REDUCED AUTOPHAGY CONTRIBUTES TO INEFFICIENT DNA DAMAGE REPAIR IN MOUSE OOCYTES

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled:

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Presented by Fei Sun,

A candidate for the degree of Master of Science,

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMPK	AMP-Activated Protein Kinase
ATG	Autophagy-Related Gene
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3-related Protein
BER	Base Excision Repair
CD	Conserved Domain
CDK	Cyclin-Dependent Kinase
CHD2	Chromodomain-helicase-DNA-binding protein 2
CMA	Chaperone-Mediated Autophagy
COC	Cumulus Oocyte Complex
CZB	Chatot, Ziomek, and Bavister Medium
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA Damage Repair
DNA-PKcs	DNA Protein Kinase Catalytic Subunit
DSB	Double Strand Break
HR	Homologous Recombination
HSP70	Heat Shock Protein 70
LAMP2A	Lysosome-Associated Membrane Protein Type-2A
LC3	Microtubule-Associated Protein 1A/1B-light Chain 3
LH	Luteinizing Hormone
MEM	Minimal Essential Medium

Met I	Metaphase I
Met II	Metaphase II
MI	Meiotic I
MMEJ	Microhomology-Mediated End Joining
MMR	DNA Mismatch Repair
mTOR	Mammalian Target of Rapamycin
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
p-ATM	Phospho-ATM
PARP-1	Poly(ADP-Ribose)Polymerase 1
PB	Polar Body
PBE	PB extrusion
PBS	Phosphate Buffer Saline
PMSG	Pregnant Mare Serum Gonadotropin
PVP	Polyvinylpyrrolidone
Rb	Retinoblastoma
ROS	Reactive Oxygen Species
RPA	Replication Protein A
SAC	Spindle Assembly Checkpoint
SSB	Single-Strand Break
TBS-T	Tris-Buffered Saline with 0.1% Tween 20
UV	Ultraviolet

ABSTRACT

The DNA damage is a major problem that, in somatic cells, leads to mutagenesis and premature aging. However, this is not the case in fully differentiated germ cells. Why DNA damage repair (DDR) machinery is not robust in fully grown mammalian oocytes is unknown. Using mouse oocyte as a model system, we found that DDR is not functional in oocytes, leading to the development of aneuploid oocytes. Our data indicate that oocyte failure to repair damaged DNA is due to the inability of DDR protein, RAD51, to access the altered, “closed” chromatin conformation in DNA-damaged oocytes. Our data also reveal that, unlike somatic cells, oocytes fail to activate autophagy in response to DNA damage, which is the cause of altered chromatin conformation and inefficient DDR. Importantly, autophagy induction rescued DDR function and decreased aneuploidy in both DNA-damaged oocytes and oocytes from maternally aged mice which are prone to severe DNA damage. Our findings provide evidence that reduced autophagy contributes to weakened DDR in mammalian oocytes, especially in those of females with advanced reproductive age. Thus, oocyte autophagy management could provide a new aneuploidy treatment option for patients of advanced reproductive age in human assisted reproductive therapy.

CHAPTER 1: Literature Review

1. Introduction

Genomic DNA is under constant threat of damage from a variety of endogenous and exogeneous factors (Ciccia and Elledge, 2010; Jackson and Bartek, 2009; Tubbs and Nussenzweig, 2017). Endogenous factors that can induce DNA damage include stress from metabolic or hydrolytic processes (Hakem, 2008). Metabolism releases DNA-damaging compounds, including reactive oxygen species (ROS), reactive nitrogen groups, reactive carbonyl groups, lipid peroxidation products, and alkylating agents, whereas hydrolysis cleaves chemical bonds in the DNA (Gates, 2009). Oxidative DNA damage by ROS exposure is particularly important and represents a persistent challenge to the genome. It naturally occurs up to 100,000 times in the mammalian cell (de Bont and van Larebeke, 2004). Exogeneous factors that can induce DNA damage include ultraviolet (UV), ionizing radiation, and genotoxic chemicals, such as alkylating agents which are used to treat proliferative disorders like cancer (Damage et al., 2006; Thoms et al., 2007; Yousefzadeh et al., 2021). Mammalian oocytes are much more vulnerable to DNA damage than somatic cells because they are arrested at prophase I stage of the first meiotic division for extended period of time (months in mice, years in cows or decades in women) until puberty age when they resume meiosis just before ovulation under luteinizing hormone (LH) cues (Bennabi et al., 2016; Hashimoto and Kishimoto, 1988; Kitajima et al., 2011; von Stetina and Orr-Weaver, 2011). Unrepaired DNA damage is extremely dangerous because in somatic cells, they may induce mutations and/or

chromosome fragmentations and rearrangements that can result in genome instability and apoptosis (Chow and Herrup, 2015; Damage et al., 2006; Gout et al., 2017; Rao, 2007; Thoms et al., 2007). Similarly, unrepaired DNA damage in oocytes can lead to abnormal embryonic development, resulting in miscarriage or and embryonic congenital disorders (van den Berg et al., 2012; Kerr et al., 2012; Zou et al., 2009).

2. Mechanisms of DNA damage repair in somatic cells

2.1 Formation and types of DNA breaks

The majority of DNA lesions results from: (1) modifications of DNA bases such as those caused by oxidation or ultraviolet light-induced pyrimidine dimers, (2) creation of abasic sites, which leave the phosphodiester backbone intact, and (3) the disruption of the phosphodiester backbone. The most common lesions occurring in cells are DNA single-strand break (SSB), which typically can be repaired easily and efficiently. The unpaired SSB typically do not induce any lethal cell effect unless they encounter DNA replication forks during the S phase, which converts SSB to DNA double strand breaks (DSBs) (Cannan and Pederson, 2016). Both SSB and DSB can be induced by ionizing radiation and DNA-damaging agents such as platinum drugs (cisplatin, oxaliplatin, and carboplatin), cyclophosphamide, chlorambucil, temozolomide and etoposide (Sancar et al., 2004; Thoms et al., 2007; Torgovnick and Schumacher, 2015).

2.2 Mechanisms of DNA single strand breaks repair

Replication protein A (RPA) is a major eukaryotic single stranded DNA-binding protein. Upon single strand breaks, RPA recruits ATR (ataxia telangiectasia and Rad3-related protein) complex to the damaged DNA sites (Byun et al., 2005; Costanzo et al., 2003; Zou, 2007; Zou and Elledge, 2003). ATR then phosphorylates and activates Chk1 and p53 followed by the initiation of SSB repair pathways (Chehab et al., 2000; Shieh et al., 2000; Smith et al., 2010). Three excision repair pathways exist in eukaryotic cells to repair SSB damage: nucleotide excision repair (NER), base excision repair (BER), and DNA mismatch repair (MMR). Nucleotide excision repair is a particularly important excision mechanism used by mammals to remove bulky DNA lesions such as those formed by UV light. This mechanism involves lesion incision, repair and ligation. Two main proteins, TFIIH and XPG, are involved in lesion incision process, leading to the removal of a short single strand DNA segment which includes the DNA lesion (O'Donovan et al., 1994; Oksenych et al., 2009). DNA polymerase then uses undamaged complemented single-stranded DNA as a template to synthesize a short new sequence. Finally, the gap will be ligated by a DNA ligase to form the double stranded DNA (Shivji et al., 1995; Friedberg, 2013). Alternatively, base excision repair mechanism depends on DNA glycosylases enzymes to remove chemically modified bases, such as oxidation, alkylation and deamination, from the DNA (Krokan et al., 1997). A DNA glycosylase is an enzyme which is capable of removing the modified base, thus forming an abasic site (apurinic/apyrimidinic site, a location in DNA that has neither a purine nor a

pyrimidine base due to DNA damage) (Krokan et al., 1997). This site is then cleaved by an AP (apurinic/apyrimidinic) endonuclease and repaired by DNA pol I and a ligase such as NER (Lindahl, 1974; Krokan and Bjørås, 2013). During DNA replication, the DNA polymerases might add wrong base at the time of strand elongation. The incorrectly added base usually results in a mismatched nucleotide, which can be recognized and repaired by MMR. A set of proteins in MMR system recognizes and repairs erroneous insertions, deletions, and mis-incorporations of bases that can arise during DNA replication and recombination, as well as repairing damaged DNA sites (Hsieh and Yamane, 2008; Pećina-Šlaus et al., 2020).

2.3 Mechanisms of DNA double-strand break repair

The DSBs are the most lethal form of DNA damage in the cells. When DSBs are not repaired, they can induce chromosome aberrations, such as chromosomal translocations and rearrangements which can induce apoptosis and cancer (Ferguson and Alt, 2001; Iarovaia et al., 2014; Richardson and Jasin, 2000). The DSBs can be caused by ionizing radiation, laser beam, bleomycin, topoisomerase II enzymes and endonucleases; they can also arise from single-stranded DNA break when unrepaired SSB encounters the replication forks(Cannan and Pederson, 2016; Jackson and Bartek, 2009). There are three mechanisms exist to repair DNA double strand breaks: non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and homologous recombination (HR). The NHEJ and HR are two major DSB repair mechanisms.

Microhomology-mediated end joining is a back-up mechanism, activated when NHEJ is not successful to repair the damaged DNA (McVey and Lee, 2008; Nussenzweig and Nussenzweig, 2007). Both NHEJ and MMEJ do not require DNA templates. Therefore, both NHEJ and MMEJ can repair DSBs independent of cell cycle. In NHEJ, once a DSB is formed, within seconds, the Ku heterodimer (Ku 70 and Ku 80) localizes to the DNA break. Ku is capable of binding the double-stranded DNA ends with high affinity (Blier et al., 1993). This binding event is facilitated by its high concentration (~400,000 molecules/cell) in the cell (Blier et al., 1993; Chiruvella et al., 2013). Ku functions as a docking site recruiting the DNA protein kinase catalytic subunit (DNA-PKcs) (Singleton et al., 1999). The DNA-PKcs then forms a complex with Ku, which allows Ku slide onto DNA end and relocates Ku inwards (Singleton et al., 1999). The nucleases involved in NHEJ remains largely unknown. Artemis is a nuclease which may be involved in processing the ends, removing any damaged or incompatible nucleotides during general NHEJ (Ma et al., 2002). Although this process removes almost all ends, a few breaks are left to be used for precise repair by recruiting two structurally similar proteins, XRCC4 and XLF (Ahnesorg et al., 2006). Both the XRCC4 and the XLF contain highly conserved regions and form filaments that bridge the DNA strands. These filaments align the ends for ligation. Ligase IV is then recruited to the two broken ends by being in a complex with XRCC4 (Ahnesorg et al., 2006). Such ligase IV recruitment leads to a conformational change to encircle the DNA and ultimately forms a phosphodiester bond to ligate the ends together (Ahnesorg et al., 2006;

Chiruvella et al., 2013). Unless there is a lack of Ku or the DNA ligase complex, the MMEJ mechanism (which joins the DNA ends by microhomology) is not activated (Sfeir and Symington, 2015; Wang et al., 2003). Due to the direct ligation of two DNA stranded ends and the lack of template, NHEJ and MMEJ is more prone to cause errors, resulting in mutations.

In contrast to NHEJ and MMEJ which are template independent, the HR does require the template. The sister chromatid serves as the template for HR in eukaryotic cells (Baumann and West, 1998). Thus, HR is inhibited during G1 and restricted primarily to specific cell cycle (S and G2 phase) in which sister chromatid is available. Because the sister chromatid can provide all genome information, HR is commonly considered to be the most accurate DNA damage repair (DDR) mechanism. The HR DDR follows a series of activated cascades. First, the DSB lesion is recognized by MRN complex (MRE11-RAD50-NBS1), that recruits the ATM (mutated in Ataxia Telangiectasia) kinase into the damaged site (Lee and Paull, 2005). The ATM kinase activates p53 which mediates cell cycle arrest to provide sufficient time to repair damaged sites (Lee and Paull, 2005).The ATM also phosphorylates the serine 139 of the histone H2AX (gH2AX) at the damaged DNA sites and nucleosomes around the DSB (Burma et al., 2001). In addition to that, ATM phosphorylates MDC1 (mediator of DNA damage checkpoint protein 1) (Liu et al., 2012). The gH2AX-ATM-MDC1 forms a tight connection which generates a positive feedback loop to amplify DSB signal along the whole nucleolus (Stiff et al., 2004; Falck, Coates, and Jackson 2005;

Stucki et al., 2005). This cascade allows RNF8 (a ubiquitin ligase) to initiate H2AX ubiquitination. Next, RNF168, an E3 ubiquitin ligase, detects and amplifies RNF8-mediated ubiquitination on histones by creating poly-ubiquitin chains (Doil et al., 2009; Uckelmann and Sixma, 2017). Histone ubiquitination then promotes chromatin relaxation, allowing DDR proteins to get access and localize to damaged sites. The HR DDR repair process involves five main steps: (1) resection, (2) strand invasion, (3) D-loop formation, (4) DNA synthesis and (5) two double strand DNA separation. After MRN complex binding to damaged sites, the 3′-DNA overhang is created by protein coordinated action of MRN complex, Sae2 protein, Sgs1 helicase, Exo1 nuclease and Dna2 nuclease. The overhang is then coated with RPA followed by its replacement with RAD51 (Cimprich and Cortez, 2008; Lyndaker and Alani, 2009). This replacement process involves several proteins including ATM, CHK2, BRCA1, BRCA2 and PALB2. RAD51 then mediates 3′-DNA overhang to invade the template strand forming the D-loop followed by DNA synthesis. After the synthesis of the broken DNA strand, a set of helicases work together to separate the two double strand DNA, resulting in crossover or non-crossover structure (Heyer et al., 2010; Krejci et al., 2012; San Filippo et al., 2008; Sung and Klein, 2006).

