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# Protein synthesis and protein degradation in mammalian oocyte development

**Ph.D. Thesis**

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This thesis was made at Institute of Animal Physiology and Genetics, Academy of Sciences of Czech Republic, v.v.i., in Libechov.

I declare that all documents, which are submitted in this Ph.D. thesis, are only for obtaining of my PhD graduation.

It was a pleasure for me to work with all the wonderful people in our department here in Libechov.

There are many people I would like to thank for their invaluable support and guidance during the process of completing this thesis. First and foremost, I would like to thank my supervisor specialist Michal Kubelka, PhD a head of Laboratory of biochemistry and molecular biology of the germ cell and professor Jan Motlik, my thesis supervisor.

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# Index

# 1Preface

The well-known dictum *ex ovo omnia* spectacularly shows that female germ cell is the cornerstone of developmental biology. Mammalian oocytes have fascinated biologists, yet it was only recently that they became objects of acute research activity, targets for biotechnology and topics of public interest.

In vitro fertilization, production of transgenic animals, cloning, and "era" of stem cells have revolutionized our study of this most rare cell in the body that spread our view in its physiology and morphology. This progress would have been impossible without the myriad scientific and technical developments. Every cell type has phenotype of its own, but only this cell can serve in the female body as a genetic hinge connecting the doors of successive generations. In this connection, oocytes and spermatocytes are the only cells in the body that undergo meiosis and fertilization. Oocytes are paradoxical cells, being at the same time both highly specialized for their role, and a totipotent, so that every other cell type body can be generated.

The mammalian oocytes have very specific regulation of the transcriptional activity. The fully grown oocytes are transcriptionally silent since the mRNA is abundantly synthesized in long prophase of the growing oocyte, where transcription activity of the nucleus is high and this process rapidly decreases as the oocyte progress to its route to be a fertilizable egg. The synthesis of new proteins is highly regulated process that allows rapid responses of meiotic cell to diverse stimuli in the absence of transcription. Synthesized mRNAs are translated, degraded or stored as a maternal „investment" to newly developing embryo that starts in a new individual after egg sperm fusion. The translational activation of dormant mRNAs, stored during oogenesis seems to be controlled by de novo polyadenylation of the transcripts. On the other hand, the phosphorylation, of the number of different key factors and their regulators, as well as structure of ribosomal proteins is associated with changes in the rate of translation. Whale translation machinery is responsible for the synthesis of proteins, their degradation or posttranslational modification involves the proteasome system.

Mechanisms of these processes are barely known hence open the fields for study of mechanisms of oocyte protein synthesis and degradation.

# 1 Introduction

This thesis is focused on general aspects of proteosynthesis and protein degradation including proteasome pathway.

    i)        Regulation of proteosynthesis in the oocyte and in the early embryo

    ii)       Protein translation regulation during oocyte meiosis

    iii)      Proteasome as a machinery for protein degradation

## 1.1   Development of Mammalian Female Germ Cell

The oogonia are the stem cells that give rise to all the oocytes in the ovary (*Rüsse 1983*). The population of oogonia goes through a predetermined, species-specific, number of mitotic cycles until they enter the prophase of meiosis. The germ cells accumulate near the outer surface of the gonad. Near time of birth, each individual germ cell is surrounded by somatic cells. The germ cells will become the ova, and surrounding cortical somatic cells will differentiate into granulosa cells (*Vanderhyden 2002, Hurk 2005*). Together, the thecal and granulosa cells form follicles that envelop the germ cells and secrete steroid hormones. Each follicle will contain a single germ cell- an oogonium, which will enter meiosis at this time.

The nucleus of diplotene oocytes is known as germinal vesicle (GV). The oocyte at the GV stage is a diploid cell (2n), which has twice the normal amount of DNA as the first meiotic prophase is stopped at diplotene in the early post-natal period. The oocytes are considered to be in the first meiotic arrest.

Oocytes remain in meiotic arrest for many months or even years. It is estimated that bovine ovaries contain an amount of 420,000 germ cells at birth that is reduced to less than 3000 by 20 years of age (*Faddy 1995*). In mammals, the ovaries are the female gonad responsible for the differentiation and release of a mature oocyte for fertilization and successful propagation of the species. The final meiotic stage ends with activation/ fertilization of egg.

## 1.2   Meiotic maturation of oocytes

Meiosis is a process of reductional division in which the number of chromosomes per cell is cut in half. In animals, meiosis always results in the formation of gametes.

Recently, has been recognized that retinoic acid provides the signal for meiotic entry in the foetal ovary (*Bowles 2007*). Male germ cells in the escape this fate because the somatic cells of the differentiating testis produce a testis-specific enzyme (Cyp26b1), that degrades retinoic acid (*Koubova 2006*). During the growth phase mammalian oocytes are arrested in vivo in the ovary at the G2/M transition in the first meiotic prophase called germinal vesicle (GV) stage. Although arrested in cell cycling, the oocytes remain synthetically active. When fully-grown oocytes are removed from their follicles, they can resume meiosis spontaneously under in vitro conditions. The meiotic maturation is characterized by morphological changes such as chromosome condensation, breakdown of the germinal vesicle (GVBD) and rearrangement of microtubule network during the first meiosis (M I), followed by extrusion of the first polar body and block of oocytes in metaphase of the second meiosis (M II). The oocytes remain arrested at the M II stage until fertilization or artificial activation. In relation to well-characterized morphological changes during oocyte maturation, the M-phase promoting factor (MPF) appears to be the main regulator. The cdc2 kinase, the catalytic subunit of MPF, is stored in oocytes as an inactive protein and its activation needs, at the first level, the association with cyclin B1, which is know as regulatory subunit of MPF and whose synthesis and degradation oscillates during the cell cycle (*Pines 1990*). Only fully matured oocytes are able to undergo successful fertilization and the initiation of zygotic development (*Moor 2001, Kubelka 2002*). Simultaneous with nuclear maturation is cytoplasmic maturation (*Rath 1995, Gershon 2006*) that is important for successful meiotic progression and further development of zygote. The process of cytoplasmic maturation includes maternal transcripts storage, reorganization of cytoskeleton, mitochondrial and cortical granule migration  (*Sun 2003, Blerkom 2004, Sun 2006, Murchison 2007, Mtango 2008, Yurttas 2008*).

Both nuclear and cytoplasmic maturation are acquired within the ovary and are necessary for developmental competence of oocyte (*Hyttel 1997*). Developmental competence is the ability of the oocyte to produce normal, viable and fertile offspring after fertilization. Developmental competence is a difficult parameter to assess since embryonic development may fail due to reasons independent of oocyte quality.

## 1.3 Translation regulation by initiation translation factors

The precise timing of protein synthesis and degradation plays an important role in controlling oocyte maturation (*Moor 2001*), where transcriptional activity is silent and the main role-play a previously synthesized mRNA and stored (*Bettegowda 2007*).

