REGULATION OF AUTOPHAGY BY NACHT, LRR & PYD DOMAINS-CONTAINING PROTEIN 5 (NLRP5) IN PREIMPLANTATION EMBRYOS

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Previous studies have shown that NACHT, leucine rich repeat and PYD domain containing 5 (Nlrp5) deficient embryos fail to develop beyond the two-cell stage. Despite this strong phenotype, little is known of the function of NALP5 and the pathways affected by its deficiency. We showed that Nlrp5 deficient oocytes and embryos exhibit a decrease in caspase activity. In addition, the kinetics of NF-κB-p65 nuclear translocation is altered, which leads to negative downstream effects. Autophagy is known to be regulated downstream of NF-κB and is a key event during the oocyte-to-embryo transition. We observed defective execution of autophagy in Nlrp5 deficient two-cells evidenced by absence of autophagosome formation and abnormal lysosomal maturation. We found that inactivating autophagy leads to an accumulation of lipid droplets and embryos lacking Nlrp5 exhibit this accumulation. Thus, NALP5 may be an integral component responsible for autophagy mediated lipid metabolism, which when compromised causes developmental arrest.
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Table of Contents

Acknowledgments .................................................................................................................................. iii

Table of Contents ................................................................................................................................... iv

List of Tables ........................................................................................................................................ vii

List of Figures ....................................................................................................................................... viii

List of Abbreviations .............................................................................................................................. ix

1.1 Infertility ....................................................................................................................................................... 2
1.2 Mammalian Embryonic Development ................................................................................................ 3
1.3 Role of Maternal-Effect Genes ............................................................................................................... 6
1.4 Nlrp5/NALP5 ............................................................................................................................................... 7
  1.4.1 Structural Analysis ............................................................................................................................................. 7
  1.4.2 Spatiotemporal Expression Profile ............................................................................................................. 9
  1.4.3 Phenotype Triggered by Nlrp5 Deficiency ............................................................................................ 10
  1.4.4 Subcortical Maternal Complex ................................................................................................................... 11
  1.4.5 NLRP Family Members .......................................................................................................................... 12
1.5 Dual Role of Caspases in Cell Fate ..................................................................................................... 13
1.6 Nuclear Factor-κB (NF-κB): A key player in development ........................................................................ 18
1.7 Autophagy .................................................................................................................................................. 21
1.8 Programmed Necrosis: Necroptosis ................................................................................................. 26
1.9 Hypothesis and Aims ............................................................................................................................. 28

Chapter 2 Materials and Methods ........................................................................................................ 29
  2.1 Mouse Husbandry ................................................................................................................................... 30
  2.2 Nlrp5 Genotyping .................................................................................................................................... 30
  2.3 MII Oocyte Retrieval .................................................................................................................................. 31
  2.4 Embryo collection .................................................................................................................................... 31
  2.5 Immunocytochemistry ............................................................................................................................. 31
  2.6 Visualization of caspases in intact embryos ......................................................................................... 34
  2.7 Inhibiting caspases in intact embryos ................................................................................................. 34
2.7.1 Progression of embryo development .......................................................... 34
2.7.2 NF-κB translocation.................................................................................... 35
2.8 Lysotracker® Red and Mitotracker® Green Staining .................................... 35
2.10 Treatments........................................................................................................ 36
2.10.1 Necrostatin-1 ........................................................................................... 36
2.10.2 Rapamycin ................................................................................................... 36
2.10.3 Analysis of NALP5 cleavage products ...................................................... 37
2.11 Western blot analysis .................................................................................... 37
2.12 Quantitative RT-PCR .................................................................................. 38
2.13 Statistical Analysis ....................................................................................... 39

Chapter 3 Results .................................................................................................. 41
3.1 Nlrp5 disruption affects D1.5 (two-cell) embryo competence ....................... 42
3.2 Nlrp5 deficient MII oocytes and early preimplantation embryos exhibit decrease in caspase activity ................................................................. 44
3.3 Inhibition of caspases disrupts early mammalian development .................... 47
3.4 Total and phosphorylated NF-κB subunit RelA/p65 is altered in Nlrp5 deficient zygotes ............................................................................................................. 49
3.5 zVAD and its effects on NF-κB subunit RelA (p65) .............................................. 52
3.6 Expression of NF-κB target genes in wildtype T and Nlrp5 tm/tm late (25-26 hr) zygotes ............................................................................................................. 56
3.7 Inhibition of necroptosis in Nlrp5 tm/tm embryos does not prevent developmental arrest ............................................................................................................. 59
3.8 Autophagy is compromised in Nlrp5 deficient preimplantation embryos ........ 61
3.8.1 Nlrp5 tm/tm two-cell embryos are autophagy deficient .................................. 61
3.8.2 Beclin-1 expression in Nlrp5 tm/tm two-cell embryos is unaltered .................. 61
3.8.3 Lysosomal content is decreased in Nlrp5 tm/tm two-cell embryos ................. 62
3.8.4 Lamp1 is increased in Nlrp5 tm/tm two-cell embryos ..................................... 62
3.8.5 p62/Sequestosome 1 expression in Nlrp5 tm/tm two-cell embryos is unaltered 62
3.9 Transcriptional regulation of autophagy related genes .................................. 67
3.10 NALP5 protein as a substrate of autophagy .................................................. 70
3.11 LC3, total and phospho-mTOR analysis of Nlrp5 deficient oocytes and embryos ....... 73
3.12 Nlrp5 tm/tm embryos display a decrease in total levels of mitochondria ........ 75
3.13 Ubiquitin-proteasome system is unaltered ...................................................... 77
3.14 Lipid clearance is compromised in Nlrp5<sup>tm/tm</sup> two-cell embryos ........................................ 79
3.15 Rapamycin induction of autophagy in Nlrp5<sup>tm/tm</sup> embryos does not prevent developmental arrest .................................................................................................................. 82
3.16 NALP5 expression in human oocytes ............................................................................................ 84

Chapter 4 Discussion .................................................................................................................................. 87
4.1 Caspases in Nlrp5 deficient mice ....................................................................................................... 89
4.2 Altered NF-κB activation in Nlrp5 deficient embryos ...................................................................... 90
4.3 Nlrp5 deficient mice display autophagic defects in embryos ......................................................... 92
4.4 NALP5 accumulation in aged human MII oocytes ......................................................................... 97
4.5 Future Directions ................................................................................................................................ 97

References Cites .........................................................................................................................................101
List of Tables

Table 1: Primary Antibodies for ICCH ................................................................. 33
Table 2: Secondary Antibodies for ICCH ............................................................... 33
Table 3: Primer Sequences for quantitative real-time PCR......................................................... 39
List of Figures

Figure 1: Stages of Mammalian Preimplantation Development ................................................................. 4
Figure 2: Mouse and Human NALP5 protein structure ........................................................................... 9
Figure 3: Non-death signaling caspase functions in mice ....................................................................... 15
Figure 4: Caspase activating complex - Inflammasome ........................................................................... 16
Figure 5: NF-κB Protein Structure ............................................................................................................. 20
Figure 6: Autophagy and Process .............................................................................................................. 26
Figure 7: Number of two-cells from Nlrp5 WT versus Nlrp5<sup>tm/tm</sup> females ......................................... 43
Figure 8: Caspase activity is reduced in Nlrp5<sup>tm/tm</sup> MII oocytes and early preimplantation embryos .......... 45
Figure 9: Effect of caspase inhibitor, zVAD-fmk on preimplantation development of mouse embryos .... 48
Figure 10: Cytoplasmic and nuclear expression of RelA/p65 in early and late WT versus Nlrp5<sup>tm/tm</sup> zygotes ... 52
Figure 11: Expression of phosphorylated RelA/p65 protein in zVAD and control treated mouse preimplantation embryos ................................................................................................................. 55
Figure 12: Relative expression of NF-κB targets in Nlrp5 WT and tm/tm late zygotes ................................. 58
Figure 13: Necrostatin-1 treated Nlrp5<sup>tm/tm</sup> early embryos do not progress beyond two-cell development .... 60
Figure 14: Defective autophagy in Nlrp5 deficient two-cell embryos ................................................................. 66
Figure 15: Relative expression of Autophagy related genes in Nlrp5 WT and tm/tm late zygotes ................. 69
Figure 16: NLRP5 is an autophagy substrate .............................................................................................. 72
Figure 17: Expression of autophagy related markers in oocytes and preimplantation embryos .................. 74
Figure 18: Total mitochondrial levels decreased in Nlrp5 deficient two-cell embryos .............................. 76
Figure 19: Ubiquitin content in Nlrp5 deficient two-cell embryos remains unaltered ............................... 78
Figure 20: Lipid droplet accumulation in Nlrp5 deficient two-cell embryos ............................................... 81
Figure 21: Rapamycin treated Nlrp5<sup>tm/tm</sup> early embryos do not progress beyond two-cell development ... 83
Figure 22: Increase in NLRP5 expression in aged human oocytes ............................................................... 86
## List of Abbreviations

<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation</th>
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<tr>
<td>Autoimmune polyendocrine syndrome 1</td>
<td>APS-1</td>
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<tr>
<td>Bovine serum albumin</td>
<td>BSA</td>
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<tr>
<td>Dimethyl sulfoxide</td>
<td>DMSO</td>
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<tr>
<td>Endoplasmic Reticulum</td>
<td>ER</td>
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<tr>
<td>Fluorescein isothiocyanate</td>
<td>FITC</td>
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<td>Human chorionic gonadotropin</td>
<td>hCG</td>
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<td>Inner cell mass</td>
<td>ICM</td>
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<tr>
<td>Kilobases</td>
<td>kb</td>
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<tr>
<td>Kilodaltons</td>
<td>kDa</td>
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<tr>
<td>Leucine-Rich Repeats</td>
<td>LRRs</td>
</tr>
<tr>
<td>Mammalian target of rapamycin</td>
<td>mTOR</td>
</tr>
<tr>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>Microtubule-associated protein 1A/1B-light chain 3</td>
<td>LC3</td>
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<td>Modified human tubal fluid</td>
<td>mHTF</td>
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<tr>
<td>Phosphate buffered saline</td>
<td>PBS</td>
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<tr>
<td>Polymerase chain reaction</td>
<td>PCR</td>
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<tr>
<td>Pregnant mare serum gonadotropin</td>
<td>PMSG</td>
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<tr>
<td>Pyrrolidine dithiocarbamate</td>
<td>PDTC</td>
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<tr>
<td>Reverse transcriptase chain reaction</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Ribonucleic acid</td>
<td>RNA</td>
</tr>
<tr>
<td>Tris-buffered saline</td>
<td>TBS</td>
</tr>
<tr>
<td>Transcription-requiring complex</td>
<td>TRC</td>
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<td>Wildtype</td>
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Chapter 1
Introduction
1.1 Infertility

It is estimated that infertility affects approximately 12-15% of couples of reproductive age worldwide (Palermo, Neri et al. 2012). Social trends in Canada have led to women postponing childbearing to a later age, often causing couples to seek medical treatment for infertility. As such, the higher prevalence of female infertility can be related to reproductive aging, combined with an increasing demand by older, less fecund women to bear children.

Millions of oocytes are harvested and cultured in *in vitro* fertilization laboratories every year with the intention of offering assisted reproductive technology (ART) to infertile couples (Mandelbaum 2000). However, ART is not always effective and has numerous factors influencing its outcomes (Winston and Handyside 1993). The progressive decline of female fecundity by the time a woman reaches her mid-30s suggests that the reproductive aging process is a major contributing factor for female infertility. This is supported by the Canadian ART Registry, which in 2007 reported a 38% clinical pregnancy rate in women under the age of 35 years, a 27% rate in women aged 35-39, and an 11% rate for women over 40 (CDC 2012). Although numerous factors may contribute to the age-related decline in reproductive potential, oocyte quality plays a predominant role (ie: aneuploidy, mitochondrial dysfunction etc.). This is in line with a number of previous reports showing that the risk of chromosomal anomalies in the embryos increases with age (Pellestor, Anahory et al. 2005; Pan, Ma et al. 2008). Additionally, women greater than 40 years of age undergoing in vitro fertilization (IVF) with oocytes received from young donors have similar pregnancy rates as young women. For non-donor IVF, live birth rates decline dramatically with age going from about 45% at age 25 to less than 5% at age 44, but can be restored in old patients by donation of oocytes from young donors (CDC 2012b). This implies that it is primarily responsible for decreased fecundity with aging.

A huge subset of failed pregnancies is believed to be attributable to the lack of high quality embryos. Repeated IVF failure in female patients is often attributed to poor quality oocytes (Margalioth, Ben-Chetrit et al. 2006). It is possible that these patients may have defective maternal factors that result in the inability to maintain early development. However, the exact mechanism by which these embryos fail to implant and develop normally has yet to be elucidated. While no known medical treatment options exist for patients with poor quality
oocytes, studies of ooplasmic transfers point to factors found in the cytoplasm as being essential for embryo development. It has been shown that injecting the ooplasm from healthy eggs improves the proficiency of embryo development emphasizing that a decrease in oocyte quality largely contributes to infertility (Muggleton-Harris, Whittingham et al. 1982). Therefore, supplementing normal cytoplasmic protein into oocytes of infertile women might restore early development and successful pregnancy.

Women have a finite number of germ cells and follicles available for a limited period during their lifetime therefore; maximizing their ability to conceive is important. New legislation in regards to ART does not allow for fetal genome alteration, therefore, ooplasmic transfers cannot be used in clinical practice. In addition, currently there are no methods for screening or treatments available for patients with poor quality oocytes. For these reasons it is essential to characterize the different factors that contribute to female infertility including molecular defects associated with poor quality oocytes to aid in the advancement of current ART methods and development of therapeutic treatments for infertile patients.

1.2 Mammalian Embryonic Development

Mammalian preimplantation development begins from the moment of fertilization whereby the sperm and the egg unite to initiate a series of cellular events in the production of a multicellular blastocyst. Prior to fertilization, oocytes enlarge with accumulation of materials for early development, but remain arrested at prophase of meiosis for most of their growth (Stitzel and Seydoux 2007). During ovulation, meiotic arrest becomes alleviated and meiotic division progresses to metaphase II. Transcription then stops, translation of mRNA is reduced and the oocyte has a sufficient supply of products stored for proper preimplantation development should fertilization occur (Kupker, Diedrich et al. 1998).

Egg activation, triggered by fertilization, completes the transformation to zygote (1-cell embryo) by signaling the completion of meiosis, the formation of the pronuclei and the first mitotic division (Schultz 2002). Prior to division, the male and female pronuclei move toward the center of the zygote, but do not fuse. During the migration DNA replication occurs, the nuclear membranes break down and the chromosomes assemble on the mitotic spindle (Nagy 2003; Wang and Dey 2006). Soon after the embryo undergoes several rounds of mitotic division
with the first cleavage producing two identical cells. The embryo will continue dividing asynchronously to produce 4-cells, 8-cells and so on, ultimately compacting to form a tightly organized cell mass called a morula. At the late morula stage, the embryo enters the uterine lumen and differentiates into a blastocyst that contains a fluid-filled blastocoel cavity with two distinct populations. This includes the inner cell mass, which will gives rise to the embryo proper and the trophectoderm that will give rise to extraembryonic tissue (Nagy 2003). Implantation into the uterine wall marks the end of preimplantation development, occurring on day 4 in mice and during days 7-9 in humans (Wang and Dey 2006).

**Figure 1: Stages of Mammalian Preimplantation Development.** During fertilization the oocyte fuses with sperm to create a zygote. The 1-cell embryo continues to asynchronously divide till the 8-cell stage followed by blastocyst formation. General gene expression patterns are observed with two main waves corresponding to the zygotic genome activation (ZGA) and a second mid-preimplantation gene activation (MGA) that contributes to blastocyst formation.
During oogenesis, the growing mammalian oocyte synthesizes and accumulates proteins and mRNAs that constitute the maternal contribution towards preimplantation development. The contribution of maternal products both supports and directs early development following fertilization, up to the time when the embryonic genome is activated (Nothias, Majumder et al. 1995; Li, Zheng et al. 2010). The maternal to zygotic transition (MZT) is the first major developmental transition and is required to allow for proper embryo development. This period is defined by two important functions: first, the elimination of maternally derived oocyte specific transcripts and second, the transcription of the zygotic genome known as zygotic genome activation (ZGA) (Tadros and Lipshitz 2009) (Figure 1).

The first phase of transition from maternal to zygotic control involves the degradation of maternal mRNA. Oocyte maturation and activation stimulate mRNA degradation. This process is selective and preferentially removes those transcripts required for maturation. By the 2-cell stage, about 90% of mRNAs become degraded and this may be one mechanism by which the developmental switch from oocyte to embryo occurs (Nothias, Majumder et al. 1995). The next phase constitutes the ZGA, which is involved in replacing maternal transcripts that are common to both the oocyte and early embryo, as well as generating novel ones that are not originally expressed in the oocyte (Schultz 1993). During this transition, the developmental program controlled initially by maternal factors gradually changes to the control of zygotic factors due to the expression of new genes. In mice, the ZGA occurs in three successive waves of zygotic gene expression (Li, Zheng et al. 2010). First, the minor ZGA is initiated at the late 1-cell stage with very weak transcriptional activity and results in synthesis of a small set of polypeptides that transiently increase at the 2-cell stage. Next, the major ZGA occurs at the 2-cell stage where a burst of transcriptional and translational activity results (Tsukamoto, Kuma et al. 2008) of novel genes not expressed in oocytes. In humans, this transition occurs by the 4- to 8-cell stages (Schultz 2002). Finally, the mid-preimplantation gene activation (MGA) peaks at the 8-cell stage, initiating morula compaction and blastocyst cavitation (Wang and Dey 2006). Proper development during early cleavage relies on this transition to prevent developmental block, known as 2-cell block due to ZGA delay or inactivation. This suggests that proper development during early cleavage stages relies on zygotic gene transcription. Developmental arrest is also a consequence of maternal lethal effect genes and will be described in Section 1.3.
1.3 Role of Maternal-Effect Genes

Maternal-effect genes encode maternal components that govern preimplantation development. There is an unequal burden on mammalian gametes to ensure the successful initiation of development (Han, Chung et al. 2005). The metabolic and homeostatic functions of the early embryo exclusively rely on maternally inherited macromolecules for survival until the activation of its own genome at cleavage stage development. Maternal factors include mRNAs, proteins, and lipids that are accumulated in the oocyte prior to fertilization and are important to the early embryo for successful development (Li, Zheng et al. 2010). One of the first roles of maternal factors is the processing of the male genome, a necessity for its participation during embryogenesis (Li, Zheng et al. 2010). Other roles involve removal of maternal RNA and proteins (Mtango, Potireddy et al. 2008), a process required for proper progression and activation of the embryonic genome.