3. DNA damage regulators in somatic cells

3.1 p53

In somatic cells, tumor protein/cellular tumor antigen p53, a critical regulator of DNA damage response machinery, mediates the DNA damage-induced cell

cycle arrest by promoting the transcription of p21, cyclin-dependent kinase inhibitor 1 (Macleod et al., 1995). The cell cycle transition requires the deactivation of retinoblastoma (Rb) protein, which is achieved via its phosphorylation by Cyclin-dependent Kinases (CDKs) (Giacinti and Giordano, 2006). The p21 is capable of inhibiting CDKs, thus blocking Rb protein deactivation and promoting cell cycle arrest (Georgakilas et al., 2017). Such arrest in the cell cycle provides enough time to repair damaged DNA. However, if the cell is unable to repair severe DNA damage, it initiates apoptosis in order to avoid further damage as a quality control mechanism (Norbury and Zhivotovsky, 2004). The p53 protein also plays an important role in initiating apoptosis after severe DNA damage by promoting the transcription of PUMA which deactivates BCL2, a major anti-apoptotic protein (Yu and Zhang, 2008). Although HR is an error-free DDR mechanism that is required to maintain genome stability, excessive HR activity can be also harmful to genome stability and may cause mutations (Richardson et al., 2004; Shammas et al., 2009; Tutt et al., 2001). Interestingly, p53 can regulate RAD51, an important protein in the HR-based DNA repair pathway, to prevent exacerbating HR activity (Arias-Lopez et al., 2006; Hasselbach et al., 2005; Hine et al., 2014).

3.2 Poly(ADP-Ribose) Polymerase (PARP)

A growing body of evidence suggest that Poly(ADP-Ribose) Polymerase 1 (PARP-1) is involved in DDR pathways by regulating DDR proteins functions (Ray Chaudhuri and Nussenzweig, 2017). The PARP-1 protein

structure consists of a DNA-binding domain, a central auto-modification domain, a carboxy-terminal catalytic domain and a conserved domain (CD). The function of PARP-1 in DDR is through its CD domain and is mainly regulated by its poly(ADP)ribosylation activity (Langelier et al., 2011). Upon DNA damage, PARP-1 responds rapidly and binds to DNA damage lesions via its DNA binding domain (Langelier et al., 2011). The PARP-1 then promotes the recruitment of DDR proteins with PAR-binding domain, such as MRE11, BRCA1 (Haince et al., 2008; Li and Yu, 2013). The recruitment of these DDR proteins is not solely dependent on PARP-1 because they can also be recruited through other pathways, such as DNA damage-mediated ubiquitination (Schwertman et al., 2016). However, depletion of PARP-1 does delay DNA damage response (Masutani et al., 2000). As mentioned previously, DNA-PKcs is required by NHEJ DNA repair pathway, and is typically recruited to the damaged sites and activated by KU70-KU80 complex (Jin and Weaver, 1997; Singleton et al., 1999b; Yue et al., 2020). Interestingly, in vitro and in vivo studies suggest that PARP-1 can stimulate the kinase activity of DNA-PKcs by its PARylation, independent of KU70-KU80 complex (Ruscetti et al., 1998).

Another critical pathway involving the activity of PARP-1 in response to DNA damage is the regulation of chromatin architecture (Ray Chaudhuri and Nussenzweig, 2017). Efficient DDR requires rapid and proper chromatin architecture remodeling, which is essential to make damaged DNA sites accessible for DDR proteins (Luijsterburg and van Attikum, 2011; Smeenk and

van Attikum, 2013). Given that hundreds of enzymes are involved in DNA repair pathways, the chromatin must be remodeled to locally “open” status and reveal the underlying DNA sequence in order to achieve efficient DDR (Bao, 2011; Dion and Gasser, 2013; House et al., 2014; Kumar et al., 2013; Luijsterburg and van Attikum, 2011; Smeenk and van Attikum, 2013; Tsompana and Buck, 2014). Indeed, in eukaryotes, the ATP-dependent chromatin remodeling complexes and histone-modifying enzymes are the two sets of factors that act to accomplish this remodeling process after DNA damage (Kumar et al., 2013; Marmorstein and Trievel, 2009; Vignali et al., 2000). The PARP-1 regulates chromatin remodeling in response to DNA damage by directly PARylating histones and recruiting other chromatin remodeling factors, such as CHD4/NuRD complex (Ray Chaudhuri and Nussenzweig, 2017). When PARP-1 is inactive, it binds to chromatin, maintaining chromatin architecture in a transcription repressed state. Upon DNA damage, PARP-1 is activated and promotes the disassociation of histone proteins from chromatin by its poly(ADP)ribosylation (PARylation) activity, allowing chromatin remodeling factors to be recruited to the local PAR scaffold (Messner et al., 2010; Poirier et al., 1982). The Chromodomain-helicase-DNA-binding protein 2 (CHD2) can modify the chromatin architecture in the DNA damaged cells by interacting with PARP-1 through the PAR chains (Luijsterburg et al., 2016). Indeed, upon DNA damage, CHD2 is recruited to damaged DNA sites rapidly, and PARP-1 inhibition abolishes such recruitment, indicating that PARP-1 is required for the recruitment of CHD2 to DNA-damaged sites (Luijsterburg et al., 2016).

3.3 Autophagy

3.3.1 Mechanisms and functions of autophagy

The word ‘autophagy’ comes from Greek word. ‘Auto’ means ‘self’, ‘phagy’ means eating. Autophagy is a survival mechanism in which a cell bulk-degrades damaged cellular components, including damaged/unnecessary proteins, lipids droplets, organelles, intracellular pathogens in response to various stress factors, such as nutrient deprivation and genotoxic stress (Klionsky and Emr, 2000; Straub et al., 1997; Tsukada and Ohsumi, 1993). The concept of autophagy was first introduced in 1960s. However, the underlying molecular mechanisms were not discovered until 1990s when the identification of autophagy-related genes (ATG genes) were identified (Ashford and Porter, 1962; Deter and de Duve, 1967; Harding et al., 1995; Klionsky, 2008; Schlumpberger et al., 1997; Straub et al., 1997; Thumm et al., 1994; Tsukada and Ohsumi, 1993). Although autophagy has been initially discovered as the major degradation mechanism activated to protect the cell against starvation, it is now commonly accepted to play important roles in cellular homeostasis in non-starved cells (Khaminets et al., 2016; Kraft et al., 2009; Rogov et al., 2014; Zaffagnini and Martens, 2016). Indeed, autophagy plays essential roles in both physiological and pathological stress responses including exercise, aging, cancers and various other diseases (Glick et al., 2010; Kundu and Thompson, 2008). Based on the method that autophagy uses to deliver substrates to the lysosome, three types of autophagy are recognized: macroautophagy, microautophagy and chaperone-mediated autophagy. All these

three types of autophagy share one common feature, i.e., the degradation of substrates within the lysosome (Glick et al., 2010; Kundu and Thompson, 2008).

Microautophagy is mediated directly by lysosome action where cytoplasmic cargo is taken directly by the lysosome through its membrane rearrangement (Ahlberg et al., 1982). To date, the underlying mechanism of microautophagy remains largely unclear (Li et al., 2012). In contrast, the mechanisms of macroautophagy has been extensively studied. Macroautophagy is an umbrella term for various types of autophagy that collectively utilize autophagosomes and degrade the membrane-engulfed cytoplasmic component through bulk degradation in the lysosome and is always referred to as “autophagy” (Feng et al., 2014). Different types of macroautophagy are named based on the substrate being targeted. For example, mitophagy means the bulk degradation of mitochondria in the autolysosome; reticulophagy means the degradation of endoplasmic reticulum in the lysosome; nucleophagy means the degradation of nucleus in the lysosome and lipophagy means the degradation of lipid droplets in the lysosome. Although different types of substrates being degraded, all of them share a very similar pathway. Macroautophagy process starts by the formation of pre-autophagosome/autophagophore which is a budding of the membrane separated from the endoplasmic reticulum. It is a developing cup-shaped separation membrane that is initiated by a set of protein complex including ULK1, ATG13, etc. (Feng et al., 2014; Jung et al., 2009; Kim et al., 2011). It is worth noting that mTOR (mammalian target of rapamycin) kinase, a major negative

regulator of macroautophagy, inhibits ULK1 and ATG13 activity through their phosphorylation (Deleyto-Seldas and Efeyan, 2021; Jung et al., 2009; Kim et al., 2011). The enlargement of pre-autophagosome membrane depends mainly on LC3 (microtubule-associated protein 1A/1B-light chain 3) which has two forms: LC3-I and LC3-II (Kabeya et al., 2004; Tanida et al., 2008). During the development of autophagosome membrane, LC3-I is conjugated to phosphatidylethanolamine, one of the most abundant phospholipids in mammalian cells, which plays an essential role in cellular membrane assembly (Kabeya et al., 2004). Such a modified LC3-I is then transformed into LC3-II after two ubiquitination-like reactions catalyzed by ATG3 and ATG7 (Kabeya et al., 2000; Tanida et al., 2004). The formation of LC3-II signifies the formation of mature autophagosome which is a double-membraned vesicle. Mature autophagosome fuses with the lysosome through SNARE protein, RAB7, forming autolysosome (Kabeya et al., 2000; Tanida et al., 2004). The LC3-II and other cellular components of autophagosome will then be degraded by lysosomal enzymes. Because LC3-II is produced in the early step of macroautophagy and is degraded in the last step of macroautophagy, LC3-II has been commonly known to represent the autophagic activity and used as a reporter/monitor thereof (Kabeya et al., 2004; Tanida et al., 2008).

Chaperone-mediated autophagy (CMA) is a highly selective degradation process that requires a high degree of coordination of various protein chaperones. During CMA, the proteins that contains KFERQ-like motif are targeted exclusively and

delivered by heat shock protein 70 (HSP70) to lysosomes (Sahu et al., 2011). The majority of KFERQ-like motif-containing proteins are cytosolic proteins, whereas organelles or membrane proteins rarely contain the KFERQ-like motif. Thus, proteins degraded by CMA are mainly cytosolic proteins (Ahlberg and Glaumann, 1985; Mortimore and Poso, 1984). During the delivery process, protein cargos are bound, unfolded and then translocated to lysosomes through a lysosomal membrane receptor, LAMP2A (lysosome-associated membrane protein type-2A) (Nishino et al., 2000). Therefore, LAMP2A is indispensable for CMA function and its deficiency leads to Danon disease, a terminal, heritable X-linked disease caused by the accumulation of autophagic vacuoles in the muscle cells and characterized by weakening of the heart muscle, skeletal muscles. (Malicdan et al., 2008; Nishino et al., 2000). The protein cargo is then degraded within the lysosome via proteases such as cathepsins (Cuervo, 2010). During the translocation process, HSP70, protein cargo and LAMP2A form the LAMP2A multimeric complex which is regulated by Glial fibrillary acidic protein (Bandyopadhyay et al., 2010). Ultimately, the CMA translocation complex is disassembled by GTP-mediated release of elongation factor-1 alpha from the lysosomal membrane (Bandyopadhyay et al., 2010).

3.3.2 The role of autophagy in DNA damage

Emerging lines of evidence indicate that autophagy is activated and plays an important role in the response of the cell to DNA damage. In mammalian cells, DNA damage induction by H_2O_2 and etoposide treatment can stimulate

autophagy activity via inhibition of mTOR or activation of AMP-activated protein kinase (AMPK) which are two main pathways initiating autophagophore formation (Alexander et al., 2010; Kim et al., 2011; Tripathi et al., 2013). The ULK1, Unc-51 like autophagy activating kinase, a critical component for autophagosome formation, is activated by AMPK through phosphorylation of Ser 317 and Ser 777, whereas it is inhibited by mTOR through phosphorylation of Ser 757 (Kim et al., 2011). The AMPK can be also activated in response to DNA damage through PARP-1 (Chen et al., 2015b). Because PARP-1 is involved in DDR mechanism, mainly by its poly(ADP)ribosylation activity which consumes ATP pool, the upregulation of PARP-1 is accompanied with increased AMP levels with subsequent activation of AMPK and autophagy (Rodríguez-Vargas et al., 2012). Moreover, the DNA damage-related proteins are capable of activating autophagic activity via both transcriptional and post-transcriptional mechanisms. For example, p53 can transcriptionally activate AMPK (Feng et al., 2007). The ATM kinase can activate tumor protein p63 isoform, ΔNp63α, which regulates the expression of several autophagy-associated proteins, such as ATG5, ATG7, and Beclin-1(Huang et al., 2012; Zhai et al., 2013).

Autophagy promotes DDR through several mechanisms and thus plays an important role in chromatin remodeling by promoting chromatin relaxation (Hewitt et al., 2016; Wang et al., 2016). Chromatin relaxation (opened chromatin state) is a critical step in almost all DDR mechanisms because it maks DDR proteins, including RAD51 and BRCA1, access to damaged DNA sites, ensuring the

efficiency of DDR process (Bao, 2011; Burgess et al., 2012; House et al., 2014; Kumar et al., 2013; Luijsterburg and van Attikum, 2011; Ray Chaudhuri and Nussenzweig, 2017; Wang et al., 2016). Autophagy promotes chromatin relaxation through (1) degrading HP1α, a chromatin crosslinker which maintains condensation of chromatin architecture (Chen et al., 2015a) and (2) degrading SQSTM1, an autophagosome cargo sequestering protein that is consumed during autophagy-mediated cargo degradation (Hewitt et al., 2016; Wang et al., 2016). Upon DNA damage, E3 ubiquitin-protein ligase, RNF168 promotes chromatin relaxation via histone ubiquitination. High levels of SQSTM1 binds to and inhibit RNF168, thereby preventing RNF168-mediated histone ubiquitination, chromatin remodeling and proper DDR protein localization to damaged DNA sites (Wang et al., 2016). The SQSTM1 also impairs DDR by promoting RAD51 degradation (Hewitt et al., 2016). Accordingly, autophagy deficiency impairs DDR due to increased SQSTM1 level (Komatsu et al., 2007; Mathew et al., 2009). Moreover, several studies indicate that reduction of CMA mediates the dysregulation of CHK1 and MRN complex, both are critical factors in HR mechanism (Liu et al., 2015; Park et al., 2015). Furthermore, it is plausible that proper autophagy activity is also essential to provide the cells with high levels of energy required to carry out DDR processes efficiently.

