to, že po překonání profázního bloku a zahájení meiotického dělení je jejich transkripční aktivita prakticky nulová a vlastní regulace průběhu meiózy je řízena na úrovni translace. V této práci ukazujeme, že po rozpadu jaderné membrány (NEBD – nuclear envelope breakdown), dochází v oocytech k výraznému navýšení aktivity protein kinázy mTOR, která je hlavním regulátorem iniciace translace a v důsledku toho je její substrát, inhibiční protein 4E-BP1 inaktivován. Tato skutečnost poukazuje na úzké propojení mezi NEBD a přesně načasovanou iniciací translace, vzhledem k tomu, že oba proteiny jsou důležitou součástí této signální dráhy.

Naše výsledky ukazují, že v oocytech pocházející ze starších samic (AF – aged females, 12 měsíců staré) dochází k rychlejšímu průběhu meiotického zrání než v oocytech pocházejících z mladších samic (YF – young females, 2 měsíce staré). To znamená, že u AF oocytů docházelo k NEBD a vyloučení polárního tělíska (PB – polar body) přibližně o 30 minut dříve než u YF oocytů. Zjistili jsme také, že rychlejší NEBD bylo doprovázeno rychlejší fosforylací proteinu jaderné laminy LAMINu A/C. V souladu s těmito zjištěními jsou i naše další výsledky, které ukazují na zvýšenou aktivitu hlavního regulátoru meiotického zrání – "zrání vyvolávajícího faktoru" (MPF – maturation promoting factor) v AF oocytech, což může vysvětlit jejich rychlejší průchod meiózou.

Kromě toho jsme v rámci této dizertace adaptovali metodu polyzomového profilování, která nám umožnila izolovat ty RNA, které jsou vázány na polyzomy (a tím i pravděpodobně aktivně translatované) z oocytů ve stádiu po NEBD jak z AF, tak i z YF oocytů, následované NGS sekvenační analýzou. S využitím této metody jsme byli schopni nalézt poměrně velké množství RNA molekul, které byly rozdílně exprimovány mezi AF a YF oocyty. Tyto rozdíly jsme

validovali v oocytech z obou věkových skupin ve stádiu po NEBD i na úrovni exprese proteinů a pro další studie jsme vybrali čtyři nejzajímavější proteiny: SGK1, CASTOR1, AIRE a EG5. Všechny čtyři proteiny byly lokalizovány v oblasti dělicího vřeténka. Navíc v případě, kdy jsme cíleně modifikovali expresi/aktivitu proteinů SGK1 či CASTOR1 v YF oocytech jsme zjistili, že u nich dochází ve zvýšené míře k defektům v uspořádání chromozomů na dělicím vřeténku a v cytokinezi, což naznačilo možnou úlohu těchto proteinů při vzniku chromozomální aneuploidie v souvislosti s věkem samic.

V souhrnu naše výsledky ukazují, že stádium vývoje oocytu těsně po NEBD (1. meiotického dělení) je kritickým bodem, ve kterém se začínají projevovat rozdíly mezi oocyty z AF a YF, což je dané s největší pravděpodobností rozdílnou úrovní translace specifických mRNA molekul v těchto oocytech. Rozdílné úrovně translace těchto specifických RNA pak mohou vysvětlit zvýšený výskyt chromozomálních aneuploidií v oocytech ze starších samic, a to i vzhledem k tomu, že cílená modifikace hladin exprese či aktivity proteinů exprimovaných těmito RNA může vést ke vzniku abnormalit v cytokinezi i v oocytech z mladších samic.

1. Introduction

1.1. The mammalian oocyte development

Mammalian oocytes are highly specialized female germ cells and they are found in the ovary, the female gonad. The origin of oocytes begins with their cellular precursors: the primordial germ cells (PGCs). PGCs differentiation takes place already in the early embryo epiblast after its implantation. In mice, it was possible to detect PGCs within the epiblast at embryonic day (E)7.25 due to an increase of alkaline phosphatase activity in their cytoplasm (Clark and Eddy, 1975; Ginsburg et al., 1990). Up to now, more markers of PGCs lineage have been described (e.g. BMP, PRDM and SMAD proteins) (Mitinori Saitou and Masashi Yamaji, 2010; Irie et al., 2015). As embryo development progresses, PGCs arrest at the G2 cell cycle stage and migrate from the yolk sac through the hindgut until they reach the genital ridge (Seki et al., 2007). Up to this point of embryogenesis, no mechanistic differences can be detected between the PGCs development of male and female. However, after reaching the urogenital ridge, male PGCs stop mitosis and do not divide further until puberty (spermatogonia), whereas female PGCs proceed their mitotic division (oogonia) (van den Hurk and Zhao, 2005).

After several mitotic rounds, oogonia switch to an incomplete meiosis leaving cells arrested at diplotene of prophase I: then they start to be called oocytes. Then primordial follicles form as somatic cells begin to surround the oocytes and a huge wave of apoptosis within oocyte population occurs (Pepling and Spradling, 2001). These somatic cells are called granulosa cells and will nurse the oocyte during future maintenance and development. Primordial follicles develop further previous to puberty and oocytes grow in size and acquire extra layers of granulosa cells (Edson et al., 2009). When puberty is reached, follicle-stimulating hormone (FSH) promotes further oocyte growth in various follicles and the dominant ones will be ovulated in response to another gonadotropin called Luteinizing hormone (LH). During this growth period, oocytes undergo remarkable structural and molecular changes in order to ensure meiotic competence. Growing oocytes change their chromatin structure from a diffuse to a more condensed state and huge amounts of molecules are synthesized and accumulated for prospective development (De La Fuente et al., 2004). Finally, dominant follicles with fully grown oocytes ovulate and, at the same time, overcome meiotic arrest in prophase I. Ovulated oocytes will then proceed though the first meiotic division up to the metaphase II stage, extruding half of their genome and a small part of cytoplasm in what is known as polar body (PB). These oocytes are considered mature (also known as eggs) and are ready to be fertilized by sperm, moment at which they will complete the second meiotic division and, finally, give rise to a zygote (Williams, 2002). See Figure 1 for a schematic representation summarizing oogenesis and the reproductive cycle.

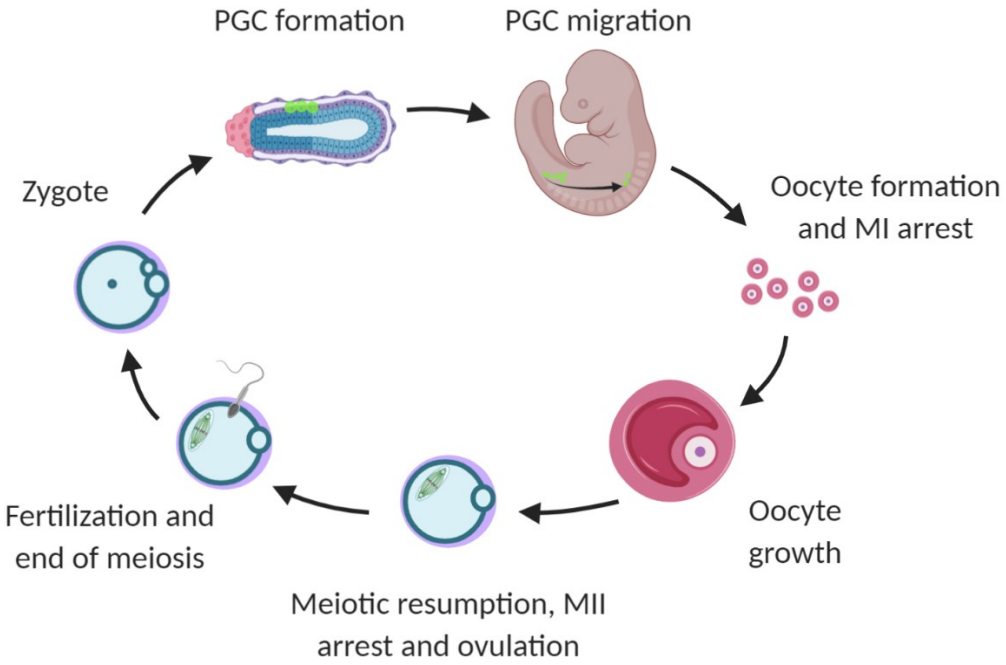

***Fig. 1*. Schematic representation of reproductive life cycle of mammalian females (mouse).** *The union of oocyte and sperm gives place to a zygote which will further develop into an embryo. During embryogenesis PGCs precursors will appear and later migrate to the genital ridge. Then, in female embryos PGC will form oocytes and arrest at MI. Later on, somatic cells will enclose the oocytes and form follicles. When females reach puberty, groups of oocytes will be selected to grow and eventually resume meiosis, reach MII stage and be ovulated. If these MII oocytes are fertilized by sperm, meiosis will be resumed and completed. The union close the circle with a new zygote. Image created with BioRender (https://biorender.com/) and inspired on a scheme published in (Edson et al, 2009).*

1.2. Oocyte maturation – first meiotic division

The period from oocyte ovulation to a ready-to-be fertilized egg is known as oocyte maturation. This transition involves both nuclear and cytoplasmic maturation. Fully grown oocytes are still arrested at prophase I of the first meiotic division inside antral follicles and their nucleus is surrounded by a nuclear membrane commonly known as germinal vesicle (GV). After LH surge, ovulation and meiotic resumption occur. This can be morphologically evidenced by the dissolution of the nuclear membrane (nuclear envelope break down – NEBD). Afterwards, chromosomes will condense, spindle will assemble and the first meiotic division will take place. Figure 2 summarises the role of the main factors involved in oocyte maturation.