Maternal products, actively accumulated during oogenesis, but utilized during embryogenesis represent genes that may be necessary for embryo development as disruption of their function leads to lethality known as maternal lethal effect genes. Nlrp5 was the first identified mammalian oocyte-specific maternal-effect gene that shows embryonic lethality at the 2-cell developmental stage (Tong and Nelson 1999; Tong, Gold et al. 2000). In addition to Nlrp5, there several maternal-effect genes have been identified recently in mammals and their importance in embryonic development has also been demonstrated through knockout and knockdown models. Functionally, many genes belonging to this category encode proteins involved in transcriptional regulation of gene expression, chromatin structure, spindle assembly and cell cycle mediators. These genes include: Zar1 (Wu, Viveiros et al. 2003), Nlrp2 (Peng, Chang et al. 2012), Floped (Ooep) (Li, Baibakov et al. 2008), Padi6 (Esposito, Vitale et al. 2007), Tle6 (Li, Baibakov et al. 2008), Filia (Zheng and Dean 2009), Hsf1 (Christians, Davis et al. 2000), Npm2 (Tschopp, Martinon et al. 2003), Atg5 (Tsukamoto, Kuma et al. 2008). A common phenotype among mouse embryos with mutations in some of these genes is arrested progression during cleavage-stage embryogenesis (ie: 4-cell arrest in Atg5 knockouts) (Li, Zheng et al. 2010). This indicates that transcription and translation of embryonic genes becomes a requirement to replace the degraded maternal factors necessary for maintaining maternal and
embryonic control over development. Elucidation of the nature of these maternal genes and their functions should be of value to both basic and clinical reproductive biology.

1.4 Nlrp5/NALP5

As mentioned previously, a number of maternal effect genes have been studied over the years to understand the molecular mechanisms governing oocyte and early embryo development. These genes encode proteins and transcripts that are essential for supporting the preimplantation embryo and a defect may lead to embryonic lethality. Nlrp5 (NLR family, pyrin domain containing 5), previously known as Mater (Maternal antigen that embryos require), was a gene initially discovered as an oocyte-specific antigen associated with autoimmune ovarian failure in neonatally thymectomized female mice (Tong and Nelson 1999). This syndrome acts as a model for human autoimmune premature ovarian insufficiency (POI), which is defined as irregular to absent ovarian function. Although Nlrp5 was one of the earliest identified maternal-effect genes in mammals, its function in oogenesis and preimplantation development is unknown. NALP5 protein may be a key player in POI as about 51% of female patients with autoimmune polyendocrine syndrome 1 (APS-1) with hypogonadism develop antibodies against NALP5 (Eisenbarth 2008). Additionally, NALP5 supports early embryonic development in mice determining female fertility. Therefore, characterization of the Nlrp5 gene and its protein will provide insight into its potential role in POI and perhaps unexplained infertility in women.

1.4.1 Structural Analysis

The NLRPs are a subfamily of the CATERPILLER (CAspase-recruitment domain (CARD) Transcription Enhancer, R (purine)-binding, Pyrin, Lots of LEucine Repeats) family of proteins that are comprised of a nucleotide-binding domain and a leucine-rich region (Tschopp, Martinon et al. 2003). The NLRP gene family constitutes of 14 members in humans and about 20 homologues in mice. Members of the NLRP family of proteins are characterized by a tripartite structure consisting of an N-terminal pyrin (PYD), followed by a nucleotide binding oligomerization (NACHT) and C-terminal leucine rich repeats (LRR) domains, each with their own complex interactions with other proteins containing similar domains (Figure 2) (Tschopp, Martinon et al. 2003; Martinon, Gaide et al. 2007). The amino-terminal PYD domain plays a role in protein-protein interactions, which are thought to function in inflammatory, apoptotic and
nuclear factor-κB (NF-κB) signaling pathways. The NACHT (neuronal apoptosis inhibitory protein. CIIA; HET-E; and terminal protein 1) domain consists of NTPase function and predicted to bind to ATP/GTP. The carboxy-terminal LRR domain is known for its involvement in protein-protein interactions that regulate different cellular functions (Tschopp, Martinon et al. 2003; Horikawa, Kirkman et al. 2005; Martinon, Gaide et al. 2007). As described, these domains are involved in complex interactions with other proteins, including caspases (Martinon, Gaide et al. 2007). Although the majority of NALP proteins function as adaptors involved in the regulation of inflammation and apoptosis, the precise mode of action for NALP5 remains undetermined.

Although discovered ten years ago, there is relatively little information regarding the function of \textit{Nlrp5}, including what pathways are affected by its deficiency. \textit{Nlrp5} is a single copy gene expressed exclusively in human and mouse oocytes. At the genomic level, it spans 32 kb DNA at the proximal end of chromosome 7 in mice and is composed of 15 exons and 14 introns with a 3.75kb transcript. The primary structure of NALP5 protein in mice has 1111 amino acids with a molecular mass of 125 kDa (Tong, Nelson et al. 2000). In humans, the transcript differs in size at 4.2kb and the protein is composed of 1200 amino acids with a molecular mass of 134 kDa (Tong, Bondy et al. 2002). Both human and mouse genes are conserved sharing a 67% homology in their cDNA nucleotide sequence, while their polypeptide chain shares a 53% similar identity of amino acids. Although they share a number of similar features in their protein structure, mouse NALP5 contains an uncharacterized amino-terminal lacking the PYD domain. Interestingly, the human NALP5 contains a nuclear localization signal present near the NACHT domain, which is not detected in mouse (Tong, Nelson et al. 2000).
1.4.2 Spatiotemporal Expression Profile

Previous expression studies in both mice and human gametes indicate that NALP5 expression is limited to oocytes. Its distribution in tissue and temporal pattern of expression is conserved across the mammalian species in which it has been studied (i.e.: mouse, human, bovine and macaques). In mice, \( \textit{Nlrp5} \) is transcribed and accumulates as oogenesis proceeds. \( \textit{Nlrp5} \) mRNA expression is highest in growing oocytes at the germinal vesicle stage and begins to degrade upon oocyte maturation and ovulation. By the two-cell stage it dramatically declines and becomes undetectable in preimplantation embryos (Tong, Gold et al. 2004). Since \( \textit{Nlrp5} \) transcripts seem to be degraded in the process of the ZGA, this suggests that NALP5 protein is maternally derived. NALP5 protein expression is detected in early growing oocytes and is present at all stages of preimplantation embryos. Although the mRNA is degraded by the onset of ZGA, the protein is present until the late blastocyst stage, suggesting a physiological role beyond the first embryonic cleavage. NALP5 protein is expressed sub-cortically in oocytes and embryos. It localizes to the cytoplasm, mitochondria, nucleoli and close to the nuclear pore complexes of mouse oocytes (Tong, Gold et al. 2004).

Analysis of MII oocytes from old mice at 42-45 weeks of age compared to oocytes from young mice at 5-6 weeks of age showed a two-fold decrease in \( \textit{Nlrp5} \) transcript expression (Hamatani, Falco et al. 2004). This finding suggests that the expression of \( \textit{Nlrp5} \) decreases in oocytes with maternal age. Additionally, Zhang et al. (2008) have compared \( \textit{Nlrp5} \) expression
levels in normal and abnormal (developmentally arrested) human preimplantation embryos showing a decrease in its expression in abnormal 4-cell embryos. However, \textit{Nlrp5} expression levels were higher in arrested embryos (abnormal) at both 8-cell and blastocyst stages than in normal ones. This altered expression of \textit{Nlrp5} in abnormal embryos further suggests that \textit{Nlrp5} has a potential role in preimplantation development in humans.

\subsection*{1.4.3 Phenotype Triggered by \textit{Nlrp5} Deficiency}

To determine the function of NALP5, we used \textit{Nlrp5}^{tm/tm} or \textit{Nlrp5}-deficient mouse lines in which homozygous females did not express either \textit{Nlrp5} transcripts or protein. By insertion of a PGK-neomycin cassette, homologous recombination in embryonic stem cells was used to mutate the \textit{Nlpr5} locus at the second intron without interrupting adjacent exons (Tong, Gold et al. 2004; Ohsugi, Zheng et al. 2008). \textit{Nlrp5} mRNA was still detected in \textit{Nlrp5}^{tm/tm} mice, although northern blot analysis showed greatly diminished protein levels compared to WT and \textit{Nlrp5} heterozygous oocytes. However, residual amounts of protein (less than \~5\%) was also detected in \textit{Nlrp5}-deficient oocytes suggesting that the neomycin cassette mutation destabilizes the \textit{Nlrp5} transcript and causes a severe hypomorph phenotype, affecting early mouse development (Ohsugi, Zheng et al. 2008).

\textit{Nlrp5} is a maternal lethal effect gene essential for preimplantation development beyond the two-cell stage in mice. Functional studies based on \textit{Nlrp5}-null females exhibit normal oogenesis, ovarian development, oocyte maturation, ovulation and fertilization. In addition, their ovarian reserve is not compromised indicating that NALP5 is not required for oogenesis. Development persists until the 2-cell stage, a critical stage for ZGA where the null females exhibit arrest (Tong, Gold et al. 2000). A similar phenotype of infertility is observed in other mammalian species such as bovine and monkey (Pennetier, Perreau et al. 2006; Wu 2009). Deficient mice respond normally to exogenous gonadotropin stimulation as they release oocytes of comparable number and morphology to wildtype females. Since the developmental arrest cannot be rescued by fertilization with wildtype sperm this implies that NALP5 is required for progression through development beyond the 2-cell stage and is a maternal-effect gene. \textit{Nlrp5}-deficient males show no phenotypic abnormalities and have normal fertility (Tong, Gold et al. 2000).
The exact function of NALP5 remains to be elucidated, although the global transcription decrease observed in two-cell embryos lacking NALP5 suggest a role in embryonic genome activation. Normally, ZGA at the two-cell stage is preceded by transcription and translation of a subset of gene products, some forming the transcription-requiring complex (TRC), a marker for ZGA (Schultz 2002). Nlrp5-deficient arrested embryos demonstrate a considerable decrease in de novo RNA transcription and are able to only synthesize 60% of the normal levels of TRC (Tong, Gold et al. 2000). Though Nlrp5 may not be critical for all transcription-translation initiation, its deficiency impacts the rate of transcription in early embryos. Further, the altered expression of Nlrp5 in developmentally arrested human embryos compared to normal embryos suggests a role in preimplantation embryo development (as described in 1.4.2).

1.4.4 Subcortical Maternal Complex

The presence of various known interaction domains in NALP5 indicates its ability to participate in the formation of protein complexes. Recently, a subcortical maternal complex (SCMC) has been identified that assembles during oocyte growth and is essential for zygotes to progress beyond the first embryonic cell divisions. In addition to NALP5, part of this complex are three newly discovered maternally encoded proteins: FLOPED (factor located in oocytes permitting embryonic development), TLE6 (transducing-like enhancer of split 6) and Filia (Li, Baibakov et al. 2008). Similarly to Nlrp5, the transcripts encoding these proteins are degraded after oocyte maturation and ovulation, although the proteins persist through preimplantation embryogenesis up until the blastocyst stage of development. The complex localizes uniformly around the cortex of oocytes and embryos, remaining excluded from regions of cell-cell contact in preimplantation embryos. It has been suggested that this complex may play a role in syngamy, mitotic spindle formation, cytokinesis or cell cycle progression. The absence of individual components of this complex significantly impedes development beyond the two-cell stage (Li, Baibakov et al. 2008).

While FLOPED, NALP5 and TLE6 directly interact with each other (Li, Baibakov et al. 2008) and Filia interacts only with NALP5 (Ohsugi, Zheng et al. 2008), the stability of the complex is dependent upon the presence of both Nlrp5 and Floped since the absence of either protein precludes its formation. In Floped-null mice, less than 20% of embryos progress beyond
the two-cell stage, thus resembling the *Nlrp5* phenotype (Tashiro, Kanai-Azuma et al. 2010). *Tle6* belongs to the *Groucho/Tle* family of transcriptional corepressors that plays a critical role in developmental processes with an unknown function in oocytes and embryos (Bajoghli 2007). Lastly, *Filia*-deficient mice have reduced fecundity with an increased rate of aneuploidy in their embryos (Zheng and Dean 2009). Ultimately, the similar expression pattern of the four genes, their physical interactions and colocalization alongside the sterile phenotype of *Nlrp5*- and *Floped*-deficient female mice define the importance of the SCMC for proper embryo progression.

### 1.4.5 NLRP Family Members

A total of 14 members (NLRP 1-14) have currently been identified in human with limited information about their expression pattern and physiological role. The expression profiles of some NALPs, together with genetic studies, suggest a crucial function for these proteins in female fertility. Besides NALP5 other NALPs such as NALP4, NALP8 and NALP9 have been shown to have exclusive oocyte and embryo expression in mice and bovine further implicating their roles in female reproduction (Ponsuksili, Brunner et al. 2006). In a study on age-associated gene expression of *Nlrp5, Nlrp9* and *Nlrp14* in mice, expression of all three was shown to decrease dramatically in aged oocytes. This suggests that *Nlrp* gene products may contribute to the decline in fertility of aging female mice. In addition, *in vitro* knockdown of *Nlrp14* in mouse oocytes caused developmental arrest between the one-cell and eight-cell stages in more than 50% of the embryos (Hamatani, Falco et al. 2004). Furthermore, mutations in another member of this gene family, *Nlrp7* cause recurrent hydatidiform moles and various forms of reproductive wastage such as spontaneous abortion, stillbirths and intrauterine growth retardation. Overexpression of this gene in humans has been linked to the development of testicular seminoma (germ cell tumour) (Zhang, Dixon et al. 2008). Also, *Nlrp2* shares a similar transcript and protein temporal profile as NALP5, highly enriched in germinal vesicles, but diminished by the two-cell stage. Recently it was discovered that embryos derived from *Nlrp2*-knockdown oocytes exhibit developmental arrest at the two-cell stage (Peng, Chang et al. 2012). Taken together these examples highlight *Nlrp* family members as essential players in female reproduction and fertility.

It is known that numerous members of the NALP family proteins mediate an innate
immune response. For instance, in response to bacterial/viral products NALPs 1-3 form protein complexes called inflammasomes that facilitate activation of caspase-1 or 5, leading to the processing of pro-interleukin 1β and NF-κB activation (Tschopp, Martinon et al. 2003; Halle, Hornung et al. 2008). A role in the regulation of apoptosis has been suggested (Rahman, Mohamed et al. 2009); however, there is no information about the downstream pathway(s) involved. It is also unknown whether the developmental failure associated with NALP5 deficiency in mouse is caused by any of these pathways. Nevertheless, the role of caspases and NF-κB in the case of NALP5 is not yet evident (see next sections). It is possible that NALPs may link some aspects of innate immunity and reproductive biology.

1.5 Dual Role of Caspases in Cell Fate

Cysteine-aspartic acid residues (caspases) are a family of cysteine proteases that cleave target proteins at specific aspartate residues and function as central regulator of cell death. Thus far, 15 members of the caspase family have been described in mammalian cells (Chowdhury, Tharakan et al. 2008). Eleven caspases have been identified in humans; caspase-1 to caspase-10 and caspase-14, whereas ten genes were identified in mouse; caspase-1,2,3,6,7,8,9,11,12 and 14 (Lu and Chen 2011). These proteins are synthesized as inactive zymogens or proenzymes, which are cleaved to an active form generating two subunits containing a prodomain, a p20 large subunit and a p10 small subunit (Sadowski-Debbing, Coy et al. 2002). They are divided into two groups based on the lengths of their N-terminal prodomains. Caspases with long prodomains are believed to be the upstream initiator caspases (caspase-1, -2, -4, -5, -8, -9, -10, -11, -12) and those with short prodomains are known as the downstream effector caspases (caspase-3, -6, -7) (Sadowski-Debbing, Coy et al. 2002). Initiator caspases contain two characteristic protein-protein interaction motifs: the death effector domain (DED) or the caspase recruitment domain (CARD). The presence of these domains ensures the interaction of procaspases with special adaptor proteins that promote caspase activation. In contrast, effector caspases depend on the upstream initiators for activation (Chowdhury, Tharakan et al. 2008).

The mechanism of upstream regulatory caspase activation depends on the assembly of recruitment platforms, such as the death-inducing signaling complex for caspase-8 and -10, the PIDDosome for caspase-2 (Tinel and Tschopp 2004), and the apoptosome for caspase-9 (Shi
2008). These platforms integrate cellular signals, promote dimerization of initiator caspases and lead to the generation of active enzymes that initiate specific signaling cascades. However, though caspases are the essential components of major physiological cell death pathways, recent findings from gene knockout studies have revealed several alternative roles for caspases including promoting processes required for life (Sadowski-Debbing, Coy et al. 2002). Beyond death signaling, they can signal through protein-protein interactions independently of enzymatic activity. They also have roles in immune function, differentiation and development, cell migration, cell proliferation, cell survival and cell cycle progression (Figure 3) (Varfolomeev, Schuchmann et al. 1998; Los, Wesselborg et al. 1999; Frost, Al-Mehairi et al. 2001). In particular, caspase 3 and 8 were shown to possess several alternative roles including macrophage differentiation (Okuyama, Nguyen et al. 2004) and T and B cell proliferation and activation (Los, Wesselborg et al. 1999). For example, caspase-8 is activated in human peripheral blood monocytes, which differentiate into macrophages during infection. While the differentiation process is accompanied by caspase activation, no morphological features of cell death are observed during this transition. The inhibition of caspases interferes with this differentiation, whereas conditional inactivation of caspase-8 in bone marrow cells prevents differentiation of myelomonocytic lineage into macrophages (Los, Wesselborg et al. 1999; Chowdhury, Tharakan et al. 2008). Caspase-8 might also play a role in embryo development because caspase-8 deficient mice exhibit defects in the development of heart muscle vasculature and have dramatically decreased pool of hematopoietic precursors. As a result, the heart is hypotrophic rather than enlarged, which may indicate that caspase-8 participates in the transmission of survival rather than death signals (Shaham 2004). Further, it has been suggested that caspases may play a role in the regulation of the cell cycle by serving as additional checkpoints that ensure only healthy cells complete the cycle. For instance, caspase-3 is responsible for cleaving and inactivating proteins like p21 and p27 that act as negative regulators of cell cycle machinery (Frost, Al-Mehairi et al. 2001).
Figure 3: Multiple caspase functions in mice. In addition to its role in cell death, caspase activation mediates non-death functions, including cell differentiation, immune response, preimplantation development, cell cycle progression and cell survival.