The initiation step of translation (i.e., the binding of the small ribosomal subunit to the mRNA) seems to be the rate-limiting step in the cap-dependent translation (*Mathews 1996, Sonenberg 1998*). This crucial process is influenced by secondary and tertiary structures within the 5' untranslated region of the mRNA and by the existence of the cap structure (m$^7$GpppN), which is characteristic for cap-dependent translation (*Thach 1992*). This structure is recognized by the cap binding protein complex eIF4F, which consists of three subunits: eIF4A (an RNA helicase, which is responsible for unwinding of mRNA secondary structures), eIF4E (the cap binding protein itself), and eIF4G, a protein required to maintain the integrity of the complex (*Hershey 1991, Sonenberg 1998*). The best studied and characterized is eIF4E, the cap-binding subunit of eIF4F. eIF4E is one of the main regulatory initiation factors, because it is present in limiting molar amounts in the cell, and as such serves as an attractive target for regulating translation. In fact, the amounts and/or activity of eIF4E are modulated at several levels: phosphorylation, translational repressors (4E-binding protein 1, 4E-BP1), and transcription (*Sonenberg 1998*).The interaction of eIF4F with repressed mRNA is absolutely necessary for efficient initiation of translation (*Sonenberg 1988*). Although direct evidence for the importance of eIF4E phosphorylation in the cap binding process is missing, many reports have shown a correlation between eIF4E phosphorylation and high translation rates (*Pain 1996, Sonenberg 1998*). Therefore, the phosphorylation of eIF4E is directly linked to the mRNA recruitment from a stored pool to actively translated polyribosomes (*Kaspar 1990*). Moreover, during somatic cell cycle, the phosphorylation state of eIF4E is generally consistent with higher rates (*Pyronnet 2001*). This protein can be phosphorylated on several sites by different protein kinases (Gingras 1999). Ellederova (*2007*) have shown recently that the phosphorylation of this protein increases during in vitro maturation of pig oocyte and this increase is associated with the release of eIE4F binding partner 4E-BP1 and activation of cap-dependent mRNA translation (*Ellederova 2007*).

Little is known, about the regulation of the cap-dependent translation initiation machinery during mammalian early embryonic development. It has been reported that transcription and translation of nascent mRNA is delayed until the 2-cell stage in mouse

embryo, when maternal zygote transition occurs (Matsumoto 1994). The porcine maternal zygote transition occurs during 4-cell stage (*Jarrell 1991, Prather, 1993*) and bovine 8cell stage (*Kopecny 1989*). Brevini (2002) have also documented, that specific changes in mRNA polyadenylation contribute to the modulation of gene expression in the early stages of bovine embryos. Abnormal polyadenylation levels of specific maternal mRNAs accompany defective developmental competence, with synchrony between polyadenylation and cleavage emerging as an apparently important factor (*Watson 2007*).

## 1.4   Protein phosphorylation

Another level of regulation of cell metabolism is represented by phosphorylation of proteins. Phosphorylation is a chemical process in which a phosphate group is added to an organic molecule. Nine amino acids, e.g. tyrosine, threonine, serine, histidine, glutamic acid, aspartic acid, arginine, cystein, and lysine can undergo phosphorylation in cells, the main targets in eukaryotic cell are threonine, serine and tyrosine. The phosphorylation state of cellular proteins is highly dynamic, i.e. it reflects control of biochemical pathways by protein kinases that catalyse phosphorylation, or protein phosphatases that mediate dephosphorylation. In addition, the phosphorylation of a protein by different kinases can occur at distinct amino acid sites thus expanding the complexity of the network (*Sebastian 1993, Fattaey 1997, Hunter 1998*).

Although the changes in protein levels are important during oocyte maturation, the contribution of protein phosphorylation cascades appears to have decisive impact on temporal and spatial organization of events during meiotic maturation. This can be exemplified by mechanisms that are involved in main regulation of M-phase, controlling MPF activity or cdc2 kinase. Those events entail activating phosphorylation on Thr161 by cdk-activating kinase and dephosphorylation of inhibitory phosphorylations on Thr14 and Tyr15 by cdc25 phosphatase. The phosphates must be removed before translocation to the nucleus where cdc2 exerts its major effects (*Solomon, 1990, Coleman 1994*).

## 1.5   Ubiquitin proteasome system

Proteasomes are large protein complexes inside all eukaryotes and archaea. They are located in the nucleus, cytoplasm and in extracellular fluids (*Peters 1994, Majetschak 2008, Sixt 2008*). The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis. The 26S proteasome is the key component of the ubiquitin-dependent pathway of protein degradation. This energy-dependent nanomachine is composed of a 20S catalytic core and associated regulatory complexes. The eukaryotic 20S proteasomes demonstrate several kinds of peptidase activities, protein-chaperone and DNA-helicase activities. This pathway controls the levels of the key regulatory proteins in the cell (*Konstantinova 2008*). A lysine residue of ubiquitin molecule attached to a substrate can itself serve as an acceptor for an additional ubiquitin molecule, and this process can be repeated so that poly-ubiquitinated proteins form. Poly-ubiquitin chains serve as recognition signals for the 26S proteasome, the major regulator of protein abundance in cells, and poly-ubiquitination thus often initiates proteolysis of the substrate (Fig. 1b,c). But poly-ubiquitination can also regulate protein function directly without affecting stability, in ways similar to mono-ubiquitination and other post-translational modifications. The mechanisms underlying proteolysis-independent regulation by poly-ubiquitination are only poorly understood but might function by changing conformation or adding or obscuring a binding site (*Hershko 1998, Hicke 2001, Pickart 2004*).
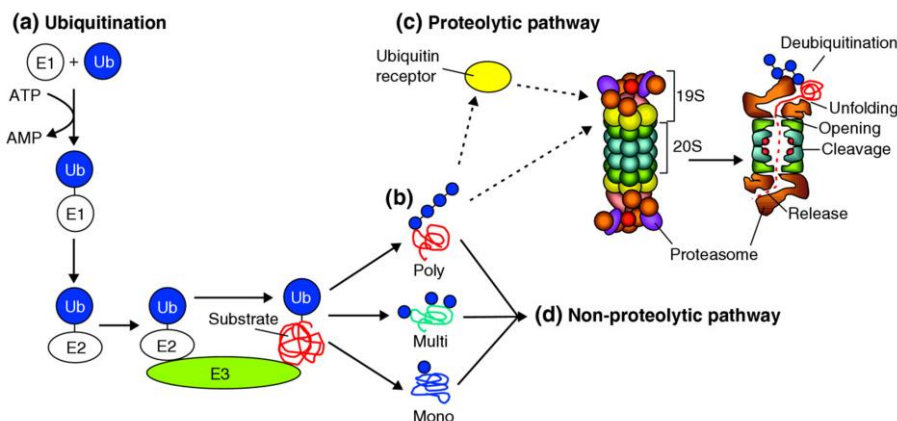


**Fig 1** The Ubiquitnin pathway (*Kaiser 2005*).

Although ubiquitin is the most well understood post-translation modifier, there is a growing family of ubiquitin-like proteins (UBLs) that modify cellular targets in a pathway that is parallel to but distinct from that of ubiquitin. These alternative modifiers include:

SUMO, NEDD8, ISG15, APG8, APG12, FAT10, Ufm1 URM1 & Hub1 (*Herrmann 2007*). Attachment of UBLs might alter substrate conformation; affect the affinity for ligands or other interacting molecules, changing substrate localization and influence protein stability (Fig. 3d). UBLs are structurally similar to ubiquitin and are processed, activated, conjugated and released from conjugates by enzymatic steps that are similar to the corresponding mechanisms for ubiquitin. These conjugates can be reversed by UBL-specific isopeptidases that have similar mechanisms to that of the deubiquitinating enzymes (*Sutovsky 2002, Herrmann 2007*). These modifiers have their own specific E1 (activating), E2 (conjugating) and E3 (ligating) enzymes that conjugate the UBLs to intracellular targets (Fig. 3a). E3 ubiquitin ligase is an anaphase-promoting complex (APC) that indirectly activate of separase enzyme that is involved in segregation of sister chromatids durng meiosis or mitosis (Fig.2; *Kudo 2006, Marangos 2008, Vogt 2008*). Proteasome is also key player in fertilization process where is responsible for regulation of extracellular receptors in gamete recognition or interaction (*Swada 2002, Sakai 2003, Yi 2007*) and is also essential for physiological development of germ cells and embryos (*Derenzo 2004, Khor 2006, Susor 2007*).
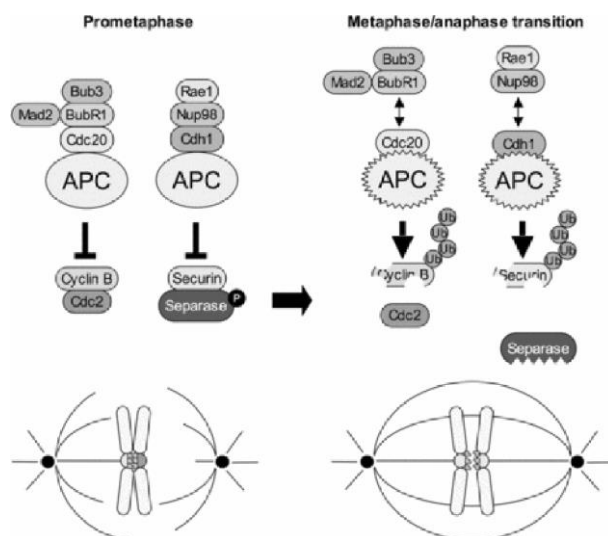


**Fig. 2**: Anaphase promoting complex is responsible for physiological segregation of sister chromatids during anaphase (*Jeganathan 2006*).

**Ubiquitin Carboxyl-C-terminal Hydrolase Isozyme L1 (UCH-L1)**

UCH-L1 is an enzyme from proteasome system (PS), a member of deubiquitinating enzymes (DUBs). DUBs are regulators of protein ubiquitination and play an important role in ubiquitin-dependent processes. Ubiquitin is a key player of ubiquitin proteasome system (UPS) where is universal marker of proteolysis, protein recycling and protein regulation,

unique because of its versatility and high degree of evolutionary conservativism. UCH-L1 is enzyme with dual function in regulation of ubiquitin level, in monomeric state with hydrolytic activity and in oligomeric form as an ubiquitin ligase (*Liu 2002*). UCH-L1 is selectively and abundantly expressed in germ cells, ovary, placenta, testis, and neuronal cells (*Day 1992, Sekiguci, 2003, Kwon, 2003, 2004, Susor 2007*). The similar protein is UCH-L3 with 51% homology but with different biochemical functions. The UCH-L3 has 200 fold higher hydrolytic activity and as well hydrolytic function for NEDD8 (*Hiroyoshi 1998*).

Our recent study (Susor 2007) unveils that UCH-L1 is abundant in porcine oocytes and is responsible for regulation of MPF activity probably due to degradation of cyclin B during meiosis. Recently it has been found that UCH-L1 deficient mouse - *gad* (*gracial axonal dystrophy*) has higher ratio of polyspermy, similarly as porcine eggs treated with inhibitor of multiple ubiquitin hydrolases (*Sekiguci 2006, Yi 2007*).

Studies reported that UPS controls the degradation of various substrates during gametogenesis and fertilization, however, relatively little is known about its function in reproduction.

# 2 Aims of thesis

**The aim of this thesis is analysis of translation regulation factors as well as protein degradation machinery in oocyte progressing through meiosis. Cytoplasmic maturation of oocyte and its contribution to successful development.**

- Regulation of eIF4E during oocyte meiosis/ correlation with protein synthesis during oocyte meiosis.

- Formation of active translation initiation complex eIF4F and overall protein synthesis during oocyte meiosis and early embryonic stage.

- Identification of protein turnover, phosphorylation and/or altered protein-protein interaction during meiotic maturation of mammalian oocytes and oocytes from lower taxa.

- Role of UCH-L1 in Ubiquitin related degradation of meiotic regulatory protein cyclin B1 and its role in fertilization process.

# 3 List of publications

4.1 Ellederova Z, Cais O, **Susor A**, Uhlirova K, Kovarova H, Jelinkova L, Tomek W, Kubelka M

**ERK1/2 Map Kinase Metabolic Pathway is Responsible for Phosphorylation of Translation Initiation Factor eIF4E During In Vitro Maturation of Pig Oocytes.**

Molecular Reproduction and Development 2008, 75: 309–317. IF 2,538

4.2 **Susor A**, Jelinkova L, Karabinova P, Torner H, Tomek W, Kovarova H, Kubelka M

**Regulation of Cap-dependent Translation Initiation in the Early Stage Porcine Parthenotes.**

Molecular Reproduction and Development 2008, 75: 1716-25. IF 2,538

4.3 Pelech S, Jelinkova L, **Susor A**, Zhang H, Shi X, Pavlok A, Kubelka M, Kovarova H.

**Antibody Microarray Analyses of Signal Transduction Protein Expression and Phosphorylation During Porcine Oocyte Maturation.**

Journal of Proteome Research 2008, 7: 2860-2871. IF 5,675

4.4 **Susor A**, Ellederova Z, Jelinkova L, Halada P, Kavan D, Kubelka M and Kovarova H

**Proteomic Analysis of Porcine Oocytes During In Vitro Maturation Reveals Essential Role for the Ubiquitin C-terminal Hydrolase-L1.**

Reproduction 2007, 134: 559-568. IF 2,962

4.5 **Susor A**, Jelinkova L, Pavlok A, Kubelka M

**Role of Ubiquitin C-terminal hydrolase L1 in antipolyspermy defence of mammalian oocyte.**

 Manuscript in preparation

# 4  Comments on publications

## 4.1  ERK1/2 Map Kinase Metabolic Pathway is Responsible for Phosphorylation of Translation Initiation Factor eIF4E During In Vitro Maturation of Pig Oocytes.

Ellederova Z, Cais O, Susor A, Uhlirova K, Kovarova H, Jelinkova L, Tomek W, Kubelka M

In this study, we have investigated the pathways responsible for phosphorylation of translation initiation factor eIF4E during in vitro maturation (IVM) of porcine oocytes. Our previous results (*Ellederova 2006*) have shown that eIF4E becomes gradually phosphorylated (*Ser-209*) during terminal stages of meiosis and similar results have been also published in mouse (*Gavin 1997*), bovine (*Tomek 2002*) oocytes. On the other hand, in parthenotes the phosphorylation of eIF4E rapidly decreases at 6 hr post activation (*Susor 2008*).

To describe the changes of eIF4E phosphorylation in pig oocytes under different culture conditions, the method of Vertical Slab Gel Isoelectric Focusing (VSIEF) combined with Western blotting with specific polyclonal eIF4E antibody was employed in the majority of experiments.

Using the VSIEF method we have been able to detect three highly acidic forms of eIF4E appearing at GVBD stage onwards, as opposed to the basic eIF4E form, which was present during the whole period of maturation (Fig. 2-A5). The middle one of the acidic forms represents eIF4E phosphorylated on Ser-209. Although eIF4E has been suggested to be a phosphorylated in vivo on a single site Ser-209 (*Joshi 1995*), two other sites have been described earlier as putative phosphorylation sites - Ser-53 and Thr-210 (*Rychlik 1987, Makkinje 1995*). The additional acidic forms of eIF4E might then represent phosphorylation on these sites or, alternatively, another posttranslational modifications of the eIF4E protein.

