REGULATION OF LINE-1 IN DEVELOPING OOCYTES AND THE IMPACT ON THE

OVARIAN RESERVE

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An Abstract of the Thesis Presented
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In humans and mice, the ovarian reserve (OR) is established during a lengthy process that starts during early embryogenesis with germ cell specification and culminates in the first weeks after birth when primordial follicles (PF) are formed. OR establishment is an important process which influences the fertile lifespan and fecundity of the individual. Fetal oocyte attrition (FOA) has been identified as a critical developmental event that regulates how many oocytes survive and contribute to the final OR. In addition to FOA, OR size and quality also depend on efficiency of meiotic recombination. Chromosome asynapsis and unrepaired meiotic double-strand breaks (DSB) lead to the exclusion of defective oocytes from the final OR by a checkpoint mechanism. There is limited understanding of how genetic factors modify these mechanisms and determine the size and quality of the OR.

LINE-1 retrotransposon elements have previously been identified as playing a critical role in FOA in female mice. Transposable elements are capable of insertion
anywhere in the genome by generating DSBs followed by insertional mutagenesis which can affect expression of genes near insertion sites. The mechanism by which LINE-1 activity affects the OR remains unknown. High levels of LINE-1 expression are thought to cause additional DSBs during meiotic recombination and negatively affect oocyte survival. We hypothesized that mouse strains with large OR might exhibit increased survival during FOA and/or quality checkpoint. Survey of the OR size among eight inbred strains, used as the founders for Genetically Diverse mice, revealed that NOD/ShiLt females have the largest and highest quality OR compared to other inbred strains including C57BL/6 strain. Here we show greater oocyte survival in the FOA window of elimination in NOD/ShiLtJ than in C57BL/6J ovaries which results in more oocytes included in the OR. To investigate one possible cause of FOA evasion, we determined LINE-1 expression levels in ovaries during prenatal stages, finding lower overall levels in NOD/ShiLtJ than in C57BL/6J ovaries. These observations support a role for LINE-1 as a regulator of OR size and suggest that unknown genetic factors acting in the NOD/ShiLtJ strain regulate LINE-1 activity and oocyte survival during development. Thus, identifying the NOD/ShiLtJ strain as a good model to investigate regulation of FOA and LINE-1 activity in female germline. Supported by R01 HD093778.
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INTRODUCTION

1. The establishment of the ovarian reserve and developmental timeline.

The ovarian reserve is a finite and non-growing population of oocytes in primordial follicles which determines the fertility and reproductive lifespan of an individual. The size and quality vary between women as reflected by the population.

![Diagram of ovarian reserve and developmental timeline](image)

Figure 1. The development of the ovarian reserve, meiosis, and LINE-1 activity in mice. (A) The developmental timeline of the ovarian reserve as it has been established in mice. At embryonic day 9.5 (E9.5) germ cells proliferate and migrate, proliferation continues as sex determination takes place at E12.5. Following sex determination female germ cells will shift to the meiotic cell cycle and the size of the germ cell population will reach its peak numbers at E13.5-E14.5. During meiotic prophase one the ovarian reserve size will decrease with three waves of germ cell elimination, the first of which is fetal oocyte attrition (FOA) between E15.5 and E18.5. As female germ cells reach the end of meiotic prophase one and arrest the meiotic checkpoint will eliminate meiotically defective cells around E18.5 and postnatal day 0 (P0). Cyst break down occurs simultaneously with the other elimination events and establishes primordial follicles completing this process at approximately P5. (B) Synapsis of homologous chromosomes can be visualized during meiotic prophase one by meiotic spreads. Staining for synaptonemal complex lateral element protein SYCP3 with immunofluorescence allows for staging of oocytes. Early meiotic prophase one is referred to as leptonema where the synaptonemal complex is beginning to be organized, but the homologous chromosomes have not yet started to pair. Zygotene is marked by the partial synapsis of homologous chromosomes. At pachynema full synapsis has been reach, followed by diplonema where chromosomes begin to desynapse. The synaptonemal complex is disassembled and the oocytes will arrest in dictyate until ovulation.
variation of clinical ovarian reserve marker Anti-Mullerian Hormone (AMH)\textsuperscript{68}, the onset of menopause, and in the rates of infertility. The ovarian reserve is established largely during fetal development which limits our ability to investigate mechanisms regulating its size and quality in human.

During early embryogenesis the differentiation of primordial germ cells (PGCs) establishes the pool of cells which will become germ cells and eventually the oocytes which make up the ovarian reserve, these cells migrate, and proliferate (Figure 1A). The process of specification and migration is completed by approximately embryonic day 10.5 (E10.5), during this time the cells will continue to proliferate\textsuperscript{1}. While the population of PGCs expand through proliferation, the embryo goes through sex determination around E12.5. The PGCs start exiting the mitotic cell cycle around E13.5 and initiate meiosis\textsuperscript{4}. As the now established female germ cells cease proliferation and become oocytes, they reach their peak number and from here the numbers decrease during the developmental process (Figure 1A). This is because many oocytes are eliminated, due to a variety of reasons which could include death by defect, death by neglect, or death by sacrifice\textsuperscript{72,73,74,75}. From E13.5 oocytes will progress through meiotic prophase I and will arrest at extended diplotene stage known as dictyate around birth (Figure 1B). Arrested oocytes will resume meiosis after puberty during ovulation cycles.

There are three main elimination events that occur during the development of the ovarian reserve. These include Fetal Oocyte Attrition (FOA), the meiotic checkpoint and cyst breakdown. Oocyte elimination during FOA in mice reduces the germ cell population by 75\%\textsuperscript{76}. FOA is a highly conserved process, but the underlying mechanisms of regulation are just being uncovered. Previous research has identified
Long Interspersed Nuclear Element-1 (LINE-1) retrotransposons as playing a role in FOA elimination\(^2\). It was observed that increase in LINE-1 expression correlates with FOA in mice from E15.5 to just before birth. Inhibiting LINE-1 activity prevented oocyte loss while increasing their expression enhanced oocyte loss\(^2,6^3\). The meiotic quality checkpoint which eliminates meiotically defective oocytes, similar to the checkpoint which also exists in mitotic cells\(^3,4\), can further reduce oocyte numbers. This mechanism of eliminating potentially defective oocytes occurs between E18.5 and postnatal day 0 (P0) when oocytes reach the pachytene and diplotene stages of meiotic prophase I. The meiotic checkpoint is reliant on the function of the CHEK2 kinase\(^5\). As both of these eliminations occur, approximately E18.5 to P5, cyst breakdown is completed and only approximately 33% of the remaining oocytes will form primordial follicles\(^7^4,6^\). This process takes place within the synchronized clusters of germ cells which are formed by a single progenitor cell and connected by intercellular bridges. Most of the remaining oocytes serve as nurse cells and die as cysts eventually break down and primordial follicles are formed, each typically containing a single oocyte\(^7^4\).

Meiosis takes place concurrently with the waves of elimination as previously described. Meiosis is the process by which haploid gametes are made from diploid precursors. The onset of meiosis takes place around E13.5 in mice and the oocytes enter meiotic prophase I in a wave\(^7\). The majority of the oocytes will enter meiosis by E15.5\(^8\). One of the main purposes of meiosis is recombination, the fidelity of recombination is critical for the maintenance of genomic integrity.