One of the best-characterized examples of non-lethal roles for caspases includes their role in regulation of the immune response. Knockouts for caspase-1 and caspase-11 develop normally, apart from defects in the production of IL-1α and IL-1β, which is likely the reason they exhibit a resistance to lethal shock induced by endotoxin (Sadowski-Debbing, Coy et al. 2002). Caspase-1 also processes IL-18 and IL-33 and is thus responsible for both inflammatory and innate immune responses. It was recently discovered that the mentioned cytokines are matured in a caspase-1 activating complex called the inflammasome (Figure 4a) and includes the apoptosis-associated speck-like protein containing caspase-recruitment domain (ASC) (Lamkanfi, Kanneganti et al. 2007). Several NALPs (NALPs 1-3) were shown to be the central scaffold of this caspase-1-activating complex (Figure 4b). As mentioned previously NALPs contain a PYD domain, this domain interacts and recruits the adapter ASC via the PYD-PYD interaction. The CARD domain within the ASC binds and recruits caspase-1 to the inflammasome, in turn activating it. This complex may also recruit other caspases, such as caspase-5 via the CARD domain of NALP1 or a second caspase-1 via the CARD domain of NALP2/3 inflammasomes (Martinon and Tschopp 2004; Li, Guo et al. 2009). In addition, various NALPs with predominant expression in sperm or oocytes including NALP1, 2, 3, 6, 7 and 12 have been proposed to form caspase-1-activating inflammasomes (Tschopp, Martinon et
al. 2003). Altogether, caspases have therefore acquired multiple activation mechanisms to control their unique signal-transducing roles in both cell death and non-cell death processes.

**INFLAMMASOME**

A

NALP1

LRR

PYD

NACHT

NAD

FIIND

CARD

ASC

Caspase-5

Caspase-1

**Foreigner Response Signal**

B

NALP3 Inflammasome

ASC – Apoptosis speck-like protein

Pro-caspase 1

Pro-IL-1β

NFκB

nucleus

Gene Transcription

Caspase 1

IL-1β
**Figure 4: Caspase activating complex - Inflammasome.** (a) CARD, caspase-recruitment domain; FIIND, F-interacting domain; LRR, leucine-rich repeats; NACHT. NAIP, CIITA, HET-E, TP1 domain; NAD, NACHT-associated domain; PYD, pyrin domain. Image modeled from (Schroder and Tschopp 2010) (b) **NALP3 inflammasome complex.** Various foreigner and internal danger signals such as pathogens, toxins, bacterial RNA and damaged organelles trigger the formation of the NALP3 inflammasome by inducing a conformational change in NALP3, which then oligomerizes. It then interacts through its PYD domain with ASC, which interacts with pro-caspase-1 through its CARD domain. This yields an active caspase-1. Meanwhile this process induces pro-IL-1β expression by NF-κB activation. Pro-IL-1β gets processed by active caspase-1 to IL-1β inducing an immune response (i.e. macropage death).

### 1.5.1 Caspases in Preimplantation Development

Although various members of the caspase family (caspases 1, 2, 3, 6, 7, 8 and 12) have recently found to be expressed in mouse embryos before the blastocyst stage, their role in preimplantation embryo development is unclear. Since the occurrence of cell death under physiological conditions is rare before implantation this suggests their possible role in non-lethal processes (Martinez, Rienzi et al. 2002; Spanos, Rice et al. 2002). General caspase inhibitors have shown a significant effect on preimplantation development where embryos arrest at the 4-to 8-cell stage and never reach the blastocyst stage. It has also been shown that caspase-8 inhibitors cause later developmental arrest (after 8-cell stage) affecting the compaction of morulae and the progression to the blastocyst stage (Zakeri, Lockshin et al. 2005; Busso, Dominguez et al. 2010). This suggests that other caspases besides *caspase-8* are involved in early stages of embryo development. Also, in mouse embryos, the appearance of mRNA for several types of caspases was reported to coincide with the onset of embryonic gene transcriptions (Martinez, Rienzi et al. 2002). Surprisingly, inactivation of individual caspases does not preclude early embryo development (Zakeri, Lockshin et al. 2005). However, the fact that chemical inhibition of caspases lead to embryo arrest points to redundancy in their function. In addition, it is also possible that they may be part of maternal effect factors necessary for ZGA
and proper embryo progression. These findings support caspase involvement in vital functions throughout preimplantation development.

**1.6 Nuclear Factor-κB (NF-κB): A key player in development**

NF-κB is a transcription factor that regulates the expression of a number of genes involved in numerous cellular processes such as inflammatory and immune responses, developmental processes, cell death and autophagy. NF-κB is composed of two different classes of the Rel family of proteins. The first class includes NF-κB1/p105 and NF-κB2/p100, and the second class of proteins includes RelA (p65), RelB and c-Rel (Izzo, Malhotra et al. 2006; Oeckinghaus and Ghosh 2009). These proteins form homo- or heterodimers that bind to DNA target sites and regulate gene expression. All members harbor a highly conserved Rel homology domain (RHD) that mediates DNA-binding, dimerization and nuclear localization. Class I members have long C-terminal domains that contain multiple copies of inhibitory ankyrin repeats while the Class II members contain C-terminal transcription activation domains (Figure 5). NF-κB1/p105 and NF-κB2/p100 are the inactive precursors of the p50 and p52 proteins, respectively. They form homo- or heterodimers with one of the three Class I proteins (Gilmore 2006). The different Rel/NF-κB proteins show a distinct ability to form dimers and bind to inhibitory subunits known as Inhibitory kappa B (IκB) proteins. Different complexes can be induced in different cell types, by distinct signals and interact in distinct ways with other transcription factors to regulate the expression of various genes. RelA and p50 heterodimers are the most common complexes present in a wide variety of cell types. In unstimulated cells, NF-κB complexes are sequestered in the cytoplasm and bound to the IκB protein family (Gilmore 2006). This masks their nuclear localization signal and prevents both nuclear translation and transcriptional activation (Schmitz and Baeuerle 1991). NF-κB can be activated in response to cytokines, bacterial products and cellular stress conditions. Pathogen-derived products such as LPS, peptidoglycan and dsRNA all lead to NF-κB activation through stimulation of toll-like receptors at the cell surface (Takeda and Akira 2005). Pro-inflammatory cytokines TNF-α and interleukin-1β (IL-1β) also induce activation through their respective cell surface receptors and distinct signaling pathways (Jacobs and Damania 2012).

Either the canonical or the non-canonical pathway is triggered based on the involvement of Class I or Class II NF-κB subunits. In the canonical pathway upon stimulation, the IκB
proteins become phosphorylated at two serine residues (S32 and S36) by the IκB kinase (IKK) complex (Izzo, Malhotra et al. 2006; Oeckinghaus and Ghosh 2009). This phosphorylation leads to further modification of the IκB, which is polyubiquitinated and targeted for degradation. IκB degradation unmasks the RHD and a nuclear localization signal. The NF-κB/IκB complex then dissociates allowing the NF-κB transcription factors to enter the nucleus, bind NF-κB responsive elements and upregulate transcription of target genes (Schmitz and Baeuerle 1991; Urban, Schreck et al. 1991). Conversely, in the non-canonical pathway, the IKK complex is activated by NF-κB-inducing kinase (NIK)-mediated phosphorylation. The active IKK phosphorylates Class II p100 to promote its proteolytic processing to p52, which can then dimerize with other NF-κB subunits and enter the nucleus (Izzo, Malhotra et al. 2006; Oeckinghaus and Ghosh 2009). We focus primarily on NF-κB-p65 since previous inhibition studies and expression analysis in preimplantation embryos target this subunit (see section 3.4 and 4.2).

It has been shown that some NLRP family members can modulate the activity of NF-κB through their role in inflammatory and immune responses. As described in section 1.5 the creation of the inflammasome protein complex that cleaves various caspases (i.e. caspase 1) to process IL-1β and can in turn activate NF-κB in response to bacterial and viral products (Mariathasan and Monack 2007). In vitro data from various studies suggests that some proteins containing CARD and PYD domains are involved in NF-κB activation. For instance, it has been shown that caspase-1 is capable of NF-κB activation, independent of its role in cytokine maturation, by recruiting RIP2 (Kersse, Lamkanfi et al. 2011). There is increasing evidence that pro-death caspases such as caspase-8 play roles beyond cell death, such as cell cycle regulation, and differentiation (Los, Wesselborg et al. 1999) Interestingly, caspase-8 has shown to signal NF-κB activation through its interaction with ASC to trigger apoptosis (Masumoto, Dowds et al. 2003).
Figure 5: NF-κB Protein Structure. There are two classes of NF-κB proteins. Class I and Class II. Both classes contain the Rel homology domain (RHD), which contains the DNA-binding domain and the dimerization domain. The C-terminal end of the RHD in near a nuclear localization signal (NLS), which is essential for transport of the NF-κB complexes into the nucleus. The ankyrin repeats of Class I proteins interact with a region in the RHD of the NF-κB proteins and by this mask the NLS preventing nuclear translocation (transrepression activity). Class II proteins have TAD (transactivation) function.

Although NF-κB plays a well-known function in the regulation of immune responses and inflammation, growing evidences support its role in key developmental processes. Various studies performed in different species during early development have shown that NF-κB DNA binding peaks around ZGA. The *Drosophila melanogaster* NF-κB homologue, Dorsal (p65 homologue), is involved in dorsoventral patterning of embryos. The proper nuclear translocation of Dorsal is responsible for the generation of the ventral structure. Its mRNA is maternally expressed and concentrated in the egg cortex (Drier, Huang et al. 1999). NF-κB genes have also been detected in *Xenopus* embryos throughout development. The homologue of p65, XrelA and RelB homologue, XrelB have been shown to regulate transcription in the early embryo (Bearer 1994). Further, during mouse spermatogenesis, NF-κB is activated in a stage-specific manner. It
appears at the pachytene stage and remains active throughout development triggering the expression of testis specific genes (Lilienbaum, Sage et al. 2000). The notion of NF-κB being a critical regulator of fertility is further supported by analysis of Rel knockout mice. Embryonic lethality is observed in female RelA knockouts (Beg, Sha et al. 1995) while severely reduced fertility occurs in female RelB knockouts (Weih, Durham et al. 1997).

In addition to investigations showing knockout embryo lethality for NF-κB, recent evidence shows expression and distribution of NF-κB during preimplantation embryo development in mice. NF-κB family members are expressed prior to and throughout preimplantation development up to the blastocyst stage (Parrott and Gay 1998). NF-κB protein is present in unfertilized oocyte and after fertilization throughout early development. The proteins are located primarily in the cytoplasm of embryos suggesting that the majority of dimers are retained in the cytoplasm by IκB proteins and are unable to regulate transcription (Parrott and Gay 1998). Therefore, their presence may be required for responses to environmental stimuli such as stress. Interestingly, a study by Nishikimi et al. (1999) has established that the activation (e.g. nuclear translocation) of NF-κB at the early one-cell stage is required for the development of mouse embryos beyond the two-cell stage. When embryos were cultured in the presence of NF-κB inhibitor PDTC from the early one-cell stage, about 91% arrested at the two-cell stage and none developed beyond the four-cell stage. Similarly, studies in bovine have shown NF-κB/p65 as an integral part of the meiotic-to-mitotic transition and activation of the embryonic genome (Paciolla, Boni et al. 2011). Along with the maternal expression pattern of NF-κB/p65, this suggests the involvement of NF-κB activation at the early one-cell stage in the proper activation of ZGA. Together these studies suggest that NF-κB is required in oogenesis and proper preimplantation development, though the role of NALP5 and NF-κB remains to be established.

1.7 Autophagy

Autophagy is an intracellular process whereby cytoplasmic components such as long-lived proteins, lipids, nucleotides and organelles are engulfed by double-membrane-bound structures known as autophagosomes, which are subsequently delivered to the lysosome for degradation (Klionsky and Emr 2000). It is considered a conserved mechanism as mammalian orthologues of Autophagy-related-genes (*Atg*) genes have shown similar functions across species. There are
three main forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Microautophagy and chaperone-mediated autophagy differ from the process of macroautophagy in that they directly engulf small portions of the cytosol via the lysosome instead of being delivered to the lysosome through an autophagosome. Since the mechanism of macroautophagy is better characterized than the other types of autophagy, it is hereafter referred to as autophagy for the purposes of this thesis (Mizushima 2007).

Autophagy was first identified in yeast as a process used in cells to survive metabolic stress. It has always been considered a nonselective bulk degradation pathway that can recycle building blocks and help restore the energy balance of the cell during starvation (Klionsky and Emr 2000). However, recent reports identify other modes of autophagy that selectively degrade aggregated proteins (aggrephagy) (Yamamoto and Simonsen 2011), unnecessary or damaged organelles (mitophagy) (de Vries and Przedborski 2012), lipids (lipophagy) (Singh and Cuervo 2012) and even invading bacterial cells. In mammalian systems, autophagy is thought to be involved in many physiological processes, including the response to starvation, cell growth control, anti-aging mechanisms and innate immunity. It occurs in low basal levels in various cell types, and is upregulated during structural remodeling or to get rid of damaging components that have accumulated in the cytoplasm (Levine and Klionsky 2004). Though autophagy is known for its role in such processes, its dysfunction is associated with certain diseases including cancer and neurodegenerative disorders (Levine and Kroemer 2008).

Generally, the process of autophagy is divided into four stages including induction, nucleation, expansion and fusion (Klionsky, Cuervo et al. 2007). Induction is the upstream signaling event that results in autophagosome formation. Autophagy occurs at basal levels in most cells for protein turnover and organelle recycling (Klionsky, Cuervo et al. 2007). Therefore, a mechanism must exist that senses these aggregates and triggers autophagy induction. One of the most studied and key regulators of autophagy is the Ser/Thr kinase mammalian Target of Rapamycin (mTOR). mTOR is an important signaling molecular that controls a variety of cellular processes, including cell cycle progression, cell growth and proliferation, and transcription (Yu, McPhee et al. 2010). Primarily, it is a major nutrient sensor of the cell, controlling cell growth in response to nutrient availability (Kim, Sarbassov et al. 2002). The pathway consists of two different complexes: mTOR complex 1 (mTORC1) and mTOR complex
2 (mTORC2). Whereas mTORC2 is not a direct autophagy regulator (Wullschleger, Loewith et al. 2006), mTORC1 is sensitive to the inhibitory effects of rapamycin. The inhibition of mTORC1 by rapamycin causes induction of autophagy through suppression (via dephosphorylation) of downstream targets, including ribosomal S6 protein kinase 1 (p70S6K1), eukaryotic initiation factor 4E-binding protein 1 (4EBP1) and UNC-51-like kinases (ULKs) (Copp, Manning et al. 2009). The mTOR pathway is regulated by diverse extracellular signals, such as amino acids and growth factors deprivation and different forms of stress (Kim, Sarbassov et al. 2002).

Atg genes that exist downstream of the mTOR complex encode proteins essential for autophagy execution. These genes control various aspects of autophagosome formation including nucleation, expansion and fusion events (Klionsky, Cuervo et al. 2007). In mammals, the formation of the autophagosome begins with a double membrane structure called the phagophore. The phagophore elongates and its edges fuse to form the autophagosome that sequesters the cytoplasmic material. The autophagosome then fuses with a lysosome to become an autolysosome that digests the cargo. A lipid kinase-signaling complex mediates vesicle nucleation. The activity of vesicular sorting protein 34 complex/phosphatidylinositol-3-phosphate kinase III (Vps34/PI3PIII) is essential for this process. Vps34 is part of the autophagy-regulating complex consisting of Atg6, Atg14 and Vps15 (Ichimura and Komatsu 2011). Vps34 associates with the phagophore membrane via Vps15 that is anchored to the phagophore. The generation of the PI3P by the kinase complex is essential for the recruitment of factors essential for autophagosome formation (Itakura, Kishi et al. 2008). Next, the expansion of the autophagosome is mediated by two ubiquitin-like conjugation systems. The first is the covalent linkage of Atg12 and Atg5. The second system involves the protein microtubule-associated protein 1 light chain 3 (LC3/Atg8). The cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine (PE) to form an LC3-PE conjugate (LC3-II), which is recruited to the autophagosomal membrane. Unlike the Atg12-Atg5 complex, LC3-II stays on the outer and inner membranes of the expanding autophagosome until its completion (Geng and Klionsky 2008). For this reason, LC3-II turnover signifies autophagic activity and therefore, can be used as an autophagosome marker. In the final stages, the outer membrane of the autophagosome will fuse with the lysosome to form an autolysosome (Ichimura and Komatsu 2011). Normal fusion and degradation is dependent upon lysosomal acidification. The inner membrane of the
autophagosome and the cytoplasmic materials contained inside are then degraded by the lysosomal hydrolases present. Specifically, LC3-II on the inner membrane is degraded, whereas LC3-II on the outer membrane is released from the lysosomes. Lastly, the resulting amino acids and lipids are then utilized for macromolecule synthesis and as an energy source (Mizushima 2007).

Besides LC3-II, other adaptor molecules implicated in targeting cargo to autophagosomes are emerging as markers for selective autophagy. Studies have shown that levels of p62 protein, also known as Sequestosome 1 (SQSTM1) are used to indicate a defect in poly-ubiquitinated protein aggregate turnover. Generally, p62 interacts with these poly-ubiquitinated aggregates and with LC3, targeting them for degradation at the autolysosome. The accumulation of p62 in autophagy-deficient cells confirms that it is selectively recognized and degraded by autophagy and therefore, can be used as a marker of autophagic activity (Ravikumar, Sarkar et al. 2010).

1.7.1 Autophagy in Development

In addition to its established roles in regulating nutrient supply and cell growth, the critical role of autophagy in early development and survival has also been studied. Several mouse models for Atg gene inactivation have revealed interesting embryonic phenotypes. Embryos devoid of Beclin1 (Atg6) exhibit developmental delay with reduced size and die during embryogenesis on embryonic day 7.5. Atg5 and 7-deficient mice die one day after birth and have reduced body weights with a decrease in amino acid levels, signifying its importance during the early neonatal starvation period (Komatsu, Waguri et al. 2005). Besides Atg genes other autophagy related molecules have shown defects in early development. For instance, disruption of mTOR causes embryonic lethality around the time of implantation (Day 5.5) in mice (Murakami, Ichisaka et al. 2004). Furthermore, an increase in autophagic induction during early preimplantation development increases the developmental competence of in vitro produced bovine embryos by reducing ER stress (Song, Yoon et al. 2012). These findings show that autophagy is a critical cellular process used during several events of development.