Two protein kinases, Mnk1 and Mnk2, phosphorylate eIF4E at the physiological site both in vitro and in vivo (*Pyronnet 1999, Waskiewicz 1999, Scheper 2001*) and are believed to be the eIF4E kinases. Mnk1 and Mnk2 can be activated by phosphorylation by the mitogen activated extracellular signal-regulated kinases (Erks) and the stress- and cytokine-activated p38 MAP kinase pathways (*Fukunaga 1997, Waskiewicz 1997, Scheper 2001*).

Although Mnk2 expression as well as its activity does not change during IVM of pig oocyte (unpublished results). Phosphorylation (activation) of Mnk1 tightly correlates with eIF4E phosphorylation (Fig. 1b,i- A4, Fig. 5- A7) both in oocytes cultured in control conditions and in presence of the inhibitors, Butyrolactone (BL I) and OA (Ocaidic Acid). We used these two inhibitors and their combinations to specify also, which of the two major M-phase kinases, which become activated during pig oocyte maturation (CDK1 kinase and MAP kinase), might be involved in phosphorylation (and activation) of Mnk1 kinase. Our results have shown that ERK1/2 MAP kinase activity, but not CDK1 or p38 MAP kinase activity, is tightly correlated and precedes phosphorylation of Mnk1 kinase in pig oocytes (Fig. 1- A4). The activity of Mnk1 also tightly correlates with eIF4E phosphorylation both in oocytes cultured in control conditions and in oocytes cultured in the presence of the inhibitors.

The obtained data suggest that ERK1/2 MAP kinase pathway is involved in eIF4E phosphorylation during IVM of pig oocytes.

## 4.2 Regulation of Cap-dependent Translation Initiation in the Early Stage Porcine Parthenotes.

Susor A, Jelinkova L, Karabinova P, Torner H, Tomek W, Kovarova H, Kubelka M
Molecular Reproduction and Development 2008, 75: 1716-25.

Primarily we result from our previous findings (Ellederova 2006). Showing that general overall protein synthesis decreases during meiosis and in this study we extend this period to the first two days of porcine embryonic development. The regulated translation of mRNAs influences a large number of biological processes, including the cell cycle (*Mendez 2001, Pyronnet 2001, Groisman 2002*), growth, embryogenesis (Riechmann 2001, Niessing 2002), and germ line development (*Morris 1995, Pain 1996, Hake 1997, Clemens 1999, Saffman 1999*). The protein synthesis in cell is regulated in various ways (*Standart 1994, Read 2002, Stitzel 2007*) and we focused our research on eucaryotic translation initiation factors (eIFs) that are associated with 5' mRNAs (*Gingras 1999*).

We document that the overall rate of protein synthesis slightly decreases (Fig. 1a-A14) after porcine egg activation despite enhanced formation of eucaryotic initiation factor (eIF4F). We found only weak affinity of eIF4E to cap structure analogue - $^7$m Sepharose during first embryonic mitosis (Fig. 5a- A16).

The binding of mRNAs to ribosomes is mediated by the protein complex eIF4F. The most known protein of eIF4F complex is eIF4E, which directly binds mRNA through its ‚cap' structure on the 5' end and ..... activity and availability. In the process of translation initiation is regulated by binding of regulatory factor 4E-PB1. 4E-BP1 normally binds eIF4E, inhibiting cap-dependent translation. Hyperphosphorylation of 4E-BP1 disrupts this binding and activates cap-dependent translation (*Pause 1994*). Fig. 4 (A15) shows rapid dephosphorylation of eIF4BP in post activation period as well as unphosphorylated form. Decrease in the overall protein synthesis is visible between 9 and 12 hr post-activation and the main decrease occurs after 15 hr post-activation, which corresponds to the first embryonic mitosis. Further decrease of protein synthesis has been also observed during 2-cell and 4-cell stage, respectively (Fig 1AB). This is in agreement with results obtained from porcine in vivo derived embryos (Fig. 1, *Jarrell 1991*). The authors showed that the uptake and incorporation of $^{35}$S-methionine rapidly decreases from unfertilised egg to morula stage, they report 67% decrease versus 80% of our results. Similar results were also reported in cattle and sheep (*Crosby 1988, Frei 1989*).
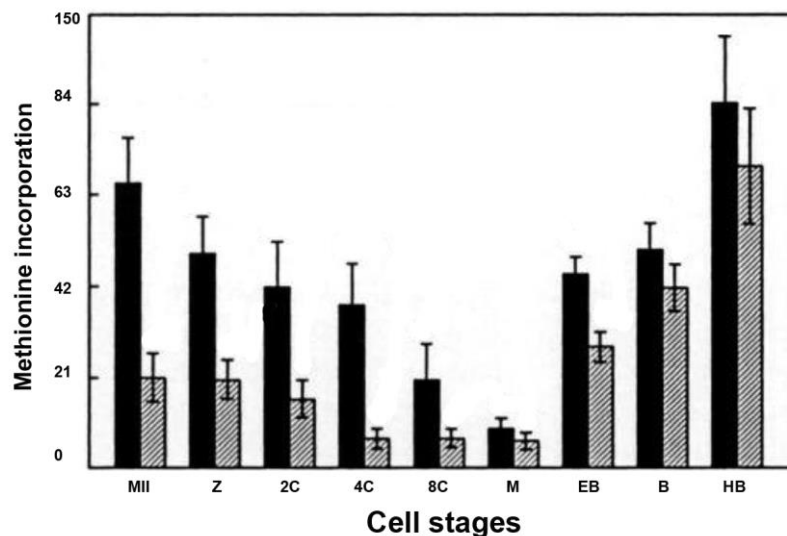


**Fig. 1**: Uptake (solid bar) and Incorporation (hatched bar) of $^{36}$S-Methionine in porcine eggs and thru pre-nidation development (*Jarrell 1991*).

Although the overall protein synthesis decreases, it is likely that specific mRNAs become translated and this requires only small amounts of an active eIF4F complex. These results and those showing that eIF4E is completely unphosphorylated at this stage (24 hr post-activation). We also suggest that phosphorylation of eIF4E is unlikely to be the major

determinant in formation of an active eIF4F complex. In later stage (4 cell), apart from phosphorylation, also the amounts of eIF4E decrease in zygotes, as well as the general translation.

Negative correlation between active eIF4F complex on one site and decreasing overall protein synthesis on the other site, in porcine zygotes suggest involvement of other regulatory pathways, such as regulation of 3' mRNA (*Brevini 2002, Lequarre 2004, Piccioni 2005, Gershon 2006, Kim 2007*) and miRNA pathway in mRNA regulation/ elimination of specific maternal transcripts is documented (*Murchison 2007, Mtango 2008, Lykke-Andersen 2008*). Camargo (*2008*) recently reported that regulation of general protein synthesis in somatic cells could be regulated via miRNA pathway.

At present, we are trying to characterize regulatory mechanism on 3'-end of mRNA that could be a key factor in repression of general translation in female germ cells.

## 4.3 Antibody Microarray Analyses of Signal Transduction Protein Expression and Phosphorylation During Porcine Oocyte Maturation.