4. DNA damage response in oocytes

During early embryonic development, mammalian female primordial germ cells divide by mitotic divisions to yield oogonia which continue proliferating to produce

primary oocytes. These primary oocytes are surrounded by a single layer of flat granulosa cells within the primordial follicles. Primary oocytes enter the first meiotic division during the fetal life. Around the time of birth, primary oocytes are arrested at the diplotene stage of prophase I of the first meiotic division until the age of puberty. During this prolonged arrest, the oocytes and the follicles enter a growth period. The single, flat, squamous granulosa cell layer within the primordial follicle converts to cuboidal epithelium, forming primary follicles and enter proliferative phase. When the number of cuboidal epithelium layers increases (≥ 2 layers), the follicles become secondary follicles. This period of oocyte and follicle growth is gonadotropin independent. However, the oocytes will not be developmentally competent until the age of puberty under the effect of gonadotropins. Only after puberty onset, the exposure of the oocyte within the ovulatory follicle to LH stimulates the primary, prophase I-arrested oocytes to resume meiosis I which is completed by the extrusion of the first polar body, reaching metaphase II stage (Bennabi et al., 2016; Hashimoto and Kishimoto, 1988; Kitajima et al., 2011; von Stetina and Orr-Weaver, 2011). Metaphase II oocyte (the egg) re-enters meiotic arrest and only resumes meiosis II after fertilization through the discontinuation of maturation promoting factor activity.

During the lengthy arrest of the oocyte at prophase I (i.e., from around the time of birth until the onset of puberty), oocytes can be exposed to various exogenous and endogenous genotoxic factors, such as ROS, ionizing radiation, chemotherapeutic drugs and environmental toxicants. These endogenous and

exogenous factors can cause DNA damage, leading to its accumulation over this prolonged arrest period. Given that females are born with a definite number of oocytes, it is extremely important and critical to repair or remove the damaged oocytes to ensure good quality oocytes and the development of healthy offspring. Indeed, nongrowing and growing prophase I-arrested mouse oocytes with damaged DNA readily undergo apoptosis, which is considered a quality control mechanism to maintain the quality of oocytes (Livera et al., 2008; Roness et al., 2014; Suh et al., 2006). In contrast to latent primary and growing oocytes, the fully grown prophase I-arrested oocytes (from sexually mature mice) do not undergo apoptosis after DNA damage (Kerr et al., 2012; Livera et al., 2008). Instead, they progress through meiosis while carrying DNA damage (Marangos and Carroll, 2012; Rémillard-Labrosse et al., 2020), increasing the risk of developing poor quality eggs. In somatic cells, unrepaired DNA damage triggers apoptosis via activation of p53, a master regulator of apoptosis. In contrast, the DNA-damaged oocytes cannot activate p53 and instead, apoptosis in latent and growing mouse oocytes is mediated by the activation of TAp63, a homology of tumor suppressor p53 (Stringer et al. 2020; Kerr et al. 2012; Suh et al. 2006). TAp63-mediated apoptosis is very sensitive in latent primary oocytes and is rapidly activated in response to a few DSBs, making it responsible for the rapid elimination of latent primary oocytes at young female age (Coutandin et al., 2016). Although TAp63 is highly expressed in the oocytes within the primordial follicles, its expression is lost in preovulatory follicles, explaining why fully grown

oocytes competent to resume meiosis are relatively resistant to the DNA damage-induced apoptosis (Collins and Jones 2016; Suh et al., 2006).

In mitotic somatic cells, the exposure to mild DNA damage is sufficient to activate ATM kinase to induce a cell cycle arrest (G2 to M transition block), necessary to provide enough time for DDR. In contrast, fully grown prophase I-arrested human and mouse oocytes cannot induce a cell cycle arrest at prophase I in response to moderate DNA damage and, therefore, oocytes progress through meiosis while having DNA damage (Marangos and Carroll, 2012; Rémillard-Labrosse et al., 2020). Only after being exposed to severe DNA damage, oocytes activate the ATM kinase and prevent meiotic resumption beyond prophase I (Marangos and Carroll, 2012). Surprisingly, mouse oocytes employ an alternative insurance mechanism, spindle assembly checkpoint (SAC), to induce a cell cycle arrest at metaphase I in response to DNA damage (Collins et al., 2015). The SAC is a cell cycle checkpoint during mitosis or metaphase I and II stages of meiosis that prevents the transition from metaphase to anaphase stage until all chromosomes are properly aligned and attached to microtubules, thereby avoiding chromosome missegregation. Induction of DNA damage by pharmaceuticals (etoposide, bleomycin, phleomycin, doxorubicin) or radiation in fully grown mouse oocytes resulted in meiotic arrest at metaphase I stage, induced by SAC activation, independent of ATM kinase activity and kinetochore microtubule attachments (Collins et al., 2015; Marangos et al., 2015). However, the underlying

mechanisms regulating SAC activation in the DNA-damaged oocytes remains largely unknow.

Although both HR and NHEJ DDR pathways can be used by growing prophase I-arrested oocytes, mouse oocytes mainly use the HR pathway in response to DNA damage. The induction of DNA damage in growing prophase I-arrested oocytes is followed by RAD51 (marker of HR pathway activity) localization to damaged DNA sites in more than 90% of oocytes, compared to DNA-PKcs (marker of NHEJ pathway) localization in 10% of oocytes (Stringer et al., 2018, 2020). This is not surprising because prophase I-arrested oocytes are tetraploid, which already have four sets of DNA material and, therefore, HR is the likely most accurate to ensure efficient DDR mechanism (Stringer et al., 2020). However, it remains unknown why, unlike growing (as opposed to fully grown) oocytes and somatic cells, fully grown human and mouse oocytes are not efficient in repairing damaged DNA using either HR or NHEJ pathway.

5. Aging in somatic cells and oocytes

5.1 DNA damage and aging

It is commonly believed that the DNA damage is continuously accumulates in both the somatic cells and the oocytes with the advancement of age, due to exogenous and endogenous genotoxic factors. In somatic cells, the accumulation of DNA damage causes proteostatic stress, genomic instability, telomeric and mitochondrial dysfunction, epigenetic alterations and altered transcription

patterns (Hakem, 2008; Jackson and Bartek, 2009; Turgeon et al., 2018; Yousefzadeh et al., 2021). The altered transcription patterns weaken the efficiency of the DNA damage response machinery, ultimately leading to cell apoptosis and cancer (Alhmoud et al., 2020; Burgess et al., 2012; Oberdoerffer et al., 2008; O'Hagan et al., 2011; Shanbhag et al., 2010).

The efficiency of SSB repair mechanism decreases with aging. One critical step for SSB mediated by the BER pathway is that the DNA glycosylase, a critical enzyme involved in BER pathway, removes the modified base to create an abasic site (Alseth et al., 2004). Interestingly, DNA glycosylase activity decreases in somatic cells of aged individuals leading to decreased abasic sites in cells from aged individuals compared to those of young ones (Atamna et al., 2000). Similarly, emerging indirect evidence suggest that MMR and NER activities are also weakened in an age dependent manner (Moriwaki et al., 1996; Yehuda et al., 2001). However, molecular mechanisms by which SSB repair is impaired in aged cells is still largely unknown.

The most serious type of DNA damage is the DSB which, if left unrepaired, triggers cancer. Like SSB, the DSB repair weakens in an age dependent manner in human lymphocytes (Mayer et al., 1989). Using in vitro plasmid rejoining assay, the NHEJ activity was found to decrease in cells of aged rats, compared to young rats (Ren and Peña De Ortiz, 2002; Vyjayanti and Rao, 2006). In fact, the age-related weakening of DSB repair mechanism with the advancement of

age contributes to the exponential increase of cancer incidence in aged individuals, compared to young individuals (Calcinotto et al., 2019).

Similar to somatic cells, the DNA damage accumulates in the oocytes from reproductively aged females. Such DNA damage accumulation contributes to the significant decline of oocytes number and quality in aged females. Indeed, DSB levels (as evidenced by increased gH2AX level) in the oocytes from aged women and mice are significantly higher than those in oocytes from young women and mice. This inefficient DSB repair mechanism in oocytes from aged individuals may be due to the decreased expression of DDR proteins such as the BRCA1, MRE11, RAD51 and ATM. Indeed, the specific depletion of BRCA1 or RAD51 from oocytes increased DNA damage levels and accelerated ovarian aging (Titus et al., 2013; Winship et al., 2018).

5.2 Autophagy and aging

The relationship between autophagy and aging has been extensively studied. It is commonly accepted that reduced autophagy is a hallmark of aging, and its activity is decreased with the advancement of age in various organisms. The fusion between the autophagosomes and lysosomes is a critical step in autophagy. Lysosomes undergo age-related changes such as decreased vesicular lysosome numbers and increased lysosome volume. Accordingly, lysosomal activity declines slower in the long-lived *C.elegans* mutants than in wild type (Chang et al., 2017; Wilhelm et al., 2017). The decrease of lysosomal

activity leads to autophagy reduction and promotes misfolded protein aggregation, further accelerating the aging process (Chang et al., 2017; Wilhelm et al., 2017). Similar changes were also observed in the muscle and intestinal cells (Cuervo and Dice, 1998; Hughes and Gottschling, 2012; Sarkis et al., 1988; Sun et al., 2020). Moreover, the expression of autophagy-related genes decreases significantly in the neural tissues from aged *Drosophila* flies. Thus, the ATG8a mutant has a reduced longevity, whereas the overexpression of ATG8a in the neural tissues extended the lifespan of aged Drosophila (Simonsen et al., 2007). The age-associated reduction of the expression of autophagy-related genes has also been observed in mice, rats and humans. Aged mice exhibited reduced autophagy activity in the hypothalamus compared to young mice as shown by the reduced expression of ATG7 and LC3-II (Ott et al., 2016). Consistent with the aging-associated autophagy reduction phenomenon, the mTOR (a negative autophagy regulator) was increased in aged mice (Baar et al., 2016). Similarly, aged rats (24 months) exhibited reduced levels of major autophagy regulators such as Beclin-1, LC3-II and ULK-1 activity (Triplett et al., 2015; Yu et al., 2017). Finally, the ATG5, ATG12, LC3-II, p62 and Beclin-1 content also declined in the aged human brain and senescent fibroblasts (Lipinski et al., 2010). Importantly, the oocytes from aged mice exhibited reduced lysosome numbers, suggesting the impairment of autophagy in aged oocytes (Peters et al., 2021). These results strongly support the conclusion that the autophagic activity is reduced with the advancement of aging, raising the

question whether reduced autophagy is the cause, at least in part, of weakened DDR in aged oocytes.

Chapter 2: Reduced autophagy contributes to inefficient DNA damage repair in mouse oocytes

1.Introduction

Infertility is a highly prevalent reproductive health problem globally. At least 10% of US women within the reproductive age experience infertility (Gerrits et al., 2017; Sun et al., 2019). Female fertility starts to decline in the early thirties, and this decline accelerates after the age of 35 (American College of Obstetricians and Gynecologists, 2020). The major cause of this decline is reduced oocyte quality. The oocytes are produced through two rounds of meiotic divisions following a single round of DNA replication. For, yet, incompletely understood reasons, oocyte meiosis is notoriously prone to errors leading to aneuploidy, the leading genetic cause of infertility (miscarriage) and congenital abnormalities such as Down's syndrome. Indeed, the incidence of aneuploidy is at least 10-times higher in oocytes than that in spermatozoa of men of comparable age, and frequently occurs in meiosis I (MI) as opposed to meiosis II (Hassold et al., 2007). This high rate of aneuploidy in oocytes is exponentially increased with the advancement of female age (Gruhn et al., 2019). Therefore, it is of great importance to understand the molecular mechanisms regulating oocytes meiosis to unveil how these mechanisms are further perturbed in oocytes from females of advanced reproductive age.

DNA damage, defined as any alterations in DNA structure that disrupts its cellular function, is a major problem leading to premature aging, genome instability, mutagenesis and cancer in somatic cells (de Bont and van Larebeke, 2004; Burgoyne et al., 2009; Hanahan and Weinberg, 2011; Kaufmann and Paules, 1996; Lindahl, 1993; Martin, 2008; Negrini et al., 2010). Mammalian oocytes enter meiosis during early fetal development. Shortly after birth, oocytes undergo an arrest at prophase I of MI. The duration of this arrest can be in the order of months (mice), years (cows) or decades (women) before these oocytes are allowed to resume meiosis prior to ovulation. Such lengthy arrests result in elevated opportunities for DNA damage accumulation in prophase I-arrested oocytes caused by various endogenous and exogenous DNA-damaging agents, such as ionizing radiation, chemotherapeutic drugs and environmental toxicants. In spite of its significance, the consequences of DNA damage in mammalian oocytes remain not fully characterized, representing a significant gap in our knowledge of understanding why oocyte MI is notoriously prone to meiotic errors.