Recently, autophagy’s role specifically in early preimplantation development has shown surprising significance. After fertilization, maternal products in oocytes are degraded and new proteins encoded by the zygotic genome become synthesized (Section 1.2). Although several
maternal proteins are degraded early in development by the ubiquitin-proteasome system (Wojcik, Benchaib et al. 2000; Mtango and Latham 2007), autophagy plays a critical role during embryo development particularly during the oocyte to embryo transition phase (Tsukamoto, Kuma et al. 2008). Mizushima’s group demonstrated that autophagy is triggered by fertilization and its induction continues until the 8-cell stage. They observed an increase in GFP-LC3 puncta number in embryos compared to unfertilized oocytes, signifying autophagic activity. To analyze the functional relevance of autophagy during early embryogenesis, oocyte-specific Atg5 KO mice were generated. Oocytes deficient in Atg5 are not able to develop beyond the 4- to 8-cell stages if fertilized with Atg5 KO sperm. This is due to the lack of autophagy in mutant 2-cell embryos that show an accumulation of GFP-LC3 in their nuclei, as opposed to wildtype embryos that generate extensive GFP-LC3 foci (e.g. autophagosomes) in the cytoplasm (Tsukamoto, Kuma et al. 2008). Therefore, complete autophagy-deficiency causes embryonic lethality; however, the precise substrates for autophagy during the preimplantation period are unknown. It is important to note that wildtype sperm is able to rescue the embryonic lethality, indicating that activation of autophagy dependent upon the zygotic genome is essential for proper preimplantation development. It may act to eliminate unnecessary proteins and organelles that accumulate within oocytes or facilitate chromatin remodeling by degrading maternal suppressors of the zygotic genes program (Tsukamoto, Kuma et al. 2008). Also, early activation of autophagy may be closely associated with proper zygotic genome activation. These findings increase the likelihood that autophagy during early preimplantation development is vital for proper progression of events yet its precise targets remain unknown.
Figure 6: Autophagy and Process. (a,b) Cytosolic proteins and organelles are sequestered in the cytoplasm and enclosed by a phagophore. (c) A double-membrane vesicle, an autophagosome forms around these constituents. (d) The outer membrane of the autophagosome fuses with a lysosome, exposing cytosolic constituents to lysosomal hydrolases. (e) Components are lysed and contents degraded.

1.8 Programmed Necrosis: Necroptosis

How cells control their own death is a fundamental question in biology. A cell can execute itself
in many different ways. Caspase-dependent cell death has always been thought as the predominant pathway to destruction; however, it is not the only cellular mechanism that regulates cell death. In the absence of caspase activation, a programmed necrosis, called necroptosis prevails (Vandenabeele, Galluzzi et al. 2010). Necrosis has for a long time been regarded as an unregulated, accidental event. Some of the morphological features of necrosis include organellar swelling, increased cell volume and loss of intracellular content (Vandenabeele, Galluzzi et al. 2010). Necrosis, however, can be executed by a molecularly regulated mechanism termed necroptosis, which is mediated through cell death receptors and a unique downstream signaling pathway. Although the exact machinery controlling necroptosis is not completely understood, several key signaling molecules downstream of the death receptor have been identified, including receptor-interacting protein kinase 1 (RIPK1) and receptor-interacting protein kinase 3 (RIPK3) (Christofferson and Yuan 2010). The phosphorylation driven assembly of RIPK1-RIPK3 necrosis complex primarily regulates necroptosis. The cell morphology under such regulated conditions is very similar to that of necrosis. Negative regulators of necroptosis include Fas-Associated protein with Death Domain (FADD) and caspase 8, which cleaves and thus inactivates RIPK1 (Green, Oberst et al. 2011). As an alternative cell death pathway in cells, necroptosis has been shown to be important for some physiological processes, including inflammatory response against virus infections and embryogenesis.

Different forms of necrotic cell death can be identified based on their initiating mechanisms. As mentioned previously, inhibition of caspases can promote a necrotic form of cell death and this death depends on the function of RIPK1. What determines RIPK1’s function is its ubiquitination status, which defines its role as a prosurvival molecule or a kinase that promotes cell death. When RIPK1 is ubiquitinated this promotes the downstream activation of NF-κB, which governs the expression of prosurvival genes. Conversely, under conditions of caspase inhibition deubiquitination of RIPK1 switches its function to that of promoting cell death. In addition, RIPK1 and RIPK3 association and their activity are also required for necrosis (Christofferson and Yuan 2010; Vandenabeele, Galluzzi et al. 2010). This necrotic form of cell death is suppressed by caspase-8, which cleaves and thus inactivates RIPK1. Therefore, blocking caspase 8 causes cells to switch to RIPK1 mediated cell death. Also, caspase-8 forms an active complex with its enzymatically inert homolog cellular FLICE-like inhibitory protein (cFLIP)
that prevents RIPK3-dependent necroptosis during development (Weinlich, Dillon et al. 2011). This negative regulatory mechanism of necroptosis is further supported by findings that deletion of RIPK1 or RIPK3 rescues the embryonic lethality in mice lacking FADD or caspase-8 (Weinlich, Dillon et al. 2011).

Understanding the modifications and regulation of RIPK1 is critical to understand how cells make critical decisions either to survive by activating or to die through programmed cell death. The availability of necrostatin-1 as a specific inhibitor of RIPK1 kinase (Christofferson and Yuan 2010) makes it possible to dissect multiple functions of RIPK1 in mediating distinct downstream signaling pathways of cell survival through NF-κB activation and cell death through programmed cell death.

1.9 Hypothesis and Aims

Discovered over ten years ago, it is unclear how NALP5 regulates early embryo development and what pathways are affected by its deficiency. Oocytes obtained from Nlrp5-deficient females show developmental failure shortly after fertilization at the two-cell stage (Tong, Gold et al. 2000). With a 53% shared homology between mice and human amino acids, investigating the disrupted Nlrp5 mouse model will help elucidate the molecular pathways involved in triggering the embryonic phenotype, as well as embryo survival in both mice and human.

We hypothesize that NALP5 plays a role in facilitating early embryo survival through the regulation of autophagy during the oocyte to embryo transition phase. We propose that the two-cell developmental arrest in Nlrp5 deficient embryos may be due to altered NF-κB activation during this transition phase, which then leads to the improper activation of autophagy related mechanisms. To address this hypothesis the specific aims of this project were as follows:

I. To investigate the biological role of Nlrp5 as a cell survival molecule, by establishing whether altered NF-κB translocation occurs during embryo development in Nlrp5-deficient zygotes due to diminished caspase activity.

II. To determine the role of autophagy in producing the Nlrp5 knockout phenotype.
Chapter 2
Materials and Methods
2.1 Mouse Husbandry

Mice strains used in experiments described include ICR (Harlan, Indianapolis, IN, USA) and Nlrp5 WT and Nlrp5<sup>tm/tm</sup> (Tong, Gold et al. 2000). ICR mice (Harlan, Indianapolis, IN, USA) were housed at the Mount Sinai Hospital animal facility. They were kept on a 12h:12h light-dark cycle with free access to food and water. Animal protocols were approved by the Hospital Animal Care Committee and met standards for the ethical treatment of animals.

Nlrp5 WT and Nlrp5<sup>tm/tm</sup> mice were housed at the Toronto Centre for Phenogenomics (TCP). Animal protocols were approved by the TCP Animal Care Committee and met standards for the ethical treatment of animals. They were kept on a 12h:12h light-dark cycle with free access to food and water. To produce heterozygous and homozygous Nlrp5 mice, Nlrp5<sup>tm/tm</sup> males were mated with Nlrp5 heterozygous females. Nlrp5 WTs originally derived from heterozygote male and female crosses were derived from WT male and female crosses.

2.2 Nlrp5 Genotyping

Nlrp5 genotypes were determined by isolating DNA from ear tissue of mice. Protein was digested overnight at 55°C from ear tissue in tail lysis buffer (100mM Tris, 5mM EDTA, 200mM NaCl₂, 0.2% SDS), supplemented with proteinase K (Roche Applied Sciences). DNA was then extracted using alcohol precipitation. PCR was performed with 2µl of DNA in 20µl total volume of a reaction mixture. The following primers sequences were used in each mixture: Nalp5 A (5’- TCA TGT CCT TGG ATG GCA TG-3’), Nalp5 B (5’- ACC GGT GGA TGT GGA ATG TG-3’), and Nalp5 C (5’- CCA CGT GCT TTC AAG ATT GC 3’). The conditions for each PCR cycle were as follows: denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 1 minute, with a total of 38 cycles. Amplified products were detected by 1% agarose gel electrophoresis (Bioshop Canada Inc., Burlington, ON, Canada) made with Tris-Acetate-EDTA buffer (Mount Sinai Hospital Media Preparation Facility, Toronto, ON, Canada) and stained with SYBR Safe DNA gel stain (Invitrogen, Burlington, ON, Canada). Nlrp5 WT and Nlrp5<sup>tm/tm</sup> genotypes corresponded to bands at 396bp and 242bp respectively.
2.3 MII Oocyte Retrieval

Six to eight week old Nlrp5 WT and KO female mice were superovulated by intraperitoneal injections with 5IU of PMSG (National Hormone and Peptide Program, NIDDK, Bethesda, MD) and 48hrs later with 5IU of hCG (Sigma, Oakville, ON, Canada). Fifteen to eighteen hours post hCG, mice were sacrificed by either cervical dislocation or carbon dioxide inhalation. Oviducts were removed and placed into mHTF (LifeGlobal, Guelph, ON, Canada) supplemented with 0.1% BSA (Sigma, Oakville, ON, Canada). Oocytes were retrieved in mHTF from oviducts and denuded of cumulus cells using hyaluronidase (Sigma, Oakville, ON, Canada). Oocytes were either fixed in fixative (formalin or PHEM) or flash frozen for further analysis.

Human MII oocytes (immature and IVF failed) were obtained from TCART (Toronto Centre for Advanced Reproductive Technology or LifeQuest) with patient consent (REB approved). They were fixed in PHEM (80mM PIPES, 5mM EGTA, 1mM MgCl₂, 25mM HEPES at pH of 7.2, 3.7% formaldehyde, 10% Triton X-100) for 1-2 hours, washed in PBS and stored at 4°C for further analysis.

2.4 Embryo collection

Six to eight week old Nlrp5 WT and KO or ICR female mice were superovulated by intraperitoneal injections with 5IU of PMSG (National Hormone and Peptide Program, NIDDK, Bethesda, MD) and 48hrs later with 5IU of hCG (Sigma, Oakville, ON, Canada). After hCG injections, females were mated with Nlrp5 WT and KO or ICR males accordingly. Those females showing vaginal plugs, as an indicator of mating, 15-18 hours post-coitum were separated and sacrificed by cervical dislocation at 19-21 (early zygotes), 26-28 (late zygotes) and 36 (two cells) hours after hCG injection. Embryos were collected in mHTF + 0.1% BSA and denuded of cumulus cells using hyaluronidase and washed in fresh mHTF + 0.1% BSA. Embryos were either fixed, used for culture or flash frozen for further analysis.

2.5 Immunocytochemistry

Human MII oocytes, Nlrp5 WT and Nlrp5<sup>tm/m</sup> embryos flushed on d0.5 and d1.5 were collected and fixed for 30-60 minutes in 10% formalin (Fisher Scientific, Fair Lawn, NJ, USA) or PHEM
(80mM PIPES, 5mM EGTA, 1mM MgCl$_2$, 25mM HEPES at pH of 7.2, 3.7% formaldehyde, 10% Triton X-100) and washed in PBS three times for ten minutes each followed by 20-30 minute incubations in nuclear permeabilization solution (0.4% Triton X-100 in antibody diluent-0.004% sodium azide and 0.0001% gelatin in PBS). Next, embryos were pre-absorbed in blocking solution (0.5% BSA in antibody diluent) for 1 hour at room temperature. Following pre-absorption, embryos were incubated overnight at 4°C with various primary antibodies (Table 1) diluted in blocking solution. As negative controls, embryos were incubated within an equivalent amount of normal IgGs or no primary antibody. The following day, embryos were washed several times in washing solution (0.1% Triton-X in PBS) and incubated for 1 hour in appropriate secondary antibody diluted in secondary antibody diluent (0.1% Triton-X + 0.0001% gelatin), at RT in the dark. After incubation, they were washed several times for 10 minutes each in PBS and transferred onto glass slides (fischer Scientific, Hampton, NH, USA). Slides were counterstained with 1µg/mL DAPI (fluochrome 4,6-diamidino-2-phenylindole; Sigma-Aldrich) for 10-15 minutes to label oocyte DNA. Embryos were then mounted with 1:1 glycerol:PBS solution and a coverslip (VWR) used to cover the samples. Samples were sealed with nail polish and slides were stored at 4°C until time of analysis.

All samples were serially scanned using the Quorum WaveFX Spinning Disk Confocal Laser-Scanning Microscope system (Leica DMI6000B; Quorum, Guelph, ON). Samples were imaged using the 20X or 40X objective with green fluorescent protein (FITC, 536nm), red fluorescent protein (TxRed, 624nm) and blue fluorescent protein (DAPI, 447nm) channels as appropriate. 20X images were optically sectioned with a spacing of 0.4µm and a total z-stack of 10 images, whereas 40X images had a spacing of 0.2µm and a total z-stack of 10 images. All groups being compared were imaged at the same time to reduce the effects of fluorescence decay. Exposure times were kept consistent between treatment groups per experiment and calculated based on the strongest fluorescence signal group. Volocity version 5.2.2 (Improvision Ltd., Coventry, UK) software was used for quantification of protein. For global quantification of fluorescence for the entire oocyte, zygote and embryos, the percent intensity protocol was used selecting the entire region of the sample. Mean fluorescence intensity values were used for all quantification measures.
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<td>PHEM</td>
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2.6 Visualization of caspases in intact embryos

Active caspases (specifically caspases 1-9) were visualized in living ICR MII oocytes, early and late zygotes and 2-cell embryos with the use of BIOMOL (Enzo Life Sciences, Farmingdale, NY, USA) FAM-VAD-FMK marker. FAM-VAD-FMK is a carboxyfluorescein analogue of pan-caspase inhibitor zVAD-FMK (benzyloxy carbonyl-valine-alanine-aspartic acid-fluoromethyl ketone) and binds irreversibly to active caspases. Samples were incubated in 150µl of HTF medium containing 1µl of 150x FAM-VAD-FMK reagent (AK-117; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA) and incubated for 2 hours at 37°C under gas phase of 5% CO₂. After incubation, samples were washed and fixed as per BIOMOL protocol instructions, counterstained with DAPI for 10 minutes, and mounted on slides using a 1:1 glycerol:PBS solution. Slides were examined by deconvolution fluorescence microscope (Olympus IX70; Applied Precision Inc., Issaquah, WA, USA) under the 20x objective using FITC and DAPI filters. Ten 1µm optical sections for each sample were obtained from each embryo and acquired images were analyzed for quantitation of fluorescence intensity with SoftwoRx software (Applied Precision Inc.). Samples were analyzed at the same time to minimize effects of fluorescence decay.

2.7 Inhibiting caspases in intact embryos

Embryos from ICR mice were recovered in pre-equilibrated Global (Life Global, Guildford, CT) media, washed and placed in 25µl of medium alone or containing 25µM of caspase inhibitor zVAD-FMK (BD Biosciences; Mississauga, ON). The general caspase inhibitor stock was prepared at a 20mM concentration in DMSO (Sigma) and diluted 1:2500 in KSOM before use.

2.7.1 Progression of embryo development

Treated and untreated (DMSO only controls) embryos were cultured in 37°C with 5% CO₂, under mineral oil (Sigma). Typically the embryos were exposed beginning at both the early (19-21hr) and late (25-27hr) one-cell zygote stage. They were exposed continuously in the presence of caspase inhibitor or placed in fresh inhibitor-free medium at the next cell division. The progression of embryo development was monitored daily to verify developmental stage and quality and was maintained until controls reached blastocyst stage, usually 3 days following
treatment when started with one-cell embryos. Progression rates were calculated as the percentage of cleaved embryos that progressed to the expected developmental stage on each day.

2.7.2 NF-κB translocation

Treated and untreated embryos (DMSO only controls) were cultured in 37°C with 5% CO₂, under mineral oil (Sigma) for 2 hours. Embryos were exposed beginning at both the early (19-21hr) and late (25-27hr) one-cell zygote stage, washed several times with PBS and fixed in 10% formalin until time of analysis. Immunocytochemistry was performed on fixed embryos as described in 2.4 using total and phosphorylated NF-κB antibody for both localization and translocation analysis. Spinning disk confocal microscopy (Leica DMI6000B; Quorum, Guelph, ON) was then used to scan embryos at 20X or 40X objective as described in 2.4.

2.8 Lysotracker® Red and Mitotracker® Green Staining

Lysotracker® Red (Molecular Probes, Invitrogen, Carlsbad, CA, USA) was used in order to monitor lysosomal production as a secondary marker for autophagy. Mitotracker® Green (Molecular Probes, Invitrogen, Carlsbad, CA, USA) was used to detect total mitochondrial content in embryos. Following *Nlrp5* WT and deficient 2-cell embryo collection, embryos were washed several times with pre-equilibrated Global media supplemented with 0.1% BSA and placed in lysotracker red or mitotracker green dye at a concentration of 1mM in DMSO diluted to a final concentration of 100µm and 200µm respectively. Live embryos were incubated for 30 minutes in 37°C, 5% CO₂ under mineral oil in the dark. Following incubation, the embryos were washed in mHTF medium supplemented with 0.1% BSA and placed in 20µl drops on a 0.2mm Hiraki depression slide and covered with a cover slip to be analyzed by spinning disk confocal microscopy as described in section 2.4.

2.9 BODIPY® Staining

Following *Nlrp5* WT and deficient oocyte, early and late zygote and 2-cell collection, samples were fixed in 10% formalin for 1 hour. Washed several times in PBS, lipid droplets were visualized using BODIPY® 493/503 (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Samples were incubated to a final concentration of 50µm for 15-20 minute at 37°C, 5% CO₂ in the dark. After incubation, samples were washed with PBS, counterstained with DAPI for 10
minutes, and mounted on slides using a 1:1 glycerol:PBS solution. Slides were examined by spinning disk confocal microscopy under 20x objective and analyzed as described in section 2.4.

2.10 Treatments

2.10.1 Necrostatin-1

Necrostatin-1 (SIH-213; StressMarq Biosciences Inc., Victoria, BC, Canada) was used as a means to determine whether inhibiting necroptosis, a non-apoptotic cell death pathway would allow for progression of Nlrp5 deficient embryos. Following Nlrp5 WT and deficient late zygote (25-27hrs) collection, samples were washed in Global media supplemented in 0.1% BSA and placed in 25µl drops of treated or control (DMSO) media. Necrostatin-1 and DMSO (0.15%) treated WT embryos were used as controls to confirm final concentration of treatment without toxic effects. Embryos were exposed at late zygote stages (25-27 hrs) to a 30µM final concentration of Necrostatin-1 (Cho, McQuade et al. 2011) and cultured in 37°C with 5% CO2, under mineral oil. The progression of embryo development was monitored every day to verify developmental stage and quality and was maintained until controls reached blastocyst stage, usually 3 days following treatment when started with one-cell embryos. Progression rates were calculated as the percentage of cleaved embryos that progressed to the expected developmental stage on each day.