Pelech S, Jelinkova L, Susor A, Zhang H, Shi X, Pavlok A, Kubelka M, Kovarova H.

Herein we describe the regulation of more than 60 proteins by protein turnover, phosphorylation and/or altered protein-protein interaction during oocyte meiotic maturation of four animal species. Kinex™ antibody microarray analysis was used to investigate the regulation of 188 protein kinases, 24 protein phosphatases, and 170 other regulatory proteins during meiotic maturation from immature germinal vesicle pig oocytes to maturing oocytes that had completed meiosis I, and fully mature oocytes arrested at metaphase of meiosis II. The regulation of these proteins was also examined in parallel during the meiotic maturation of bovine, frog (*Xenopus laevi*s) and sea star oocytes (*Pisaster ochraceus).*

In the case of porcine and frog oocyte systems, we have estimated validation of microarray results using immunoblot analyses, and determined that only about a third of the changes in either protein levels or phosphorylation inferred by the antibody microarray analysis could be validated. Some of the best characterized phosphoprotein changes in frog oocyte occurring at the time of onset of GV breakdown are the increased phosphorylations of MEK1, ERK2, RSK1 and ribosomal S6 protein, which were not evident from the results of

antibody microarray analysis (Fig. 6-A25), but were very prominent when immunoblotting was used (Fig. 8b-A27).

Serious concern that has been raised from our work is the large number of false negatives that were generated by the antibody microarray. This is the case of the number of antibodies that failed to show changes greater than 24% by the antibody microarray analysis, but did in fact show large alterations in protein expression or phosphorylation by Western blotting. In this study, this corresponded to 20 of 23 antibodies that are shown in Fig. 7 (A26) and Fig. 8 (A27).

By western blotting analysis we confirmed altered expression levels of Bub1A, IRAK4, MST2, PP4C and Rsk2, and the phosphorylation site changes in the kinases Erk5 (T218 + Y220), FAK (S722), GSK3-beta (Y216), MEK1 (S217 + S221) and PKR1 (T451), and nucleophosmin/B23 (S4) during pig oocyte maturation.

Despite the aforementioned caveats, the low cost and sample requirements for antibody microarray analysis make this powerful technology very attractive for broad-based proteomics to discover potentially important players in signalling pathways.

## 4.4 Proteomic Analysis of Porcine Oocytes During In Vitro Maturation Reveals Essential Role for the Ubiquitin C-terminal Hydrolase-L1.

Susor A, Ellederova Z, Jelinkova L, Halada P, Kavan D, Kubelka M and Kovarova H
Reproduction 2007, 134: 559-568.

In this study we choose a powerful tool to study a proteome of pig oocytes during meiosis. Proteomic analysis is effective method to identify proteins that take a role in physiological or pathological processes of the cells (*Calvert 2005, Aitken 2007, Gestel 2007, Hanrieder 2007, Katz-Jaffe 2007, Vitale 2007, Gorla 2008, Kim 2008*). We analysed de novo protein synthesis by uptake of [$^{35}$S]-methionine. Proteomic approach to analyse the porcine oocytes at the initial GV and final MII stage of meiosis, identified candidate proteins that were differentially synthesized during in vitro progression of meiosis. Among them, the ubiquitin C-terminal hydrolase-L1 (UCH-L1) was identified by mass spectrometry and further studied for its role during oocyte maturation.

To study the role of UCH-L1 in the process of meiosis in pig oocyte model, we used a specific inhibitor of this enzyme; UCH-L1 inhibitor (marked as C30 or LDN-57444),

belonging to the class of isatin O-acyl oximes with 28 fold greater selectivity over UCH-L3 (*Liu 2003*). Using UCH-L1 specific inhibitor as a modulator of UCH-L1 activity resulted in retention of GVBD and meiotic block at MI stage. The block of metaphase I–anaphase transition was not completely reversible, probably due to insufficient degradation of proteins in oocytes treated with inhibitor. Treatment of oocytes with potent proteasomal inhibitor MG132 leads to a similar phenotype as were published (*Josefsberg 2000, Chmelikova 2004*). Proteasome is essential for oocyte maturation and we focused on degradation of cyclin B1 that is regulatory subunit of MPF and its degradation in meiotic cycle goes via ubiquitin degradation system (*Glotzer 1995*). Our result from immunoblot analysis shows a significant decrease in monoubiquitin pool in eggs treated with UCH-L1 specific inhibitor (Fig. 5b-A38). In summary, a proteomic approach coupled with protein verification study revealed an essential role of UCH-L1 in the completion of the first meiosis and its transition to anaphase.

The biological function/relevance between UCH-L1 and its homologue UCH-L3 is unclear, but the functional/structural differences might lead into differences in function or regulation and will be important aim of our further research.

## 4.5   Role of UCH-L1 in antipolyspermy defence of mammalian eggs.

Susor A, Jelinkova L, Pavlok A, Lopatarova M, Kubelka M

**Abstract**

Protein degradation is essential for oogenesis and embryogenesis. The ubiquitin-proteasome system regulates many cellular processes via the rapid degradation of specific proteins. Ubiquitin carboxylterminal hydrolase-L1 (UCH-L1) is exclusively expressed in neurons, testis, ovary, and placenta. The recently published data show that insufficiency in UCH-L1 activity is coupled with polyspermy. However, the functional role of UCH-L1 in this process remains unknown. Here, we report that the expression pattern and localization of UCH-L1 is similar on pig and mouse egg. Using two UCH-L1 specific inhibitors we obtained a high polyspermic rate after IVF of bovine eggs. To further investigate the antipolyspermic mechanism of UCH-L1 in eggs, we analysed the migration of Cortical Granules (CG) in UCH-L1-deficient eggs. Our results documented that in UCH-L1-deficient eggs CGs pattern does not changing from GV stage to MII, as it does control group. These results suggest that formation of oligomeres of UCH-L1 is involved in cytoskeletal reorganization and as such in inhibition of physiological function of CGs in antipolyspermy defence.

## Introduction

The Ubiquitin C-terminal hydrolase-L1 (UCH-L1) is a member of the family of deubiquitinating enzymes belonging to the proteasome system. The ubiquitin-proteasome system regulates many cellular processes via specific protein degradation (*Etlinger 1989, Muller 1995, Sutovsky 2003*). Our proteomic analysis of porcine oocytes demonstrated that UCH-L1 was one of the most abundant oocyte proteins (*Susor 2007*). It was also suggested that UCHL-1 plays an important role in apoptosis. UCH-L1 was described as a proapoptotic enzyme that stabilizes p53 in somatic cells (*Yu 2008*). On the other hand, in the *gad* mouse (*Saigoh 1999*), the lack of functional UCH-L1 results in the absence of a physiological apoptotic wave in testes that is important for maintenance of spermatozoa development (*Kwon 2005, Wang 2006*).

In our recent research we focus on the role of UCH-L1 in bovine eggs in order to expand current understandings of physiology and pathology of fertilization in mammals, in particular in polyspermy defence.

## Methods

To further investigate the role of UCH-L1 in the oocyte, we analysed the oocytes by SDS PAGE, Western blotting, immunocytochemistry/ confocal microscopy, microinjection of *in vitro* synthesized mRNA, for UCH-L1 (Fig. S1), and *in vitro* fertilization (IVF).