The major player triggering these events is the maturation-promoting factor (MPF), comprised by Cyclin dependent kinase-1 (CDK1) and CYCLIN B, generally regulating the G2/M transition (Gautier et al., 1990). MPF is kept inactive in the GV prophase I arrested oocytes by signals sent from granulosa cells, as removing oocytes from antral follicles fosters spontaneous maturation. Granulosa cells provide an environment of high levels of cyclic adenosine monophosphate (cAMP) in the oocytes by producing and providing cyclic guaosine monophosphate (cGMP), which inhibits phosphodiesterase 3A (PDE3A) ability to degrade cAMP. This way, granulosa cells maintain protein kinase A (PKA) pathway activity in order

to ultimately keep MPF inactivated (and therefore meiosis is arrested) (Norris et al., 2009; Vaccari et al., 2009). While PKA is active, myelin transcription factor 1 (MYT1) and WEE1 protein kinases phosphorylate the Thr14 and Tyr15 inactivation sites of CDK1, whereas when PKA is inactivated, cell division cycle 25 (CDC25) can dephosphorylated the same sites (Coleman and Dunphy, 1994).

Another key regulator of meiosis arrest/resumption is the anaphase-promoting complex/cyclosome (APC/C). To reinforce meiotic arrest in GV oocytes, APC/C acts together with cadherin 11 (CDH11) to degrade CYCLIN B1 and keep low levels of MPF (Reis et al., 2006). After GVBD, APC/C^{CDH1} slowly fades and it turns to APC/C^{CDC20} activity (Seki et al., 2007). Moreover, APC/C activity has been shown responsible for MPF decrease during anaphase after meiotic resumption, which would allow the oocyte to proceed and culminate the first meiotic division and extrusion of PB (Ledan et al., 2001; Marangos et al., 2007). After PB has been extruded, CDK1 activity is re-established and meiosis becomes arrested one final time at metaphase II stage until fertilization occurs (Polanski et al., 1998).

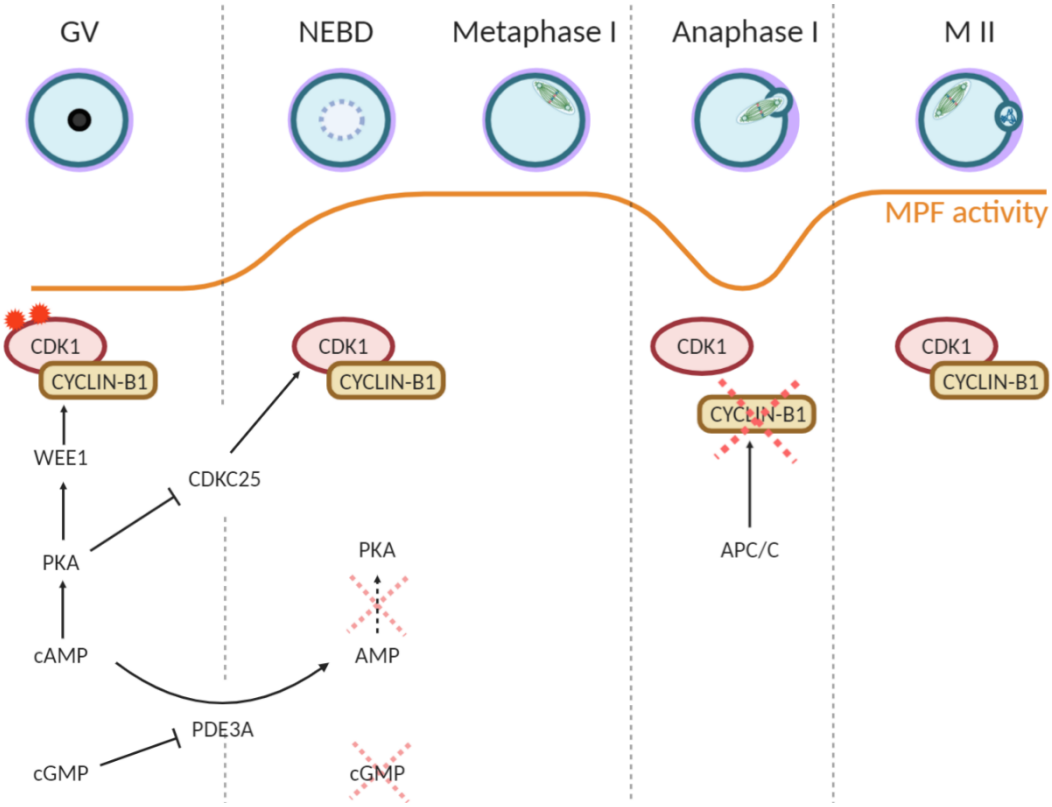

Fig. 2. The activity of MPF during meiotic maturation. *Inside ovarian follicles, granulosa cells maintain a constant flux of cGMP to the MI arrested oocytes. cGMP blocks PDE3A activity, keeping cAMP levels high enough for PKA to be active. PKA inactivates CDC25 and activated WEE1, which is responsible for the inhibiting phosphorylation of CDK1 on Thr14 and Tyr15. Upon resumption of meiosis, cGMP levels decrease and PDE3A is able to degrade cAMP. Without the presence of cAMP, the effects of PKA are no longer active and CDK25 can dephosphorylate the inhibiting sites from CDK1. At this point MPF is active and the oocyte is released from meiotic arrest. To be able to proceed through anaphase I, MPF activity drops once more as APC/C degrades CYCLIN B1. After the first meiotic division, MPF becomes active again and meiosis is arrested at metaphase II. Image created with BioRender (https://biorender.com/).*

Oocyte cytoplasmic maturation starts even before nuclear maturation. During oocyte growth, these cells accumulate huge amounts of molecules including substrates, RNAs and proteins to reach meiotic competence for the first cellular division (Hyttel et al., 1989; Sirard et al., 2006). Oocytes which are not fully-grown, can go through NEBD but will not be able to extrude PB. The material accumulated for cytoplasmic maturation is such that oocyte can grow 200 to 300 times in size. All this material will be used for the future meiotic events and even during early embryo development.

1.3. Transcription silencing and translational control

A very important and characteristic attribute of mammalian oocyte meiosis is that it partly occurs in the absence of transcription. After the growth period, transcription in oocytes is almost absent and it is not until the Zygote Genome Activation (ZGA) when it becomes once again fully active, which in mice happens at the 2-cell stage (De La Fuente et al., 2004). Due to this transcriptional silencing, oocyte RNAs (also known as maternal RNAs) must be uncommonly stable to be ready for use at the right time of meiosis or early embryonic development (Bachvarova and De Leon, 1980). Apart from maternal RNAs stabilization, gene expression is ultimately controlled by translation and post-transcriptional protein modification, which obtain a paramount role (Stitzel and Seydoux, 2007).

There are three mechanisms through which oocytes efficiently control protein expression and distribution: RNA localization, cap-dependent translation and polyadenylation (Bettegowda and Smith, 2007; Ma et al., 2013; Susor and Kubelka, 2017). Although RNA distribution into subcellular compartments occurs prior to translation, it has been demonstrated to be an efficient way to control local protein activity (Weatheritt et al., 2014). A clear and radical example can be found in *Xenopus Laevis* oocytes, in which several RNAs are limited to either the animal or vegetal pole (e.g. *Xcat2*, *Vg1* or *An1-4*).

However, there is still a long way to fully understand the reason for RNA localization in mammalian oocytes. So far, it is known that within the subcortical cytoplasmic membrane regions there is an accumulation of groups of RNAs and RNA-binding proteins (RBPs) (Li et al., 2008). These subcortical maternal complexes (SCMC) may be acting as RNA reservoirs as they include several RBPs related to RNA stabilization or translational repression (e.g. DDX6, MSY2, CPEB1). When meiosis resumption takes places they move to other parts of the oocyte and dissolve (Flemr et al, 2010). It is also important to note that without the SCMC, further development is affected and stalls at the 2-cell stage (Li et al., 2008). Another RNA compartmentalization has been recently identified in the nucleus of mouse oocytes, where a significant population of poly(A)-RNA is concentrated when compared to the rest of the cytoplasm (Susor et al., 2015). Such distribution of poly(A)-RNA has been proposed to be a mean to provide the necessary proteins in a more co-ordinate and effective way to all the cellular events taking place at the nucleus and later quickly forming spindle.

In the case of translation initiation, it is controlled in a cap-dependent way through the eukaryotic initiation factor 4F (eIF4F) cap-binding complex. The eIF4F consists of three subunits: eIF4E (recognises and binds the 7-methyl-guanosine cap structure on 5'UTR of mRNAs), eIF4A (helicase that unwraps RNA

secondary structures) and eIF4G1 (maintains the whole complex integrity). The major regulators of eIF4E activity in binding RNAs for translation initiation are 4E-binding proteins (4E-BPs), which compete with eIF4G1 to bind eIF4E (Mader et al., 1995). The best example and most studied is 4E-BP1, which when hyperphosphorylated stops binding to eIF4E and enables it to initiate the complex formation and RNA translation (Gingras et al., 1998). And the responsible for 4E-BP1 phosphorylation is the mammalian target of Rapamycin (mTOR, a protein kinases regulating translational initiation), which is in turn regulated by the phosphoinositide 3-kinase and protein kinase B (PI3K/AKT) signalling pathway (Scheper and Proud, 2002).