Erroneous recombination can lead to aneuploidy and approximately half of spontaneous abortion is due to chromosomally abnormal pregnancy\(^9,10\). Meiotic
Prophase I (MPI) is split into four different stages which describe the progression of recombination and synapsis. These stages are leptotene, zygotene, pachytene and diplonema. During the leptotene phase meiotic double-strand breaks (DSBs) are induced by SPO11, and these DSBs are made in non-random and identifiable recombination hotspots designated by PRDM911,12,13. During zygotene homologous chromosomes begin pairing and synaptonemal complex (SC) proteins are recruited14. The SC resembles a zipper and is comprised of two lateral elements connected by transverse filaments which overlap in the middle and form the central element. During zygotene, SYCP2 and SYCP3 organize along the axis of the cohesion core which make up the lateral elements and SYCP1 begins to connect them forming the transverse element15,16,17. The central element is comprised of many proteins including SYCE1, SYCE2, and TEX1218,19,20.

DNA repair and recombination proteins are recruited to DSBs sites including RAD51, which direct homology search and chromosome pairing21,22,23. DSB repair occurs through homologous recombination and involves many specialized proteins including yH2AX, BRCA1, and RAD5124,25,26. Meiotic DSBs repair is completed during pachytene when full synapsis has been reached. The completion of recombination and cross over formation are important for increasing genetic diversity and proper chromosome segregation during first meiotic division. Failure to fully synapse a pair of homologous chromosomes can lead to recombination failure and lack of crossover connecting homologues. Asynapsed chromosome axes and persistent DNA damage are marked by the presence of BRCA1, yH2AX, and Rad5127. These defects in pachytene oocytes activate checkpoint mechanisms and lead to elimination of defective
oocytes. However, some defective oocytes may survive resulting in inclusion of low-quality oocytes in the ovarian reserve. Asynapsis and consequently insufficient number of crossovers can lead to aneuploidy in ovulated eggs due to mis-segregation of the chromosomes\textsuperscript{28}. However, if the cell properly synapses then it will continue to diplotene which is where the synaptonemal complex starts to disassemble but homologous chromosomes will stay connected by crossovers. Late diplotene oocytes arrest at a stage called dictyate and will stay arrested for months in mice and for years in humans. As mentioned, oocytes with asynapsis or persistent DNA damage may be eliminated by the meiotic checkpoint.

2. **Genetically Diverse mice models better reflect the phenotypic variation seen in human population.**

The establishment of the ovarian reserve is a process that is highly conserved across species. Studies of the human ovarian reserve are very important for the understanding of human fertility disorders. Studies by which to conduct this research mostly involve testing for hormone levels and imaging ovarian follicles by ultrasound. More specifically, Anti Mullerian Hormone (AMH) is typically used for approximating follicle numbers in humans and used as a diagnostic tool. Poly-cystic ovarian syndrome is the most common cause of non-ovulating infertility and affects approximately 5-10\% of people at reproductive age\textsuperscript{69}. Recently it has been shown that AMH can be used as a diagnostic tool for PCOS, because individuals have increased levels of AMH\textsuperscript{70}. The cause(s) of PCOS have yet to be identified, however, it is thought that it is the results of different genetic interactions as well as environmental factors\textsuperscript{71}.
There are many factors which can influence the size and quality of the developing ovarian reserve, including environmental and genetic factors. There is a large gap in the understanding of how genetic background and allelic variation impact the development of the ovarian reserve, and how they could predispose individuals to premature menopause or fertility problems. Recently the reproductive field has made advances in personalized medicine looking more specifically at genetic markers for infertility risk, however, known markers are limited. Advances in our understanding of human disease are limited by indirect methods used in human studies and therefore other models of study are critical in the advancement of medical diagnosis and potential therapeutic treatment. Ovarian reserve establishment is highly conserved therefore mouse models are indispensable in furthering our knowledge and understanding of these processes and the underlying mechanisms that control them.

Germ cell development and meiosis have been studied in mice in depth but most research has been performed in genetically homogeneous models. Genetic homogeneity makes for more reproducible research where genetic diversity would make that difficult, however this does not reflect genetic heterogeneity of the human population which we are trying to model in order to understand diseases. Traditionally research has used inbred mouse strains to study developmental processes, which while it allows for systematic and reproducible studies, it has created a blind spot around genetic diversity and allelic variation. Much of the established knowledge in this field has used C57BL/6 or 129S1/SvImJ inbred strains. While using these strains and the gene knockout approach are helpful in establishing the role of individual genes in
disease etiology, it misses the translational applicability in the human population which is not genetically identical.

To achieve better simulation of human genetic and phenotypic diversity, new mouse models have been developed including the Diversity Outbred and Collaborative Cross mice\textsuperscript{30}. Diversity Outbred mice are a genetically heterogeneous population similar to human with the advantage of minimizing environmental factors in a study. However, like humans each genome is unique and therefore not reproducible. The Collaborative Cross is a collection of recombinant inbred lines each being a unique mosaic of eight founder strains genomes\textsuperscript{31}. Both of these populations were established using the same eight founder inbred strains: A/J, C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, NZO/HiLtJ, CAST/EiJ, PWK/PhJ, WSB/EiJ. While the Diversity Outbred mice are genetically diverse, they are only reproducible as a population; the Collaborative Cross mice are recombinant inbred strains with increased diversity they offer higher reproducibility.

The need for a better understanding of gene-gene and allele-allele interactions is crucial in understanding nearly any complex traits and disease. It is repeatedly observed in human disease onset and presentation, this is described as penetrance and expressivity\textsuperscript{77}. Penetrance is described as the portion of the population which carries a disease-causing mutation and has the disease phenotype\textsuperscript{78}. Expressivity is the degree to which a disease presents phenotypically\textsuperscript{79}. There are various reasons for these differences seen between individuals including environmental factors, allelic variation, and modifier genes.

Modifier genes can act as a protecting factor in some cases, by modifying the activity of the mutant transcript either directly or indirectly\textsuperscript{80}. Allelic variation accounts for
the presences of alleles which produce different gene products or differences in expression\textsuperscript{81}. These allelic variations can often be masked by canalization, which is the lack of phenotypic change despite genetic or environmental factors\textsuperscript{82}. This can delay the identification of the role of different alleles in disease, due to canalization by environmental factors, which makes understanding allelic variation in humans difficult since people experience a large variety of environmental factors over their lifespan.

This makes model organisms indispensable in the investigation of allelic variation as a driver of phenotypic variation. Studies in mice have revealed that genetic interactions in heterozygotic animals can drive specific traits when expressed in specific backgrounds\textsuperscript{84}. The inbred founder strains used to establish the genetically diverse mouse panels also have characteristic SNPs and phenotypes of their own. These strains have established uses however we do not know what more broad study of the inbred founder strains may reveal phenotypically or how their unique genetic backgrounds may interact when combined with one another\textsuperscript{32}. The study of these models in addition to CC lines and DO mice present and opportunity to better model and study on various levels how genetic background and allelic variation can impact processes which may be well known or established in a single model. In fertility research to best understand human diseases, we need studies in genetically diverse models where we can identify how and why such large variation is seen across individuals.
3. **LINE-1 retrotransposable elements and their known roles and regulatory mechanisms.**

Transposable elements make up a large portion of the mammalian genome and are a class of repetitive DNA sequence which can translocate\(^3\). Transposons are separated into two categories which are known as retrotransposons and the DNA transposons\(^34,35\). Retrotransposons, specifically LINE-1 have been implicated in oocyte elimination during FOA\(^2\). Retrotransposons represent the largest number of the transposons present in the mammalian genome and they move via an RNA intermediate inserting themselves into new locations of the genome\(^34\).