2.10.2 Rapamycin

Rapamycin (sc-3504; Santa Cruz, CA, USA) was used as a means to determine whether inducing autophagy would allow for progression of Nlrp5 deficient embryos. Following Nlrp5 WT and deficient late zygote (25-27hrs) collection, samples were washed in Global media supplemented in 0.1% BSA and placed in 25µl drops of treated or control (DMSO) media. Rapamycin and DMSO (0.15%) treated WT embryos were used as controls to confirm final concentration of treatment without toxic effects. Embryos were exposed at late zygote stages (~25-27 hrs) to a 100nM final concentration of Rapamycin (Song, Yoon et al. 2012) and cultured in 37°C with 5% CO2, under mineral oil. The progression of embryo development was monitored daily to verify developmental stage and quality and was maintained until controls reached blastocyst stage, usually 3 days following treatment when started with one-cell embryos. Progression rates were
calculated as the percentage of cleaved embryos that progressed to the expected developmental stage on each day.

2.10.3 Analysis of NALP5 cleavage products

For analysis of NALP5 cleavage products ICR 2-cell embryos were collected and snap frozen for further analysis. Following ICR late zygote collection, samples were washed in Global media supplemented with 0.1% BSA and exposed to 3-methyladenine (3-MA; Sigma), to a final concentration of 1.0mM, 2.0mM (Song, Yoon et al. 2012) and control DMSO overnight at 37°C with 5% CO₂, under mineral oil. The next day, embryos that advanced to the next developmental stage of 2-cells were collected, washed in PBS and snap frozen for WB analysis as described in 2.10.Embryo progression to 2-cells was recorded.

2.11 Western blot analysis

After collection, batches of oocytes, early (19-21hr) and late (25-27hr) zygotes, and 2-cell embryos were washed several times with PBS and snap frozen in 5µl of PBS + 0.1% PVP. Collections were resuspended on ice in 10-12µl of oocyte lysis buffer (25mM Hepes, pH 7.3, 10mM EDTA, 0.1% SDS, 125mM NaCl, 10mM NaF, 0.5% sodium deoxycholate + 1% Triton) supplemented with 1x protease inhibitor cocktail (Sigma). Whole oocyte lysates were snap frozen and thawed 2x before being solubilized in 1x SDS loading buffer (10mM Tris-HCL, pH6.8, 2% SDS, 10% glycerol, + 0.002% bromophenol blue) and boiled for 5 minutes. Proteins were resolved on a 4-12% polyacrylamide 0.1% SDS gel at a constant voltage (100V) for about 1.5 hours. Separated proteins were transferred onto PVDF membrane (Bio-Rad; Hercules, CA, USA) in transfer buffer (12.5mM Tris, 96mM glycine + 20% methanol) for 1.5 hours at 100V. Membranes were washed in TBS-T (1x TBS + 0.1% Tween 20 (Fischer Scientific)) and nonspecific sites were blocked by incubation for 1 hour in blocking buffer (TBS-T + 5% nonfat dry milk) at RT. After blocking, membranes were incubated overnight at 4°C in blocking buffer, with anti-NALP5 rabbit polyclonal antibody (10% gel; sc-134842; 1:200; Santa Cruz, CA), anti-BECN1 rabbit polyclonal antibody (10%; sc-11427; 1:500 or 1:1000; Santa Cruz, CA), anti-rat microtubule-associated protein 1 light chain 3 (LC3) rabbit polyclonal antibody (4-10% gradient gel; PM046; 1:1000; MBL, MA) anti-mTOR rabbit monoclonal antibody (4-10% gradient gel; #2983S; 1:1000; Cell Signaling), anti-phospho-mTOR rabbit monoclonal antibody (4-10%
gradient gel; #2976S; 1:1000; Cell Signaling) or anti-β-Actin goat polyclonal antibody (10% gel; sc-1616; 1:400; Santa Cruz, CA). Following incubation, membranes were washed three times, for 10 minutes each, in TBS-T and proteins were tagged by incubation with horseradish peroxidase conjugated anti-rabbit or anti-goat IgG secondary antibody (for NALP5, BECN1 and LC3, anti-rabbit 1:5000; for β-Actin, anti-goat 1:1500) diluted in blocking buffer for 1 hour at RT. After washing membranes three times for 10 minutes each in TBS-T, antibody binding was detected with Novex ECL Chemiluminescent Substrate (Invitrogen), according to manufacturer’s instructions. PageRuler Prestained Protein Ladder (Fermentas) was used as molecular weight markers.

### 2.12 Quantitative RT-PCR

*Nlrp5* WT and deficient late zygotes were collected and loaded in groups of 5 into 50µl of guanidinium isothiocyanate (GITC) solution for storage at -80°C. Total nucleic acid was precipitated with 100% ethanol, 7.5 mM ammonium acetate and glycogen. For quantitative RT-PCR (qRT-PCR) analysis cDNA was prepared following the procedures as outlined in Rambhatla *et al.*, 1995. Random hexamer primers were used for the mRNA population. RNA extracted from zygotes was processed without the use of primer or RT enzyme and used as a negative control. Expression levels of the cDNA for all genes were assessed by qRT-PCR using Low Strip Tubes, White (BioRad, Mississauga, ON, Canada) on the Mastercycler® Eppendorf Realplex (VWR Canlab, Mississauga, ON, Canada) using Mastercycler ep realplex software (VWR Canlab). Quality of prepared cDNA was measured by analyzing the housekeeping gene β-actin. Real-time PCR for target genes (*Fabp5, Lamp1, Beclin, Ripk1, Atg4B* and *Atg5*) and housekeeping gene β-actin were carried out in 5µl reactions. Sequences for selected mRNAs are listed in Table 2. All gene expression experiments utilized the SYBR Green PCR mix (Applied Biosystems, Foster City, CA, USA). SYBR Green reactions consisted of 1µl template, 0.1µl of 20mM forward and reverse primer mix (Table 2), 2.5µl SYBR Green and H₂O to a total of 5µl reaction. All qRT-PCR conditions were as follows: 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, cycling for a total of 40 cycles. Comparisons of expression levels were determined by normalizing to β-actin. CT value or cycle threshold value is a numerical value indicating the number of PCR cycles required before amplification of specific cDNA reaches the exponential phase. Lower values are thereby indicative of greater gene expression. Fold change
in expression was calculated relative to WT zygotes, by averaging the ratio of \( \beta\text{-actin} \) CT values to the target gene CT values.

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</table>

2.13 Statistical Analysis

All statistical analysis was performed using SigmaPlot Version 11 (Systat Software Inc., San Jose, CA, USA). Analysis was performed either with a two-way ANOVA following Tukey’s post test, a Mann-Whitney Rank Sum Test or the Student’s t-test, where appropriate. When two groups or treatments were compared, the Student’s t-test was used if the data passed both the Shapiro-Wilk test for normality and equal variance test. When either failed, Mann-Whitney Rank Sum Test was used. Data comparing Lamp1 in WT and KO 2-cells was transformed relative to WT fluorescence intensity levels. This was done to complete datasets from different days to
increase sample size. When progression rates were compared in caspase inhibition experiments, two-way ANOVA was used and included two factors: timepoint (early zygote vs. late zygote) and treatment type (DMSO vs. zVAD). For each experiment, oocytes/zygotes/2-cells were collected and pooled from many female mice to ensure a representative sample population. All data are represented as mean ± SEM with p<0.05 being defined as significant.
Chapter 3
Results
3.1 *Nlrp5* disruption affects D1.5 (two-cell) embryo competence

Whether *Nlrp5* deficiency impacted the number of two-cells flushed from oviducts was determined. It has been shown originally that the number of zygotes and two-cell embryos from *Nlrp5* deficient females (Tong, Gold et al. 2000) were similar to wildtype females. Our results mirror these previous findings in that there was no significant difference observed in number of two-cell embryos between *Nlrp5* WT (n=37) and deficient females (n=31) (Figure 1A). However, embryos flushed from *Nlrp5* deficient females contained a significantly (p<0.05) higher proportion of abnormalities, including fragmented or irregular-sized blastomeres (Figure 1B). About 6% of WT two-cells were abnormal compared to 44% of the deficient two-cells. It is thus likely that fragmentation and abnormal cleavage is not a consequence of embryo arrest but a contributing factor behind developmental failure in this model.
Figure 7: Number of two-cells from \textit{Nlrp5} WTs versus \textit{Nlrp5}^{tm/tm} females.

(A) Total number of two-cells retrieved from WT and \textit{Nlrp5}^{tm/tm} pregnant females at Day 1.5 from oviducts. Data are shown as the total number of embryos collected per female. (B) Proportion of abnormal embryos retrieved from WT and \textit{Nlrp5}^{tm/tm} pregnant females at Day 1.5. Data are shown per females as percentage of abnormal embryos with fragmented or irregular size blastomeres. Numbers on bars represent n-value and different letters represent significant difference (p<0.05). Man-Whitney Rank Sum Test.
3.2 Nlrp5 deficient MII oocytes and early preimplantation embryos exhibit decrease in caspase activity

With recent evidence suggesting a role for caspases in non-lethal functions during preimplantation development, I wanted to establish the caspase activity profile during early stages of normal development. I investigated caspase activity in WT oocytes (15-18 hrs post-hCG), early zygotes (19-21 hrs post-hCG), late zygotes (26-28 hrs post-hCG) and two-cell embryos (42 hrs post-hCG) using a cell permeable pan-caspase substrate. This method detects active caspases in living cells by binding of carboxyflourescein (FAM) labeled substrate, which irreversibly binds to activated caspases. My data showed no change in caspase activity within oocytes (n=47) and early zygotes (n=19), however, a significant increase (p<0.05) in activity within oocytes compared to both late zygotes (n=19) and two-cell embryos (n=43) was observed. A significant increase in activity was also detected among early zygotes and two-cells, as well as between late zygotes and two-cells. No difference was observed between early and late zygotes [Figure 2A]. These data show a steady and significant increase in caspase activity throughout preimplantation development, suggesting that caspase activity is required for the proper development of early embryos.

Members of the NALP5 protein family are known for their role in regulating caspase activity by forming complexes known as inflammasomes. Therefore, I asked whether caspase activity could be affected in Nlrp5 deficient oocytes and preimplantation embryos. Active caspases were visualized by the pan-caspase fluorescent assay described above. I observed a significant decreased in caspase activity in Nlrp5<sup>tm/tm</sup> oocytes, zygotes and two-cell embryos in comparison to WT [Figure 2B]. This suggests that Nlrp5 may be required for activation of caspases during development and this may be one of the reasons for the Nlrp5 deficient embryo arrest phenotype.
**Figure 8:** Caspase activity is reduced in $Nlrp5^{tm/tm}$ MII oocytes and early preimplantation embryos.

**(A)** Pan-caspase activity in oocytes, early zygotes, late zygotes and two-cells analyzed by deconvolution fluorescence microscope. Groups were compared using Kruskal-Wallis one-way ANOVA on ranks with Dunn’s post-hoc test. Representative images are shown below the graph. **(B)** $Nlrp5$ tm/tm oocytes, zygotes and 2-cells show a decrease in caspase activity. Deconvolution fluorescence microscopy was used to determine caspase activity. Values represent relative fluorescence ±SEM. Student’s t-test. For both figures, numbers above bars represent n-value and different letters represent significant difference (p<0.05) (Roxanne Fernandez).
3.3 Inhibition of caspases disrupts early mammalian development

Data presented above indicate that \textit{Nlrrp5} may play a possible role in regulating caspase activation. Previous work in our laboratory have shown a decrease in overall caspase activity in \textit{Nlrrp5} deficient oocytes, zygotes and two-cell embryos compared to WT. In mammalian preimplantation embryos (i.e. bovine, human), cell death begins at about the 1-cell stage with further death observed by the blastocyst stage. However, caspases 1, 2, 3, 6, 7, 8 and 12 are all expressed in oocytes and early embryos prior to blastocyst stage. Since caspase activity is observed at these stages I wanted to determine the role of caspases during preimplantation mouse development, a time when cell death is not a factor. Developing embryos at the early (21hr) and late (26hr) zygote stage were exposed to zVAD-FMK, a cell permeable pan caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases and can inhibit the induction of cell death (Busso, Dominguez et al. 2010). I show that this inhibitor has major effects on mammalian preimplantation development, especially when transiently applied at the early one cell stage but no effect when applied at the late one-cell stage. In both early (n=54) and late (n=45) control groups, respectively, 75% and 79% of the embryos that that cleaved to the two-cell stage continued developing and reached the blastocyst stage. Exposure to zVAD at the early (n=45) one-cell stage prevented most embryos from reaching blastocyst stage. Major developmental arrest was observed at the two-cell stage. About 36% of embryos that had reached the two-cell stage progressed to the early blastocyst stage. On the other hand, embryos treated at the late (n=46) one-cell stage showed similar results as the control groups with about 74% of embryos reaching the blastocyst stage. Experiments were repeated five times with consistent results as represented in Figure 3. In all early treated cases development was blocked between the two-cell and early blastocyst stage. These results suggest that caspase activity in early development must be important in situations other than cell death. Interestingly, the timing of developmental arrest at the two-cell stage correlates with the activation of the zygotic genome, as well as, the arrest observed in \textit{Nlrrp5} deficient embryos. This further strengthens the role of caspases for setting up events leading to proper initiation of embryonic development.
Figure 9: Effect of caspase inhibitor, zVAD-fmk on preimplantation development of mouse embryos.

One-cell embryos collected at 20hrs post-hCG were cultured in GLOBAL medium with or without 10µm zVAD. Embryos were added to the medium at early 1-cell (20hrs) and late 1-cell (27hrs) stages. Results are based on five separate experiments. Values are percentage ±SE of embryos developed to the indicated stages. n-values are as indicated; early DMSO n=58; early zVAD n=54; late DMSO n=45; late zVAD n=46. Within each category, values with different letters are significantly different. p<0.05, as per two way ANOVA followed by Tukey’s post-hoc test.
3.4 Total and phosphorylated NF-κB subunit RelA/p65 is altered in Nlrp5 deficient zygotes

Nikishikimi et al. were first to identify the activation of NF-κB during mouse preimplantation development. The presence of RelA (p65), a strong transactivator of various genes was confirmed in MII oocytes and embryos during preimplantation development. Similar to my studies with caspase inhibitors, their experiments showed that treatments of early one-cell embryos with NF-κB inhibitors led to embryo arrest at the two-cell stage; however, no effect was observed when treatment was applied at later developmental stages. The phenotype of arrest in early inhibitor treated embryos resembled that of Nlrp5 deficient embryos. Therefore, I was interested in determining whether the Nlrp5 phenotype could be due to the NF-κB inhibition. As it is well established that localization of NF-κB in the nucleus is evidence for its activation, I used immunocytochemistry and detected expression of NF-κB with a laser confocal microscope. I wanted to see the expression of total NF-κB (p65) in Nlrp5 WT compared to Nlrp5<sup>tm/tm</sup> early (21hr) embryos using total NF-κB p65 (A) antibody. Expression of p65 was cytoplasmic and nuclear in both genotypes similar to previous reports. There was a significant (p=0.025) overall decrease in p65 expression in Nlrp5<sup>tm/tm</sup> (n=22) compared to WTs (n=28) (Figure 4A). We next evaluated the extent of nuclear translocation of NF-κB in early (21hr) and late (27hr) zygotes using phosphorylated NF-κB (p65) antibody (Ser536). This antibody only detects p65 when phosphorylated at serine 536, which enables translocation of p65 to the nucleus. Immunocytochemistry showed altered phosphorylated p65 (p-p65) protein expression levels between WT and Nlrp5<sup>tm/tm</sup> female pronuclei with a significant decrease of expression in early Nlrp5<sup>tm/tm</sup> zygotes (p<0.001) and a significant increase of expression in late Nlrp5<sup>tm/tm</sup> zygotes (p=0.002). Although there was no significant difference of p-p65 expression between male pronuclei, there was a trend towards a decrease in expression in Nlrp5<sup>tm/tm</sup> and then similar to the female a significant increase in expression in Nlrp5<sup>tm/tm</sup> zygotes (p<0.001). These data indicate that kinetics with respect to timing of NF-κB translocation may be delayed in Nlrp5 deficient zygotes.
A

Ratio of Total Fluorescence:DAPI

WT Male tm/tm Male

B

Relative Expression of Total NFκB

WT Male +/+ tm/tm +/+ tm/tm

C

Ratio of Total Red Fluorescence:DAPI

WT Female tm/tm Female

/+/+ tm/tm
D) WT Male vs tm/tm Male

WT Female vs tm/tm Female

Ratio of Total Fluorescence:DAPI

0.2

0.4

0.6

0.8

1.0

1.2

1.4

1.6

0.25

0.5

0.75

1.0

1.25

1.5

1.75

2.0
Figure 10: Cytoplasmic and nuclear expression of RelA/p65 in early and late WT versus Nlrp5<sup>tm/tm</sup> zygotes.

Embryos were collected at the early (19-21hrs) and late (25-27hrs) zygotes stage and stained by immunofluorescence using anti-RelA antibody as specified in each figure. (A) Total endogenous RelA/p65 expression levels are decreased in Nlrp5<sup>tm/tm</sup> early zygotes. Total NF-κB p65 (A) antibody was used against p65. Values represent relative fluorescence to WT expression ±SEM for early and late zygotes (n=3 independent experiments). Numbers above bars represent the total number of embryos from all experiments. Different letters represent significance. p=0.02, as per Student’s t-test. (B) Phosphorylated RelA/p65 (S536 Cell Signaling) expression levels do not show a significant change in male pronuclei in Nlrp5<sup>tm/tm</sup> early zygotes. (C) Female pronuclei in Nlrp5<sup>tm/tm</sup> early zygotes show a significant decrease in p-p65 expression levels. p<0.001, as per Student’s t-test. (D) Male pronuclei in late zygotes show a significant increase in p-p65 expression levels. p<0.001, as per Mann-Whitney rank sum test. (E) Female pronuclei in late zygotes show a significant increase in p-p65 expression levels. p=0.002, as per Mann-Whitney rank sum test. For figures B, C, D and E values are expressed as a ratio of mean fluorescence to DAPI. Numbers above bars represent n-value. Different letters on bars represent significant difference. Eight mated females contributed the total number of embryos used. Representative images are shown below the graph.
3.5 zVAD and its effects on NF-κB subunit RelA (p65)

As stated earlier, \(Nlrp^{tm/tm}\) oocytes, zygotes and two-cell embryos show a significant decrease in overall caspase activity. Since altered RelA expression in \(Nlrp^{tm/tm}\) early zygotes was observed (Section 3.4) this could be due to decreased caspase activity. Studies have previously shown that several NLRPs can form protein complexes known as inflammasomes that cleave specific caspases and trigger NF-κB activation (Schroder and Tschopp 2010). Therefore, I wanted to determine whether caspase inhibition would affect NF-κB translocation (ie: activation) during normal development and phenocopy NF-κB results observed in \(Nlrp^{tm/tm}\). I used phosphorylated RelA antibody and immunofluorescence analysis to determine NF-κB translocation. There was a significant increase of RelA expression in early (21hr) treated zVAD embryos (\(p=0.002\)). In late (27hr) treated zVAD embryos there was no significant difference. This implies that without the full activation of all caspases, NF-κB function might be altered. In assessing pronuclear RelA expression no difference was observed in treated early and late embryos. It is important to note that I used a different \(p\)-p65 (Santa Cruz) antibody, which had both cytoplasmic and nuclear staining. Our results from section 3.4 were based on \(p\)-p65 antibody from Cell Signaling, which show a better representation of phosphorylated/active p65 with sole nuclear expression. Despite our findings, these experiments must be repeated before firm conclusions can be made.
Figure 11: Expression of phosphorylated RelA/p65 protein in zVAD and control treated mouse preimplantation embryos.