Ellederova et al., (2006) reported that porcine oocytes display a gradual increase in 4E-BP1 phosphorylation during meiotic progression. Surprisingly, this was accompanied by a general decrease of translation. Later on, this trend of increased 4EBP1 phosphorylation and decreased translation was also seen in mice oocytes after NEBD, together with a specific localization of phopho-4EBP1 at the meiotic spindle (Romasko et al., 2013; Susor et al., 2015). This suggests that, during meiotic maturation, only specific mRNAs are translated in a cap-dependent manner while general protein synthesis decreases. Moreover, this localization was shared with mTOR, moving from cytoplasm to spindle during maturation; reinforcing the importance of mTOR orchestrating cap-dependant translation events during meiosis resumption (Yang et al., 2009; Kogasaka et al., 2013). Mammalian oocyte treatment with either Rapamycin or Torin 2 prevented a proper maturation, spindle migration and asymmetric PB extrusion (Mayer et al., 2014).

The length of RNA poly(A) tail is another factor controlling translation as, in general, mRNA with larger poly(A) tails are more prone to be translated whereas mRNAs with shorter tails are repressed and prone to degradation. In oocytes, cytoplasmic polyadenylation is a common mechanism to control which transcripts will undergo translation (Stebbins-Boaz et al., 1996). For this kind of control, mRNAs should contain the cytoplasmic polyadenylation element (CPE) and the polyadenylation signal 'AAUAAA' (McGrew et al., 1989). CPE can organise translation by binding to CPE-binding proteins (CPEB-1-4) (Theis et al., 2003). The importance of cytoplasmic polyadenylation in oocytes is reflected by the fact that CPEB1 knockout mice are arrested at pachytene with a disrupted synaptonemal complex (Tay and Richter, 2001). Moreover, CPEB1 inhibition in porcine oocytes results in inhibition of CYCLIN B1 synthesis and meiotic resumption (Nishimura et al., 2010).

1.4. Polysome profile analysis

Translation control plays a substantial role in determining protein abundance in the transcriptionally silent oocytes but it is also important in other cells during specific physiological situations (e.g. stress and apoptosis) (Holcik and Sonenberg, 2005). Therefore, in those circumstances the analysis of the trascriptome might not be accurate enough to mirror gene expression on the protein level. To that end, polysome profiling has become a standard method in assaying those mRNAs bound to ribosomes and prone to translation.

The molecular machinery responsible for mRNA translation is the 80S ribosome, which can be translating along one mRNA together with several other ribosomal units at the same time (what is called polysome). Polysomal profiling technique allows separating RNAs depending on the amount of ribosomes attached to them (Mašek et al., 2011). This has made this technique very appealing not only for assessing mRNA translation but also for ribosome biogenesis and the study of other translation related proteins (Sydorskyy et al., 2005; Choudhuri et al., 2013; Androsavich et al., 2016). Polysomal profiling should not be confused with the similar technique named ribosome profiling, which is used to determine the exact position of ribosomes within the RNAs coding sequence instead of focusing on the entire RNAs (Brar and Weissman, 2015). The protocol for polysome profiling starts with the stabilization of ribosomes on the RNAs as they are loaded at a specific time followed by cell lysis. The cellular lysate is then loaded onto a sucrose gradient, which contains different continuous densities. Upon centrifugation, RNAs are distributed along the sucrose density gradient according to their ribosomal load. Ribosome free RNAs and small ribosomal subunits (40S) will be then retained on the top, less dense, sucrose gradient layer followed by bigger 60S subunits in the next layers. Consecutive layers will contain RNAs with assembled 80S ribosomes, which would have travelled further followed by RNAs with two ribosomes assembled (already considered polysomes) and so on. Finally, the RNAs separated by the distinct layers can be collected and each layer can be analysed independently.

However, despite the clear advantage that polysome profiling could bring to the study of translatome in a transcriptionally silent cell as the mammalian post-NEBD oocyte, not many studies have used it so far. The technique has an inherent complexity and it is time consuming, however, the main reason behind its low success in the mammalian female germ cell study is its requirement for a large size of samples (Zhao et al., 2019). The most common methods usually apply 45 $OD_{260\ nm}$ units of cell extract, which can be roughly extrapolated to more than 10000 oocytes. Taking into account that 20-25 meiotically competent oocytes can be obtained from one single mouse female, the amount needed for conventional polysome profiling would be far too many (it would take hundreds of mice to sacrifice). Therefore, there was still a need to develop a suitable technique to be applied on scarce samples such as maturing oocytes. Nonetheless, there have been some efforts to apply polysomal fractionation on mammalian oocytes despite their scarcity with subsequent microarray approaches. In the few experiments performed in the past, different strategies have been proposed to try to overcome this problem (each one with its own drawbacks): spiking the oocyte sample with non-homologous RNA (Scantland et al., 2011), by performing simultaneous fractionation of oocytes with a more abundant type of cells (Potireddy et al., 2006) or by creating a 'RiboTag' mouse in order to be able to immuno-precipitate the ribosomes and the RNAs bound to it (Martins and Conti, 2018).

1.5. Segregation of chromosomes during meiosis I and maternal ageing

One of the main differences between mitosis and meiosis is the segregation of chromosome homologs during the first meiotic division rather than the segregation of sister chromatids (which occurs in both

mitosis and second meiotic division). The chromosomes of prophase I arrested oocytes have already undergone synapsis and recombination of their homologs (Rodrigues et al., 2008). At this stage chromosomes are relatively decondensed and the GV is still intact. For a successful segregation of the homologs, the connections between homologs must be maintained during the long meiotic arrest until anaphase I and a stable physical union of the sister chromatids must be kept in order to form attachments to same spindle poles in the first meiotic division (Smith and Nicolas, 1998).

Meiotic errors during the segregation of chromosomes may lead to oocytes with chromosomal imbalance, also known as aneuploidy. Aneuploid oocytes are usually unviable and in most organisms it is a rare event. For example, the probability of meiotic mal-segregation in *Saccharomyces cerevisiae* is 1 in 10000 times (Sears et al., 1992) and in Drosophila melanogaster 1 in 1700-6000 (Koehler et al., 1996). However, in mammals, the frequency of meiotic aneuploidy is much higher. In young mice, the occurrence of aneuploidy in fertilized eggs is estimated to be between 1-2%, which is similar in young humans (Jones and Lane, 2013; Capalbo et al., 2017). This high incidence in humans has put oocyte aneuploidy in the scope of many studies as it has severe consequences such as embryo lethality (miscarriages) or developmental disabilities in the new-borns (e.g. trisomy of 21, 18 and 13 chromosomes cause Down, Edward and Patau syndromes, respectively) (Savva et al., 2010).

There are two different mechanisms through which chromosomes can wrongly segregate during meiosis: non-disjunction, where chromosome segregation fails between either homologs in meiosis I or sister chromatids in meiosis II; and premature separation of sister chromatids, where one of the sister chromatids separates from the other during the first meiotic division. It is also possible that chromosomes undergo a so-called reverse segregation during meiosis I as sister chromatids segregate instead of the homologous chromosomes. Although these matured oocytes may have the correct number of chromosomes, further errors can occur in meiosis II because they lack centromeric cohesion (Lee, 2019).

Moreover, there is a factor, which in some mammalian species triggers aneuploidy rates even higher: advanced maternal age. In humans, women of 32 years old have already a 20% incidence of oocyte aneuploidy and it can further increase to 60-80% when they reach 42 years of age (Kuliev et al., 2011; Jones and Lane, 2013; Capalbo et al., 2017). This fact has been a growing concern in the last years as women from modern societies tend to postpone childbearing in their lives (Molina-García et al., 2019). Although not all mammals display such a relation between aneuploidy and maternal age, mice have proven to be a good model for this case as they have a similar segregation error incidence and also suffer from age-related aneuploidy as human oocytes. For example, women around 20 years old and 3 month old mice have a likelihood for aneuploidy of 1-5%, whereas in 40 year old women it increases to more than 50% in a similar way as in 12 month old mice female (Merriman et al., 2012; Jones and Lane, 2013). Therefore, aged female mice have been used as a model in the studies directed to the better understanding of this phenomenon.

The factors driving age-related aneuploidy are still being elucidated, however, some evidence already exists pointing towards some suggestions. One of the main factors proposed is the loss of cohesin, a

protein complex that glues sister chromatids together. It has been proposed that GV oocytes are lacking in cohesin protein turnover, leading to a slow degradation of the cohesion between sister chromatids throughout the females life (Tachibana-Konwalski et al., 2010). It has been reported that meiotic recombination protein REC8 homolog (REC8), which is a protein of the cohesin complex, is hardly detectable in 13 month old mice, while it is clearly present in 3 months old mice (Chiang et al., 2010). Moreover, total loss of cohesin in mice lead to premature sister chromatid segregation (Tachibana-Konwalski et al., 2010). However, it still remains to be determined whether the loss of cohesin during aging is also a trait of human oocytes. Furthermore, cohesin loss could only attain for those cases of aneuploidy derived from premature sister segregation but not for the non-disjunction ones. Therefore, there might be other factors that could be involved in age-related chromosomal aneuploidy.