LINE-1 retrotransposable elements are autonomous non-LTR transposons, because they encode their own mobility proteins. LINE-1 contains two open reading frames (ORFs) which are ORF1 and ORF2. ORF1 contains the sequence for a protein containing the RNA recognition motif (RRM). ORF2 encodes for a protein with endonuclease activity and reverse transcription. LINE-1 mRNA is transcribed in the nucleus and then will be transported to the cytoplasm where the two ORFs will be translated into proteins, and these two proteins then form a ribonucleoprotein which binds with RNA. This ribonucleoprotein containing now LINE-1 RNA will return to the nucleus, and through endonuclease activity create DSBs in the genome and the LINE-1 RNA will be inserted and reverse transcribed.

![Figure 2. LINE-1 activity and retrotransposition](image)

(A) LINE-1 retrotransposable elements are made up of an internal promoter and two open reading frames. ORF1 contains the sequence for a protein containing the RNA recognition motif (RRM). ORF2 encodes for a protein with endonuclease activity and reverse transcription. LINE-1 mRNA will be transcribed in the nucleus and then will be transported to the cytoplasm. In the cytoplasm the two ORFs will be translated into proteins, and these two proteins then form a ribonucleoprotein which binds with RNA. This ribonucleoprotein containing now LINE-1 RNA will return to the nucleus, and through endonuclease activity create DSBs in the genome and the LINE-1 RNA will be inserted and reverse transcribed.
frames: ORF1 which encodes a protein with the RNA recognition motif (RRM) and the ORF2 which encodes a protein for reverse transcription and the endonuclease which allows for insertion into the genome\textsuperscript{36,37,38} (Figure 2A). LINE-1, similar to a virus, has a life cycle in which it must transcribe itself into RNA (Figure 2B), be exported to the cytoplasm (Figure 2C), translate its two protein components ORF1 and ORF2 (Figure 2D), form a ribonucleoprotein which is made up of the two ORF proteins and LINE-1 RNA (Figure 2E), be transported back into the nucleus, and go through target primed reverse transcription in which it is inserted into a new location in the genome (Figure 2F)\textsuperscript{39,40}.

Active copies of LINE-1 throughout the genome can be beneficial or detrimental and impact many processes\textsuperscript{42,43}. There are several genetic disorders directly connected to mutation by LINE-1 retrotransposons including a specific haemophilia type A due to insertion directly into a gene\textsuperscript{88}. However, the presences of LINE-1 and other transposable elements has vastly diversified the human genome\textsuperscript{42}. Through the process of insertion, the LINE-1 is creating DSBs by endonuclease activity. In mice it has a much higher rate of insertion compared to humans, which is thought to impact the rate of mutation. This may be a drawback of using mice to model ovarian reserve development and LINE-1’s role, however, biological differences are expected between model organisms. Due to the mutagenic potential regulation of LINE-1 in the germline is critical.

In most cells, DNA methylation is the most ubiquitous method of regulation which silences LINE-1 transcription (Figure 2 I)\textsuperscript{44}. DNA methyltransferases (DNMTs) and chromatin helicases (LSH) have known roles in LINE-1 suppression in male
germline\textsuperscript{47,48}. However, primordial germ cells undergo global DNA demethylation in which LINE-1 elements are derepressed allowing their expression\textsuperscript{45,46}. In the male germline, the PIWI/piRNA pathway (Figure 2 III) is the major mechanism for transposon silencing\textsuperscript{85}. The PIWI/piRNA pathway is also known to interact with an extensive network of proteins which make up nuage or mitochondrial cement in germ cells\textsuperscript{86,86,86,87}. In male germline high levels of retrotransposon expression due to mutation in PIWI genes was shown to be detrimental and interferes with progression of meiotic recombination\textsuperscript{51,52,53,54}. Ribonucleoproteins produced by LINE-1 are double stranded RNA (dsRNA), and it is not surprising that the RNA interference (RNAi) system and RISC complex has been identified in playing a role in LINE-1 suppression by RNA degradation (Figure 2 II)\textsuperscript{87}.

Across various species in both sexes, retrotransposable elements are often found in germ cell granules\textsuperscript{51,55,56,57}. Germ cell granules are defined by being a membrane-less “organelle” which have been found to contain RNPs and have been identified across mammals, \textit{Drosophila}, \textit{C. elegans}, \textit{Xenopus}, and \textit{Zebrafish}\textsuperscript{101}. PIWI/piRNAs are known to be important in the formation of nuage also referred to as mitochondrial cement, which are a site of transposon RNA degradation (Figure 2 IV)\textsuperscript{89}. In \textit{C. elegans} P-granules function similarly to nuage in retrotransposon regulation (Figure 2 V)\textsuperscript{90}. LINE-1 RNA has also been associated with stress granules and autophagy (Figure 2 VI)\textsuperscript{91}. At the protein level Balbiani bodies are a method of RNP aggregation which contains the retrotransposons from the nucleus (Figure 2 VII)\textsuperscript{92}. However, much less is known about mechanisms for retrotransposon suppression in fetal oocytes. Oocytes enter meiotic prophase I with derepressed LINE-1 elements
which leads to their increased expression throughout meiotic prophase one (MPI).

In oocytes many of the known germline retrotransposon repression mechanisms are dispensable. When knocked down in male mouse germline PIWI protein, piRNA, and other nuage factors show a sterile phenotype, while in female germline the mice remain fertile.

Previous work from the Bortvin lab, showed LINE-1 elements as playing an important role in FOA. This work also confirmed that when LINE-1 is inhibited during Meiotic prophase I there are short lasting protective effects where oocyte survival was increased. Continued work also shows that the role of FOA is potentially to preliminarily eliminate poor quality oocytes from the OR. The LINE-1 driven mechanism acting in FOA reverse transcription intermediates and endonuclease activity. Reverse transcription intermediates have been linked to immune system activation and the complement system and which could induce apoptosis in oocytes. Endonuclease activity the process by which LINE-1 is inserted into the genome creates DSBs which can trigger apoptosis.

Retrotransposable elements are abundant in the genome and LINE-1 are the only active retrotransposons in humans and in mice. They have been linked to many diseases (PMID: 32850797) therefore it is important to understand how they are regulated. Despite extensive knowledge about LINE-1 regulation in somatic cells and male germline, the mechanisms of LINE-1 suppression during oogenesis in mice remain poorly understood.
4. Variations in ovarian reserve development seen in different genetic backgrounds of mice and the role of LINE-1.

Meiosis is an essential developmental process, that must occur for the development of oocytes. During meiosis a variety of processes occur including the elimination of oocytes. These elimination events undergone by the oocyte population are critical, because this determines the size and quality of the ovarian reserve for the lifespan of an individual. The first of these events is FOA, which as described previously is driven by LINE-1\(^2,63\). Many studies have been performed to investigate the process of ovarian reserve development, however the models used to perform these studies are unable to provide information on what could cause ovarian reserve variation as seen in the human population.