Embryos were collected at the early (19-21hrs) and late (25-27hrs) zygote stages and treated with 25µM caspase inhibitor zVAD or DMSO for 2 hours. Immunofluorescence using anti-phosphorylated RelA antibody (S536 Santa Cruz) was used to determine effects of treatment on NF-κB expression/translocation. (A) In early zVAD treated embryos there is an increase in p-p65 expression compared to controls. p=0.002, as per Student’s t-test. (B) Late zVAD treated embryos display no significant change in p-p65 levels. (C) Pronuclear expression analysis shows no significant difference in p-p65 in zVAD treated early embryos. (D) Pronuclear expression analysis shows no significant difference in p-p65 in zVAD treated late embryos. For all figures, values represent relative fluorescence to p-p65 expression of DMSO treated zygotes ±SEM for early and late zygotes (from 2 independent experiments). Different letters above bars represent significant difference.
3.6 Expression of NF-κB target genes in wildtype T and Nlrp5 \textsuperscript{tm/tm} late (25-26 hr) zygotes

Embryos lacking \textit{Nlrp5} show reduced levels of embryonic transcription, which may result from abnormalities in transcription machinery. Though NF-κB appears to be essential for development beyond the two-cell stage, the target genes it regulates in preimplantation embryos remain unknown. Therefore, we explored the mRNA expression of possible NF-κB target genes using quantitative real-time PCR. Since we did not know which targets may be regulated by NF-κB at this stage, I used microarray data from the GEO profiles database found on the NCBI (National Centre for Biotechnology Information) website to aid in the process. We selected \textit{Beclin-1}, \textit{Fabp5} and \textit{Ripk1} based on the significant difference observed in their expression in oocytes and the one- to two-cell transition period, a critical stage for proper EGA development. (Figure 6A). All of these genes are known targets of NF-κB in somatic cells (Park, Zhao et al. 2009; Hwang, Huh et al. 2011; Ogawa, Owada et al. 2011) \textit{Beclin-1}, \textit{Fabp5} and \textit{Ripk1} displayed no significant alteration in gene expression between \textit{Nlrp5} WT and \textit{Nlrp5} \textsuperscript{tm/tm} late (25hrs post-hCG) zygotes (Figure 6B). These results indicate that the selected genes may not be the NF-κB targets of interest in zygotes or that the timepoint of zygote collection is too early to see any change in transcription. In addition, since there is no difference in expression between WT and tm/tm zygotes I know that these embryos are not transcriptionally inactive.
Expression of NF-κB Targets in Late Zygotes (25-26hrs)

**A**

**Beclin-1**

- Intensity
- oocyte, 1-cell, 2-cell

**Fabp5**

- Intensity
- oocyte, 1-cell, 2-cell

**Ripk1**

- Intensity
- oocyte, 1-cell, 2-cell

**B**

Relative Ratio

- Beclin-1
- Fabp5
- Ripk1

- Nlrp5 WT (n=4/5)
- Nlrp5 tm/tm (n=5/6)
Figure 12: Relative expression of NF-κB targets in Nlrp5 WT and tm/tm late zygotes.

(A) Microarray data of mouse preimplantation embryo development from GEO profiles on NCBI website for following genes: Beclin-1 (GDS814 / 1430358_at / Becn1), Fabp5 (GDS813 / 1416022_at / Fabp5) and Ripk1 (GDS813 / 1449485_at / Ripk1). Expression intensity was used to determine significance of expression between oocytes, one-cell and two-cell embryos (n=4). Different letters represent significance. p=0.029, as per Mann-Whitney rank sum test. (B) Quantitative real-time PCR analysis of NF-κB targets in WT and Nlrp5tm/tm late (25-26hrs) zygotes. The mRNA levels were normalized against the corresponding levels of mouse β-actin. No significant difference in expression of Beclin-1, Fabp5 and Ripk1 is observed between WT and Nlrp5 deficient zygotes. Bars represent the fold change in comparison to Nlrp5 ±SEM. Sample sizes range from pools of four to six containing five zygotes each.
3.7 Inhibition of necroptosis in Nlrp5<sup>tm/tm</sup> embryos does not prevent developmental arrest

In the absence of caspase activation, a necrotic form of cell death prevails known as necroptosis caused by the activation of RIPK-1. As the exact mechanism of death in developmentally arrested Nlrp5<sup>tm/tm</sup> two-cell embryos remains unclear, I wanted to determine if embryo demist at the two-cell stage could be mediated by this mechanism. Since I have shown that caspase activity is decreased in Nlrp5<sup>tm/tm</sup> zygotes and two-cell embryos, the form of cell death that occurs in these embryos may be necroptotic. I treated Nlrp5<sup>tm/tm</sup> zygotes with RIPK1 inhibitor Necrostatin-1 to determine whether blocking its activity would help Nlrp5 deficient embryos progress beyond the two-cell stage. There was no significant difference observed between control DMSO (n=59) treated and Necrostatin-1 (n=48) treated Nlrp5<sup>tm/tm</sup> zygotes. In one trial, one Nlrp5<sup>tm/tm</sup> embryo from each treatment group reached the blastocyst stage. A total of three Necrostatin-1 treated Nlrp5<sup>tm/tm</sup> embryos reached 4-cells while five DMSO treated Nlrp5<sup>tm/tm</sup> embryos reached 4-cells. In general these differences were not significant to conclude that blocking necroptosis helps deficient embryos progress beyond the two-cell arrest. Nlrp5 WT zygotes were treated with DMSO (n=40) and Nectrostatin-1 (n=37) as controls to show that treatments do not affect normal development. There was no significant difference observed between WTs indicating that Necrostatin-1 had no effect on normal development. Although there is a significant difference observed between WT and Nlrp5<sup>tm/tm</sup> zygotes, this difference implies that WTs were able to progress through development undisturbed while the deficient embryos remained stagnant at two-cells. These data suggest that necroptosis may not be why Nlrp5<sup>tm/tm</sup> embryos arrest.
Figure 13: Necrostatin-1 treated Nlrp5<sup>tm/tm</sup> early embryos do not progress beyond two-cell development.

Nlrp5 WT and Nlrp5<sup>tm/tm</sup> early (21 hrs post hCG) zygotes were treated with 30µm of Necrostatin-1 and observed for progression up to D4.5. No significant difference between Necrostatin-1 treated tm/tm embryos was observed compared to DMSO treated tm/tm embryos. Nlrp5 WTs were treated with both DMSO and Necrostatin-1 as controls to signify no treatment effects on normal development. Results are based on three separate experiments with Nlrp5 WTs and five separate experiments with Nlrp5<sup>tm/tm</sup> embryos. Values are percentage ±SEM of embryos developed to the indicated stages. n-values are as indicated; WT DMSO n=50; WT Necrostatin-1 n=37; Nlrp5<sup>tm/tm</sup> DMSO n=59; Nlrp5<sup>tm/tm</sup> Necrostatin-1 n=48. Within each category, bars with different letters represent significant difference. p<0.05, as per two way ANOVA with Holm-Sidak method.
3.8 Autophagy is compromised in Nlrp5 deficient preimplantation embryos

Among other factors that have shown to be regulated downstream of NF-κB, autophagy is one. Activation of autophagy has been recently shown to occur at the two-cell stage and disruption of ATG5, a key molecule in its regulation causes early developmental arrest. As NALP5 protein levels decline during preimplantation embryo development, I investigated if NALP5 could be a substrate of autophagy and if deficient embryos display autophagic deficiencies.

3.8.1 Nlrp5<sup>tm/tm</sup> two-cell embryos are autophagy deficient

Initially synthesized in an unprocessed form known as proLC3, LC3 is converted to a proteolytically processed form known as LC3-I. It is then modified into the PE-conjugated form, LC3-II and is the only protein marker reliably associated with completed autophagosomes as it is localized to the outer membrane of these structures. I took an immunofluorescence approach to determine autophagosome presence (puncta) by staining with anti-LC3 antibody. Nlrp5 WT two-cell embryos displayed numerous LC3 immunoreactive dots in their cytoplasm while Nlrp5 deficient two-cell embryos displayed approximately 5-6 dots per embryo. Interestingly, the deficient embryos showed an accumulation of LC3 in the nucleus (Figure 3A). These events are often observed in other cell types under autophagy-inactive conditions, although their physiological importance is unknown (Tsukamoto, Kuma et al. 2008). These results parallel those of Atg5 deficient embryos that show a similar accumulation of LC3 dots in their nuclei and are autophagy inactive (Tsukamoto, Kuma et al. 2008).

3.8.2 Beclin-1 expression in Nlrp5<sup>tm/tm</sup> two-cell embryos is unaltered

Beclin-1 is required for the initiation of the formation of the autophagosome. Autophagic induction often but not always upregulates Beclin-1 levels. Therefore, Beclin-1 can be used as another marker of autophagy. To determine Beclin-1 levels, western blot analysis of Nlrp5 WT and Nlrp5<sup>tm/tm</sup> embryos was used but no significant difference in protein levels was observed between genotypes (Figure 3B). To determine if distribution of Beclin-1 was altered, I performed immunocytochemistry. Similar to western blot analysis, overall immunofluorescence levels showed no difference in Beclin-1 expression in Nlrp5 WT and deficient two-cell embryos (Figure 3C). However, subcellular distribution of Beclin-1 was different. While diffuse
expression in the cytoplasm of Nlrp5 deficient two-cell embryos was detected (n=19/23), punctate cytoplasmic localization of Beclin-1 was observed in WT embryos. WTs also showed an enrichment of subcortical localization of the Beclin-1 punctae, which is reminiscent of LC3 expression in normal two-cells. These data indicate that total Beclin-1 levels are not altered, however, its clustering into autophagosome-like foci is defective.

3.8.3 Lysosomal content is decreased in Nlrp5\textsuperscript{tm/tm} two-cell embryos

LysoTracker Red is known to accumulate in acidic organellar compartments such as lysosomes and autophagosomes. We used this probe to label lysosomes in two-cell embryos. LysoTracker Red staining showed a decrease in lysosomal content in Nlrp5 deficient two-cell embryos compared to WTs (Figure 8D). The lysosomes in deficient embryos appeared smaller. These results are parallel to the lack of autophagy induction observed in deficient two-cells. The decrease in acidic components in Nlrp5 deficient embryos signifies that the autophagy defect in these embryos may be lysosome and/or autophagosome related.

3.8.4 Lamp1 is increased in Nlrp5\textsuperscript{tm/tm} two-cell embryos

Lysosomal-associate membrane protein-1 (LAMP-1) is a transmembrane protein that is localized primarily in lysosomes. Therefore, alongside LysoTracker staining, I used immunofluorescence to determine LAMP-1 expression in WTs and Nlrp5 deficient two-cell embryos. We expected to see a decrease in LAMP-1 expression in Nlrp5 deficient embryos. Results show a significant increase in LAMP-1 expression in Nlrp5 deficient two-cells compared to WTs (Figure 8E). This finding contradicts my previous result of decreased lysosomal content in Nlrp5 deficient two-cells. There are no studies reported with similar results, therefore, I most likely need to repeat and implement other lysosome related experiments to confirm our findings.

3.8.5 p62/Sequestosome 1 expression in Nlrp5\textsuperscript{tm/tm} two-cell embryos is unaltered

In addition to the markers above, p62 can be used to aid in assessing autophagy impairment. It interacts with ubiquitinated protein aggregates and links them to LC3, targeting them for autolysosomal degradation. Therefore, if Nlrp5\textsuperscript{tm/tm} embryos are autophagy defective then we would expect to see an increase in p62 levels. There was so significant difference observed in p62 fluorescence intensity in Nlrp5\textsuperscript{tm/tm} embryos compared to WT (Figure 8F). These results are
contrary to our findings with LC3 and Lysotracker. However, p62 results are context dependent and other processes may affect its expression. For instance, its involvement in proteasomal degradation can affect its expression levels when the autophagic pathway is inhibited.

**A**

![Bar graph showing the number of LC3 dots](image)

**B**

![Western blot images](image)

- **Beclin-1**: N1 and N2 with a 60 kDA band
- **β-actin**: N1 and N2 with a band
Figure 14: Defective autophagy in Nlrp5 deficient two-cell embryos.

(A) Quantification of LC3 dots per embryo image is shown. Autophagosome formation is observed in Nlrp5 WT two-cell embryos and but not in deficient embryos. Accumulation of LC3 in nuclei of deficient embryos is observed. Arrows point to LC3 puncta. Bars represent mean number of dots per embryo ±SEM. Autophagosomes are indicated by an arrow. Different letters above bars represent significant difference and numbers represent n-value for one experiment. This experiment was performed three times with similar results. p<0.001, as per Student’s t-test. Representative images are shown below the graph. (B) No change in Beclin-1 expression is observed in Nlrp5 deficient two-cell embryos compared to WTs. Beclin-1 distribution is altered in deficient embryos with no punctae formation but a more diffuse localization of the protein. Arrows point to Bars represent mean fluorescence values ±SEM. Numbers above bars represent n-value for one experiment. This experiment was performed four times with similar results. (C) Representative immunoblot of Beclin-1 (60 kD) protein levels in Nlrp5 WT and deficient two-cell embryos. Whole lysates from 70 two-cell embryos were loaded in each lane. N1: WT two-cells; N2: Nlrp5tm/tm two-cells. Beclin-1 levels do not change in deficient embryos. (D) Nlrp5 deficient two-cell embryos exhibit a decrease in lysosomal content with LysoTracker Red staining. Bars represent mean fluorescence values ±SEM. Different letters above bars represent significant difference and numbers represent n-value. p=0.002, as per Mann-Whitney rank sum test. Representative images are shown below the graph. (E) Lamp1 levels are increased in Nlrp5 deficient two-cell embryos. Bars represent mean fluorescence values ±SEM. Different letters above bars represent significant difference and numbers represent n-value. p<0.05, as per Mann-Whitney rank sum test. Representative images are shown below the graph. (F) No change in p62/Sequestosome 1 expression is observed in Nlrp5 deficient two-cell embryos compared to WTs. Bars represent mean values ±SEM. Numbers above bars represent n-value. Representative images are shown below the graph.
3.9 Transcriptional regulation of autophagy related genes

The induction of autophagy in certain scenarios can be followed by an upregulation in mRNA expression of certain autophagy related genes. Therefore, determining mRNA levels of a few genes may provide data on induction of autophagy. For instance, significant changes in \textit{Atg4B}, \textit{Atg5} and \textit{Atg7} gene transcription in \textit{Drosophila melanogaster} salivary gland shows an increase in autophagy (Lee, Clough et al. 2003). We looked at \textit{Atg5} and \textit{Atg4B} for their role in regulating LC3 conversion. \textit{Atg4B} cleaves the C-terminus of LC3 to produce LC3-I and \textit{Atg5} participates in the conversion of LC3-I to LC3-II. With an increase in \textit{Lamp-1} protein levels in \textit{Nlrp5} deficient embryos determining whether this alteration is due to an earlier transcriptional problem was of interest. These genes are expressed throughout preimplantation development up to blastocyst stage as shown from microarray data acquired from the GEO profiles database (GDS813 / 1418236_s_at / Atg5; GDS812 / 94551_at / Atg4b; GDS812 / 160089_at / Lamp1) (Figure 9A). \textit{Atg5}, \textit{Beclin-1}, \textit{Atg4B} and \textit{Lamp-1} genes displayed no significant alteration in gene expression between \textit{Nlrp5} WT and \textit{Nlrp5}^{tm/tm} late (25hrs post-hCG) zygotes (Figure 9B). These results suggest that the selected genes are not transcriptionally compromised in autophagy deficient embryos. However, it is important to note that autophagy is induced during fertilization and is transiently suppressed between the late one-cell and middle of two-cell stage. Therefore, perhaps transcriptional analysis of early (36-38 hrs post hCG) or late two-cells (42-46 hrs post hCG) is more of an appropriate timepoint for detection of autophagy since the presence of its activity during these times has been previously shown (Tsukamoto, Kuma et al. 2008).
Expression of Autophagy Related Genes in Late Zygotes (25-26hrs)

A

Atg5

Atg4b

Lamp-1

B

Relative Ratio

Atg5

Beclin

Atg4B

Lamp1

Nlrp5 WT (n=5/6)  Nlrp5 tm/tm (n=4/5)
Figure 15: Relative expression of Autophagy related genes in \textit{Nlrp5} WT and \textit{tm/tm} late zygotes.

Quantitative real-time PCR analysis in WT and \textit{Nlrp5}^{tm/tm} late (25-26hrs) zygotes. The mRNA levels were normalized against the corresponding levels of mouse $\beta$-actin. No significant difference in expression of \textit{Atg5}, \textit{Beclin-1}, \textit{Atg4B} and \textit{Lamp1} is observed between WT and \textit{Nlrp5} deficient zygotes. Bars represent the fold change in comparison to \textit{Nlrp5} ±SEM. Sample sizes range from pools of four to six containing five zygotes per sample.
3.10 NALP5 protein as a substrate of autophagy

Levels of NALP5 protein have previously been reported to diminish during preimplantation embryo development (Tong, Gold et al. 2004) through an unknown mechanism. As autophagy is massively induced at late 2- and 4-cell stages, it was important to determine whether NALP5 could be a substrate of autophagy. Initial western blot analysis of NALP5 expression using anti-NALP5 antibody in Nlrp5 WT, heterozygote and deficient oocytes showed two specific protein immunoreactive bands (Figure 10A). The presence of the 125kD band was expected; however, an additional specific band appeared at 55kD, implying a possible splicing product or post-translational modification of the protein and a nonspecific band at ~65kD. Nlrp5 deficient oocytes showed virtually no NALP5 expression confirming the Nlrp5<sup>tm/tm</sup> model of both specific bands. To determine if NALP5 levels could be altered in autophagy inhibited conditions I treated early (21 hrs post-hCG) embryos with autophagy inhibitor, 3-MA (1mM; see section 2.10.3). I evaluated NALP5 expression through western blot analysis in untreated and treated two-cell embryos. The 55kD NALP5 band expression was increased in the treated group compared to the control (Figure 10B). This suggests that it is the 55kD isoform that may be regulated by autophagy; however, further experiments must be conducted to conclude this result.
Figure 16: NALP5 is an autophagy substrate.