Reduced quality of mitochondria has been observed in advanced age human oocytes and even reduced ATP production, making it another possible aneuploidy factor (Müller-Höcker et al., 1996; Van Blerkom, 2011). Furthermore, meiotic spindle defects and aberrations in assembly have also been reported to be more common in advanced maternal age oocytes, mainly involving chromosome failure to attach to microtubules (Battaglia et al., 1996). There have been also many efforts to obtain answers by studying and comparing the whole transcriptomes of oocytes from different maternal ages in several species (Labrecque and Sirard, 2013). Interestingly, very small differences were detected between GV oocytes from young females, however, oocytes at the metaphase II (MII) stage showed consistently different transcript levels, suggesting that during the first meiotic division important changes in translation and RNA degradation might have occurred. These differential transcripts found at the MII stage included genes involved in spindle formation, spindle assembly checkpoint (SAC), cell cycle control and mitochondrial metabolism (Hamatani et al., 2004; Pan et al., 2008; Steuerwald et al., 2001; Grøndahl et al., 2010). Thus, age-related aneuploidy in oocytes seems to be multifactorial and more research should be performed in order to better define its causes.

2. Aims of the thesis

Mammalian oocytes become transcriptionally silent after resumption of meiosis until ZGA. Therefore, gene expression at these stages relies mainly on the translational control of the RNAs previously stored by these germ cells. During this period, chromosomal segregation errors may arise. This chromosome mis-segregation takes place usually during the first meiotic division, after NEBD, and its incidence is profoundly increased with maternal age (in the case of mice and human).

The objective of this thesis is to investigate the mechanisms of translational regulation at the crucial stage of oocyte meiosis, i.e. post-NEBD, as well as elucidation of possible differences in translated RNAs between oocytes from young and aged mice females. Through this, the thesis should contribute to a better understanding of the translation governing NEBD and adds valuable information about the problems in aged-related chromosomal aneuploidy in oocytes.

The specific aims of this work are:

To learn more about 4E-BP1 and cap-dependent translation in the NEBD oocyte.

- To study the relationship between 4E-BP1 and mTOR localization within the NEBD oocyte and to better understand how it affects the cell cycle regulator CDK1.
- To deepen the knowledge about the role of 4E-BP1 cap-dependent repressor by expressing dominant negative mutants during oocyte meiotic progression.

To detect any anomalies in nuclear membrane dissociation between maturing young and aged females.

- To investigate if MPF levels/activity are altered in the oocytes from aged females compared to their young counterparts.
- To detect any possible differences in the dissociation of the nuclear membrane during maturation of the two mentioned oocyte groups.

To study any differences in the pattern of translated RNAs between post-NEBD oocytes from young (2 months old) and aged (12 months old) mice females.

- To develop a modified method of polysomal profiling to extract polysome-bound RNAs from a reasonable small amount of oocytes.
- To apply polysomal profiling to post-NEBD oocytes from young (2 months old) and aged (12 months old) females for a subsequent translatome screening utilizing next generation sequencing (NGS) and detect possible differences.
- Validate at the RNA and protein levels the most significant differences in polysome-bound RNAs revealed from the data obtained from sequencing.
- Study the possible effects of altering the protein levels of the validated genes on the first meiotic division.

3. Comments on publications

This thesis is based on the research carried out and presented in four scientific articles. Three of them have been published in peer reviewed journals and one has been recently submitted (currently under review). The articles are listed chronologically together with a description of my contribution:

3.1. Jansova D, Koncicka M, Tetkova A, Cerna R, Malik R, Del Llano E, Kubelka M, Susor A. 2017. Regulation of 4E-BP1 activity in the mammalian oocyte. Cell Cycle. 16(10):927-939. doi: 10.1080/15384101.2017.1295178.

- E.L. contributed with collection and preparation of samples as well as in writing the manuscript.

3.2. Koncicka M, Tetkova A, Jansova D, Del Llano E, Gahurova L, Kracmarova J, Prokesova S, Masek T, Pospisek M, Bruce AW, Kubelka M, Susor A. 2018. Increased expression of maturation promoting factor components speeds up meiosis in oocytes from aged females. International Journal of Molecular Sciences. 19(9). pii: E2841. doi: 10.3390/ijms19092841.

- E.L. performed polysomal fractionation and contributed to manuscript writing.

3.3. Masek, T.; del Llano, E.; Gahurova, L.; Kubelka, M.; Susor, A.; Roucova, K.; Lin, C.-J.; Bruce, A.W.; Pospisek, M. Identifying the Translatome of Mouse NEBD-Stage Oocytes via SSP-Profiling; A Novel Polysome Fractionation Method. Int. J. Mol. Sci. 2020, 21, 1254. doi: 10.3390/ijms21041254.

- E.L. helped in developing the method and collaborated in all experiments (oocyte collection, cell tissue cultivation, polysomal fractionation, library preparation, qPCR), analysed part of the data and contributed in writing the manuscript.

3.4. Del Llano E, Masek T, Gahurova L, Pospisek M, Koncicka M, Jindrova A, Jansova D, Iyyappan R, Roucova K, Bruce AW, Kubelka M, Susor A. Age-related differences in the translational landscape of mammalian oocytes. Aging Cell. Submitted the 6th May 2020.

- E.L. designed and performed most experiments (oocyte collection, polysomal fractionation, library preparation, qPCR, western blot, immunocytochemistry, life cell imaging) analysed the data and wrote the manuscript.

During my Phd, I also contributed to another scientific article, the scope of which is not the same as the subject of this thesis and therefore I just mention it here.

Tetkova A, Susor A, Kubelka M, Nemcova L, Jansova D, Dvoran M, Del Llano E, Holubcova Z, Kalous J. Follicle-stimulating hormone administration affects amino acid metabolism in mammalian oocytes, Biology of Reproduction, Volume 101, Issue 4, October 2019. doi: 10.1093/biolre/ioz117

- E.L. contributed writing and editing the manuscript.

3.1. Regulation of 4E-BP1 activity in the mammalian oocyte

During meiotic maturation, mammalian oocytes become transcriptionally silent. As transcription loses its role over protein expression control, translation gains more relevance in taking over this function. Cap-dependent translation is also promoted and it is mainly regulated at the initiation step. The eIF4F complex is capable of binding to the cap-structures of mRNAs and is responsible for their translation initiation. One of the essential proteins forming the eIF4F is the eIF4E, which can be controlled by binding to 4E-BPs. 4E-BP1 is the most studied of them and through binding to eIF4E it prevents the formation of the whole complex (and translation initiation). Only after hyperphosphorylation of 4E-BP1, its binding to eIF4E is disabled and the eIF4F complex can be assembled.

In this study we first checked the RNA levels of the three known *4e-bps* (*4e-bp1, 4e-bp2 and 4e-bp3*) in GV and MII oocytes through qRT-PCR. All three transcripts were present in oocytes of both stages at similar levels. However, when their protein levels were checked by western blot (WB), only one out of the three was present: 4E-BP1. Moreover, 4E-BP1 showed a clear and complete phosphorylation shift from GV to MII, while post-NEBD oocytes contained both phosphorylated and unphosphorylated populations. This indicated that 4E-BP1 phosphorylation occurred after meiotic resumption and nuclear envelope breakdown.

We also elucidated 4E-BP1 and its phosphorylated forms localization within the oocyte during maturation. After performing immunocytochemistry (ICC), we were able to detect global 4E-BP1, as well as its phosphorylated form (Thr 37/45) distributed throughout the whole cytoplasm of GV oocytes with a more intense signal in the nucleus, which was similar at the following stages up to MII with a slight increase in the spindle area. Consistent with the WB data, none of the phosphorylated forms was present in GV stage oocytes. On the other hand, the signals from 4E-BP1 phosphorylated at Ser65 and Thr70, had a marked localization at the newly forming spindle at NEBD and continued to be around the chromosomal area up to MII.

As in a previous study we showed that suppression of mTOR activity by Rapamycin significantly reduced 4E-BP1 phosphorylation and since some other studies suggested that CDK1 can phosphorylate 4E-BP1, we wanted to find a possible relation between them in the mouse oocyte. To that end, we treated NEBD oocytes with Rapamycin (mTOR inhibitor), Roscovitine (CDK1 inhibitor) or BI2536 (Polo like kinase 1 (PLK1) inhibitor). Both treatments with Rapamycin and Roscovitine suppressed 4E-BP1 phosphorylation, suggesting that CDK1 indeed inhibited this protein's phosphorylation and it did it through mTOR, as documented by descreased mTOR phosphorylation at Ser2448 after Roscovitine treatment. On the other hand, BI2536 had no effect on 4E-BP1 inactivation suggesting that PLK1 did not play the role in mTOR inactivation and/or 4E-BP1 phosphorylation.

To further investigate the effects of 4E-BP1 phosphorylation in maturing oocytes, we created a RNA coding for a 4E-BP1 protein with the above mentioned phospho-sites mutated into Alanine, which should act as a dominant negative when injected into oocytes. Oocytes expressing the mutant protein and which reached MII, showed the presence of both phosphorylated and unphosphorylated forms of 4E-BP1 on

WB, while wild type MII oocytes had only phosphorylated protein. Furthermore, by using a *Renilla* Luciferase reporter containing a TOP motif we were able to report a decreased cap-dependent translation in the oocytes with mutant 4E-BP1. Nonetheless, despite the oocytes microinjected with mutant RNA could extrude PB, ICC revealed they had superior incidence of aberrant spindles (59% compared to 3% of wild type).