Our study aims to begin to uncover underlying factors which drive these variations. While models for genetic diversity have been developed for the express purposes of studies like this, the Collaborative Cross and Diversity Outbred mice\(^30\). The genetic background for these two panels were established by the eight inbred founder strains: A/J C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, NZO/HiLtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ. While the Collaborative Cross and Diversity Outbred panels allow for

![Ovarian reserve size differs across inbred founder strains.](image-url)
studies of genetically diverse mice at various scales, there is a unique opportunity to get an understanding of the contribution from each of the founder lines. An understanding of the characteristics of the inbred founder strains ovarian reserve development will allow for more informed studies of outbred panels of mice. This will better model the variation of the ovarian reserve size and quality seen in the human population.

Using inbred founder strains preliminary data from our lab shows variation among ovarian reserve size (Figure 3). These results highlight interesting differences between found strain ovarian reserve development indicating potential differences in mechanisms which regulate the processes which shape the ovarian reserve. Specifically, NOD/ShiLtJ which has the largest ovarian reserve at P5, and additionally FOA (E15.5-E18.5) shows reduced loss in comparison with other strains. This study aims to gain an understanding of the differences in driving mechanisms of FOA in NOD/ShiLtJ, A/J, and C57BL/6J.
RESULTS

Differences in oocyte survival during FOA in C57BL/6J and NOD/ShiLtJ suggest differences in potential regulatory mechanisms. To understand how differences in ovarian reserve size arise among genetically different backgrounds we analyzed oocyte numbers during three waves of elimination in three founder strains; C57BL/6J, NOD/ShiLtJ and A/J. These were previously identified as having significant differences in oocyte numbers and development. Using whole mount immunofluorescent staining (WMIF) and microscopy of whole ovaries, we estimated oocyte numbers in ovaries at multiple developmental stages. We counted oocytes at E15.5 (early FOA), E18.5 (late FOA), and postnatally at birth (P0), P2, and P5 which will inform us of how many oocytes survive FOA, pass the meiotic checkpoint, and arrest forming the ovarian follicle reserve.

Results show that overall NOD/ShiLtJ has the largest ovarian reserve at P5 with an ovarian reserve of 4111±516 (<0.0001) oocytes per ovary compared to C57BL/6J at 2295±454 and A/J 2684±369 (Figure 4A). At E15.5 oocyte numbers were not significantly different between strains. Interestingly, we observed a minimal oocyte loss in NOD/ShiLtJ between E15.5 and E18.5 (non-significant difference), which accounts for only 3% compared to 25% in C57BL/6J (E15.5 vs E18.5; <0.0001). Reduced oocyte loss during this period of FOA makes NOD/ShiLtJ strain particularly interesting as this suggest that mechanisms triggering oocyte loss may not function in NOD/ShiLtJ. In A/J, we see an intermediate loss of oocytes at 14% (E15.5 vs E18.5; P=0.0076). Interestingly, NOD/ShiLtJ and A/J strains share a defective allele for Hemolytic Complement 5. The complement system has been previously linked to the response to
reverse transcription intermediates produced by retrotransposable elements which are a driver of FOA. This could suggest an unexpected role of the Complement System in oocyte development which future studies will investigate. Here we investigate the potential differences of regulatory mechanisms related to LINE-1 during meiosis and FOA in these three strains.

Although we see only a minimal oocyte loss over the 3-day span of FOA it is possible that either NOD/ShiLtJ strain has more oocytes entering MPI or that FOA takes place earlier than in C57BL/6J. We analyzed female germ cell numbers earlier in embryonic development at E13.5 and E14.5 to see if their numbers differ at the onset of MPI. At E13.5 A/J and C57BL/6J have similar numbers of germ cells 3726±747 and 3012±794 respectively (P= 0.1475), while NOD/ShiLtJ has significantly higher number 4979±574/ovary (P= 0.0054) (Figure 4A). By E14.5 the number of oocytes have increased across all three strains as female germ cells continue to proliferate until E15.5103. C57BL/6J and NOD/ShiLtJ have similar germ cell numbers (7490±251 and 6730±373, respectively; P= 0.1658) and A/J has significantly less than C57BL/6J (5690±586; P=0.0003). Between E14.5 and E15.5 all strains lost germ cells with the highest loss in C57BL/6J at 28%, NOD/ShiLtJ 20%, and A/J 11%, while germ cell numbers are similar at E15.5 when all female germ cells are expected to enter MPI.
Figure 4. Differences in oocyte survival during FOA in C57BL/6J and NOD/ShiLtJ suggest differences in potential regulatory mechanisms. (A) Average numbers of oocytes per ovary, for C57BL/6J, AJ, and NOD/ShiLtJ represented with timepoints and STDEV. (B) shown as the percentage of oocytes remaining in the ovarian reserve during development after E14.5 (100%) to the establishment of the final ovarian reserve at P5. (C) The percentage of oocytes positive for meiotic cell cycle marker SYCP3. (D) Immunofluorescent images of whole ovaries with GCNA, SYCP3, and γH2AX. Whole ovary images are 3D renderings, magnification represents one single Z-slice at higher magnification from region indicated by the box. (E) Oocyte meiotic spreads at E15.5 analysis showing percentage of cells in meiotic stages: leptotene, zygotene, pachytene, and abnormal. N>3, error bars represent standard deviation from the mean.
Results indicate that when female germ cells reach peak numbers, NOD/ShiLtJ does not have significantly higher number of oocytes than C57BL/6J to explain later difference. It is possible that differences we observed at specific timepoints are due to differences in meiotic entry and progression dynamics.

We hypothesized that differences in onset of meiosis could affect when oocytes. To assess if NOD/ShiLtJ enters meiosis earlier, we investigated the percentage of cells with meiotic cell cycle markers SYCP3 (SC component) and γH2AX (DSBs marker) (Figure 4C-D). The percentage of SYCP3 positive oocytes is now in all samples indicating that they are just entering meiosis but there are more oocytes positive for SYCP3 in NOD/ShiLtJ at E13.5 than in C57BL/6J and AJ (11% vs 6% and 7% respectively). However these differences are not significant due to variability across samples (Figure 4C). Similarly, high variation of SYCP3 colocalization is observed at E14.5, with NOD/ShiLtJ and AJ showing similar numbers of SYCP3 positive cells. At E14.5 C57BL/6J had more oocytes colocalized with γH2AX, whereas at E13.5 NOD/ShiLtJ had more γH2AX positive oocytes (Figure 4D). Our results from whole mount immunostained ovaries show that NOD/ShiLtJ has slightly higher percentage of oocytes in meiosis prior to E15.5, suggesting that it may be advancing faster than C57BL/6J and A/J. However, these changes are not large enough to suggest that in NOD/ShiLtJ FOA occurs earlier between E14.5 and E15.5.