Double-stranded DNA breaks (hereafter referred to as DNA damage) are considered the most lethal form of DNA damage which occurs constantly in almost all types of cells due to the assaults by endogenous and environmental agents. As a result, cells have evolved a DNA damage response that involves a complex network of signals responsible for activating specific machineries mediating DNA damage sensing, cell cycle regulation, DNA damage repair (DDR) and apoptosis (Ciccia and Elledge, 2010; Kastan, 2008). Upon DNA

damage, the cell activates a DNA damage checkpoint, an essential step to allow the required time for DDR by inducing cell cycle arrest. If the DNA damage is not repaired, apoptosis will be triggered to induce a cell death (Jackson and Bartek, 2009; Lindahl and Barnes, 2000; Pailas et al., 2022). In mitotic cells, the exposure to mild DNA damage is sufficient to induce cell cycle arrest, necessary to provide enough time for DDR. In contrast, mammalian oocytes can progress through meiosis while having a moderate level of DNA damage, leading to the risk of generating developmentally incompetent gametes (Marangos and Carroll, 2012). Surprisingly, studies in mouse and human oocytes suggest that the DNA damage response is further weakened in oocytes from aged females compared to young adult females (Horta et al., 2020; Oktay et al., 2015; Rémillard-Labrosse et al., 2020; Titus et al., 2013). Why DNA damage response is not robust in mammalian oocytes, especially in those from females with advanced age, remains an open question.

Autophagy is a cellular quality control mechanism which plays an important role in maintaining cellular homeostasis by degrading and recycling unnecessary cytoplasmic proteins and organelles in response to diverse stress conditions, such as nutrient deprivation, infection and genotoxic stress(He and Klionsky, 2009; Kundu and Thompson, 2008). Autophagy includes three major forms: microautophagy, chaperon-mediated autophagy and macroautophagy(Filomeni et al., 2015; Klionsky and Emr, 2000). Macroautophagy, hereafter referred to as autophagy, is the most prevalent form of autophagy. In macroautophagy, a

double-membrane vesicle known as an autophagosome forms which will target and isolate damaged cytoplasmic components such as protein aggregates and organelles from the rest of the cell. The autophagosome will then fuse with a lysosome to form autolysosome to accomplish degradation of the content by lysosomal enzymes (Gozuacik and Kimchi, 2007; Kroemer et al., 2010). An emerging body of evidence suggests that DNA damage in somatic cells stimulates autophagy which in turn plays a critical role in DNA damage response and repair (Eliopoulos et al., 2016; Gillespie and Ryan, 2016; Gomes et al., 2017; Wang et al., 2016). Recent data showed that autophagy induction by rapamycin treatment improved the developmental competence of bovine oocytes (Li et al., 2020). Moreover, transient treatment of porcine and murine oocytes with MG132, a proteasomal inhibitor that is known to stimulate autophagy, improved oocyte developmental competence (Sutovsky and Prather, 2004; Whitworth et al., 2009; Zhou et al., 2003). On the other hand, autophagy inhibition during oocyte maturation impaired the developmental potential of porcine oocytes (Shen et al., 2018). However, the role of autophagy in DNA damage response in mammalian oocytes is largely unknown.

Moreover, our data uncovered a molecular pathway of DNA damage in oocytes and demonstrated that DNA damage is a cause of aneuploidy in oocytes. We found that DNA damage alters chromatin architecture and induces chromosome fragmentation caused by microtubule-mediated tension. Importantly, we found that oocytes behave differently than somatic cells in response to DNA damage:

(1) the oocyte is inefficient in repairing damaged DNA due to the failure to localize DDR protein, RAD51, and (2) autophagy is not activated in response to oocyte DNA damage and further decreased in oocytes from aged mice. Induction of autophagy in DNA-damaged oocytes rescued DNA damage, altered chromatin architecture, chromosome fragmentation and the incidence of aneuploidy. Collectively, these results provide the first evidence that reduced autophagy contributes to weakened DNA damage response in mouse oocytes.

2. Material and methods

2.1 Ethics

Mice were kept and experiments were performed in accordance with the Animal Care and Use guidelines of the University of Missouri (Animal Care Quality Assurance Reference Number, 17180).

2.2 Mouse strains

Sexually mature CF1 female mice (Envigo, Indianapolis, IN, USA) were used in this study. Mice were housed at 21 °C and 55% humidity in 12 h/12 h light/dark cycle and ad libitum access to food and water. Unless otherwise specified, oocytes were collected from 6-8-week-old mice. Aged oocytes were collected from 12-14-month-old mice.

2.3 Oocyte collection and in vitro maturation

Fully grown, prophase I-arrested (GV-intact) oocytes were collected from CF1 mice previously primed (44-48 h before collection) with pregnant mare serum gonadotropin (PMSG, Lee BioSolutions, Maryland Heights, MO, USA #493-10-10), as previously described(Schultz et al., 1983; Stein and Schindler, 2011). Cumulus oocyte complexes (COCs) were denuded mechanically by pipetting and cultured in bicarbonate-free minimal essential medium (MEM) supplemented with 3 mg/ml polyvinylpyrrolidone (PVP) and 25 mM HEPES (pH 7.3) under mineral oil (MilliporeSigma, St. Louis, MO, USA #P2307, #H3784 and #M8410). Prophase I-arrested oocytes were then transferred to Chatot, Ziomek, and

Bavister (CZB) medium and cultured at 37˚C with 5% CO_2 in humidified air for either 3 (arrested at prophase I), 7 (Met I), or 16 h (Met II). To prevent meiotic resumption, milrinone, a phosphor diesterase inhibitor, was added to the medium (2.5 μM, MilliporeSigma #M4659) (Tsafriri et al., 1996).

Etoposide (MilliporeSigma #E1383), rapamycin (MilliporeSigma #553211), MG132 (MilliporeSigma #474790), spautin-1 (Selleckchem, Houston, TX, USA #S7888), niraparib (Selleckchem #S7625), nocodazole (MilliporeSigma #M1404) and monastrol (MilliporeSigma #M8515) were dissolved in DMSO and added to CZB culture medium at a final concentration of 50 μg/ml, 100 nM, 10 μM, 20μM, 10 μM, 5 μM, and 100 μM, respectively. DMSO was added to CZB culture medium as a vehicle control at 0.1% concentration. SiR-DNA was added to the maturation medium at a final concentration of 500 nM to label the DNA during time-lapse confocal live imaging.

2.4 Immunocytochemistry and confocal microscopy

Oocytes were fixed for 20 min at room temperature in a freshly prepared 2 % paraformaldehyde solution (MilliporeSigma #P6148) dissolved in phosphate buffer saline (PBS). After fixation, oocytes were permeabilized using 0.1% Triton X-100 in PBS solution for 20 min. Oocytes were then incubated in blocking solution (PBS containing 0.3% BSA and 0.01% Tween-20) for an additional 20 min. The oocytes were then incubated in primary antibody for 1 h at room temperature prior to 3 successive washes in blocking solution (8 min each).

Oocytes were then incubated in secondary antibody for another 1 hour at room temperature followed by washing in blocking solution (3 times, 8 min each). The oocytes were mounted on slides using Vectashield containing 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Vector Laboratories, Burlingame, CA, USA) to label the DNA, under a coverslip with gentle compression. Omission of primary antibodies and the use of isotype-specific immunoglobulins served as negative controls. Fluorescence signals were observed under a 63X oil objective using Leica TCS SP8 confocal microscope. Oocytes were captured using 1, 3 or 5 µm Z-intervals.

All oocytes in the same experiment were imaged and processed simultaneously. The laser power for imaging all groups was adjusted to a level where the signal intensity is just below the saturation point in the group showing the highest fluorescence intensity. Fluorescence intensity was quantified using NIH Image J software (National Institute of Health, Bethesda, MD, USA) with same processing parameters. The number of gH2AX foci was analyzed and calculated automatically using isosurface spot analysis feature of Imaris software (Bitplane, Zürich, Switzerland). Same processing parameters were applied for each experimental analysis.

Antibodies: The following primary antibodies were used: conjugated α-tubulin-AlexaFluor 488 (Life Technologies #322588; 1:75), CREST autoimmune serum (Antibodies Incorporated, Davis, CA, USA # 15-234; 1:25), anti-RAD51

(MilliporeSigma #ABE257), anti-p-ATM (Santa Cruz Biotechnology, Dallas, TX, USA #sc47739), anti-gH2AX (Abcam, Boston, MA, USA #ab11174), anti-PARP1 (Cell Signaling, Danvers, MA, USA #9532), anti-α-Tubulin antibody (MilliporeSigma, St. Louis, MO, USA # T6074), LC3A/B antibody (Cell Signaling, #4148).

2.5 Time-lapse confocal microscopy

Oocytes were transferred to milrinone-free CZB medium. Brightfield and SiR-DNA image acquisition was started following nuclear envelope breakdown using Leica TCS SP8 confocal microscope equipped with a microenvironmental chamber to maintain the oocytes at controlled CO_2 (5%) and temperature (37 °C) in a humidified air. Images of single oocytes were captured at 5 μm Z-intervals every 40 min. Images were processed using NIH Image J software.

2.6 In Situ chromosome counting

Met II stage oocytes (14 h) were transferred to CZB medium supplemented with 100 μM monastrol, a cell-permeable Eg5 kinesin inhibitor to induce monopolar spindle formation with subsequent chromosome dispersion, for additional 2 h (Balboula and Schindler, 2014; Duncan et al., 2009; Londoño-Vásquez et al., 2022). Oocytes were then fixed in a freshly prepared 2 % paraformaldehyde solution followed by immunostaining using CREST autoimmune serum antibody (to label kinetochores). Oocytes were imaged under a 63X oil objective using Leica TCS SP8 confocal microscope at 0.7-μm Z-intervals to capture all

kinetochores. The total number of kinetochores were acquired after analyzing all confocal sections using NIH Image J software.

2.7 Western blotting

Oocytes or granulosa cells (after COC denudation) or were lysed in 1% SDS, 1% β-mercaptoethanol, 20% glycerol and 50 mM Tris–HCl (pH 6.8). Lysed oocytes were heated at 95°C for 10 min. Proteins were then separated by electrophoresis using SDS-PAGE (10% SDS polyacrylamide precast gel) and transferred to nitrocellulose membranes using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 2% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at room temperature and incubated overnight at 4°C with primary antibodies diluted in a blocking solution. After washing in TBS-T (3 times for 10 min each), the membranes were incubated in secondary antibodies (anti-Rabbit IgG-HRP, ThermoFisher Scientific, Waltham, MA, USA #A16104 and anti-mouse IgG-HRP, Bio-Rad #1706516) diluted in blocking solution for 1 h at room temperature followed by 3 times washing in TBS-T solution. Protein signals were detected using the Clarity Max Western ECL Substrate (Bio-Rad #1705062S) following the manufacturer's protocol. NIH Image J software was used to do protein quantification.

Antibodies: anti-PARP1 (Cell Signaling #9532), anti-gH2AX (Abcam #ab11174), anti-α-tubulin antibody (MilliporeSigma #T6074) and LC3A/B antibody (Cell Signaling #4148).

2.8 Detection of autophagic activity

Autophagy was assessed using Cyto-ID autophagy detection kit (Enzo Life Sciences, Farmingdale, NY, USA #ENZ-51031) following the manufacturer's protocol with some modifications. Briefly, oocytes were incubated with the reaction mix (1 µL in 500 µL CZB medium) at 37˚C with 5% CO_2 in humidified air for 15 min. DNA was stained by adding Hoechst 33342 (25 µg/mL) to the same medium for an additional 5-7 min. Oocytes were then rinsed in PVP-PBS before imaging live oocytes using Leica DMI8 fluorescence microscope. The images were analyzed using NIH Image J software.

2.9 Alkaline comet assay / DNase sensitivity assay

Fully grown prophase I-arrested oocytes were permeabilized in 0.1% Triton X-100 in PBS for 10 min followed by transfer to 50 µl (5 Units) DNase I enzyme (ThermoFisher Scientific #18047019) or DNase I reaction buffer (non-DNase-treated). The LMAgarose (Trevigen, Gaithersburg, MD, USA #4250-050-02) was preheated in a tube in boiling water for 30 min before cooling down to 37 °C. Oocytes treated with or without DNase I were mixed with LMAgarose on comet assay slides (Trevigen #4250-050-03). The slides were incubated at 4 °C in the dark with high humidity for 30 min followed by incubation in lysis solution (Trevigen #4250-050-01) overnight at 4 °C. The slides were then immersed in a freshly prepared alkaline unwinding solution (pH>13, 200 mM NaOH, 1mM EDTA in dH_2O) for 1 h at 4 °C in the dark prior to submerging in 4°C alkaline electrophoresis solution (pH>13, 200 mM NaOH, 1 mM EDTA in dH_2O).

Electrophoresis was run at 21 V for 30 min using the Comet Assay ES unit. The slides were then immersed twice in dH_2O for 5 min each followed by another wash in 70% ethanol for 5 min. Slides were dried at 37° C for 15 min. SYBR-Gold (ThermoFisher Scientific, #S11494) was placed on dried agarose for 30 min at room temperature before complete drying at 37° C. Samples were imaged using Leica DMI8 fluorescence microscope. Tail moment was calculated based on the following equation: tail moment = tail length x (fluorescence intensity of the DNA in the tail part /total fluorescence intensity of the DNA in both the head and tail). The images were analyzed using NIH Image J software.

2.10 Quantification and statistical analysis

One-way ANOVA, Student t-test and chi-square contingency test were used for the evaluation of experimental statistical significance using GraphPad Prism software (GraphPad v9, San Diego, CA, USA). ANOVA was followed by the Tukey post hoc test. The data were expressed as means ± SEM. p-value less than 0.05 was considered statistically significant.