In summary, we show that out of the three 4E-BPs NEBD oocytes express only 4E-BP1 protein and this is phosphorylated through an mTOR-CDK1 pathway. In addition, the study documents that 4E-BP1 phosphorylation is essential for a proper spindle formation and, as a consequence, a proper meiotic progression.

3.2. Increased expression of maturation promoting factor components speeds up meiosis in oocytes from aged females

It is well known that in some mammalian species (such as mice and human) the oocytes from aged females (AF) have increased aneuploidy rates compared to oocytes from young females (YF). As the reasons for this feature are not yet clear, we decided to further investigate the differences between these two groups of oocytes.

In this study, the first and most obvious difference we identified was a faster meiotic progression in oocytes from AF. In general, these oocytes underwent NEBD and PB extrusion around 30 minutes before the oocytes from YF. To further support this evidence we performed ICC to score the number of stable microtubule end-on attached kinetochores at the metaphase stage. In accordance with the previous finding, AF oocytes showed more microtubule-kinetochore attachments than YF oocytes. We also checked if there were any differences in the protein levels of LAMIN A/C, the phosphorylation of which is necessary to degrade the nuclear membrane. We found no differences in the levels of total LAMIN A/C protein between the two age groups; however, LAMIN A/C phosphorylated at Ser22 was indeed increased during the NEBD and post-NEBD of AF oocytes. Moreover, through electron microscopy of GV oocyte cryosections, AF oocytes showed different shapes of nuclear membrane compared to YF. The membrane of AF had a higher number of invaginations, which decreased its compactness and increased the circumference area.

As LAMIN A/C is phosphorylated by an active CDK1, we wondered if an alteration of the MPF components could be behind the faster meiotic progression of AF oocytes. mRNA expression experiments using qRT-PCR showed that there was a significantly higher amount of *cdk1, ccnb1 and ccnb2* transcripts in AF oocytes than in YF. These levels correlated well with the amount of protein as seen also on WB. On the WB we detected that CDK1 (Thr161) phosphorylated form was significantly increased in AF oocytes as well. In addition, we treated oocytes with the CDK1 inhibitor Roscovitine and detected the reduction of LAMIN A/C (Ser22) phosphorylation, confirming the previously mentioned involvement of CDK1 in this protein. In order to induce a phenocopy of aged oocytes, we injected RNA coding for CCNB

into GV oocytes from YF. In accordance with our hypothesis, these injected oocytes showed a faster meiotic progression and increased kinetochore attachment such as AF oocytes.

We also analysed and compared the transcripts in polysomal fractions. For this analysis the RNAs, which were bound to polysomes were separated from the free RNAs in each YF and AF oocyte samples. Those groups of transcripts were sequenced and we found that several RNAs with a role in translation regulation were upregulated in the AF.

In this study, we show that, compared to YF, oocytes from AF have altered expression and activation of MPF. This leads to a faster meiotic progression, including nuclear envelope dissociation and PB extrusion. This imbalance in timing could be tightly related to the increased aneuploidy which the AF oocytes suffer as they would have less time to mature and go through the quality checkpoints.

3.3. Identifying the Translatome of Mouse NEBD-Stage Oocytes via SSP-Profiling

Polysome profiling has been for the last decades a method of choice to assay cellular translational features at a precise time point. The fact that mammalian oocytes become transcriptionally silent and possess a huge pool of stored (not always in use) RNAs, makes polysomal profiling a very appealing technique to identify their actual translatome at a given time point. However, fully grown mammalian oocytes are a particularly scarce source of sample and this has hindered their study using this method. Therefore, to surpass this issue, we have successfully adapted a polysomal fractionation method for scarce samples which we named SSP-profiling (Scarce samples polysomal profiling).

Nowadays, the most common way to produce polysome profiles is through the ultracentrifugation of the sample loaded on a sucrose gradient using 12,1ml tubes and SW41Ti rotor. To minimize gradient volume and concentrate the sample loaded on it, we optimized the ultra-centrifugation conditions to obtain the same high-quality polysome profile in much smaller tubes (5,5ml) and a SW55Ti rotor. Once we figured out the best ultra-centrifugation conditions (45,000 RPM (246,078x g) for 65 min) for a full profiling, we compared the new profiles with those obtained with the classic SW41Ti rotor and no differences in quality could be found.

We performed several polysomal profilings with both rotor methods using different decreasing amounts of Hek-293 cell samples. This experiment showed that the profiles from the SW55Ti rotor were more sensible, as a loaded volume of one OD_{260} nm unit was roughly equivalent to 2,5 OD_{260} nm units on the conventional SW41Ti rotor, proving that our new variant of the method possessed increased sensibility.

In the next step, we applied this new technique on oocytes. We loaded a number of 200 post-NEBD stage oocytes as we considered it a reasonable relatively low amount. However, as expected, we were not able to detect any peaks/profile through UV absorbance due to the low amount of sample. We also included a regular Hek-293 cell sample as a control, which, despite showing nice peaks/profile, we did not consider as a fully conclusive confirmation that polysome profiling happened in oocytes as well. Therefore, we decided to design a model to visualize the profiling through the qRT-PCR of 18S and 28S rRNA, to

emulate the peaks of ribosomes seen by UV absorbance in conventional profiling. After ultra-centrifugation, we collected the samples along the gradients in 10 fractions, being the first fractions lower in sucrose density (containing free RNA), middle fractions medium sucrose density (containing free ribosome subunits and monosomes) and final fractions having high density sucrose (containing RNA bound to polysomes. As a control we also added a sample of 200 post-NEBD oocytes treated with 0,5M Ethylenediaminetetraacetic acid (EDTA) in order to induce polysome disruption. After polysome fractionation and qRT-PCR, we could clearly see that EDTA treated samples (compared to non-treated oocytes) had an increased amount of rRNA in the first fractions. This demonstrated that the qRT-PCR was a valid method for polysome profile validation of scarce samples. Moreover, the same procedure was applied to abundant Hek-293 samples with/without EDTA treatment and a similar shift of rRNA to the firsts fractions could be detected by qRT-PCR method.

Finally, we performed RNA-sequencing analysis of post-NEBD oocyte profiles. The ten fractions obtained from fractionation were pooled into two: non-polysomal (NP, containing fractions 2-5 with free RNAs or bound to monosomes) and polysomal (P, containing fractions 6-10 with RNAs bound to polysomes). We created libraries and sequenced both NP and P using NGS. We found that several transcripts were expressed exclusively in NP or P fractions. Moreover, 'Gene Enrichment Analysis' showed that the overrepresented genes in the polysome-bound fractions were related to pyruvate and glucolytic/gluconeogenesis metabolic pathways. Also categories of genes related to microtubule cytoskeleton, mitochondria, translation control factors and meiosis. All of them were in good accordance with the post-NEBD oocyte characteristics.

In this study we presented an adaptation of a polysomal profile method to be used on very scarce samples, such as mammalian oocytes. The new method was as functional as the currently common one, however, the the use of the new SW55Ti rotor substantially improved the sensitivity. Moreover, we successfully performed RNA-seq on polysomal fractionated oocyte samples. The method was also tested on other oocyte stages, zygotes and 2-cell embryos.

3.4. Age-related differences in the translational landscape of mammalian oocytes

Aneuploidy incidence in some mammalian oocyte species significantly increases with age, such as in human and mice. Women of 32 years of age already have a 20% incidence of oocyte aneuploidy and it can further increase to 60-80% when they reach 42 years of age. To uncover the reasons behind this age-related aneuploidy in oocytes, there have been several studies looking for differences in their whole transcriptome. However, only few of them focused on the post-NEBD stage – the stage when chromosomes condense and meiotic spindle assembles. In this study, in order to better understand the causes of age-related oocyte aneuploidy, we analysed and compared the polysome-bound RNAs of post-NEBD oocytes from YF and AF by using a novel polysomal profiling technique specially adapted to scarce samples followed by RNA-sequencing.

First we collected samples of 200 post-NEBD oocytes from YF and AF and performed polysomal fractionation. No significant changes were detected in the polysomal profiles of both oocyte groups. Each sample was therefore divided into two sub-samples: non-polysomal (NP, containing free RNAs or monosome-bound RNAs) and polysomal (P, containing polysome-bound RNAs). Fractionated RNA samples were then amplified and sequenced by NGS. Sequencing data revealed that among the detected transcripts with (FPKM)>1, up to 1006 transcripts had a fold change (FC) bigger than 10 between the two groups (623 higher in YF and 383 higher in AF). Furthermore, we found 37 transcripts with significant difference in expression by using Deseq2. Interestingly, after 'Over-Representation Analysis' (ORA) of the 1006 genes with FC>10 some of the enriched categories of biological function included genes related to cell division, microtubule cytoskeleton and mRNA metabolism. This pointed towards a possible different translation of certain genes in the AF oocytes, which makes them more prone to chromosome segregation errors.