Identification of MPI stages is difficult in whole mount stained ovaries, therefore to determine if meiotic progression is accelerated in NOD/ShiLtJ, we staged meiotic spreads at E15.5 using cytological method (Figure 4E). In A/J 58% of oocytes were still in the leptotene stage at E15.5, 37% were in zygotene while only 4% had reached
pachytene. In C57BL/6J the majority of oocytes were in zygotene (54%), 45% in leptotene and 1% in pachytene. Only 18% of oocytes were in leptotene in the NOD/ShiLtJ with 59% in zygotene and 22% in pachytene. This confirms that NOD/ShiLtJ oocytes are cytologically more advanced than both C57BL/6J and A/J at this time in development. Whether the accelerated meiotic progression in NOD/ShiLtJ is linked to decreased oocyte elimination during FOA remains unclear, therefore we set out to investigate the regulatory mechanisms which may be driving the differences observed between these three strains.

**LINE-1 protein levels and localization differ between strains during FOA.**

The differences in oocyte survival during FOA observed in these three strains of mice, suggest differences in underlying mechanisms regulating oocyte elimination. LINE-1 retrotransposons are an important driver of FOA, therefore we investigated LINE-1 expression at the protein level in ovaries from embryonic day 14.5 (E14.5) through postnatal day 0 (P0) (Figure 5A). Protein levels were compared by Western Blot probed for LINE-1 as well as the germ cell maker MVH and housekeeping gene β-Actin. L1 levels detected by WB in ovaries from single females were variable but on average C57BL/6J had higher levels at E17.5 and E18.5 than NOD/ShiLtJ and A/J. This variation may reflect true biological differences between individual animals within strain and more sensitive method is needed to confirm that. The overall lower levels of LINE-1 in NOD/ShiLtJ and A/J suggest that this may be the underlying cause of higher survival of oocytes. The differences in protein levels measured in whole ovarian extracts may not reflect LINE-1 activity because it’s the localization of LINE-1 in the nucleus that results in DNA damage and retrotransposition.
Next, we investigated LINE-1 subcellular localization by immunostaining on sections and evaluating % of oocytes with cytoplasmic and nuclear localization patterns of LINE-1. Intriguingly, we detected LINE-1 in large cytoplasmic granules in addition to diffused signal in cytoplasm or, nucleus (Figure 5B). These granules have not been previously reported in fetal oocytes and may be involved in sequestration of LINE-1 away from the cytoplasm and nucleus to minimize their damaging effect on DNA and potential for reinsertion. Abundant cytoplasmic LINE-1 has the potential to transported into the nucleus, causing DSBs through its endonuclease activity but how much LINE-1 is transported may be regulated by unknown factors (Figure 2F).

Compared to NOD/ShiLtJ, A/J and C57BL/6J had overall more oocytes with nuclear LINE-1 localization (Figure 5D). NOD/ShiLtJ had peak diffuse cytoplasmic (28.2%) LINE-1 localization at E16.5 (Figure 5C). Whereas in C57BL/6J and A/J diffuse

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**Figure 5** LINE-1 protein levels and localization differs between strains. (A) Graph of LINE-1 protein levels normalized to MVH. Example of Western blot probed for β-actin (42kDa), MVH (76kDa), and LINE-1 (41kDa) with the ponceau staining for loading control. (B) immunofluorescent image of NOD/ShiLtJ ovary with GCNA (nuclear germ cell marker) in purple and LINE-1 in green. Image shows diffuse cytoplasmic LINE-1 localization and LINE-1 granules. (C) Graph of the percentage of oocytes with diffuse cytoplasmic LINE-1. (D) Graph of the percentage of oocytes with LINE-1 nuclear signal. (E) Graph of the percentage of oocytes with LINE-1 granule(s). *Protein level below detection. N≥2, error bars represent standard deviation from the mean. Best fit curve calculated with non-linear fit Gaussian curve.
cytoplasmic LINE-1 localization increases to E17.5 (respectively C57BL/6J 29.4% and A/J 26.1%). All three strains had the highest % of oocytes with nuclear LINE-1 localization at E16.5 (respectively A/J 2.8%, C57BL/6J 2.8%, NOD/ShiLtJ 0.9%) (Figure 5D). Analysis of localization shows that C57BL/6J had the highest % of oocytes with diffuse cytoplasmic and the most presence of nuclear LINE-1 localization. Additionally, the presence of diffuse cytoplasmic localization of LINE-1 persists for longer in C57BL/6J and A/J. This data shows that in NOD/ShiLtJ has fewer oocytes which have nuclear LINE-1 localization when oocytes typically undergo FOA.

We observed novel granules containing LINE-1 which were not previously reported (Figure 5B). Germ cell granules such as nuage or P-granules have been seen across many species and in both sexes during germ cell development. These granules are often associated with mechanisms regulating retrotransposons expression and activity. However, previous reports show that germ cell granules and their components have not been detected in mouse fetal oocytes.

To better understand the possible function of LINE-1 granules we analyzed their numbers and dynamics. We hypothesized that sequestration to granule is a regulatory mechanism to reduce the entry of LINE-1 into the nucleus. Analysis shows presence of granules in all strains at E15.5. However, at E15.5 they are present in lower levels in C57BL/6J in comparison to A/J and NOD/ShiLtJ (respectively A/J 4.8%, C57BL/6J 3.4%, and NOD/ShiLtJ 14.1%) (Figure 5E). The number of oocytes with LINE-1 granule localization peaks in A/J and C57BL/6J at E17.5 (26.1% and 30.7% respectively), where in NOD/ShiLtJ granular presence peaks earlier at E16.5 (31.7%).
To compare the dynamics of LINE-1 localization changes between strains we generated and compared best-fit curves (Figure. 5C-E). Statistical significance was calculated by comparing fits (C57BL/6J vs. NOD/ShiLtJ and C57BL/6J vs. A/J), using the extra sum-of-squares F test. There was no significant difference between C57BL/6J and A/J in LINE-1 localization parameters. For C57BL/6J vs. NOD/ShiLtJ, there was a significant difference in LINE-1 cytoplasmic and granule localization (P=0.0077 and P=0.0205, respectively), while the difference in nuclear localization was not significant.

These results show that LINE-1 granules form earlier in NOD/ShiLtJ which could result in earlier and more efficient restriction of LINE-1 and thus increased survival of oocytes during FOA. However, as shown in Figure 4E, NOD/SHiLtJ oocytes show accelerated MPI progression which may be linked to earlier appearance of granules. Due to the unknown nature of the LINE-1 granules their function remains unknown.

**Protein interactors suggest sequestration and degradation of LINE-1 in embryonic oocytes.** To determine mechanisms regulating LINE-1 and the composition of LINE-1 granules we investigated which proteins are interacting with LINE-1 in C57BL/6J and NOD/ShiLtJ fetal ovaries. With the observed changes in LINE-1 protein localization and levels, we wanted to know what are the differences and similarities in protein interactors between C57BL/6J and NOD/ShiLtJ at E17.5. This timepoint was selected because it’s in the middle of FOA and before many oocytes are eliminated in C57BL/6J. Using Immunoprecipitation of LINE-1 protein and subsequent mass spectrometry we identified candidate LINE-1 interacting partners shared and specific to C57BL/6J and NOD/ShiLtJ with significant enrichment over IgG p-value <0.05 (Table 1).
Anti-LINE-1 ORF1p antibody successfully pulled down peptides mapping to LINE-1 annotated proteins (P11260, O54849, and C6EQK4) which confirmed antibody specificity. The other protein candidates from the sample can be classified into several categories: Transcriptional regulation, RNA degradation, protein degradation, and transport. Among shared interactors, the top three most abundant proteins in the C57BL/6J samples were SPATSL2, SNX16, and SLC29A1 which are involved in protein degradation and protein transport (Figure 6A). SPATSL2 was the most abundant (abundance ratio 54.941 P=3.85E-06) and has been previously linked to stress granules\(^7\). SNX16 (abundance ratio 24.392 P=0.0002) plays a role in protein transport, with known roles in the transport of proteins to lysosomes\(^10\). SLC29A1 also known as ENT1 (Equilibrative nucleoside transporter 1) has been linked to RNA transport (abundance ratio 14.197 P=0.001)\(^9\). These results suggest that in C57BL/6J, LINE-1s are preferentially sequestered to granules during this period of time.