3. Results

3.1 DNA-damaged oocytes progress through MI leading to the development of aneuploid gametes

To investigate the consequences of DNA damage on oocyte MI, we used etoposide to induce DNA damage in fully grown prophase I-arrested (germinal vesicle/GV) oocytes. Etoposide is a topoisomerase II inhibitor, commonly used as a DNA damage inducer in somatic cells (Álvarez-Quilón et al., 2014; Tamamori-Adachi et al., 2018) and mammalian oocytes (Collins et al., 2015; Leem et al., 2019; Marangos and Carroll, 2012; Rémillard-Labrosse et al., 2020). Upon inducing DNA damage, histone H2AX is rapidly phosphorylated on Ser139 (γH2AX) by Ataxia Telangiectasia Mutated (ATM) kinase to trigger signaling for DDR (Bassing et al., 2002; Burma et al., 2001; Stiff et al., 2004). As soon as damaged DNA is resolved and repaired, γH2AX decreases (Chowdhury et al., 2005). Therefore, γH2AX has been recognized as a sensitive molecular marker for DNA damage in somatic cells and oocytes (Lin et al., 2014; Ma et al., 2013; Mah et al., 2010; Sharma et al., 2012). Similar to previous reports (Marangos and Carroll, 2012; Marangos et al., 2015; Rémillard-Labrosse et al., 2020), treating prophase I-arrested oocytes (incubated with milrinone, a phosphodiesterase inhibitor, to prevent meiotic resumption) with etoposide (50 mg/ml for 3 h (Leem et al., 2019)) induced DNA damage as evidenced by the significant increase of γH2AX foci numbers and fluorescence pixel intensity, compared to DMSO-treated oocytes (Figure 1A and Figures 2). This result was further confirmed by Western blot analysis where γH2AX was significantly increased in etoposide-

treated oocytes, compared to DMSO-treated controls (Figure 3). Induction of DNA damage in prophase I-arrested oocytes does not completely prevent meiotic resumption and progression when the oocytes are allowed to mature in vitro (Marangos et al., 2015; Rémillard-Labrosse et al., 2020). Indeed, similar to previous reports (Leem et al., 2019), approximately 38% of DNA-damaged oocytes extruded the first polar body (PB) and reached metaphase II stage (Figure 4A). However, there was a significant delay in the timing of PB extrusion (PBE) in the etoposide-treated oocytes when compared to control oocytes (Figure 4B). This PBE percentage in the etoposide-treated oocytes is relatively higher than that reported previously (Collins et al., 2015; Marangos et al., 2015). This discrepancy might be due to the use of different etoposide concentrations (100 mg/ml in (Marangos et al., 2015) vs. 50 mg/ml in this study), the use of different mouse strains or the difference in maternal age (3-4-week-old mice in (Collins et al., 2015) vs. sexually mature 4-6-week-old mice in our study). Although DNA damage exposure after meiotic resumption leads to aneuploidy, to our knowledge, it is not known whether DNA damage induction for a limited time specifically in fully grown prophase I oocytes can induce aneuploidy when the oocytes are allowed to resume meiosis. Using in situ chromosome counting technique, we found that ~80% of etoposide-treated oocytes were aneuploid, compared to ~3% aneuploidy in controls (Figure 5). Thus, DNA-damaged oocytes are capable of progressing through MI leading to the development of aneuploid gametes. Because most chromosome segregation errors during MI arise from mistakes occurring during metaphase I (Antonarakis et al., 2004), we

examined metaphase I stage in DNA-damaged oocytes in detail. Consistent with previous reports (Marangos et al., 2015; Rémillard-Labrosse et al., 2020), we observed higher rates of chromosome misalignment (a phenotype associated with aneuploidy) in etoposide-treated oocytes when compared to control oocytes (Figure 6A, B). Importantly, we found that chromosome architecture is altered in DNA-damaged oocytes. In contrast to control oocytes in which chromosomes have normal bivalent morphology (Figure 7A_a), etoposide-treated oocytes had significantly higher rates of compact chromosome morphology (Figure 7A_b; 1J) and chromosome fragmentation (Figure 7A_c, d; 1K). Interestingly, we found that almost all chromosome fragments are those containing kinetochores (Figure 7A_c, d). Kinetochores are large protein complex that link spindle microtubules to centromeric DNA of chromosomes. Therefore, we asked whether chromosome fragmentation in etoposide-treated oocytes is induced by microtubule-induced tension on chromosomes. Importantly, treating DNA-damaged oocytes with nocodazole, a microtubule depolymerizing drug (Eichenlaub-Ritter and Boll, 1989), rescued the chromosome fragmentation phenotype, but not chromosome compaction phenotype (Figure 6C and Figure 7A, C), indicating that microtubule-induced tension on chromosomes is the reason of chromosome fragmentation in DNA-damaged oocytes. Our results are consistent with previous observations that microtubules can establish stable attachments with kinetochores in DNA-damaged oocytes (See ref (Lane et al., 2017) and our unpublished data). During MI, homologous chromosomes must be bioriented and correctly attached (at kinetochores) to opposite spindle poles, necessary for faithful chromosome

segregation during anaphase I and telophase I. The presence of separated kinetochores in DNA-damaged oocytes (Figure 7A_c, d) suggests that homologous chromosomes will not properly segregate during anaphase/telophase I. Indeed, using time-lapse confocal imaging, we observed lagging chromosomes during anaphase I/telophase I in all DNA-damaged oocytes (Figure 8), the phenotype never observed in control oocytes and highly correlates with chromosome missegregation (Ganem and Pellman, 2012; Mihajlović et al., 2021). These results demonstrate that early exposure of prophase I-arrested oocytes to DNA damage can have a detrimental consequence on oocytes by increasing their risk of developing aneuploid eggs.

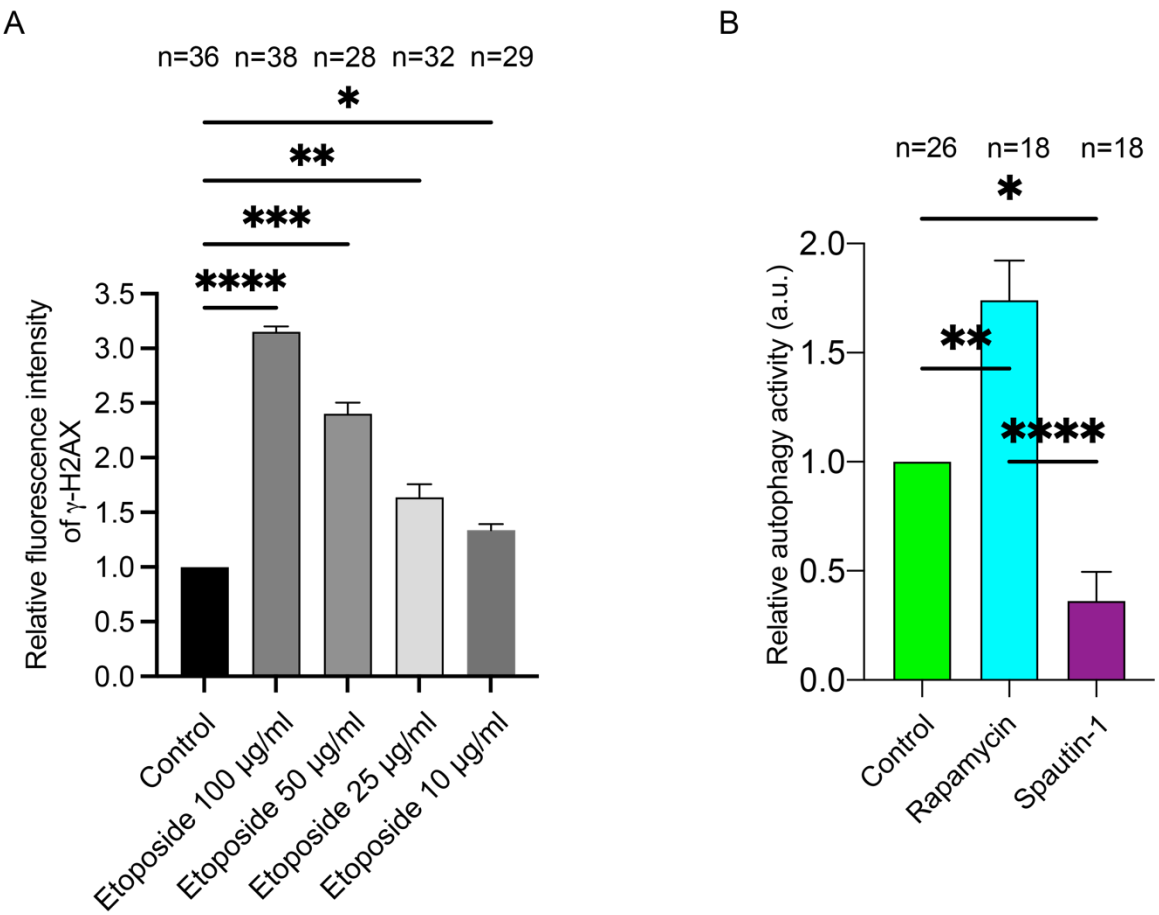

Figure 1: Confirmation of chemicals' effects in oocytes (etoposide, rapamycin, spautin-1).

(A) Fully grown prophase I-arrested oocytes were incubated for 3 h in milrinone-containing CZB medium supplemented with etoposide at the indicated concentrations. Oocytes were fixed and immune-labeled with γH2AX antibody. Quantification of γH2AX fluorescence intensity is shown. (B) Fully grown prophase I oocytes were incubated in milrinone-containing CZB medium supplemented with the indicated treatments for 3 h followed by assessing autophagic activity by using Cyto-ID detection kit. Relative autophagy activity quantification is shown. Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

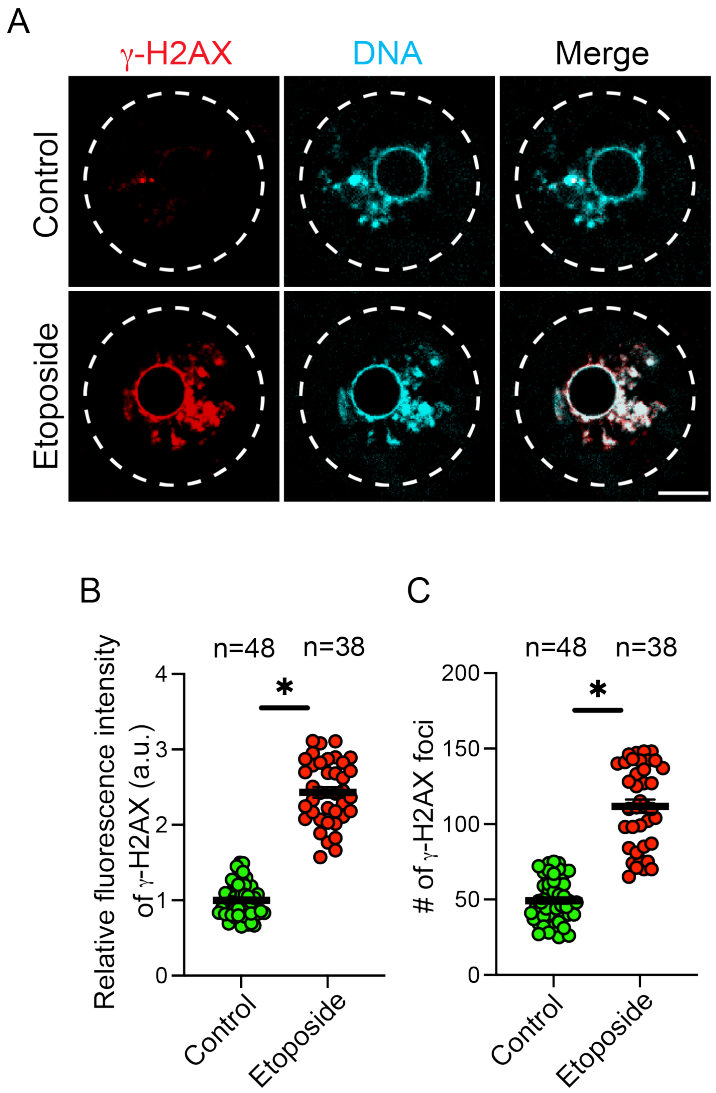

Figure 2: Etoposide induce DNA damage in mouse oocytes

(A) Fully grown prophase I-arrested (germinal vesicle) oocytes were incubated in CZB medium supplemented with DMSO (control) or etoposide for 3 h. Milrinone was added to the medium to prevent meiotic resumption. Oocytes were fixed and immune-labeled with γH2AX antibody. Representative images are shown. (B) Quantification of γH2AX fluorescence intensity in "A". (C) Quantification of γH2AX foci numbers in "A". Scale bars represent 10 μm. DNA was stained with DAPI (blue). Data are expressed as mean ± SEM. Student-t-test were used to analyze the data. Values with asterisks are significantly different, $*p < 0.05$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph/column.