To study the role of UCH-L1 in the process of fertilisation on bovine model we used two specific inhibitors of this enzyme, (UCH-L1 inhibitor/ C30- isatin O-acyl oxime derivate - *Liu 2003;* #16-3-Amino-2-keto-7H-thieno[2,3-b]pyridin-6-one derivate- *Mermerian, 2007*).

## Results

### Localization of UCH-L1

To study the precise localization of UCH-L1 in the oocytes we have microinjected the porcine cRNA (in vitro synthetized mRNA from cDNA template) of UCH-L1 that was fused with enhanced green florescent protein (EGFP-UCH-L1). For generation of UCH-L1 cRNA we used porcine UCH-L1 (clone:OVR010079H10 from Dr. Uenishi H) sequence highly homologous with bovine sequence and we detected the identical localization pattern as in porcine oocyte (Fig. S2). Figure 1 shows the sub-cortical localization of  EGFP-UCH-L1 in GV stage and in fully matured bovine oocyte (MII stage). We verified the localization of fusion protein by the immunocytochemistry with specific anti-UCHL-1 antibody (Fig. 2), which shows similar pattern.
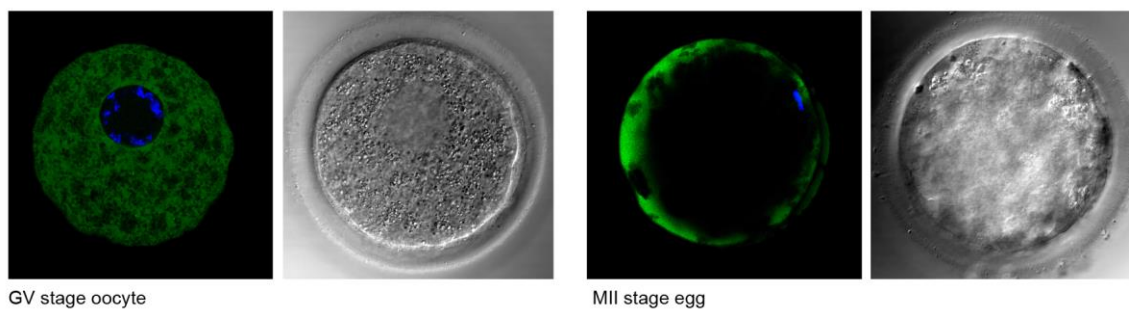
GV stage oocyte                    MII stage egg

**Fig.1:** Confocal images of bovine oocytes microinjected with cRNA for pig EGFP-UCH-L1.
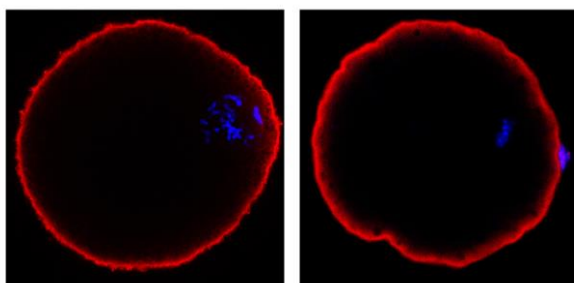


**Fig. 2:** Native UCH-L1 protein was visualised with polyclonal antibody (Chemicon) in GV and MII stage of bovine oocytes.

Inhibition of UCHL-1 with chemical inhibitors in bovine oocytes

The role of UCHL-1 in fertilization was investigated with specific UCH-L1 inhibitors (C30, #16). Eggs treated with 20uM specific inhibitors for 24hrs show more than 3 fold higher rate of polyspermy after IVF (Fig. 3). In addition the level of ubiquitinated proteins and monoubiquitin was detected by immunoblot analysis. The level of ubiquitinated proteins and monoubiquitin in bovine eggs treated with inhibitors was increased in comparison with control (Fig. 4) or recently published results from porcine oocytes (*Susor 2008*).

The amount of monoubiquitin after treatment of oocytes with UCH-L1 specific inhibitors is slightly elevated and this might suggest that level of monoubiquitin is substituted with *de novo* synthesis from stored mRNA, as egg is deficient in ubiquitin. On the other hand the level of ubiquitinated proteins increased in comparison with control (Fig. 4). Fig. 5 suggesting increased formation of UCH-L1 oligomers in presence of inhibitors.
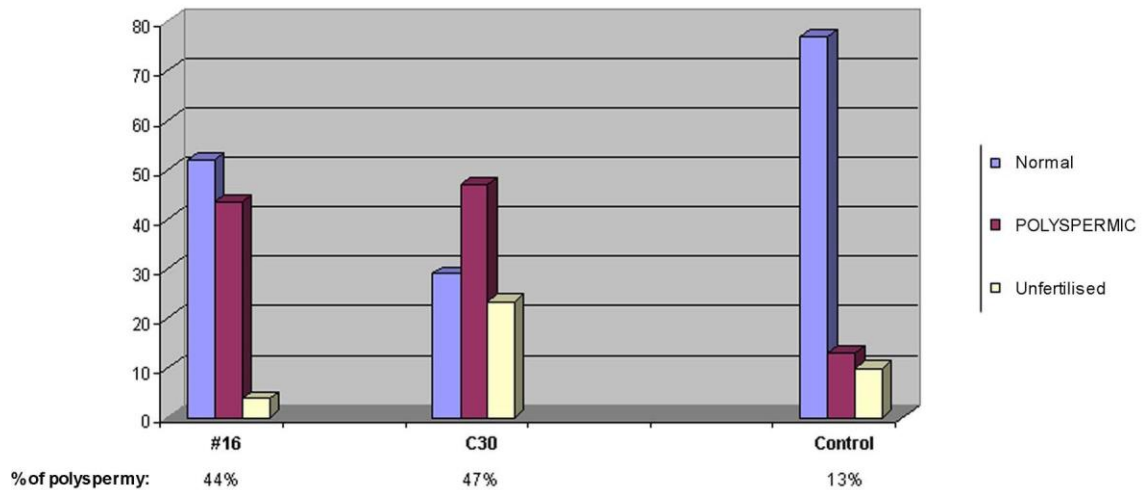
%of polyspermy:    44%           47%                   13%

**Fig. 3:** Evaluation of effect on spermatozoa-egg penetration ability was used 20μM concentration of UCH-L1 specific inhibitors. IVF was done after 24hr inhibitors treatment during oocyte maturation.
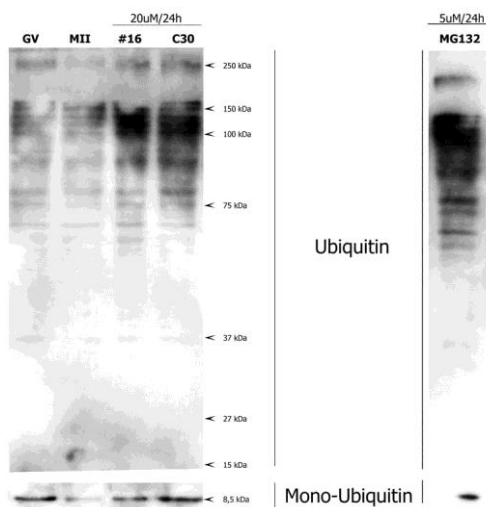


**Fig. 4:** Western blot analysis of monoubiquitin pool and ubiquitination of proteins in oocytes, a specific monoclonal ubiquitin antibody (Santa Cruz) was used. Oocytes were treated with UCH-L1 inhibitors and proteasomal inhibitor MG132.