Figure 6 Protein interactors of LINE-1 indicate sequestration and degradation. Volcano plot of the results of proteins pulled down with LINE-1, Log abundance ratios (X = Log2 abundance ratio, Y = -Log10 P-Value) of protein abundance plotting top overlapping candidates between C57BL/6J and NOD/ShiLtJ samples. (A) Candidates from C57BL/6J E17.5. (B) Protein interactors pulled down with LINE-1 in NOD/ShiLtJ E17.5. N=3
Protein interactors present in the NOD/ShiLtJ sample fall under RNA degradation, transport, and protein degradation, which includes DICER, SLC29A1, and SNX16 (Figure 6B). The most abundant interactor in NOD/ShiLtJ was DICER (abundance ratio 11.838 P=1E-17), which has known roles in male germline and transposon regulation by RNA degradation\textsuperscript{95}. DICER was present in C57BL/6J IP samples although at much lower abundance. SLC29A1 and SNX16, interacted with LINE-1 in NOD/ShiLtJ (abundance ratio 11.091 P=1E-17 and abundance ratio 7.272 P=5.64E-13, respectively), both act as transporters, although one for RNA and the other for protein transport for degradation\textsuperscript{98,100}. These results suggest that in NOD/ShiLtJ, LINE-1s are preferentially regulated by RNA and/or protein degradation.

Other proteins of interest which did not overlap between samples include proteins from the coatomer protein complex family, in C57BL/6J this was COPE (abundance ratio 21.313 P= 0.0007) and in NOD/ShiLtJ COPZ was detected (abundance ratio 2.979 P= 6.6E-05). The coatomer protein complex is involved in vesicle formation and protein transport. Additionally, subunits of the Eukaryotic translation initiation factor (EIF) family were also detected. In C57BL/6J this included EIF3A (abundance ratio 3.04 P= 0.15) and EIF4A2 (abundance ratio 4 P= 0.08). NOD/ShiLtJ samples contain EIF1 (abundance ratio 3.06 P= 0.006). The EIF proteins are not only involved in the regulation of RNA translation, but also have been detected in stress granules, which are known to regulate retrotransposons in other systems\textsuperscript{104}. The results from analysis of interacting partners suggest that LINE-1 degradation occurs
more efficiently in NOD/ShiLtJ than in C57BL/6J.

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Table 1. Immunoprecipitation and mass spectrometry results. Proteins pulled down with LINE-1 retrotransposon protein ORF1 in C57BL/6J and NOD/ShiLtJ at E17.5 which were significant overlap between samples. Accession numbers from uniprot are associated with protein names, the values plotted in Figure 6 with \( x = \log_{2} \) abundance ration and \( y = -\log_{10} \) abundance ratio.

LINE-1 transcription among strains.

Differences observed in LINE-1 protein levels could be due to post-transcriptional regulation by mRNA and protein degradation as revealed by interacting partners. But can also arise by mechanisms acting on the transcriptional level or be due to differences in LINE-1 copy number. To determine if there are differences in LINE-1 on the transcript levels between C57BL/6J, A/J and NOD/ShiLtJ strains in fetal ovaries we performed RT-qPCR, using primers for housekeeping gene Gapdh and for Line-1, with timepoints from E14.5-E18.5 (Figure 7). Transcript levels in A/J and NOD/ShiLtJ were compared to C57BL/6J by calculating fold change using \( \Delta\Delta CT \) method (1). As observed for LINE-1 protein, we see variable Line-1 transcript levels in ovaries across the timepoints and between ovaries from different females. This suggests that Line-1 mRNA levels are variable, or a more sensitive method is needed for quantification. From E14.5-17.5 A/J and NOD/ShiLtJ Line-1 expression seems lower although difference were not statistically significant, additional samples are needed to complete the
analysis. Future analyses of LINE-1 copy number and distribution in the genome are needed to determine the predominant mode of LINE-1 regulation in mice.

Figure 7. **Line-1 expression changes across development.** The fold change (FC) calculated as $\Delta\Delta CT$ from qPCR analysis performed on C57BL/6J, AJ, and NOD/ShiLtJ with timepoints from E14.5-E18.5. C57BL/6J was used as a control across all timepoints ($\Delta\Delta CT = 1$). N=3. Statistical testing using ANOVA Friedman test. Differences are not significant.
DISCUSSION

The aim of this study was to determine the mechanisms and genetic factors that regulate the ovarian reserve size. We investigated differences in oocyte development between mouse strains with large and small ovarian reserves. Our results suggest that increased ovarian reserve size in NOD/ShiLtJ females is mostly due to decreased oocyte elimination during FOA. We show that the key regulators of FOA, LINE-1 retrotransposons localize to novel granules and are present at overall lower levels in NOD/ShiLtJ. Among LINE-1 interactors we identified proteins involved in RNA and protein degradation in addition to known components of stress granules and regulators of protein transport. These findings suggest that in oocytes LINE-1s are regulated at multiple levels by mechanisms involving RNA and protein sequestration to granules and degradation, and that these processes occur faster and/or more efficiently in NOD/ShiLtJ oocytes.

Ovarian reserve development has been studied in depth in animal models using functional studies. These studies revealed how this process occurs and many of the key regulators, but how genetically diverse backgrounds and allelic variants impact this process and drive differences in ovarian reserve among individuals remains unknown. Using two founder strains contributing to genetically diverse mice panels we show that ovarian reserve differs between strains and that these differences arise by different efficiency of oocyte elimination mechanisms during early stages of oocyte development. NOD/ShiLtJ strain has the highest number of oocytes in the ovarian reserve compared to reference strain C57BL/6J and other inbred founder strains analyzed, therefore we chose this strain to determine the mechanism that regulates ovarian reserve size.
LINE-1 retrotransposons are known drivers of FOA in mice, and previous studies report that inhibition of LINE-1 reverse transcription (but not endonuclease activity) increases oocyte survival during embryonic stages coinciding with FOA\(^2\). However due to endonuclease activity surviving oocytes still acquire additional DNA damage which can be detrimental to meiotic recombination. Previous studies using AZT an antiviral reverse transcription inhibitor showed that the reverse transcription intermediates, RNA:DNA hybrids, are the driver of FOA (E15.5-E18.5). When production of intermediates is blocked oocyte survival increases temporarily. However, many oocytes are still eliminated later by meiotic checkpoint, possibly due to the accumulated non-meiotic DNA damage induced by endonuclease activity\(^63\). In NOD/ShiLtJ overall levels of LINE-1 are reduced, which would suggest that both reverse transcriptase products and DNA damage would be lower. Not only does NOD/ShiLtJ show overall lower levels of LINE-1, the localization patterns of protein also show differences from that of C57BL/6J. NOD/ShiLtJ oocytes show less abundant cytoplasmic signal than C57BL/6J. This is critical, because active LINE-1s are damaging to oocytes when they are transported to the nucleus where they induce DNA DSBs in the process of reinsertion\(^39,40\). These non-meiotic DSBs occur in random genomic locations and may interfere with chromosome synapsis which relies on programmed meiotic DSBs and homologous recombination repair. We observed that NOD/ShiLtJ oocytes progress through MPI faster than C57BL/6J which could be due to the lack of interreference from LINE-1 induced DSBs. This supports the idea that minimizing LINE-1 induced damage improves oocyte survival and efficient meiotic recombination. Future studies are needed to confirm that indeed NOD/ShiLtJ oocytes have less non-meiotic DSBs. Due to the
multicopy nature of LINE-1, it is impossible to generate a mouse model without LINE-1, therefore NOD/ShiLtJ as a strain offers a naturally occurring model with low LINE-1 expression to further study the impact of LINE-1s and their regulation on ovarian reserve development.