Afterwards, we selected 9 differentially expressed genes with a potential role in meiosis, cell division or spindle localization according to up to date literature. Using RT-qPCR we tested whether the polysomal RNA levels of the selected targets were indeed different between YF and AF. Unfortunately, the polysome-bound RNA extracted was of very low amount and it was hard to see clear differences. Nevertheless, our results suggested that three of the selected transcripts followed the trend of our seq-data. The genes were Serum/Glucocorticoid Regulated Kinase 1 (*Sgk1*), Cytosolic Arginine Sensor For mTORC1 Subunit 1 (*Castor1*) and Kinesin-5 protein (*Eg5* or *Kif11*). Moreover, to investigate the possibility that these differences were due to differences in the whole transcriptome and not only in the polysome-bound RNAs, we also checked these RNAs levels in the total transcriptome of oocytes and no differences were found. Afterwards, we tested the actual protein levels of the candidates by WB, as well as their localization in the oocytes by ICC. In addition, we also tested Autoimmune Regulator (AIRE) protein as it was the most significant differentially expressed gene in our seq-data. The results correlated well with the seq-data; SGK1 was lower in AF oocytes while CASTOR1, AIRE and EG5 were higher. Moreover, all proteins were localized on the meiotic spindle, partially supporting the hypothesis of their role in chromosome mal-segregation.

To further test that changes in these proteins could potentially lead to aneuploidy in oocytes, we decided to modify the expression levels or activity of two of them in YF oocytes towards the AF oocyte trend. This way, we overexpressed CASTOR1 by injection of ds-RNA or we repressed SGK1 protein activity by adding a specific inhibitor to the media. Overexpression of CASTOR1 did not affect maturation rates. Nonetheless, thanks to the co-injection of histone *h2b* fused with *gpf* RNA, we could track the chromosomes by life cell imaging and detect an increased number of oocytes displaying certain abnormalities; namely, chromosomal misalignment or full DNA extrusion into small PBs. On the other hand, treating YF oocytes with SGK1 inhibitor (GSK-650394; 10μM) significantly reduced their ability to extrude PB. Moreover, from those oocytes that extruded PB, most of them suffered characteristic abnormalities such as symmetric division or non-split spindle between oocyte and PB.

In this study we produced a data-set of polysome-bound RNA transcripts at the post-NEBD stage of oocytes from YF and AF and showed that there are several transcripts with altered expression. Moreover, we have validated the differences of several of these transcripts/proteins and detected their localization on the meiotic spindle. Moreover, altering the expression levels or activity of CASTOR1 or SGK1 resulted in cytokinesis or chromosomal alignment errors. This suggests that there are differences in the translational program of post–NEBD oocytes dependent on maternal age, which might be tightly related to the increased chromosomal aneuploidy observed in AF oocytes.

4. Discussion

In this dissertation, my goal was to uncover potential molecular mechanisms causing age-related aneuploidy in mammalian oocytes. It has been reported that in the case of human and mice oocytes (both displaying age-related aneuploidy) the differences in the transcriptome of GV oocytes from YF and AF are extremely minimal, to the point they could be considered equal (Pan et al., 2008; Reyes et al., 2017). However, at the more advanced stage of MII, much larger differences in the transcriptome become obvious. Interestingly, most erroneous chromosomal segregations take place during the first meiotic division, which starts when those transcriptome levels are relatively similar (Hassold and Hunt, 2001). It is also after meiotic resumption that translation takes over transcription for gene expression control. Therefore, the principal question I addressed is whether translation in oocytes from AF differs somehow from the YF oocytes during the first meiotic division, potentially leading to inappropriate chromosomal segregation.

Prior performing any experiments using oocytes from AF, we first focused on oocyte translation itself. Recently, the importance of the mTOR role in meiotic maturation has been described, showing that it is responsible for cap-dependent translation activation together with the eIF4F complex (Richter and Sonenberg, 2005; Thoreen et al., 2012; Susor et al., 2015). This mechanism of initiation of translation was proven to be essential during meiotic maturation as inhibition of the mTOR–eIF4F axis did not affect NEBD but it impaired proper meiotic division, many times resulting in aneuploidy. Despite this evidence pointing towards the post-NEBD stage to be the turning point of translational control, we decided to analyse another piece of the puzzle to reassure this statement: the 4E-BP proteins. Mayer et al (2014) reported that only 4E-BP1proteins were found in bovine oocytes (not 4E-BP2-3) in a similar way as we found in mouse oocytes, suggesting that only 4E-BP1 is important during meiosis although the other two proteins might still have a rescue or later role as their RNA levels persist all along (Tsukiyama-Kohara et al., 2001).

More importantly, our results indicate that 4E-PB1 is controlled by mTOR and CDK1. The activity of these two proteins is tightly regulated after meiotic resumption and NEBD, which further stresses the importance of translational control at this stage. According to our results showing decreased mTOR phosphorylation (Ser2448) after CDK1 inhibition, its seems that CDK1 is at least one of the kinases phosphorylating mTOR, and activated mTOR is the kinase responsible for4E-BP1 phosphorylation as similarly reported by Heesom et al (2001). While others already reported increased phosphorylation of 4E-BP1 after NEBD in mice, bovine and porcine oocytes (Hampl and Eppig, 1995; Mayer et al., 2014; Susor et al., 2015), we also show that the protein phosphorylated at the Ser65 and Thr70 sites localize to the forming spindle. This is in good agreement with previous findings showing the presence of active mTOR in the same spindle area, documenting that both activator and active substrate share the same cellular compartments (Susor et al., 2015).

The fact that 4E-BP1 is actively phosphorylated at the onset of meiotic maturation to release eIF4E and allow cap-dependent translation activation, together with its dependency on CDK1 activation through

mTOR, making the post-NEBD oocyte as a key stage, in which translation has already become paramount for meiotic progression control. Therefore we decided to look for possible age-related aneuploidy causes at the post-NEBD stage of oocytes from YF and AF.

When starting to analyse and compare the NEBD of oocytes from the two maternal age groups, their difference in timing was the first to be noticed. AF oocytes were going through NEBD (and PB extrusion) before the oocytes from YF in an average of 30 minutes. Despite some authors reported similar trends in the past (Eichenlaub-Ritter et al., 1988; Sebestova et al., 2012), others also could not find any differences in timing of meiotic progression (Duncan et al., 2009; Lister et al., 2010). It is hard to establish a clear reason for these discrepancies but the possibilities include a different system to select meiotically competent oocytes, different protocol for oocyte handling or simply due to different strains of mice. Moreover, we have also found that AF oocytes are more meiotically competent than YF as a larger percentage of them reached MII. A reason for both faster timing and increased competence could be intrinsically related and explained by a reduced effectivity of the spindle assembly checkpoint (SAC) in aged oocytes. The SAC is a mechanism to ensure proper chromosome attachment before anaphase to avoid chromosomal segregation mistakes and SAC must be silenced before the oocytes can progress further beyond this stage (Marston and Wassmann, 2017). The SAC has been already reported not to be very efficient in mammalian oocyte meiosis compared to mitosis as it is not able to detect unaligned chromosomes if they are already attached to spindle fibres (Gui and Homer, 2012; Sebestova et al., 2012; Kolano et al., 2012). However, it is possible that in AF oocytes the SAC is altered and becomes even less effective, increasing meiotic competence and perhaps faster progression. There is still research to be done in order to understand the reasons behind a possible hindered SAC in AF but some authors have pointed towards an altered gene expression of SAC proteins (or related proteins) (Eichenlaub-Ritter, 2012).

While further investigating the faster NEBD of AF oocytes, we found out that phosphorylation of LAMIN A/C at Ser22 also took place earlier in these oocytes. The phosphorylation of this protein is known to be dependent of CDK1 (Peter et al., 1990). And indeed, we showed that MPF (CDK1 (Thr161) and CCNB) components, responsible for meiotic resumption, are significantly increased in AF oocytes and probably causing the faster NEBD. Knowing that the increased ccnb leads to higher CDK1 activation, Davydenko et al, (2013) microinjected oocytes with *ccnb* RNA and reported precocious spindle formation and lagging chromosomes at anaphase I. In our case, we also injected oocytes with *ccnb* RNA and saw a faster meiotic progression compared to control oocytes, which was similar to the faster progression of AF oocytes. Curiously, we also detected higher number of *ccnb2* RNAs in AF oocytes compared to YF. Although the reason for this finding is not so clear, it is known that CCNB2 may take on the role of CCNB as a regulatory subunit of CDK1 in case of CCNB failing somehow (Félix et al., 1990; Solomon, 1993; Li et al., 2018).

Therefore, we were able to prove that already at the post-NEBD stage oocytes from YF and AF differed significantly and even AF oocytes exhibited a slight phenotype. The next step was to consider a possible differential translation of mRNAs between the two groups.

In order to find those actively translating RNAs during NEBD, which could be involved in the increased aneuploidy rates of advanced maternal age oocytes, we had to adapt a polysomal profiling technique for scarce samples such as oocytes. Polysome profiling allows separating those RNAs bound to ribosomes (more prone to translation) from those which are not. However, this technique is usually applied on millions of cells (2-4x10^7 cells approximately) to verify polysomal peaks and this is not a feasible amount to be obtained from oocytes. Although oocytes are larger in size that the average somatic cell, it is possible to collect only 20-30 meiotically competent oocytes from each sacrificed female. According to our calculations based on 18S and 28S copy numbers between samples of 200 oocytes and Hek-cell lysates, we estimated that 10000 oocytes would be needed to visualize by UV absorbance the polysomal peaks after fractionation for just one sample. Moreover, the case of AF oocyte samples is even more dramatic, for the females need to be grown for one year in advance and, besides, they will provide no more than 3-5 oocytes each. Therefore, the need for adapting polysomal profiling to such kind of scarce samples was evident.