(A) Representative immunoblot of NALP5 expression in Nlrp5 WT, heterozygote and tm/tm MII oocytes. N1: Nlrp5 WT oocytes; N2: Nlrp5 heterozygote oocytes; N3: Nlrp5tm/tm oocytes. Whole lysates from 100 oocytes were loaded in each lane. Representative immunoblot of NALP5 expression in control (DMSO) and 3-MA treated embryos at two-cell stage. Whole lysates from 160 two-cell embryos were loaded in each lane. An increase NALP5 is observed in one of its cleavage products (55kD).
3.11 LC3, total and phospho-mTOR analysis of *Nlrp5* deficient oocytes and embryos

As discussed previously, LC3 is often used as a marker for autophagy detection. Western blot analysis using anti-LC3 antibody with *Nlrp5* WT, heterozygote and deficient oocytes was completed. Results show total LC3 (LC3-I) in WT, heterozygote and deficient oocytes. LC3-II appears in both WTs and deficient oocytes (Figure 11A).

When mTOR is activated (phosphorylated at serine 2448), autophagy is inhibited and can be monitored by phosphorylation status of p70S6K, a protein kinase downstream of mTOR. Total mTOR levels and levels of phosphorylated mTOR (S-2448), were measured in *Nlrp5* WT and deficient oocytes, zygotes and two-cell embryos. Total mTOR was present in all WT and deficient oocytes and embryos. Phosphorylation of mTOR was present in WT oocytes but absent in *Nlrp5* deficient oocytes. Interestingly, no difference in mTOR phosphorylation status was observed in the two-cell stage embryos (Figure 11B). In evaluating mTOR, the oocytes appear as though they are trying to perform autophagy, they may be sensing an accumulation of products and trying to get rid of them. This is contrary to previously reported results of no autophagy activity being observed in oocytes. By the two-cell stage there should be a massive flux of autophagy, however, in both WT and deficient embryos we see mTOR phosphorylation. Though mTOR phosphorylation signifies autophagy inhibition, the more commonly used check for autophagy inhibition is its downstream targets p70S6K and 4E-BP1. Unfortunately, the immunoblot was unable to detect p70S6K due to inefficient transfer or retaining of proteins onto the membrane at this lower molecular weight.
Figure 17: Expression of autophagy related markers in oocytes and preimplantation embryos.

(A) Representative immunoblot of LC3-I and LC3-II expression. N1: WT oocytes; N2: Heterozygote oocytes; N3: tm/tm oocytes. Whole lysates from 100 oocytes were loaded in each lane. (B) Representative immunoblot of total and phosphorylated mTOR expression. Analysis of Nlrp5 WT and tm/tm oocytes, zygotes and two-cell embryos. Whole lysates from 170 two-cell embryos were loaded in each lane.
3.12 *Nlrp5*<sup>tm/tm</sup> embryos display a decrease in total levels of mitochondria

It was previously shown that NALP5 protein localizes to mitochondria, but the significance of this observation is not clear. Increasing evidence indicates that autophagy is involved in selective degradation of organelles including mitochondria. We wanted to determine whether mitochondria were targets for degradation. Since *Nlrp5* deficient two-cell embryos lack autophagy, mitochondria would be expected to accumulate. We compared the total mitochondrial content and distribution in two-cell embryos. MitoTracker Green (Fernandes, Tsuda et al. 2012) staining revealed a significant decrease in total mitochondrial levels of *Nlrp5*<sup>tm/tm</sup> two-cell embryos. We observed a high enrichment of organelles in the subcortex of *Nlrp5*<sup>tm/tm</sup> embryos (76%; n=21), whereas all WT embryos exhibited cytoplasmic distribution (Figure 12). The decrease in mitochondrial content in deficient embryos signifies that the mitochondrial insufficiency is independent of autophagy. The compromised mitochondria in *Nlrp5* deficient embryos observed in a previous study (Fernandes, Tsuda et al. 2012) may be a result of excessive mitochondrial activity and thus accumulation of reactive oxygen species.
Figure 18: Total mitochondrial levels decreased in \textit{Nlrp5} deficient two-cell embryos.

Mitochondrial pol as measured by Mitotracker Green in \textit{Nlrp5} WT and \textit{Nlrp5}^{tm/tm} two-cell embryos. Bars represent mean fluorescence values ±SEM. Different letter above bars represent significant difference and numbers represent n-value. p<0.05, as per Student’s t-test. Representative images are shown below the graph.
3.13 Ubiquitin-proteasome system is unaltered

Besides autophagy, the ubiquitin-proteasome system (UPS) is one of the main routes for protein clearance. For proteasomal degradation, the substrate proteins are required for tagging by ubiquitin. When autophagy is inactivated in otherwise normal KO mice of *Atg5* and *Atg7*, aggregates and ubiquitinated proteins accumulate in various tissues. Further, the involvement of protein degradation by UPS during onset of ZGA supports its vital role during preimplantation development (Shin, Tokoro et al. 2010). If autophagy were defective in *Nlrp5<sup>tm/tm</sup>* and Ub proteins are destined to be degraded via this pathway we would expect to an accumulation of ubiquitinated proteins. Immunostaining of *Nlrp5* WT and *Nlrp5* deficient two-cells with an antibody that recognizes mono and polyubiquitinated complexes revealed that there is no significant difference between these groups (Figure 13). These data parallel that of p62 expression, signifying that most likely the protein aggregates are cleared properly.
Figure 19: Ubiquitin content in Nlrp5 deficient two-cell embryos remains unaltered.

Immunofluorescence staining of Nlrp5 WT and deficient two-cells with anti-Ub antibody. There is no significant difference in ubiquitin expression in WTs compared to deficient embryos. Bars represent mean fluorescence values ±SEM. Numbers above bars represent n-value. Representative images are shown below the graph.
3.14 Lipid clearance is compromised in Nlrp5<sup>tm/tm</sup> two-cell embryos

In addition to the degradation of accumulated protein aggregates and organelles, autophagy plays a role in selectively degrading lipids. High lipid content has shown to interfere with embryo developmental potential as is evident in embryos of obese mice (Ma, Yang et al. 2012). Also, since we observed no difference in protein and mitochondrial clearance this suggests that maybe it is not the lack of protein or organelle degradation that leads to the developmental arrest of Nlrp5 deficient embryos but some other component in embryos that must be degraded through autophagy. Therefore, we evaluated lipid content in Nlrp5 deficient versus WT embryos. Initially, we confirmed that lipids are autophagy targets during normal preimplantation development. Oocytes, zygotes and two-cell embryos were stained with BODIPY and their lipid droplet content was evaluated. There was a significant decrease in lipid content through preimplantation development (Figure 14A). This implies that lipids are being used in early development and if not used or cleared could alter proper development. Conversely, we wanted to see whether treating embryos with autophagy inhibitor, 3-MA would cause an increase in lipid content. A significant increase in lipid droplets was observed in 3-MA treated two-cell embryos compared to normal two-cell embryos, implying that the lipid increase is a direct result of autophagy inhibition (Figure 14A). In other words, this confirms that autophagy targets lipids during development.

Previously Nlrp5 deficient GV oocytes have shown an increase in lipid content (Tashiro, Kanai-Azuma et al. 2010). We determined whether Nlrp5<sup>tm/tm</sup> two-cell embryos exhibit similar results. There was a significant increase in lipid droplet content in Nlrp5 deficient two-cell embryos compared to WTs (Figure 14B). Droplets appeared larger and aggregated. This buildup of lipids may contribute to the two-cell arrest observed in Nlrp5 deficient embryos.
**A**

Bar chart showing Relative Fluorescence Units for different stages:
- MIIs
- Zygotes
- 2-cells
- 3-MA treated 2-cells

**B**

Bar chart showing Relative Fluorescence Units for different genotypes:
- WT
- tm/tm
Figure 20: Lipid droplet accumulation in Nlrp5 deficient two-cell embryos.

(A) Lipid droplet content was measured across different stages of development including oocytes, zygotes and two-cell embryos. Early zygotes were treated with 1mM 3-MA to disrupt autophagy and allowed to progress to two-cell stage for further lipid analysis. Lipid content is decreased throughout preimplantation with an increase in two-cells after 3-MA treatment. Bars represent mean fluorescence values ±SEM. Different letter above bars represent significant difference and numbers represent n-value. p<0.01, as per Kruskal-Wallis one way ANOVA on ranks with Dunn’s post-hoc test. (B) Nlrp5 deficient two-cell embryos exhibit an increase in lipid droplet content. Bars represent mean values ±SEM. Different letter above bars represent significant difference and numbers represent n-value. p<0.05, as per Student’s t-test. Representative images are shown below the graph.
3.15 Rapamycin induction of autophagy in Nlrp5\textsuperscript{tm/tm} embryos does not prevent developmental arrest

Rapamycin is a well-known inhibitor of mTOR function and strong inducer of macroautophagy in some cell types (Ravikumar, Sarkar et al. 2010). As discussed previously, it has been demonstrated that the signaling pathways activating the mTOR complex can in fact inhibit autophagy, whereas the signals that inhibit mTOR activity stimulate autophagic degradation. Rapamycin inhibits complex 1 of mTOR kinase activity, resulting in mTOR inhibition and autophagic influx. Since I suspect a lack of autophagy in Nlrp5\textsuperscript{tm/tm} embryos, I wanted to determine whether treatment of these embryos at early (21 hrs post hCG) zygotes would help trigger their progress beyond the two-cell stage. There was no significant difference observed between control DMSO (n=39) treated and Rapamycin (n=37) treated Nlrp5\textsuperscript{tm/tm} zygotes. In one trial, one Nlrp5\textsuperscript{tm/tm} embryo from each treatment group reached the blastocyst stage. A total of nine rapamycin treated Nlrp5\textsuperscript{tm/tm} embryos reached 4-cells while six DMSO treated Nlrp5\textsuperscript{tm/tm} reached 4-cells. The rapamycin treated Nlrp5 deficient group had five 8-cells by day 3.5 but stalled beyond this stage. These differences were not statistically significant to conclude that inducing autophagy helps deficient embryos progress beyond the two-cell arrest. Nlrp5 WT zygotes were treated with DMSO (n=11) and Rapamycin (n=10) as controls to show that treatments do not affect normal development. There was no significant difference observed between WTs indicating that Rapamycin had no negative impact on normal development.
Figure 21: Rapamycin treated Nlrp5<sup>tm/tm</sup> early embryos do not progress beyond two-cell development.

Nlrp5 WT and Nlrp5<sup>tm/tm</sup> early (21 hrs post hCG) zygotes were treated with 100µm of Rapamycin and observed for progression up to D4.5. No significant difference between Rapamycin treated Nlrp5 deficient embryos was observed compared to DMSO treated deficient embryos. Nlrp5 WT's were treated with both DMSO and Rapamycin as controls to signify no treatment effects on normal development. Results are based on three separate experiments for Nlrp5 deficient embryos and one experiment of Nlrp5 WT's. Values are percentage ±SEM of embryos developed to the indicated stages.
3.16 NALP5 expression in human oocytes

It has been previously reported that levels of several Nlrp transcripts, including Nlrp5 are decreased in aged mouse oocytes (Hamatani, Falco et al. 2004). Accordingly, our laboratory has shown a negative correlation between aged human oocytes and Nlrp5 transcript levels. This decrease in expression may be one reason for decreased developmental potential of human oocytes of advanced maternal age. Also, similar to mice, NALP5 is expressed in human oocytes. We first performed western blot analysis using antibody generated by J. Dean (National Institutes of Health, Bethesda) to confirm specificity for human NALP5(Figure 16A). To determine whether a cohort of human oocytes exhibit altered NALP5 protein expression with age, I analyzed unfertilized human oocytes that matured to metaphase II in vitro from patients of three different age categories. Despite the decrease in mRNA levels observed in aged mice and human oocytes, NALP5 protein levels show a significant increase in aged, >39 years old human oocytes compared to oocytes retrieved from female IVF patients under 38 years (Figure 16B). This result goes hand in hand with previously reported data from our laboratory in aged mouse oocytes showing an increase in NALP5 protein expression. These data suggest that the predisposition towards abnormal embryonic development with aging is associated with elevated NALP5 protein expression.
A

**NLRP5** 135 kD

B

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>Relative Fluorescence Units</th>
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Figure 22: Increase in NALP5 expression in aged human oocytes.

(A) Representative immunoblot of NALP5 expression. Whole lysate from 15 oocytes was loaded into lane. (B) Failed IVF oocytes different age groups were stained by indirect immunofluorescence using anti-NALP5 (Jurrien Dean) antibody. An increase in NALP5 expression is observed in oocytes retrieved from females greater than 39 years old. Bars represent relative fluorescence ±SEM. Student’s t-test. Numbers above bars represent n-value and different letters represent significant difference. p<0.001, as per Kruskal-Wallis one-way ANOVA on ranks with Dunn’s post-hoc test.
Chapter 4
Discussion
Early pregnancy loss in humans, often occurring due to defects that occur before implantation, is a worldwide concern. Millions of oocytes are harvested each year and cultured in IVF laboratories with the aim of offering infertile couples the help of ART. Although many underlying causes of infertility have been overcome by these techniques, the amount of couples experiencing infertility continues to rise due to late age conception. There is, therefore, a continued need to unravel the complications of preimplantation embryonic development and to develop novel technologies that might help improve fertility and fertility-associated issues in women.

At least 10% of infertile couples experience repeated IVF failure, which in part stems from compromised embryo developmental potential. This can be attributed to poor quality oocytes produced by the female. The mechanisms leading to poor quality of oocytes vary. Generally, successful fertilization and early development is dependent upon multiple oocyte factors, such as RNA and proteins involved in regulation of preimplantation embryo development. These oocyte factors are known as maternal factors and play a critical role throughout early development. Maternal-effect genes have shown to be crucial for normal embryonic development. For instance, genetic ablation of certain genes (i.e. Nlrp5, Zar1 etc.) has shown female infertility due to arrest during early stages of preimplantation embryo development (Tong, Gold et al. 2000; Wu, Viveiros et al. 2003). Thus, it is obvious that investigating the effects of diminished maternal effect factors/genes will help elucidate information about the molecular mechanisms of oocyte quality.

Besides being expressed in human oocytes, there is limited information regarding the molecular mode of action of Nlrp5. Female mice lacking Nlrp5 are capable of normal oogenesis, maturation and fertilization; however, they remain infertile due to embryonic arrest at the two-cell stage. We have shown that although ovulation rates of deficient females are comparable to wild-type littermates, a higher rate of fragmented and/or irregularly shaped two-cell embryos are retrieved. This supports the idea that early events required for embryo survival are compromised. For instance, activation of the embryonic genome is altered and accompanied by a decrease in protein synthesis (Tong, Gold et al. 2000). In this thesis, I have examined the involvement of autophagy as a possible contributor to the preimplantation defect that is observed in Nlrp5 deficient embryos.
4.1 Caspases in Nlrp5 deficient mice

While the majority of NLRP proteins are adaptors involved in the regulation of inflammation, the precise function and mechanism of NALP5 remains unclear. My results confirm the requirement of caspase activity, which serves as a developmental role independent of cell death during the maternal to zygotic transition. I observed an overall downregulation of caspase activity in Nlrp5 deficient oocytes, zygotes and two-cell embryos. It is plausible that Nlrp5 and specific members of the caspase family may form a complex, which may be involved in activating caspases for regulatory functions in development. Various NALPs have previously shown the formation of a cytoplasmic protein complex called an inflammasome, to facilitate the maturation and secretion of proinflammatory cytokines, including IL-1β, IL-18 and IL-33 (Mariathasan and Monack 2007; Halle, Hornung et al. 2008; Li, Guo et al. 2009). Caspases, particularly caspase-1 and -8, have a well-established role in inflammasome activation as their cleavage triggers cytokine processing. NALP5 shares many of the domains necessary for the recruitment of this complex and as such may function through a similar pathway contributing to the formation of an inflammasome. It is unknown whether the developmental failures associated with NALP5 deficiency in the mouse are caused by deregulated inflammasome activation and consequent underproduction of 1L-1β. In addition, there have been studies in human, which proposed that increase of cytokines (i.e. IL-1β) in in vitro cultures is a hallmark of more successful early embryo development (Karagouni, Chryssikopoulos et al. 1998; Sharkey 1998), which would support cytokine processing by such a complex. Therefore, the decrease in caspase activity in deficient oocytes and embryos could affect the formation of such a complex and activation of downstream targets leading to the two-cell arrest phenotype. This finding would support a hypothesis that the inflammasome complex is a reflection of the formation of a successful signaling platform. This would support the activation of caspases and subsequent cytokine processing by such a complex. It is, however, unlikely that 1L-1β itself is the key factor in this pathway, as 1L-1β and caspase-1 deficient females are fertile and do not exhibit embryo arrest.

Previous studies have shown that non-lethal caspase activity is present before implantation (Zakeri, Lockshin et al. 2005; Busso, Dominguez et al. 2010). In agreement with this study, our results showed that zygotes treated with zVAD, a general caspase inhibitor
significantly impacted development, showing an arrest at the 4- to 8-cell stage with only few embryos reaching blastocyst. Furthermore, we find that earlier zygotes exposed to the inhibitor exhibit the more severe outcome of arrest. Inhibitor treatment at the early 1-cell stage caused embryos to arrest; however, no effect was observed when treatment was performed at the later zygote stage. This early effect of zVAD in embryos suggests that the main process affected is either proliferation or execution of autophagy in blastomeres during the first or second division. It also emphasizes that caspase activity is most likely required during the period of time when pronuclei are starting to form. Interestingly, individual caspase knockout models do not have early preimplantation issues. However, the fact that general caspase inhibition leads to embryo arrest points to the redundancy in their function. For instance, *caspase-12* was shown to compensate for the lack of *caspase-2* and *-3* by facilitating apoptosis in oocytes (Takai, Matikainen et al. 2007). Specific caspases essential for preimplantation development, however, still need to be investigated particularly in *Nlrp5* deficient embryos.

The exact mechanism that drives *Nlrp5* deficient two-cell embryos to death has not been determined. In the absence of caspase activity, necroptotic cell death is known to prevail. Since *Nlrp5* deficient zygotes display normal levels of *Ripk1* expression and overall decreased caspase activity, we treated them with inhibitor of RIPK1, a known stimulator of necroptosis (Cho, McQuade et al. 2011). Although blocking necroptosis did not help null embryo progress beyond the two-cell stage, further analysis is required to confirm whether deficient embryos undergo necroptosis.