Despite a good understanding of LINE-1 regulation in male germ cells and somatic cells, much less is known about the regulation of LINE-1 during ovarian reserve establishment in mice, in particular during fetal stages when LINE-1s are derepressed due to global genome demethylation in germ cells. In the male germline, factors such as PIWI proteins, piRNAs, and other nuage factors, regulating LINE-1, are necessary for fertility. When knocked out these factors produce a male-sterile phenotype. However, when these factors are lost in oocytes, female mice remain fertile, suggesting that they are not the main mechanisms by which retrotransposons are regulated in females unlike in males. This includes well known pathways in RNA regulation like the piRNA pathway, RNAi, and the RISC complex.

Known germ cell granules like P-granules, nuage, and Balbiani bodies have not been identified in oocytes prior to primordial follicle formation. We have identified novel LINE-1 containing granules in the cytoplasm of fetal oocytes. We hypothesized that these granules may be a form of LINE-1 sequestration. Using immune precipitation and mass spectrometry of LINE-1 complexes we identified LINE-1 interacting proteins in C57BL/6J and NOD/ShiLtJ ovaries. Interacting proteins revealed possible mechanisms for sequestration and degradation of LINE-1 RNA and protein. Some of the candidates we identified, such as DICER1, have been previously implicated in other systems in transposon regulation. Because LINE-1 proteins make ribonucleoprotein (RNP)
complexes with its own mRNA it is possible that DICER1 contributes to LINE-1 mRNA degradation. Other proteins identified include SPATS2L, SNX-16, and SLC29A1 with known roles that can be linked to LINE-1 RNP regulation. SLC29A1 is an RNA transporter which may be involved in LINE-1 RNA transport within the oocyte\textsuperscript{98}. SNX-16 is a known transporter involved in protein shuttling to lysosomes and degradation mechanisms\textsuperscript{100}. SPATS2L is a serine-rich protein which could be involved in regulating formation of LINE-1 granules in oocytes, as it has been identified in stress granules as being important in rRNA processing and transport\textsuperscript{97,106}. Eukaryotic initiation factors (eIFs) are involved in the initiation of translation and have been previously associated with stress granules\textsuperscript{104}. EIF protein subunits were pulled down with LINE-1 in both strains and may be playing a role in the assembly of the LINE-1 containing granules. LINE-1 interacting proteins identified in both strains showed differences in their abundance in IP, which may suggests differences in processes involved in LINE-1 regulation. For example, DICER and SLC29A1, most likely involved in RNA transport/degradation, were most abundant in NOD/ShiLtJ while stress granule protein SPAST2L was most abundant in C57BL/6J. DICER1 is an RNA degradation protein, which has known roles in the RISC complex\textsuperscript{95}. This could indicate a possibility that degradation of LINE-1 is more efficient in NOD/ShiLtJ and therefore decreases the
LINE-1 levels (Figure 8). Follow up studies to confirm interactors and their roles in LINE-1 regulation are needed to determine how they regulate LINE-1 dependent oocyte elimination during FOA.

It is possible that LINE-1 protein levels differ across strains due to regulation occurring at the transcription level or even the number of active copies in the DNA itself. Results from this study show variability in transcript levels, and further investigation is required to clarify this point. However, if RNA transcript levels differ between strains, protein candidates pulled down with the LINE-1 RNPs (Figure 6) suggest potential

**Figure 8** Working model of LINE-1 regulation in female germ line during ovarian reserve establishment. Based on protein interactors we hypothesize that following translation and prior to or following RNP formation LINE-1 is sequestered to granules in the cytoplasm reducing the levels of RNP that can reenter the nucleus. Following granule formation regulatory proteins degrade LINE-1 proteins preventing further LINE-1 accumulation in the nucleus.
regulatory mechanisms for RNA degradation. This includes DICER1, which is known to be part of the RISC complex and is critical in transposon regulation in other systems including in the male germ line\textsuperscript{87}. Future studies will investigate whether lower levels of LINE-1 in some strains are due to fewer active copies present in the genome of A/J and NOD/ShiLtJ, or that strain-specific variants of transcription factors are involved in regulating LINE-1 transcription\textsuperscript{107,108,109}.

In addition to LINE-1 regulation, the immune system differences in A/J and NOD/ShiLtJ strains, carrying defective Complement Component 5 allele, may play an important role in oocyte survival in both of these strains. Previously retrotransposons have been linked to immune response, therefore LINE-1 activity may elicit immune system response. During the LINE-1 lifecycle reverse transcription creates intermediates DNA-RNA hybrids which could trigger an immune response involving complement cascade and lead to oocyte elimination. Increased oocyte survival observed in NOD/ShiLtJ and A/J strains supports this idea. It could potentially be a multifactor system, where the combination of reduced levels of LINE-1 and immune system differences combined allow for increased oocyte survival in NOD/ShiLtJ and A/J.

Overall our findings suggest that regulation of LINE-1 in fetal oocytes involves mechanisms based on RNA/protein degradation, and sequestration to granules away from the nucleus. Through this study new pathways and genes were identified as being involved in regulation of the ovarian reserve size. Future studies are needed to determine their role in oocyte development. Moreover, these genes may provide potential candidates for predicting ovarian reserve in the human population. This study
identifies NOD/ShiLtJ as a new model for the study of female fertility and ovarian reserve development, and highlights the significance of studying genetically diverse strains to better understand genetic regulation of developmental processes.
MATERIALS AND METHODS

Mouse models: All animal experiments have been conducted according to AALAC and IACUC guidelines and have been approved by the Jackson Laboratory Animal Care and Use Committee (Animal use #15001). C57BL/6J (#000664), NOD/ShiLtJ (#001976), and A/J (#000646) females and males between 6–8 weeks of age were obtained from production or repository colonies at The Jackson Laboratory (Bar Harbor, ME). Timed pregnancies were determined by vaginal plug observation in the mornings after matings were set up, with midday time of plug observation counted as E0.5.