**Fig. 5:** Using a polyclonal anti UCH-L1 antibody were visualized forms of UCH-L1 in oocytes. In presence of UCH-L1 found a increasing formation of oligomeric form of UCH-L1.
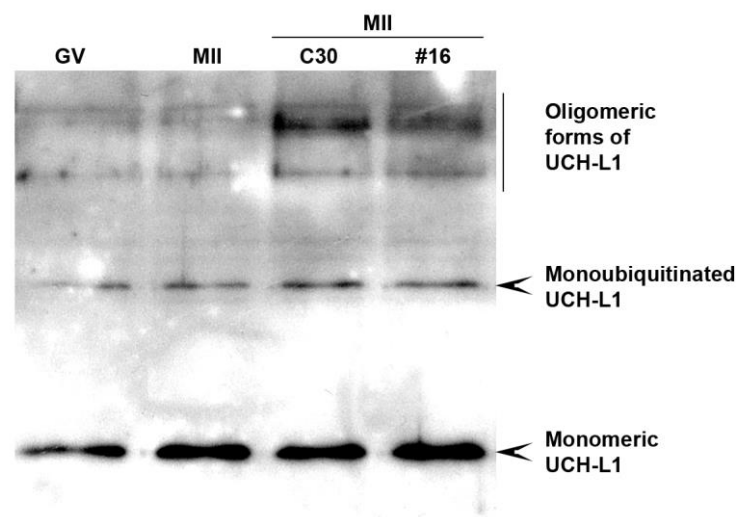
Cortical granules migration in UCHL-1 inhibited Eggs

To study important marker of proper fertilizable MII stage oocytes, we focused on the distribution of the cortical granules. Interestingly we have found that cortical granules (CGs) migration is influenced in UCH-L1 inhibitors treated oocytes. CGs migrates during meiosis to the cortex of oocytes and show similar pattern as was observed in GV stage (Fig. 6). In control groups the localization of CGs is uniformly cortical in MII stage oocyte however in inhibitors treated MII oocytes the pattern remains similar as it is in freshly isolated GV oocytes. We used classification from Connors (*1998*) of CGs migration as oocyte like, intermediate and egg like (Table 1).

| | | CGs status (%) | | |
|---|---|---|---|---|
| | n | Oocyte like | Intermediate | Egg like |
| GV | 10 | 10 (100) | 0 (0) | 0 (0) |
| MII | 12 | 1 (8) | 3 (25) | 8 (67) |
| 20µM C30 | 18 | 14 (78) | 1 (5) | 3 (17) |
| 20µM #16 | 26 | 22 (84) | 2 (8) | 2 (8) |

**Table 1:** Cortical granules classification in bovine oocytes. Results are form two independent experiments.
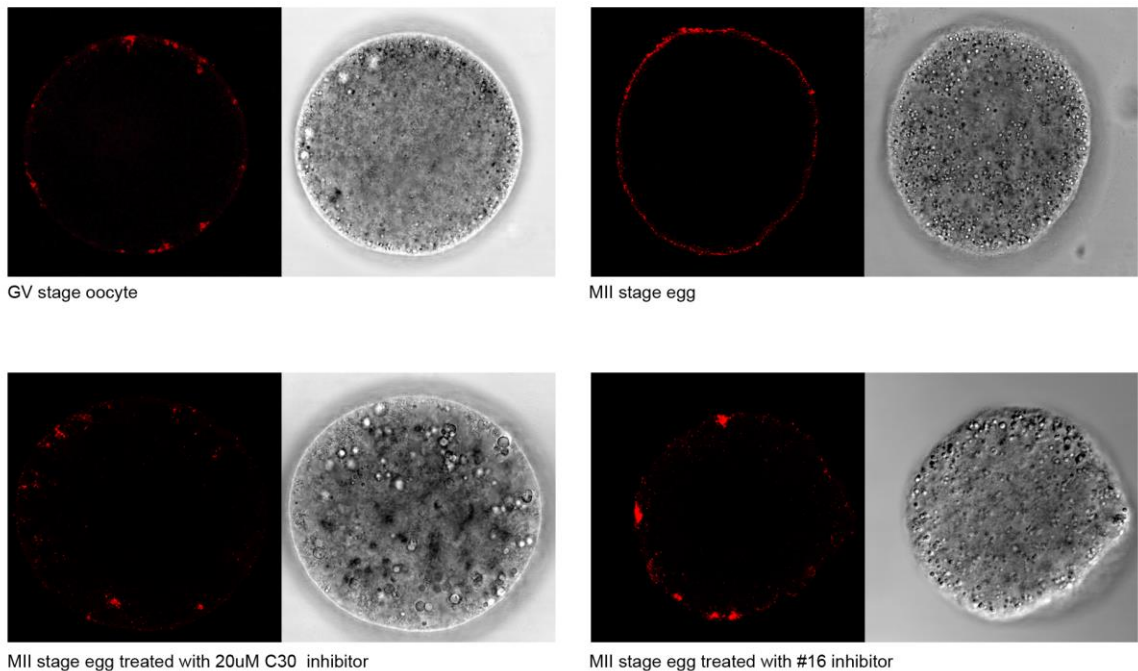


**Fig. 6:** Visualisation of CGs in bovine oocytes using *Lens Culinaris Agglutinin* -Rhodamine (Vector Laboratories). Oocytes were treated with 20µM concentration of UCH-L1 specific inhibitors for 24hrs, in control a DMSO was used as inhibitor vehicle.

**Discussion**

In the present study, we have demonstrated that the UCH-L1 is involved in the regulation of fertilization of bovine eggs coupled with specific CGs migration. We also documented the subcortical localization of UCH-L1.

Using immunocytochemical staining we have shown subcortical localization of native UCH-L1 in oocyte during maturation. Similar results were obtained when EGFP-UCH-L1 cRNA has been expressed in the oocytes. The subcortical localization of this protein was previously described in mouse and pig (*Sekigucchi 2006, Sutovsky 2007*).

Successful nuclear progression through meiosis in bovine oocytes might be caused by fact that the monoubiquitin pool that is responsible for APC activity is not depleted in inhibitor treated oocytes (*Hershko 1999, Morgan 1999*) Inhibition of UCH-L1 in pig oocytes leads to decreased levels of monoubiquitin and consequently to MI block (*Susor 2007*).

Immunoblots show increasing level of UCH-L1 oligomers. Liu (*2002*) documented that UCH-L1 has two enzymatic functions; in monomeric form has weak hydrolytic activity, whereas in oligomeric state has function as ligase. This enzymatic functions could answer the question about regulation of ubiquitin in oocytes, where we see the increasing level of monoubiquitin pool and on the other hand the increasing amounts of ubiquitinated proteins in the presence of specific inhibitors during cultivation period. This finding is consistent with recently published results in neural cells (*Tan 2008*), where inhibition of UCH-L1 leads to increased ubiquitination of proteins. In oocytes treated with inhibitors the formation of UCH-L1 oligomers increases comparised with other UCH-L1 forms, this result might suggest that in the presence of inhibitors UCH-L1 ligase function is not impaired or even stimulated.

Our data show significant differences in spermatozoa penetration ability in eggs with inhibited UCH-L1. The higher penetration of egg by spermatozoa is in agreement with the results from *gad* (*gracial axonal dystrophy*, *Mukoyama 1989*) mice, in *gad* mice allele encodes a truncated Uch-l1 lacking a segment of 42 amino acids containing a catalytic residue (*Larsen 1996*). The *gad* mice, has impaired fertility with lower number of pubs per litter (*Yamazaky 1988*).