### 4.2 Altered NF-κB activation in *Nlrp5* deficient embryos

The *Nlrp5* deficient phenotype of two-cell embryo arrest resembles results of NF-κB inhibition experiments (Nishikimi, Mukai et al. 1999). These experiments have shown that NF-κB activation (i.e. translocation from the cytoplasm to nucleus) occurs during the early zygote stage and transient treatment with NF-κB inhibitor at the early but not late one-cell stage causes two-cell arrest. In addition, the expression of RelA (p65) in MII oocytes and embryos during development has been observed and progressively decreases by the blastocyst stage (Parrott and Gay 1998) depicting a similar temporal expression as NALP5 protein. Therefore, in light of these findings determining whether the *Nlrp5* phenotype could be due to inefficient NF-κB activity became evident.
The induction of NF-κB involves its regulated nuclear localization by cytoplasmic modifications that can directly activate NF-κB subunits. In addition to NF-κB nuclear translocation, subunits like NF-κB-p65 are post-translationally modified (Perkins 2006). Phosphorylation of NF-κB-p65 at serine 536 regulates its activation, nuclear localization and reflects increased transcriptional activity (Schmitz and Baeuerle 1991). Therefore, in order to determine NF-κB-p65 induction in Nlrp5 deficient early embryos, NF-κB-p65 at Serine 536 was studied. Initially, expression of total NF-κB-p65 (unmodified and modified) expression levels showed an overall decrease in NF-κB-p65 expression in early Nlrp5 zygotes. Similarly, NF-κB-p65 at Serine 536 levels was decreased in Nlrp5 deficient early female pronuclei. However, in late Nlrp5 deficient zygotes, an increase in both the male and female pronuclei was observed. There appears to be a defect in kinetics of NF-κB-p65 nuclear translocation in deficient embryos with a delay in activation. Also, the decreased expression in early embryos signifies overall decreased phosphorylation of NF-κB-p65 at Serine 536. This defect in phosphorylation of p65 could potentially contribute to the NF-κB impaired shuttling. A similar defect in nuclear translocation of NF-κB-p65 is evident in mouse caspase-8 knockout B-cells (Lemmers, Salmena et al. 2007).

It has been shown that delayed nuclear translocation of NF-κB-p65 can compromise NF-κB mediated gene expression and cell function in myeloid differentiation deficient dendritic cells (Kaisho, Takeuchi et al. 2001). Our observation highlights the importance of the delayed nuclear translocation of NF-κB in Nlrp deficient embryos, in causing possible defects in gene transcription. Altered NF-κB in Nlrp5 deficient embryos could compromise transcription of target genes that are required for normal early embryo development. As stated earlier, several NLRPs form inflammasomes that cleave caspases and trigger the activation of NF-κB, which drives proinflammatory gene transcription (Schroder and Tschopp 2010). We propose that NLRP5 forms a similar complex in embryos, which is involved in the timely activation of NF-κB-p65 downstream signals in response to its activation. A delay in translocation is enough to impair NF-κB activity, which would affect the transcription of target genes necessary for development.

As a transcription factor, NF-κB’s active participation in preimplantation development is likely to be mediated through its target genes. Nlrp5 deficient one- and two-cell embryos have
shown a global decrease in *de novo* RNA transcription. Further, only 60% of the transcription-related complex (TRC), regulated by EGA is synthesized in null embryos (Tong, Gold et al. 2000). This decrease signifies a possible defect in transcriptional machinery. Though it has been reported that numerous genes are regulated by NF-κB in immune system functions (Li and Verma 2002; Hayden, West et al. 2006), the target genes it regulates in preimplantation embryos remain unknown. Therefore, we set out to examine the expression pattern of several NF-κB target genes (i.e. *Beclin-1*, *Fabp5* and *Ripk1*) that are known to have differences in expression between oocytes and the one- to two-cell transition period. This is the critical period for EGA, as well a period where there is an upward surge in transcriptional activity. No difference was observed in transcript levels between WT and deficient embryos, which indicates that either the selected genes may not be NF-κB targets of interest in early preimplantation or that NF-κB transcriptional activity is not decreased despite its apparent delay in translocation. These genes may also be downstream targets of the signaling pathways. In addition, since we know these genes are expressed throughout early preimplantation, the fact that the deficient embryos are expressing them at least signifies that the embryos are not completely compromised at the transcriptional level.

### 4.3 *Nlrp5* deficient mice display autophagic defects in embryos

Degradation of maternal factors is essential in diverse aspects of normal cell physiology and development. In the early embryo, degradation of maternal factors (i.e. mRNA, proteins etc.) is crucial for the proper transition from maternal to embryonic control of development (Tadros and Lipshitz 2009; Walser and Lipshitz 2011). The molecular target(s) that must be eliminated is currently unclear. It has been shown that paternal mitochondria entering the oocyte cytoplasm after fertilization are eliminated by autophagy during early development (Sato and Sato 2011). However, it is unlikely that the lack of their degradation would cause early embryo arrest, as paternal mtDNA are occasionally detected in later developmental stages (Schwartz and Vissing 2002). Thus, degradation must have some selectivity in order to maintain the maternal factors necessary for the transition from maternal to embryonic genome control. Protein degradation serves to inactivate proteins that are needed in the early stages of transition but that would be harmful to the embryo later. For instance, in early Xenopus embryo, elimination of cytoplasmic polyadenylation element binding protein (CPEB) is necessary for mitosis to proceed (Bowerman
and Kurz 2006). Similarly, oocyte specific mRNA that is not subsequently expressed from zygotic genes may not only be unnecessary, but also detrimental to early development after fertilization. This can be exemplified by c-mos protein, which is essential for regulating meiosis, yet detrimental to embryo progression after fertilization as it inhibits its cleavage (Watanabe, Vande Woude et al. 1989). Therefore, a mechanism that selectively degrades a subset of mRNAs/proteins that are detrimental to preimplantation development must exist. The exact mechanism(s) by which these maternal factors are degraded after fertilization remains unclear. Since autophagy has a role in quality control maintenance by disposing of damaged, aggregated proteins and organelles, we presume that the selective degradation of maternal factors in early embryos for normal developmental progression functions through this mechanism. It is likely that this pathway will not involve broad, global macroautophagy, but perhaps chaperone-mediated autophagy, where specific target cargo is delivered to the lysosomes (Mizushima 2007).

In this study, we have described defective autophagy in Nlrp5 preimplantation embryos further supporting the importance of this process during early embryo development. In mammals, autophagy is activated shortly after fertilization but does not rely on the presence of sperm. It has been shown that maternal, together with zygotic inactivation of Atg5 has a dramatic effect on early development with embryos not progressing beyond the four- to eight-cell stage. This is due to the lack of autophagy observed in deficient two-cell embryos that show no GFP-LC3 punctas in the cytoplasm but an accumulation of GFP-LC3 in their nuclei. My results resemble these findings and clearly demonstrate the importance of autophagy during early embryo development. In deficient Nlrp5 two-cell embryos we see an accumulation of LC3 in nuclei similar to the Atg5 phenotype and developmental arrest at the two-cell stage. Though LC3 is thought of as a cytoplasmic protein, its nuclear localization has been observed in a number of published studies (Ciechomska and Tolkovsky 2007; Drake, Kang et al. 2010). This suggests that shuttling of protein between the nucleus and cytoplasm may be molecularly regulated. These results are reminiscent of Beclin-1 behaviour. The presence of a nuclear export signal (NES) in Beclin-1 plays a crucial role in its function in autophagy and its tumor suppressor activity. The loss of NES switches Beclin-1 from a primary localization in the cytoplasm to the nucleus blocking autophagy function (Liang, Yu et al. 2001). This supports the lack of autophagy that is
observed in \textit{Nlrp5} deficient embryos with enhanced nuclear LC3 accumulation and no cytoplasmic puncta.

I observed a diffuse expression of Beclin-1 compared to the obvious punctate form observed in the subcortical region of WTs. In mammalian cells, a similar finding where pancreatitis-induced vacuole membrane protein 1 induced autophagy Beclin-1 produces punctae, however, the mutant form abolishes autophagy activity and has a diffuse expression (Ropolo, Grasso et al. 2007). Moreover, another member of the NALP family – NALP4 has shown to regulate autophagy through its interaction with Beclin-1 by the NACHT domain (Jounai, Kobiyama et al. 2011). Thus, my data indicate that that NALP5 is essential for autophagosome formation and may functionally interact with Beclin-1 to trigger the autophagic process. Therefore, \textit{Nlrp5} deficient embryos may lack the ability to form autophagosomes, which will affect autophagic flux. As such, my results show a decrease in lysosomal induction in deficient two-cell embryos. Interestingly, an increase in LAMP-1 (lysosomal associating membrane protein 1) expression is detected as well with concomitant decrease in Lysotracker Red staining, which marks mature acidified lysosomes. The process of autophagy leads to fusion of lysosomes and autophagosomes to form autophagolysosomes, which engulf, deliver and degrade cytoplasmic material for proper cellular recycling (Klionsky and Emr 2000). LAMP-1 increase may be a hallmark of early lysosomes, which are not maturing and acidifying. It is thus possible that autophagic flux is inhibited in deficient embryos, in part due to improper autophagosome maturation and therefore, immature lysosomes may accumulate and remain unused. The LAMP-1 increase may be due to this accumulation.

On occasions where autophagy is inhibited, studies have shown an accumulation of protein aggregates, as well as in p62 and ubiquitin-bound targets (Viiri, Hyttinen et al. 2010; Johansen and Lamark 2011). For instance, in \textit{Atg5} and \textit{Atg7} deficient neurons, autophagy inhibition is accompanied by an increase in ubiquitin and p62 levels, due to an increase in ubiquitinated protein aggregates (Johansen and Lamark 2011). The increase in ubiquitin levels when autophagy is inactive suggests a role for autophagy in targeting ubiquitinated proteins for degradation. However, it is also possible that compensation exists in a form of the ubiquitin-proteasome system (UPS). Moreover, recent evidence suggests that p62 shuttles proteins for degradation through the UPS (Wooten and Geetha 2006). Our results show no change in levels
of p62 and ubiquitin in Nlrp5 deficient embryos, which indicates that these substrates are most likely, cleared by the proteasome. It has been shown that proteasomal degradation is important for the transition from maternal to zygotic control (Mtango and Latham 2007). In addition, studies have reported a mandatory increase in autophagy activity in cases where the UPS is inhibited (Pandey, Nie et al. 2007). Therefore, the UPS could be taking over in Nlrp5 deficient embryos as a means to compensate for the lack of autophagy. As such, protein clearance does not seem to be altered in deficient embryos.

Evaluating whether the autophagic defect in deficient embryos may be due to a clearance defect in mitochondrial pool was also an unexplored interest. Autophagy plays a role in eliminating and recycling damaged organelles to maintain the well being of cells (Ravikumar, Sarkar et al. 2010). It has previously been shown that NALP5 protein localizes to the mitochondria (Tong, Gold et al. 2004). In addition, we have reported that Nlrp5 deficient oocytes produce an excess of reactive oxygen species (ROS), which is detrimental to the cell (Fernandes, Tsuda et al. 2012). As we observe a lack of autophagy in Nlrp5 deficient two-cells with a decrease in the mitochondrial pool, this suggests that the mitochondria are compromised and probably disintegrating. The defect is biogenesis related rather than a recycling issue, since we would expect to see an increase in mitochondria. The mitochondrial defects observed in the oocyte, prior to fertilization suggests the possibility of these damages carrying through early development (post-fertilization) and affecting the proper biogenesis of mitochondria.

Lipids play important roles in energy metabolism during oocyte maturation, fertilization and early preimplantation development (Hillman and Flynn 1980; McKeegan and Sturmey 2011). Autophagy plays a role in lipid breakdown through lipophagy, increasing free fatty acid availability for beta-lipid oxidation (Singh and Cuervo 2012). Our results in treating normal embryos with 3-MA and observing an increase in lipid droplet content further strengthen the importance of lipids as autophagic targets. Since an increase in lipid droplet (LD) volume has been reported previously in Nlrp5<sup>tm/um</sup> GVs (Kan, Yurttas et al. 2011) it was hypothesized that this increase would carry through early development. Indeed we observed an increase in lipid droplet volume in Nlrp5 deficient two-cells. LD accumulation due to autophagic inhibition has been previously reported in Atg5 and hepatocyte specific Atg7 knockout models, which further confirms the possible involvement of Nlrp5 in regulating lipid clearance. This suggests that the
increase in lipid content in embryos and the lack of their clearance may be the cause of the developmental arrest. In this thesis, I define a role for \textit{Nlrp5} by showing that this maternal lethal effect gene product appears to be required for proper lipid clearance. I propose that deficient embryos are unable to breakdown lipid droplets to free fatty acids for β-oxidation due to lack of autophagy. Evaluating ATP content in two-cell embryos would allow for a better understanding of the effects of this pathway.

To determine the mechanism through which NALP5 might regulate autophagy in preimplantation development, mTOR emerged as a possible candidate. Results showed mTOR expression in oocytes and early embryos. Since mTOR activation halts autophagy, using a potent inhibitor of this pathway called rapamycin will reverse its activation (Sarkar, Ravikumar et al. 2009). Therefore, if \textit{Nlrp5} deficient two-cell embryos work through this pathway to induce autophagy then inhibiting mTOR with rapamycin treatment should have rescued the two-cells and triggered development. Our results did not show any rescue effect, which suggests that this process may be triggered through another mechanism.

It is tempting to regard both NF-κB and autophagic dysfunction as independent events in an attempt to uncover pathways associated with normal early embryo development. However, it is important to realize that they are interrelated though their link is a matter of debate. According to some studies, NF-κB can induce autophagy by increasing the expression of autophagy-triggering protein Beclin-1 (Copetti, Bertoli et al. 2009). A number of indirect pieces of evidence also suggest an activating role on autophagy (Paul, Kashyap et al. 2012) Conversely, NF-κB has also been reported as a negative regulator of autophagy, for instance in the context of tumor necrosis factor-α ROS production (Djavaheri-Mergny, Amelotti et al. 2006). Additionally, autophagy might itself contribute to the inhibition of the NF-κB pathway. It has shown to mediate the degradation of the IKK complex (Qing, Yan et al. 2007) and mediate the depletion of IKK activator p62 (Duran, Linares et al. 2008). Therefore, it is not surprising that a deficiency in \textit{Nlrp5} in embryos impacts various pathways. Unraveling the connection between these pathways and how they interact during preimplantation development should be the next step of analysis.
4.4 NALP5 accumulation in aged human MII oocytes

Among infertile women seeking IVF not all reach the desired outcome of pregnancy. These women produce embryos, which fail to undergo normal early embryo development. It is possible that maternal factors necessary for supporting normal development are defective or insufficiently endowed in their fertilized ova. We hypothesized that NALP5 is an essential maternal factor in humans, which causes developmental failure in its absence.

Despite a decrease in Nlrp5 mRNA expression in aged mice and human oocytes, we observed an increase in NALP5 protein levels in oocytes retrieved from aged IVF patients (>39 years old). Similarly, aged mouse oocytes have shown an increase in NALP5 protein expression (unpublished data, Velummailum and Chong). As NALP5 is eliminated during preimplantation stages through an unknown mechanism, the increase in protein levels with concomitant decrease in transcript levels suggests an accumulation of NALP5 protein in oocytes of older females due to a defect in its degradation. Work from our laboratory also indicates that old oocytes are defective in their execution of autophagy, which strengthens the possibility of NALP5 as a substrate of autophagy. Intracellular accumulation of DNA, protein, lipids and cellular organelles is one of the common features of age-related changes. It has been observed that autophagic activity declines with age, and this is correlated with accumulation of damaged proteins and organelles (Rezzani, Stacchiotti et al. 2012). Though it has been previously shown that autophagy is absent in oocytes, our results have shown the opposite (Section 3.11). In addition, our laboratory has also shown that germinal vesicles retrieved from older mice display an increase in mitochondrial endowment but not because of increased biogenesis. Therefore, the increase in NALP5 protein levels observed might be a consequence of decreased autophagic flux in older human oocytes. If autophagy were inefficient with age, it would fail to clear the accumulated NALP5 protein, which may interfere with proper development. This alludes to the fact that autophagic machinery may be altered prior to the two-cell stage in Nlrp5 deficient mice.

4.5 Future Directions

Nlrp5 is a maternal-effect gene that is essential for the development of mouse embryos. We have provided evidence that Nlrp5 has a role in autophagy, in maintaining clearance of accumulated
lipids to allow for proper embryo development. Evaluating ATP levels in Nlrp5 deficient two-cells would help in explaining the possible lack of lipid metabolism in these embryos since defective autophagy is preventing the breakdown of lipid droplets. The build up of lipids may interfere with proper embryo development and lead to two-cell arrest.

Exploring whether NALP5 protein interacts with autophagy related proteins would help outline the mechanistic pathway associated with the autophagy defect. This will be achieved by immunoprecipitation experiments after transfections of epitope-tagged proteins of interest in cell lines. We propose to use HEK293 cells as this cell line has high transfection efficiency. It is important to note that these cells do not endogenously express NALP5 protein, as it is an oocyte specific protein. Therefore, our ultimate goal would be to create permanent NALP5 transfected cell lines. Determining whether Beclin-1 and NALP5 interact would be of great interest, since another NALP family member (i.e. NALP4) has shown to regulate autophagy through its interaction with this protein. As well, interaction of NALP5 with apoptosis associate speck protein (ASC) could tease out the possibility of NF-Kβ regulation through this docking molecule. ASC, which contains PYD and CARD domains, has many functions such as activation of caspases and modulation of NF-Kβ (Srinivasula, Poyet et al. 2002; Hasegawa, Imamura et al. 2005). It has been shown to act through NALP inflammasomes and therefore, may be of interest in interrogating possible NALP5 pathways. Furthermore, we will treat NALP5 transfected cells with autophagy inhibitors like 3-MA, chloroquine and bafilomycin, all compounds known to inhibit various stages of autophagy, to determine NALP5 processing pattern. We would like to determine whether NALP5 itself is a substrate of autophagy and therefore, if it would accumulate in the absence of autophagy.

Western blot analysis of various autophagy related proteins in WT and Nlrp5 deficient two-cell embryos is also essential including cleaved LC3, LAMP-1 and downstream mTOR targets. When mTOR is activated, autophagy is inhibited and can be monitored by phosphorylation of its downstream targets like p70S6K and 4E-BP1. Determining the phosphorylation status of these proteins through western blot analysis would help confirm at what point in the pathway autophagy is defective in Nlrp5 deficient embryos.

My findings primarily focus on Nlrp5 phenotypes in mice. My immunofluorescence data suggests an increase in NALP5 in human oocytes retrieved from aged females. Western blot
analysis of oocytes from young and old females will further confirm the increased NALP5 expression in older oocytes. Ultimately, by determining the role NALP5 plays in humans, the proposed studies could significantly improve current ART techniques and alleviate some unexplained causes of infertility.
References Cited