Whole Mount Immunofluorescence: Embryonic and postnatal ovaries were dissected into 1X PBS and fixed in 4% PFA for 24 hours. After 24 hours the ovaries were changed into 70% ethanol and stored at 4C. Prior to staining, ovaries were equilibrated for a minimum of 4 hours in 1X PBS. Immunostaining was performed as described in Boateng et al. 202165. Primary antibodies used: GCNA (Abcam) at 1:1000, SYCP3 (Abcam) at 1:1000, γH2AX (Cell Signaling) at 1:1000, and Ki67 (Biocare) at 1:250. Secondary antibodies used: antirat-647 (Alexafluor), antimouse-594 (Alexafluor), and antirabbit-488 (Alexafluor).

Imaging and analysis of whole ovaries to quantify oocyte numbers performed as described in Boateng et al. 202165.

Paraffin Histology Sections and Immunofluorescence: Embryonic and postnatal ovaries were dissected into 1X PBS and fixed in 4% PFA for 24 hours. After 24 hours the ovaries were changed into 70% ethanol and stored at 4C. Samples were embedded in histogel prior to paraffin embedding and serial sections were cut at 5μm thickness.
Primary antibodies used: GCNA (Abcam) at 1:1000, MVH (Abcam) at 1:1000, LINE-1 (Abcam) at 1:1000. Secondary antibodies used: antirat-647 (Alexafluor), antimouse-594 (Alexafluor), and antirabbit-488 (Alexafluor). Immunostained sections were imaged with Leica DM5500 Microscope at 40X oil objective. Image analysis was performed in ImageJ. Maximum projections were used to count oocyte numbers indicated by oocyte markers GCNA and MVH. LINE-1 subcellular localizations were categorized as cytoplasmic signal, nuclear signal, and granule signal however a single oocyte could be characterized by more than one of these categories (i.e. cytoplasmic and granules).

**Meiotic Spreads:** Embryonic and newborn ovaries were dissected into 1X PBS. Meiotic spreads were performed as described by Reinholdt, et al., 2004. Primary antibodies used: SYCP3 (Abcam) at 1:1000, γH2AX (Cell Signaling) at 1:1000, SYCP1 (Abcam) at 1:500, BRCA1 (Namekawa102) at 1:500 and RAD51 (Abcam) 1:500. Secondary antibodies used: antimouse-594 (Alexafluor), and antirabbit-488 (Alexafluor).

Spreads were imaged with LEICA DM5500 Microscope at 40X oil objective. Image analysis was performed using OMERO platform. Meiotic stages were characterized using SYCP3 marker for synaptonemal complex.

**Primary Antibodies used in this study:**

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Western Blot: Embryonic and postnatal ovaries were dissected into 1X PBS and then moved into cryotubes and flash frozen in liquid nitrogen and stored at -80C. Samples were lysed in RIPA extraction buffer using Bioruptor with 10 cycles (30s on/30s off). Samples in Laemmli SDS buffer were boiled for 10 minutes at 95C. Samples were run for 45 minutes on an 8-16% Criterion TGX precast gel. Biorad TGX-midi gel rapid transfer machine was used. Antibodies used: MVH (Abcam) at 1:1000, β-Actin (Cell Signaling) at 1:1000, LINE-1 (Abcam) at 1:1000. Secondary antibody against rabbit - HRP (Thermofisher) at 1:5000. Blot was then developed with Immobilon Forte and detected using chemi luminescence on film.
Analysis of western blot was performed in ImageJ using set region of interest to capture each band. Bands for MVH and LINE-1 were measured collecting area, mean, min, max, integrated density, and raw integrated density, with corresponding background measurements. These data were then used to calculate the Correlated Total Fluorescence (CTF) value by multiplying the area of the band by the mean of the background and then subtracting that from the integrated density of the band. The LINE-1 CTF value was then divided by the MVH CTF value to normalize the LINE-1 signal.

**qPCR:** Embryonic and postnatal ovaries were dissected into 1X PBS and then moved into cryotubes and flash frozen in liquid nitrogen and stored at -80°C. RNA was extracted using the RNeasy Micro Kit following manufacturers protocol. Following RNA extraction mRNA was isolated using the RNeasy Pure mRNA Bead Kit, and RNA levels were measured using Nanodrop. SuperScript IV First-Strand Synthesis System was used to synthesize cDNA. cDNA was used for qPCR with PowerTrack SYBR Green Master Mix and run in the Viia 7 Real-Time PCR System using primers listed below. Expression fold change in NOD/ShiLtJ and A/J strain was calculated using ΔΔCT method and normalized to C57BL/6J. Statistical analysis was performed using ANOVA Friedman test and Dunn’s multiple comparison test.

**qPCR Primers:**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Gapdh⁸⁸</td>
<td>TCCATGACAACCTTGGCATTG</td>
<td>CAGTCTTCTGGGTGGCAGTGA</td>
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</table>
**Immunoprecipitation and Mass Spectrometry:** Embryonic day 17.5 ovaries were dissected into 1X PBS, flash frozen in liquid nitrogen and stored at -80°C. Samples were lysed in RIPA buffer and extracts were prepared using Bioruptor on/off for 30s for 10 cycles. N= 14 females were used for each strain for immunoprecipitation for each replicate sample. Three independent IP replicates were performed with two technical replicates per Mass Spectrometry analysis.

Immunoprecipitation was performed using standard protocol with A/G Magnetic beads. Beads were blocked in 2% BSA at 4C for 1 hour. Protein lysates were precleared with beads at 4C for 1 hour. Blocked beads were then bound with antibodies LINE-1 (Abcam) at 2μg and IGG (MP Biomedicals) 1μg at 4C for 1 hour. Ovarian lysates were added to the antibody bound beads and incubated overnight at 4C. Samples were eluted from the beads with 100mM ammonium bicarbonate and stored at -80°C prior to mass spectrometry using Eclipse Tribrid Orbitrap Mass Spectrometer coupled to a Thermo UltiMate3000 nano-LC system with an EasySpray C18 Column.

All data analysis was performed in Proteome Discoverer software. Mass Spectrometry data was normalized prior to statistical analysis by total peptide amount for each IP. Quantification ratios were created by dividing the experimental IP peptide precursor intensity by the IgG control IP peptide precursor intensity. Pairwise ratio-based comparisons of the peptide data were then used to calculate protein ratios. Sample data
for runs within a given group were then grouped together to create average values. Unique hits have a fold-change of 100 (the maximum allowed by the software).

All data was filtered for a False Discovery Rate (FDR) <0.05 and the p-values were calculated using a background-based t-test in Proteome Discoverer software.

**Statistical Analysis**: Statistical analyses were performed in PRISM Graph Pad 9.4 software using appropriate testing methods to determine the statistical significance between strains and time points with P<0.05. Differences in oocyte numbers between C57BL/6J, NOD/ShiLtJ, and A/J at specific timepoints were analyzed using ANOVA Kruskal-Wallis test with Dunn's multiple comparison. Oocyte loss during FOA was analyzed by comparing oocyte numbers between E15.5 vs. E18.5 for each strain using Mann-Whitney non-parametric test. For LINE-1 localization during development were analyzed by generating fitted curves using non-linear least squares regression and testing for differences in best-fit parameters between strains. Statistical significance was calculated by comparing fits (C57BL/6J vs. NOD/ShiLtJ and C57BL/6J vs. A/J), using the extra sum-of-squares F test.
REFERENCES


BIOGRAPHY

She is a candidate for Master of Science degree in Biochemistry from the University of Maine in August 2022.