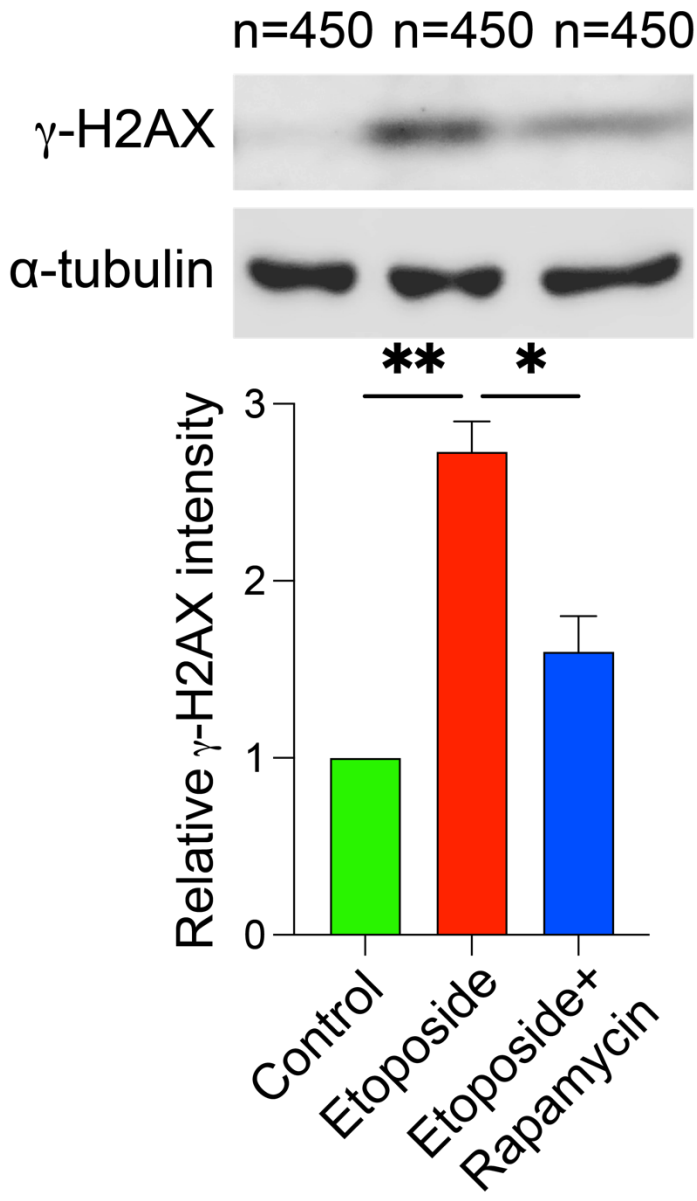

Figure 3: Autophagy induction rescues DNA damage in oocytes

Fully grown prophase I oocytes were incubated with DMSO (control), etoposide or etoposide + rapamycin for 3 h in milrinone-containing CZB medium followed by Western blot analysis using γH2AX and α-tubulin antibodies. Representative blot images of the same membrane (upper panel). Quantification of γH2AX (lower panel). one-way ANOVA were used to analyze the data. Values with asterisks are significantly different, $*p < 0.05$, $**p < 0.01$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph/column.

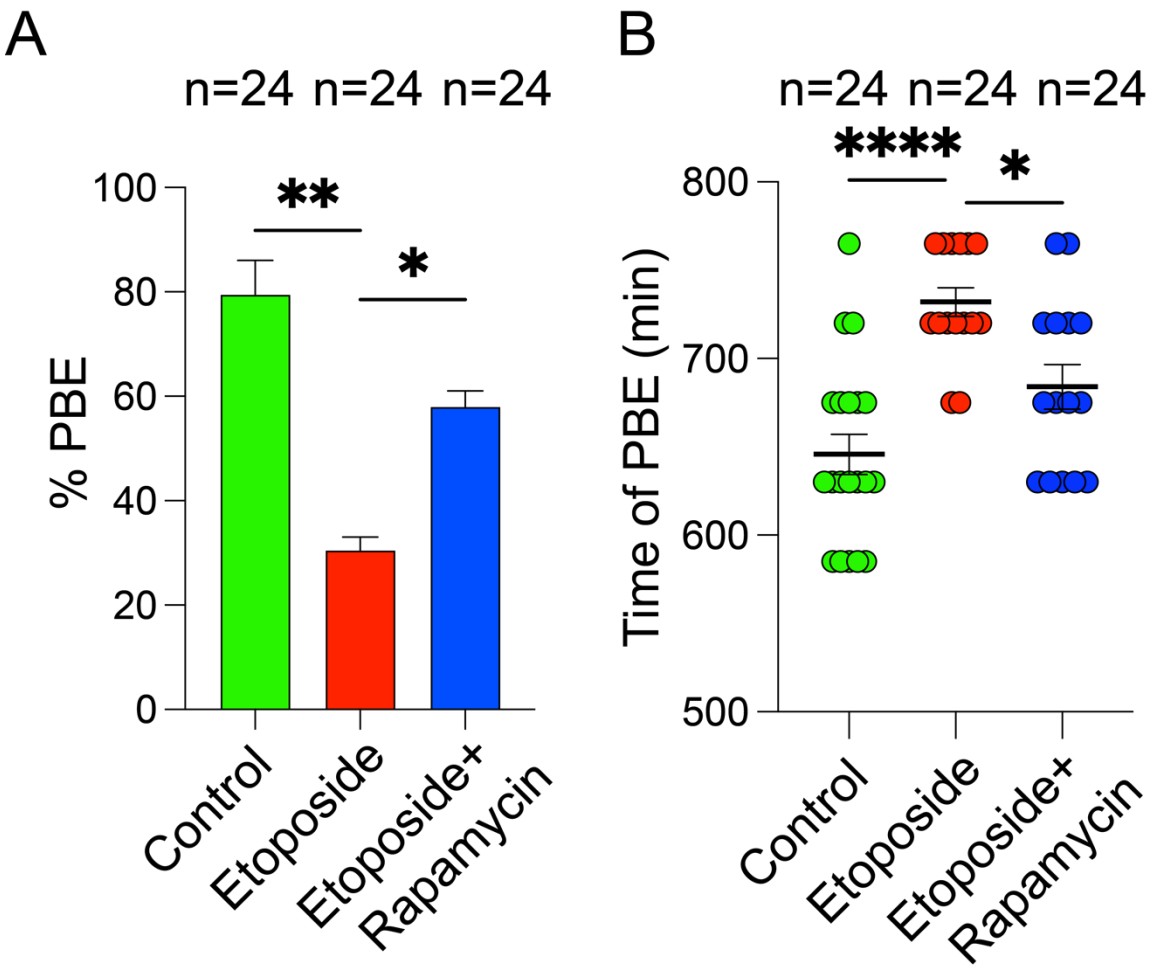

Figure 4: Comparative rates of polar body extrusion

Fully grown prophase I oocytes were incubated with the indicated treatment in milrinone-containing CZB medium for 3 h followed by washing and time-lapse imaging during *in vitro* maturation in CZB medium. (A) Quantification of first polar body extrusion (PBE) percentage at 16 h after maturation. (B) Quantification of PBE timing. one-way ANOVA were used to analyze the data. Values with asterisks are significantly different, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph/column.

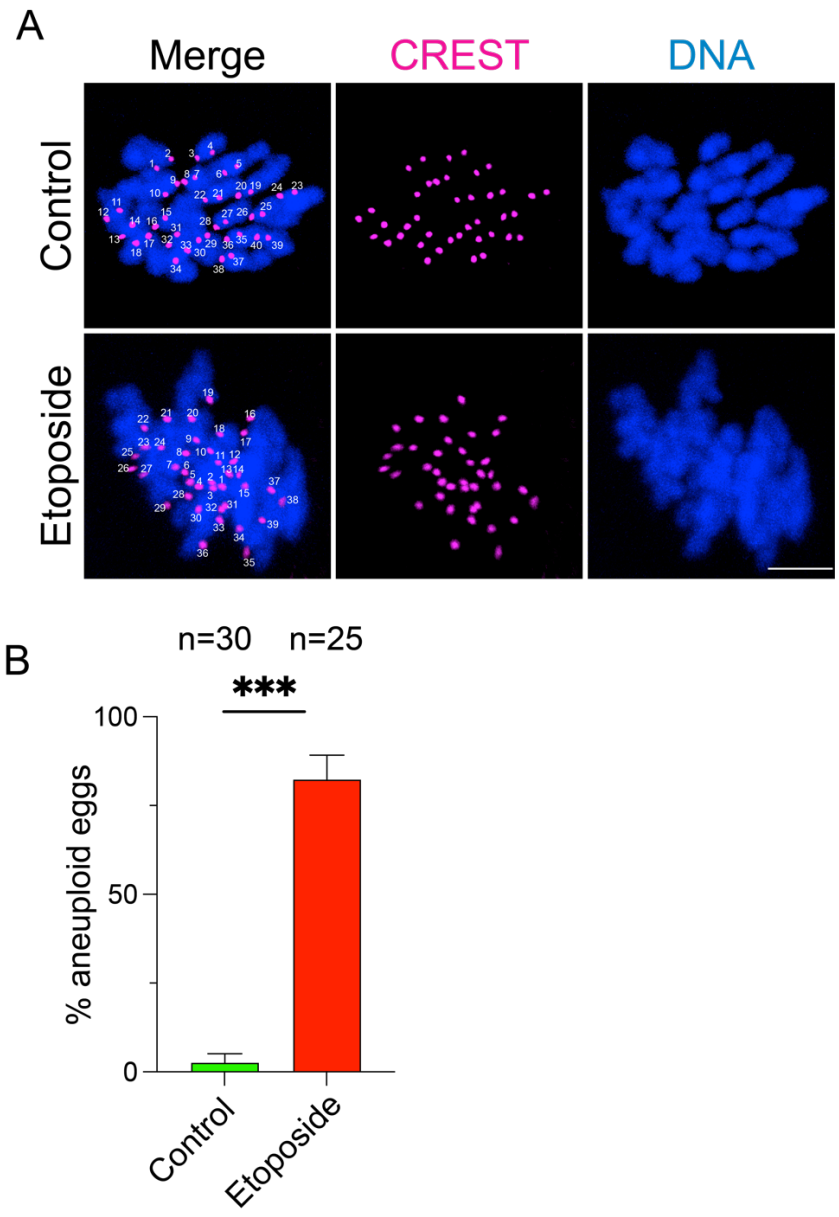

Figure 5: DNA damage induction in GV oocytes significantly increases the incidence of aneuploidy at Metaphase-II

(A) Fully grown prophase I oocytes were incubated with DMSO (control) or etoposide for 3 h in milrinone-containing CZB medium followed by washing and in vitro maturation for 14 h (metaphase II). Oocytes were treated with monastrol for 2 h, fixed and kinetochores were immune-labeled with CREST antibody. Oocytes were scored either as euploid (containing 40 kinetochores) or as aneuploid (containing less or more than 40 kinetochores). Representative images are shown. (B) Quantification of aneuploidy percentage in “A”. Scale bars represent 10 µm. DNA was stained with DAPI (blue). Data are expressed as mean ± SEM. Values with asterisks are significantly different, ***$p < 0.001$.

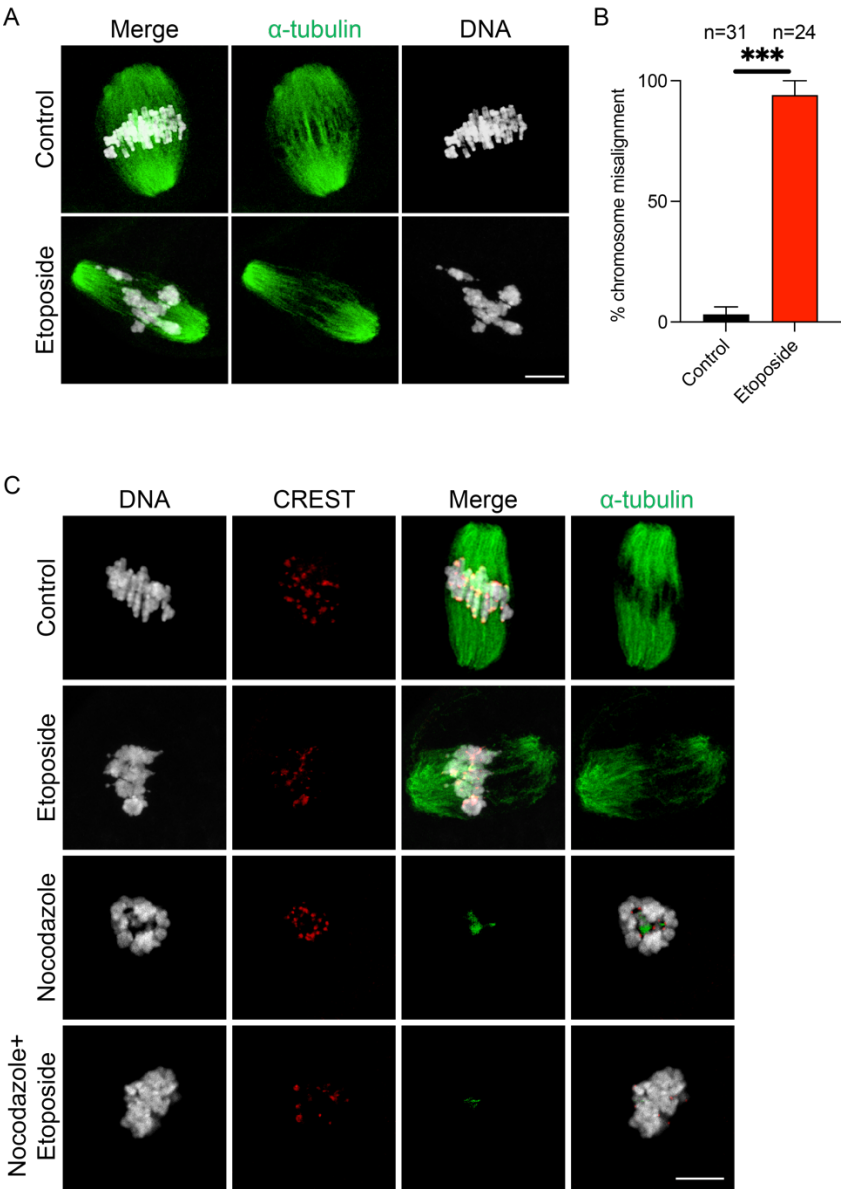

Figure 6: Observations of oocytes chromosome morphology after DNA damage induction

Fully grown prophase I oocytes were incubated with the indicated treatments for 3 h in milrinone-containing CZB medium followed by washing and in vitro maturation for 7 h (metaphase I) in CZB medium. Metaphase I oocytes were fixed and immune-labeled with anti-α-tubulin (A, C) and anti-CREST (C) antibodies. DNA was stained by DAPI. Representative images are shown. (B) Quantification of chromosome misalignment percentage in "A". Scale bars represent 10 μm. Data are expressed as mean ± SEM. Student-t-test was used to analyze the data. Values with asterisks differ significantly, ***$p < 0.001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