It has been reported that impaired function of UCH-L1 in mammalian eggs increase incidence of polyspermy (*Sekiguci 2006, Sutovsky 2007*). However, the effects of UCH-L1-specific inhibitors on several fertilization processes have not been systematically investigated. Recently it also been reported that inhibition of UCH-L1 has detrimental effect on embryonic development in mouse (*Yamazaki 1988, Sekiguci 2006*) and pig (*Sutovsky 2007*). Our IVF experiments show that inhibition of UCH-L1 increases polyspermy in bovine oocytes.

We suggest that impaired proteasomal activity; specifically through highly abundant ubiquitinated proteins in eggs with inhibited UCH-L1 is responsible for high polyspermy ratio in zygotes. Mechanism responsible for sperm egg fusion is proteasome dependent (*Sun 2004, Sutovsky 2004, Yi 2007*) and deubiquitination/ ubiquitination process is responsible for physiological function of cortical granules that is one of the most important factors for embryo developmental competence (*Membenek 2007*). Exocytosis of cortical granules in mammalian eggs is required to produce the zona pellucida block of polyspermy (*Hoodbhoy 1994, Ducibella 1996*). It has been observed that cortical granules migrate to the periphery of the oocyte during maturation (*Wang 1997, Connors 1998*). Inhibition of UCH-L1 blocks migration of CGs that is physiological process as oocyte progress through meiosis (*Connors 1998*). Migration of CGs is dependent on oocyte cytoskelet, specifically by microfilaments (*Sun 2001, Sun 2006*). While in control oocytes the CGs migrate to the cortex, in the eggs treated with inhibitor the CGs are not translocated and remain in the same position similar to GV stage and thus impaired the role of CGs.

Very little is known about role the of proteasome in cytoskelet. Most results come from somatic cells; Csizmadia (*2008*) have report that experimental proteasome inhibitor induced reorganization and relocation of non-ubiquinated actin microfilaments and microtubules. Burgess (*2004*) suggests that ubiquitin attached to Lys118 adopts one or a few conformers, stabilized by a small interaction with actin. Ardley (*2004*) have reported that in neural cells with mutation in UCH-L1 forms protein aggregates structured from tubulin, chaperone, ubiquitin and vimentin.

Yi (2008) has documented that proteasome is involved in regulation of cytoskelet of extracellular matrix. Our finding is first to date, showing that UPS is involved in the process responsible for migration of CGs in oocyte. Mutations in the ubiquitin C-terminal hydrolase gene (UCH, *Kaitna 2002*) in *C. elegans* cause embryonic lethality (*Kaitna 2002*). UCH mutant embryos fail to polarize the actin cytoskeleton properly, fail to segregate germline determinants and segregation of P-granules. In addition, they fail to assemble an intact cleavage furrow, which is in good agreement with our results showing that 100uM concentration of C30 causes defects in extrusion of polar body (Fig. S3).

Inhibition of UCH-L1 during oocyte *in vitro* maturation decrease developmental competence of zygotes, due to increasing number of penetrated spermatozoa per egg. Presently, it is not clear how the ubiquitin or ubiquitin related pathway is involved in regulation of cytoskeletal dynamics.

**<u>Conclusion</u>**

Our results suggest that impaired function of UCH-L1 correlates with reduced migration of CG to the cortex and thus lead to insufficient antipolyspermy defence. In another words, polyspermy in bovine oocytes is due to insufficient ZP-mediated polyspermy block in response to CG exocytosis.
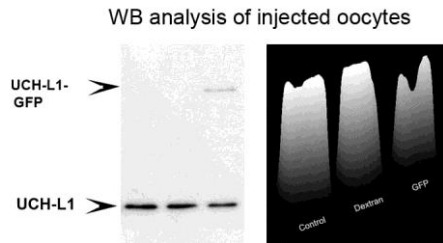
**Supplementary figures**



**Fig. S1:** Western blot and Image J analysis of oocytes microinjected with EGFP-UCH-L1 cRNA construct.
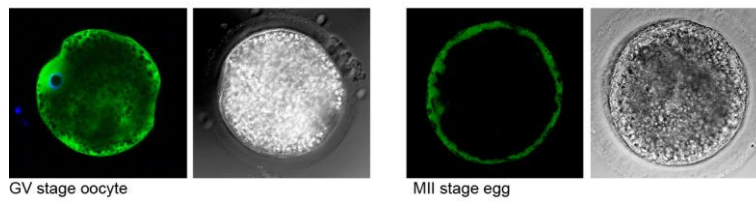


**Fig. S2:** Confocal images of porcine oocytes microinjected with cRNA for EGFP-UCH-L1.
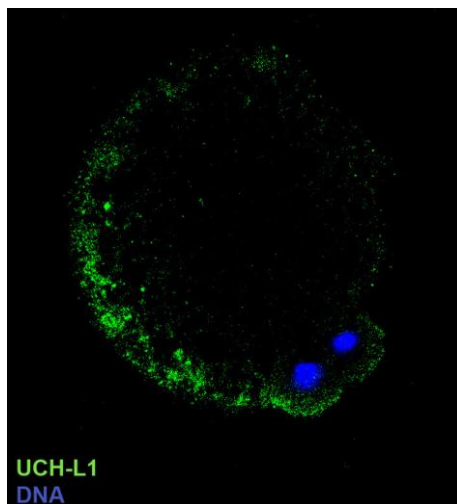
**Fig. S3:** Confocal image of cow egg treated with 100µM concentrations of UCH-L1 inhibitors stained for UCH-L1 and DNA. As mentioned in discussion, 30% of oocytes extrude two polar bodies with all chromatin content.

# 5 Conclusion

We analysed eucaryotic initiation translation factors that are responsible for protein synthesis in mammalian oocytes. We examined also the affinity of those factors to $^7$mCap Sepharose during *in vitro* maturation. The protocol for generation of porcine parthenotes was optimalised in our laboratory that serve us as a model for analysis of translation regulation in the early embryonic stage

Antibody microarray analysis was used to investigate the regulation of signalling pathways primarily during meiotic maturation of pig oocytes and subsequently in correlation with other species (bovine, frog and sea star).

On the basis of our proteomic studies, where was detected UCH-L1 as one of the most abundant proteins, whose expression changed during maturation of porcine oocytes. We have focused our further experiments on detailed study of UCH-L1 expression, location and function in mammalian oocytes.

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**ERK1/2 Map Kinase Metabolic Pathway is Responsible for Phosphorylation of Translation Initiation Factor eIF4E During In Vitro Maturation of Pig Oocytes.**

Ellederova Z, Cais O, Susor A, Uhlirova K, Kovarova H, Jelinkova L, Tomek W, Kubelka M.

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**Regulation of Cap-dependent Translation Initiation in the Early Stage Porcine Parthenotes.**

Susor A, Jelinkova L, Karabinova P, Torner H, Tomek W, Kovarova H, Kubelka M.

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**Pages A10-19**

**Antibody Microarray Analyses of Signal Transduction Protein Expression and Phosphorylation During Porcine Oocyte Maturation.**

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**Pages A20-A31**

**Proteomic Analysis of Porcine Oocytes During In Vitro Maturation Reveals Essential Role for the Ubiquitin C-terminal Hydrolase-L1.**

Susor A, Ellederova Z, Jelinkova L, Halada P, Kavan D, Kubelka M and Kovarova H.

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