In the past, there have been only few attempts to modify the method to suit this need, all followed by microarray assays. Potireddy et al, (2006) used a method to separate ribonucleoprotein particles (RNPs) into 'subribosomal' (<80S) and 'polyribosomal' (>80S) from samples ranging 160-500 MII oocytes. This method was previously published by De Sousa et al (1993) and consisted on loading the scarce samples onto small volume tubes containing 50µl of 40% sucrose followed by ultracentrifugation. However, the main verification of the actual success on separating the RNPs was performed on a sample containing a large amount of cultured rat C6 glioma cells. Therefore, the separation of each single oocyte sample was not directly tested and doubts about its success may arise. A different approach was taken by Scatland et al, (2011) by loading 75 bovine oocytes/embryos on sucrose gradients of 10-60% sucrose and centrifuged on a SW60Ti rotor (slightly smaller than the SW55Ti rotor we used). While they had a similar approach as we did by using a smaller rotor than the conventional SW44Ti to concentrate the samples in the sucrose gradient, their strategy to visualise the polysomal peaks was totally different. They spiked their samples of study with cross-linked heterologous carrier polysomes from *Drosophila* SL2 cells. The spiking material was abundant enough to be visualised by UV absorbance and verify the proper polysomal fractionation. However, the presence of spiking RNA, despite being from a heterologous source, interfered significantly with the microarray probes forcing them to find an intermediate compromise between *Drosophila* RNA contamination and over-stringent conditions for bovine RNA on the array. A more conventional approach for polysomal fractionation has been also published, in which big groups of mouse oocytes (500-600) were loaded onto 12ml tubes containing 15-50% sucrose gradients (Chen et al., 2011). However, validation of the correct fractionation was not proven. In comparison to these mentioned protocols, our new adaptation of the method (SSP-Profiling) has proven to successfully separate the RNAs from as few as 100-200 mouse oocytes for a variety of subsequent applications (such as qPCR or NGS). Moreover, by being able to check the levels of 18S and 28S in each fraction, we could validate the fractionation quality for each sample. Recently, a completely different set-up has been proposed by Martins and Conti (2018) which consists of expressing the HA-tag on the bigger ribosomal subunits of oocytes followed by its immunoprecipitation together with the bound RNAs. The advantages of this

technique are avoiding both the validation of the polysomal profile and the long polysomal fractionation protocol. On the other hand, the disadvantages of using the ´Ribo-tag' method compared to our SSP-profiling is that it requires to create transgenic mice model and hold on to them, whereas SSP-profiling enables the use of any kind of sample without any previous genetic modification (we tested it on somatic cells, oocytes and embryos from different animal and plant species).

Once our adapted polysomal fractionation technique was designed and established, we used it to separate the polysome bound-RNAs from the rest in samples containing 200 NEBD oocytes from either YF or AF. So far, there have been several studies focusing on the comparison between the whole transcriptome of oocytes from different maternal age (Hamatani et al., 2004; Pan et al., 2008; Grøndahl et al., 2010), however, the actual translatome has yet not been compared. Previous studies on oocyte whole transcriptome analysis related to maternal aged mainly focused on the stages of GV (before meiotic maturation) and/or MII (after meiotic maturation). In Pan et al (2008), the whole transcriptome analysis by microarray showed that in GV stage only 5% of the analysed transcripts were significantly different between the two maternal age groups with just a FC>2 whereas in the MII stage the differences were much evident with a 33% of transcripts having a FC>2. Reyes et al, (2017) further confirmed this trend by transcriptome analysis of human oocytes. However, as we have previously discussed, already in the post-NEBD stage we have seen evident differences between AF and YF oocytes based on meiotic progression and MPF activity. Therefore, despite the whole transcriptome being very similar in GV between the two age groups, we expected the differences to be more biologically relevant in the translatome of post-NEBD oocytes. Our NGS data revealed 1006 genes with a FC>10 between YF and AF polysomal RNAs and when 'Over Representation Analysis' (Liao et al., 2019) was performed, the most relevant categories included transcripts related to cell division and cytokinesis. Moreover, we were able to validate the differential translation of four transcripts derived from sequencing data: *Sgk1, Castor1, Aire* and *Eg5*. Expression of SGK1 protein was lower in AF oocytes and CASTOR1, AIRE and EG5 expression was higher in AF compared to YF oocytes. Interestingly all four proteins showed spindle localization in the oocyte, which might indicate a possible role in cellular division and, as a consequence, a possible role in oocyte aneuploidy. Moreover, we excluded the possibility of the different expression of these four proteins to be a consequence of a faster meiosis rather than intrinsic overall translation. Since we previously showed that AF oocytes underwent NEBD circa 30 minutes earlier, we confirmed by ICC that EG5 levels were still higher in AF oocytes even when we cultured YF oocytes for 30 minutes more. We reassured this fact by a further WB experiment where AIRE levels were also significantly different between the AF and the extra 30 min cultured YF oocytes.

EG5 (or KIF11) is a motor Kinesin-5 protein involved in spindle bi-polarity formation and microtubule sliding, the role of which has been thoroughly studied in mitosis and meiosis (Mann and Wadsworth, 2019). Moreover, this protein seems to gain importance in i*n vitro matured* oocytes, as these cells express much more EG5 during maturation than the i*n vivo matured* oocytes (Kovacovicova et al., 2016). Thus, it was not exceptionally surprising to find that *Eg5* mRNA was actively translated in the NEBD stage in different rates between oocytes from YF and AF. Previous experiments comparing whole transcriptomes

in MII oocytes did not mention any changes in *Eg5* levels but it is important to remark that they were carried on different species, as well as at different stages (Hamatani et al., 2004; Pan et al., 2008; Grøndahl et al., 2010). On the other hand, in the study using mouse model, Pan et al (2008) did report several kinesins (p.e. *Kif21a, Kif13* and *Kif5b*) to be upregulated in MII oocytes from aged females, similarly as the *Eg5* kinesin in our experiments. Furthermore, it is quite well established that overexpression of EG5 in certain tissues causes aneuploidy and fosters tumorigenesis (Liu et al., 2010; Castillo et al., 2007). It is then plausible that oocytes from aged females have higher amount of EG5, probably creating extra pulling forces on the spindle, which would lead to faster meiosis and even premature sister chromatid separation. This could be especially true in the NEBD stage, as EG5 inhibition by monastrol delays or arrests oocytes in GV stage (Mailhes et al., 2004). Nonetheless, to prove this theory, more direct experiments on oocytes should be conducted.

Another of the proteins we found to be upregulated in AF oocytes was AIRE. Although we were not able to clearly see significant different levels of *Aire* mRNA in the polysomal fractions between young and aged female oocytes due to scarcity of RNA, our sequencing data clearly indicated that *Aire* mRNA was indeed a more frequently actively translated mRNA in the aged group. This protein has been deeply studied for its role as a transcription factor in immune tolerance control, mainly expressed in thymic epithelium cells rendering unnoticed in a transcriptionally silent cell as the fully grown meiotic oocyte (Björses et al., 1998). Even though it has been reported that AIRE -/- females display infertile traits it is not possible to link oocyte quality with ARIE as the follicular loss in this case is due to autoimmune responses over the ovary rather than the lack of AIRE itself in this organ (Jasti et al., 2012). Our data is in agreement with that of Pan et al, (2008) who has shown that *Aire* transcripts are also higher in MII oocytes from aged females, which means this unbalance might be affecting meiosis during further stages and not just during the first division. Moreover, our further experiments demonstrate that AIRE is more abundant in aged oocyte spindles. Recently, Gu et al, (2017) showed that the interaction of AIRE with other spindle-associated proteins was essential for spindle assembly and maintenance in stem cells, which also raised our interest in its possible significant role in meiosis. Interestingly, in the same study, a stronger phenotype of defective spindles in cells with an overexpressed truncated version of AIRE compared to cells in which the protein was depleted. The reason for this is not entirely clear, but is in agreement with our data and it suggests a detrimental role of overexpressed AIRE on the spindle in AF oocytes.

CASTOR1 is the third protein we validated to be more expressed in AF oocytes. This protein acts as an arginine sensor for the mTOR1 pathway by binding to arginine and losing its inhibitory effect over GATOR2, eventually allowing mTORC1 activation (Saxton et al., 2016). Otherwise, when arginine levels are low, CASTOR1 forms homodimers or heterodimers with CASTOR2, inhibiting GATOR2 and the mTORC1 pathway. Our data not only showed that CASTOR1 had higher expression in AF oocytes compared to YF counterpart, but also that it was localized on the spindle, sharing its localization with mTOR (Susor et al., 2015). It is possible that this shared localization is to ensure a better control over mTOR activity during meiosis as its inhibition/downregulation leads to meiotic defects(Susor et al., 2015; Guo et al., 2018). To study further the effects of increased CASTOR1 in oocytes, we decided to

overexpress this protein by mRNA injection in the GV stage in oocytes from YF to mimic the increased protein levels seen in AF oocytes. While there were neither differences in the amount of oocytes reaching MII stage, nor in the timing of NEBD and PB extrusion, we were actually able to detect that some specific abnormalities were more common in the CASTOR1 overexpressing cells compared to controls. The most frequent abnormality was chromosomal misalignment, with usually one or two chromosomes orbiting around the spindle instead of aligning in the centre until the moment of chromosome segregation. Another less frequent phenotype was the extrusion of two PBs with DNA content, leaving the bigger cell devoid of DNA. This last phenotype is obviously deleterious for the oocyte while chromosome misalignment is detrimental and might lead to aneuploidy (Liu and Keefe, 2008). Altogether, these results reveal CASTOR1 as a possible player in aged related chromosomal aneuploidy in oocytes.