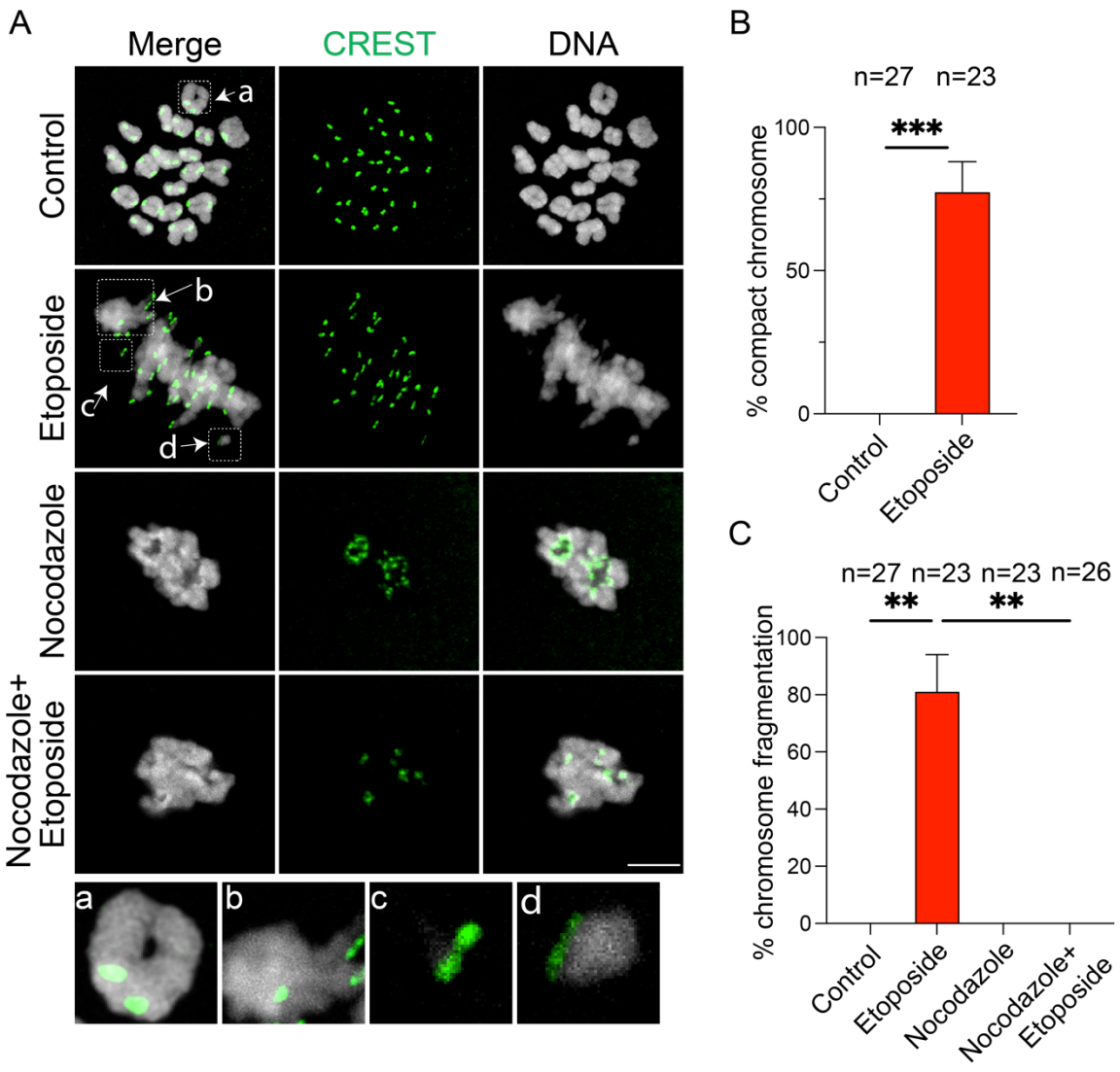

Figure 7: Metaphase-I chromosome morphology is significantly altered in DNA-damaged oocytes

(A) Fully grown prophase I oocytes were incubated with DMSO (control) or etoposide for 3 h in milrinone-containing CZB medium followed by washing and in vitro maturation for 7 h (metaphase I) in CZB medium with or without nocodazole. Metaphase I oocytes were fixed and immune-labeled with CREST antibody to label kinetochores. Representative images are shown. White arrows represent chromosome morphology (a, b) and fragmentation (c, d). (B) Quantification of compact chromosome percentage in "A". (C) Quantification of chromosome fragmentation percentage in "A". Scale bars represent 10 μm. DNA was stained with DAPI (blue). Data are expressed as mean ± SEM. Student-t-test or one-way ANOVA were used to analyze the data. Values with asterisks are significantly different, $**p < 0.01$, $***p < 0.001$.

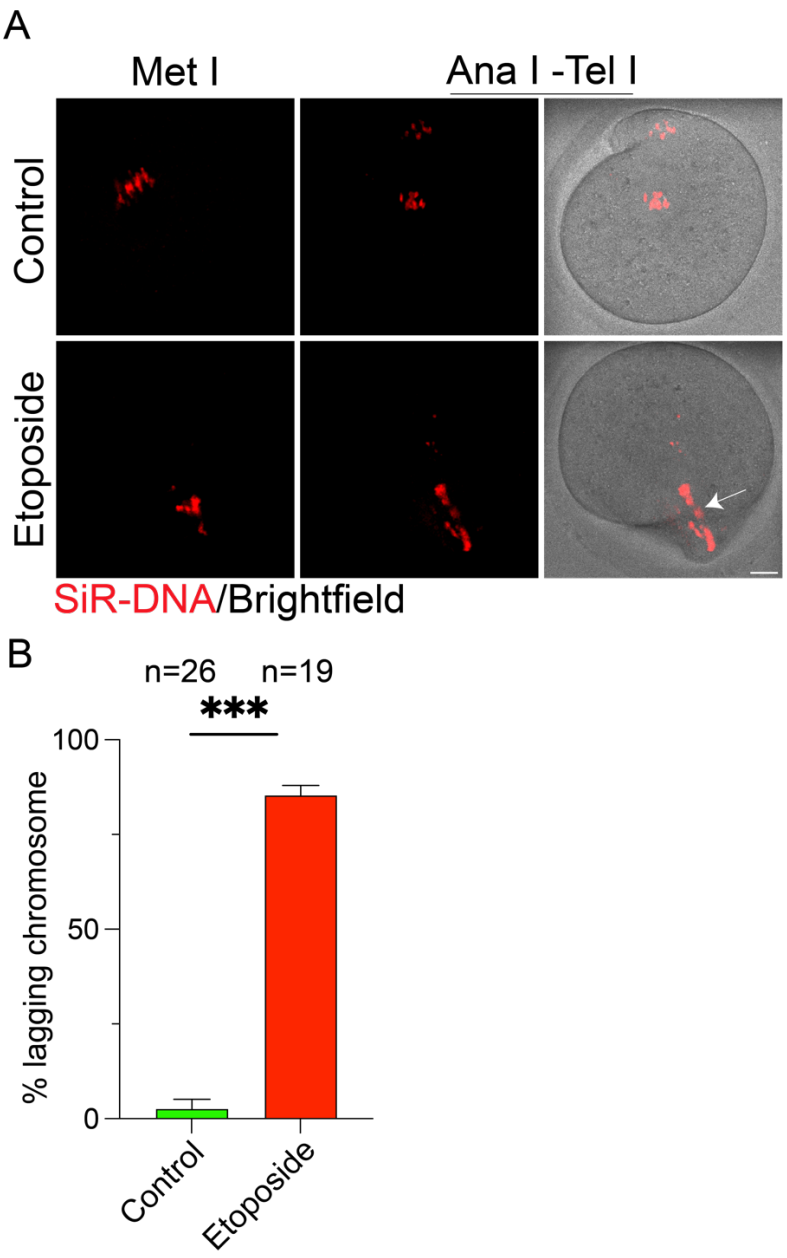

Figure 8: DNA damage induction in GV oocytes significantly increases the incidence of lagging chromosome

(A) Fully grown prophase I oocytes were incubated with DMSO (control) or etoposide for 3 h in milrinone-containing CZB medium followed by washing and in vitro maturation in CZB medium containing SiR-DNA (to label the DNA). Shown are representative time-lapse confocal images. The white arrow represents lagging chromosomes. (B) Quantification of the percentage of lagging chromosomes in “A”. Scale bars represent 10 μm. DNA was stained with DAPI (blue). Data are expressed as mean ± SEM. Values with asterisks are significantly different, $**p < 0.01$, $***p < 0.001$.

3.2 Mouse oocytes inefficiently activate autophagy in response to DNA damage

Somatic cells can efficiently repair most DNA damage if they are given longer time for recovery (Yang et al., 2019). We asked whether providing DNA-damaged oocytes with longer recovery time will also allow for efficient DDR. Following a 3 h etoposide treatment, prophase I-arrested oocytes (by milrinone) were cultured in etoposide-free medium for an additional 16 h followed by assessing DNA damage. Interestingly, prophase I-arrested oocytes were not able to repair damaged DNA as γH2AX did not decrease after the extended incubation in etoposide-free medium (Figure 9). This contrasts with a previous study showing that mouse oocytes have the capacity to repair damaged DNA over a period of 10 h (Collins et al., 2015). This discrepancy may be explained by the lower concentration of DNA-damaging agent (25 mg/ml etoposide) that was used in the aforementioned study, compared to 50 mg/ml etoposide in our study. These findings suggest that prophase I oocytes have a limited capacity to repair certain levels of DNA damage and, at that stage, one or more of DDR mechanisms are not fully functional.

Autophagy plays a critical role in maintaining cellular homeostasis (Kundu and Thompson, 2008). Emerging evidence suggests that, in somatic cells, autophagy is stimulated in response to DNA damage and plays important role in repairing damaged DNA (Eliopoulos et al., 2016; Gillespie and Ryan, 2016; Gomes et al., 2017; Yang et al., 2016). In somatic cells, autophagy can regulate chromatin

conformation by reducing histone ubiquitination, making DNA damaged sites accessible to DDR proteins. Therefore, autophagy-deficient cells have altered DDR mechanism (Wang et al., 2016). However, the role of autophagy during DNA damage response is largely unknown in mammalian oocytes. Given altered chromatin morphology in DNA-damaged oocytes (Figure 7A), we asked whether autophagy-mediated DDR mechanism is disabled in oocytes. To this end, fully grown prophase I-arrested oocytes were collected and treated with etoposide to induce DNA damage followed by assessing autophagy activity. Strikingly, in contrast to somatic cells in which autophagy is stimulated in response to DNA damage (Eliopoulos et al., 2016; Gillespie and Ryan, 2016; Gomes et al., 2017; Yang et al., 2016), autophagy activity (assessed by an autophagy Cyto-ID detection assay) was significantly decreased in live DNA-damaged oocytes, compared to controls (Figure 10). When autophagy is activated, cytosolic form of the pro-autophagic, microtubule-associated protein 1 light chain 3 (LC3-I) becomes conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes(Tanida et al., 2008). Because LC3-II is specifically correlated with autophagosomes and autolysosomes, it is a reliable marker of autophagic activity in both somatic cells and oocytes (Kabeya et al., 2004; Lee et al., 2014; Tsukamoto et al., 2008). Consistent with Cyto-ID assay results, LC3-II expression levels assayed by Western blot densitometry were significantly decreased in DNA-damaged oocytes when compared to controls (Figure 11). This contrasts with granulosa cells, in which LC3-II was significantly increased

following DNA damage induction by etoposide (Figure 12). Thus, activation of autophagy in DNA-damaged oocytes is not functional, unveiling a notable difference between somatic cells and fully grown oocytes, and raising the question of whether inefficient autophagy activation is the cause, at least partially, of weakened DDR in oocytes.