We also decided to study the effects of altered SGK1 expression in oocytes as we found out that this protein had lower abundance in AF oocytes compared to YF. SGK1 is a serum- and glucocorticoid-regulated kinase mainly studied for its role in regulating ion channels, carriers, enzymes and transcription factors. Its activation depends on insulin, follicle stimulation hormone (FSH), corticosterone and thrombin, and this pathway includes PI3K, PDK1 and mTORC2 (Lang et al., 2018). Surprisingly, in spite of its known role in the membrane, we found that SGK1 was localized in the spindle region of maturing fully grown oocytes. Interestingly, it has also been already reported that SGK1 expression declines with age (Harries et al., 2012), however, we are the first to report it in oocytes. To investigate its role in oocyte meiosis, we used a specific inhibitor of SGK1 in YF oocytes to try to mimic the lower levels of SGK1 in aged oocytes. We used the same concentration, which was reported to allow A549 human lung cells to grow for 48h without reducing their viability (Alamares-Sapuay et al., 2013). Interestingly, inhibition of SGK1 significantly reduced the number of oocytes reaching MII with many oocytes arrested in the GV or NEBD stage. Moreover, most of the oocytes, which were able to extrude PB, displayed cytokinetic errors (unsplit spindle between oocyte and PB or symmetric division). Recently, Hiraoka et al, (2019) demonstrated that SGK protein of starfish oocytes phosphorylates CDC25 and MYT1, which results in MPF activation during the G2/M transition phase. Although this agrees with our data regarding the importance of SGK1 during meiotic maturation, it is somehow opposed to our results related to maternal aging. According to Hiraoka et al, (2019) SGK1 activity enhances MPF activation, however, in our results we found that SGK1 levels are lower in AF oocytes in which we have also detected higher MPF activity levels (leading to faster meiosis). A possible explanation could be that due to higher MPF activity in AF oocytes, these cells try to counteract the effect by decreasing SGK1 translation. It is also possible that SGK1 has a different role in mammalian oocytes than SGK in starfish as mice have three types SGK protein: SGK1, SGK2 and SGK (Lang et al., 2003). Although we have proven that SGK1 is involved in meiotic progression, it is unknown whether SGK2 and SGK3 also could also be involved in MPF activation. More research needs to be conducted to understand these contradictions and to increase our knowledge about SGK1 role in mammalian oocyte meiosis.

Altogether, we have shown that the post-NEBD stage in oocytes is a crucial inflexion point, in which oocytes from YF and AF start to manifest their differences basically through different translation patterns

of specific RNAs. These differences in the translation of RNAs could be very well involved in the higher aneuploidy rates seen in AF oocytes, since we have shown the alteration of some of them lead to cytokinetic abnormalities.

5. Conclusions

- 4E-BP1 is the only 4E-BP protein expressed during oocyte meiosis and it is regulated in a cell-cycle dependent manner by CDK1 activity post-NEBD.
- The inactivation of 4E-BP1 through phosphorylation is necessary for the initiation of the cap-dependent translation post-NEBD.
- Phosphorylated forms of 4E-BP1 (Ser65 and Thr70) localize at the newly forming spindle after NEBD.
- Oocytes from AF have a faster meiotic progression than their counterparts from YF.
- Expression of MPF components on a protein level (CDK1 and CYCLIN B) is increased in AF oocytes, probably speeding up NEBD and the rest of meiotic progression.
- After NEBD, oocytes from YF and AF display differential translation of several specific RNAs, four of them: *Sgk1, Castor1, Aire* and *Eg5* being validated by us.
- Altering the levels/activity of CASTOR1 and SGK1 in GV oocytes resulted in several cytokinetic defects, reinforcing the idea of their differential translation to be related with increased aneuploidy in AF oocytes.
- Our modified version of polysomal profiling (termed SSP-profiling) is suitable for successful analyses of the ribosomal profiling of small input samples without compromising neither them nor future applications (e.g. NGS).

This work provides new information regarding translation in the post-NEBD stage of mammalian oocytes. Moreover, it contributes to deepen the current knowledge on the causes of mammalian oocyte age-related aneuploidy. Some interesting targets have been revealed, which are not only related to oocyte aneuploidy but could also have important roles in meiosis itself. Further research should be conducted using these targets if these possible roles are to be elucidated. Last but not least, the new SSP-profiling method could be used by other researchers to study polysomal profiling on a large variety of minute and valuable samples.

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7. Abbreviations

4E-BP	4E binding protein
AF	Aged females
AKT	Protein kinase B
APC/C	Anaphase-promoting complex/cyclosome
cAMP	Cyclic adenosine monophosphate
CASTOR1	Cytosolic Arginine Sensor For mTORC1 Subunit 1
CDC25	Cell division cycle 25
CDH11	Cadherin 11
CDK1	Cyclin dependent kinase-1
cGMP	Cyclic guanosine monophosphate
CPE	Cytoplasmic polyadenylation element
CPEB	CPE binding protein
EDTA	Ethylenediaminetetraacetic acid
EG5	Kinesin-5 protein
eIF4A	Eukaryotic initiation factor 4A
eIF4E	Eukaryotic initiation factor 4E
eIF4F	Eukaryotic initiation factor 4F complex
eIF4G1	Eukaryotic initiation factor 4G1
FC	Fold change
FSH	Follicular stimulating hormone
GFP	Green fluorescent protein
GV	Germinal vesicle
ICC	Immunocytochemistry
LH	Luteinizing hormone
MII	metaphase II oocytes
MPF	Maturation promoting factor

mTOR	Mammalian target of rapamycin
MYT1	Myelin transcription factor 1
NEBD	Nuclear envelope break down
NGS	Next generation sequencing
NP	Non-polysomal
ORA	Over-representation analysis
P	Polysomal
PB	Polar body
PDE3A	Phosphodiesterase 3A
PGC	Primordial germ cell
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PLK1	Polo like kinase 1
RBP	RNA binding protein
REC8	Meiotic recombination protein REC8 homolog
RNP	Ribonucleoprotein particle
SAC	Spindle assembly checkpoint
SCMC	Subcortical maternal complexes
SGK1	Serum/Glucocorticoid Regulated Kinase 1
SSP-profiling	Scarce simple polysomal profiling
WB	Western Blot
YF	Young females
ZGA	Zygote genome activation

8. Curriculum Vitae

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Education/Qualifications

2015-Present	Charles University in Prague, Czech Republic; Faculty of Science
	A PhD student of the Study Program Developmental and Cell Biology
	PhD thesis: Age-related differences in translation of mammalian oocytes
2013-2014	University of Nottingham, United Kingdom
	Master Med. Sci. in Assisted Reproduction Techniques
	Internship to conduct diploma thesis at the department of Biology of Development and Reproduction, INRA, Paris, France
	Diploma thesis: The effects of TSA on the development and pre-patterning of mice embryos
2011-2012	Autonomous University of Barcelona, Spain
	Master Sci. in Advanced Genetics
	Internship for conduct diploma thesis at the CIBBIM Nanomedicine, VHIR, Barcelona, Spain
	Diploma thesis: The role of EPHB6 in colorectal cancer

Presentations and conferences

2019

- MiniRNA meeting, Zeliv, Czech Republic. Oral presentation.
- RNA Club 2019, Prague, Czech Republic. Poster presentation.

- Awakening of the genome: The maternal-to-zygotic transition, Prague, Czech Republic. Poster presentation.

2018

- RNA Club 2018, Prague, Czech Republic. Poster presentation.
- Visegrad Group Society for Developmental Biology, Inaugural Meeting, Brno, Czech Republic. Oral and poster presentation.
- ESHRE annual meeting (2018), Barcelona, Spain. Oral presentation.

2017

- EMBO Protein synthesis and translational control, Heidelberg, Germany. Poster presentation.

2016

- RNA club, Brno, Czech Republic. Poster presentation.

Publications

- Masek T, **Del Llano E**, Gahurova L, Kubelka M, Susor A, Roucova K, Lin CJ, Bruce AW, Pospisek M. Identifying the Translatome of Mouse NEBD-Stage Oocytes via SSP-Profiling; A Novel Polysome Fractionation Method. Int J Mol Sci. 2020;21(4):1254. Published 2020 Feb 13. doi:10.3390/ijms21041254
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Pending publication

- **Del Llano E**, Masek T, Gahurova L, Pospisek M, Koncicka M, Jindrova A, Jansova D, Iyyappan R, Roucova K, Bruce AW, Kubelka M, Susor A. Age-related differences in the translational landscape of mammalian oocytes. Aging Cell. Submitted the 6th May 2020.

9. Annex: Research papers