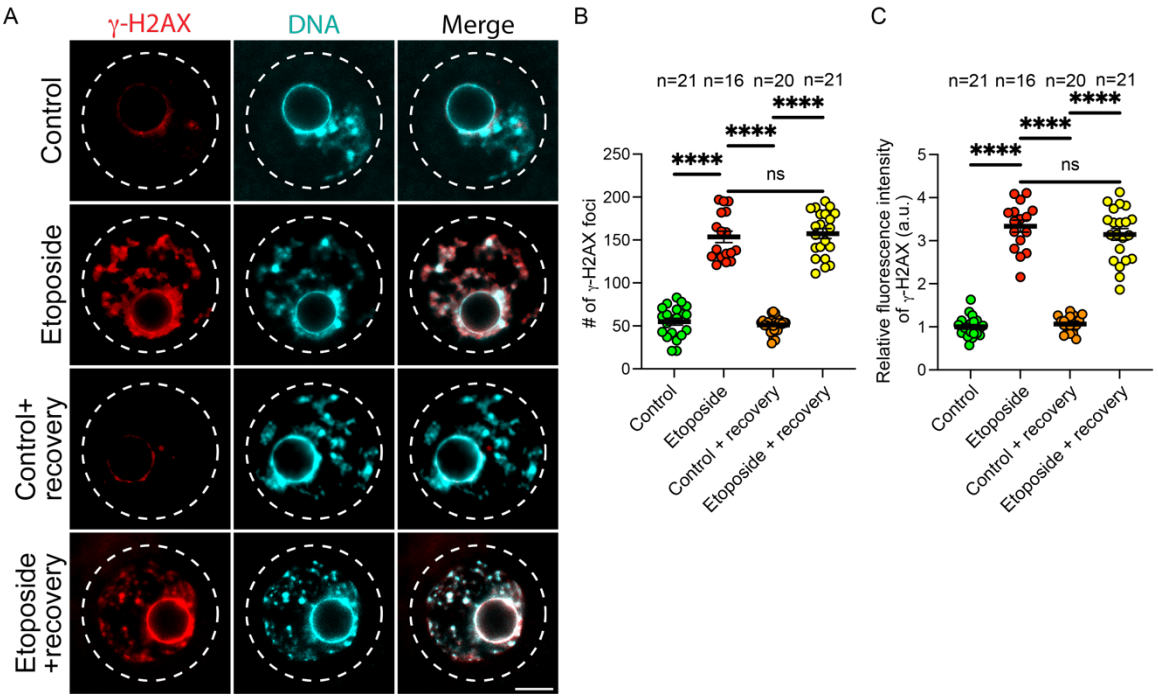

Figure 9: Culturing DNA-damaged oocytes overnight in etoposide-free medium did not significantly reduce γ-H2AX levels

(A) Fully grown prophase I oocytes were incubated in milrinone-containing CZB medium supplemented with DMSO (control) or etoposide for 3 h. A subset of control and etoposide-treated oocytes were fixed after 3 h, whereas the remaining oocytes were released from etoposide and cultured in milrinone-containing CZB medium (to prevent meiotic resumption) for 16 h (recovery) prior to fixation. All fixed oocytes were immuno-labeled at the same time with γH2AX antibody. DNA was labeled with DAPI. Representative images are shown. Scale bar represents 10 μm. (B) Quantification of γH2AX foci numbers in "A". (C) Quantification of γH2AX fluorescence intensity in "A". Data are expressed as mean ± SEM. One-way ANOVA were used to analyze the data. Values with asterisks differ significantly, ****$p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

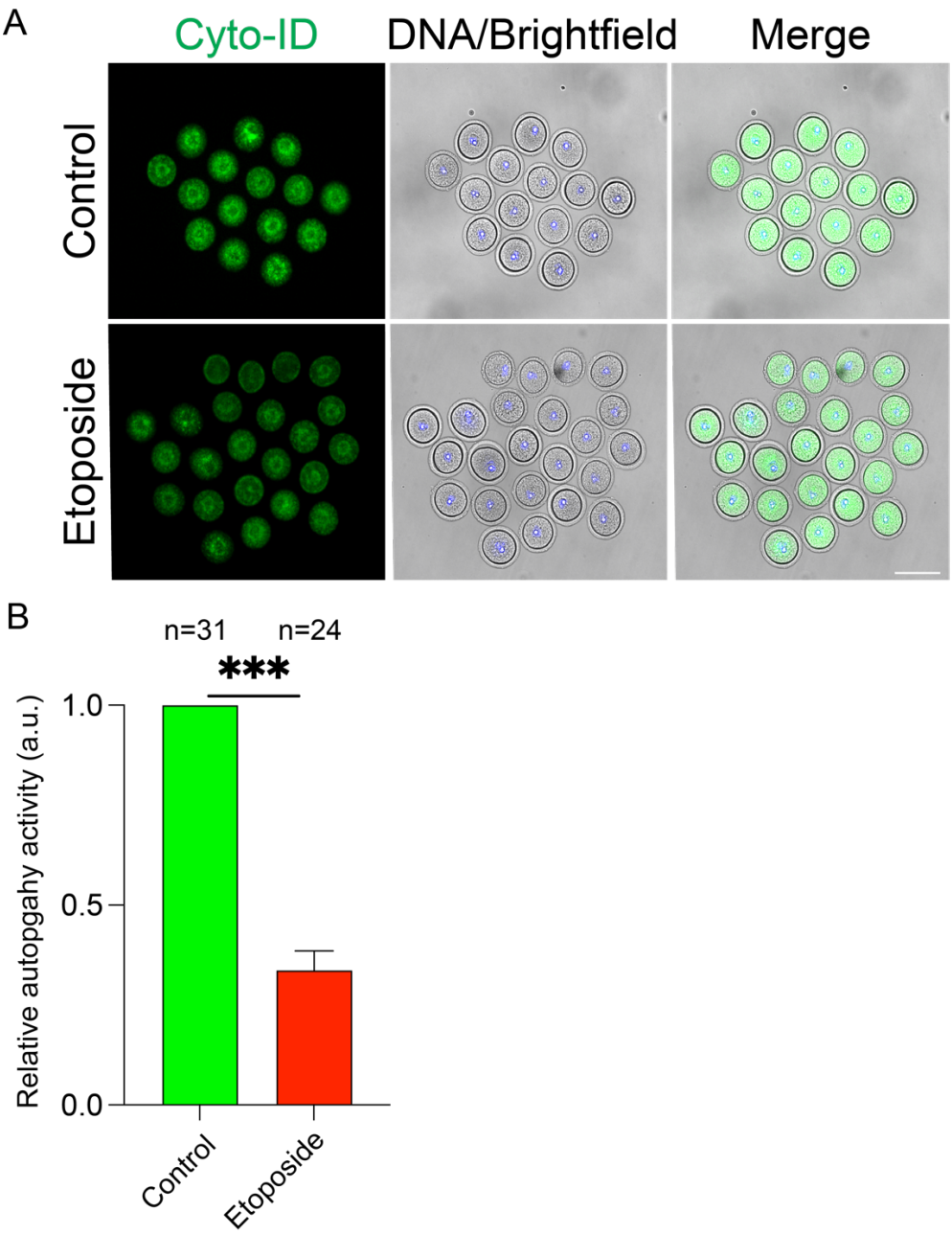

Figure 10: Autophagy activity was significantly reduced upon DNA damage in mouse oocytes

(A) Fully grown prophase I oocytes were incubated in milrinone-containing CZB medium supplemented with DMSO (control) or etoposide for 3 h followed by autophagic activity measurements by using Cyto-ID detection kit. Representative images are shown. Scale bar represents 100 µm. (B) Quantification of autophagic activity in "A". Data are expressed as mean ± SEM. Student-t-test were used to analyze the data. Values with asterisks differ significantly, ***$p < 0.001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

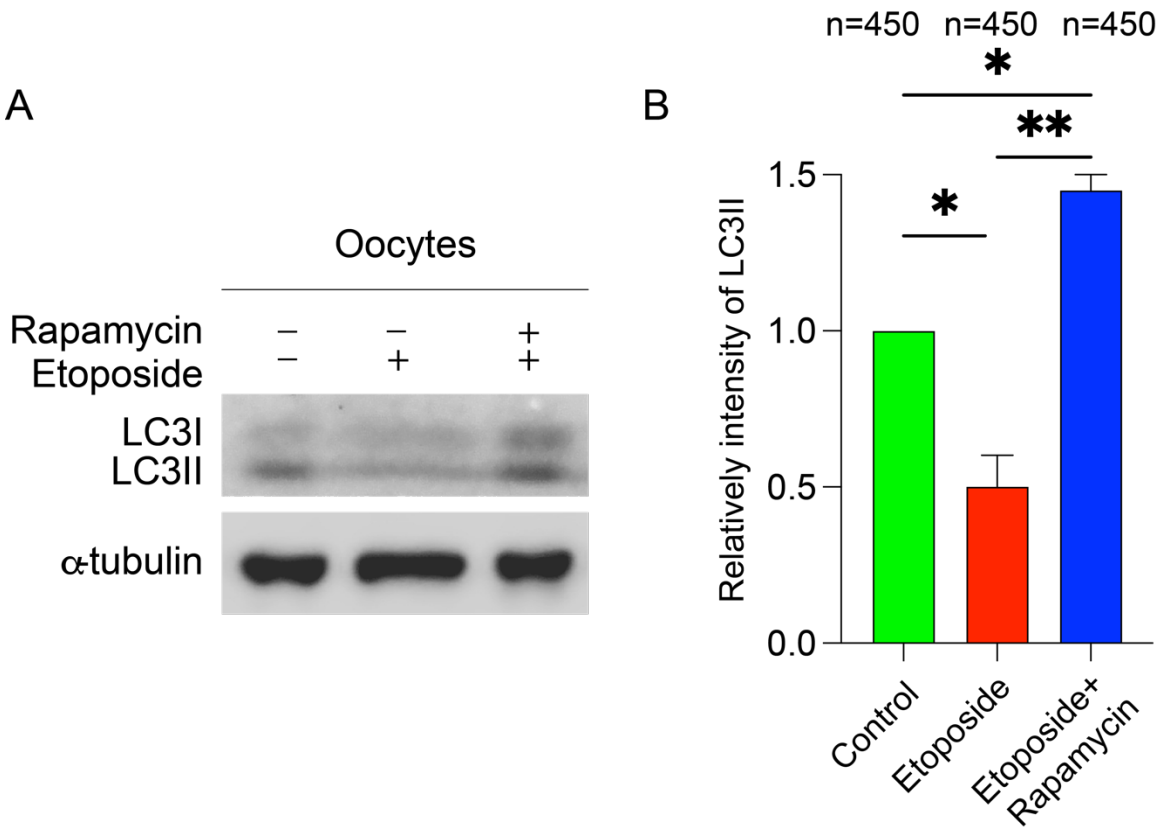

Figure 11: Western blots results of autophagy activity in oocytes

(A) Fully grown prophase I oocytes were incubated in milrinone-containing CZB medium supplemented with the indicated treatments for 3 h followed by Western blot analysis with LC3 and α-tubulin antibodies. (B) Quantification of LC3II in "A". Data are expressed as mean ± SEM. One-way ANOVA were used to analyze the data. Values with asterisks differ significantly, $*p < 0.05$, $**p < 0.01$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

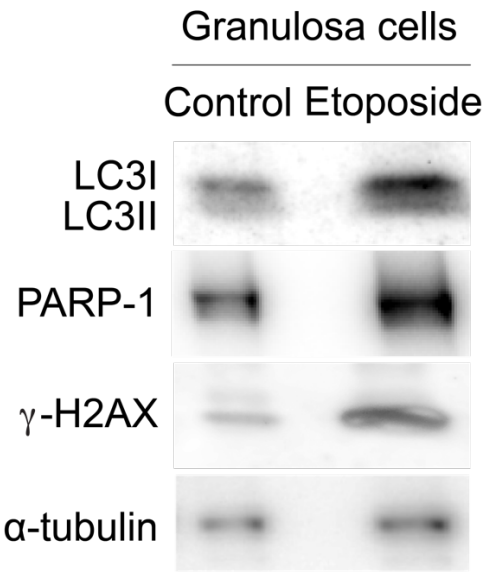

Figure 12: Western blot results of somatic cells.

Cumulus oocyte complexes (COCs) were cultured in milrinone-containing CZB medium supplemented with DMSO (control) or etoposide for 3 h. COCs were denuded mechanically, and cumulus granulosa cells were lysed prior to Western blot analysis by using LC3, PARP-1, γH2AX and α-tubulin antibodies. Representative images are shown (same membrane relabeled).

3.3 Reduced autophagy is the likely cause of weakened DNA damage response in oocytes

To investigate whether autophagy induction can rescue DNA damage in oocytes, we used rapamycin, a well-established autophagy inducer in both somatic cells and oocytes (Li et al., 2020; Noda and Ohsumi, 1998; Sarkar et al., 2009). Rapamycin is a potent inhibitor of mammalian target of rapamycin (mTOR) which negatively regulates autophagy. Indeed, treating mouse oocytes with rapamycin stimulated autophagy as evidenced by the significant increase ($p< 0.01$) of autophagy activity in live oocytes, compared to control oocytes (Figure 1B) and its ability to rescue decreased autophagy (LC3-II) in etoposide-treated oocytes (Figure 11). Importantly, autophagy induction during etoposide treatment decreased DNA damage as evidenced by decreased γH2AX foci numbers and fluorescence intensity compared to etoposide group (Figure 13). To confirm this finding, we employed the alkaline comet assay, a potent method to detect DNA fragmentation/damage in eukaryotic cells where increased DNA tail moment and length (refer to material and methods for more detail) correlates with DNA damage (Olive and Banáth, 2006). Again, autophagy induction by rapamycin significantly decreased DNA damage in etoposide-treated oocytes as evidenced by decreased DNA tail moment and length (Figure 14). To further confirm our conclusion, we induced DNA damage by exposing prophase I oocytes to ultraviolet (UV) radiation, instead of etoposide treatment. Compared to control group, a brief exposure of the oocytes to UV radiation (302 nm for 30 seconds) significantly increased ($p<0.0001$) DNA damage as shown by increased γH2AX

(Figure 15) and increased both DNA tail length and moment (Figure 16). Importantly, rapamycin treatment was able to significantly reduce DNA damage in UV-exposed oocytes (Figures 15, 16). These results suggest that autophagy induction by rapamycin rescues DNA damage in mouse oocytes.

Rapamycin induces autophagy by inhibiting mTOR (Sarkar et al., 2009) which can regulate other multiple cellular functions in addition to autophagy (Castellanos et al., 2016). To confirm that rapamycin rescues DNA damage through autophagy induction, we inhibited autophagy using mTOR-independent pathway. Spautin-1 is a specific and potent small molecule inhibitor that efficiently inhibits autophagy by degrading Beclin-1, an indispensable protein required for autophagy initiation (Schott et al., 2018). Treating mouse oocytes with spautin-1 efficiently inhibited autophagy (Figure 1B). Consistent with our conclusion that autophagy regulates DDR in DNA-damaged oocytes, inhibiting autophagy with spautin-1 increased DNA damage in etoposide-treated oocytes (Figure 17). Moreover, inhibition of autophagy independently of mTOR pathway using spautin-1 abolished the rescue effect of autophagy induction by rapamycin in DNA-damaged oocytes (Figure 13). Proteasomal inhibitor, MG132, is known to induce autophagy by increasing LC3-I to LC3-II conversion (Ge et al., 2009; Seguin et al., 2014). Consistent with our observations, MG132 significantly decreased DNA damage in etoposide-treated oocytes as evidenced by decreased γH2AX (Figure 18). Thus, reduced autophagy is the likely cause of weak DDR in oocytes.

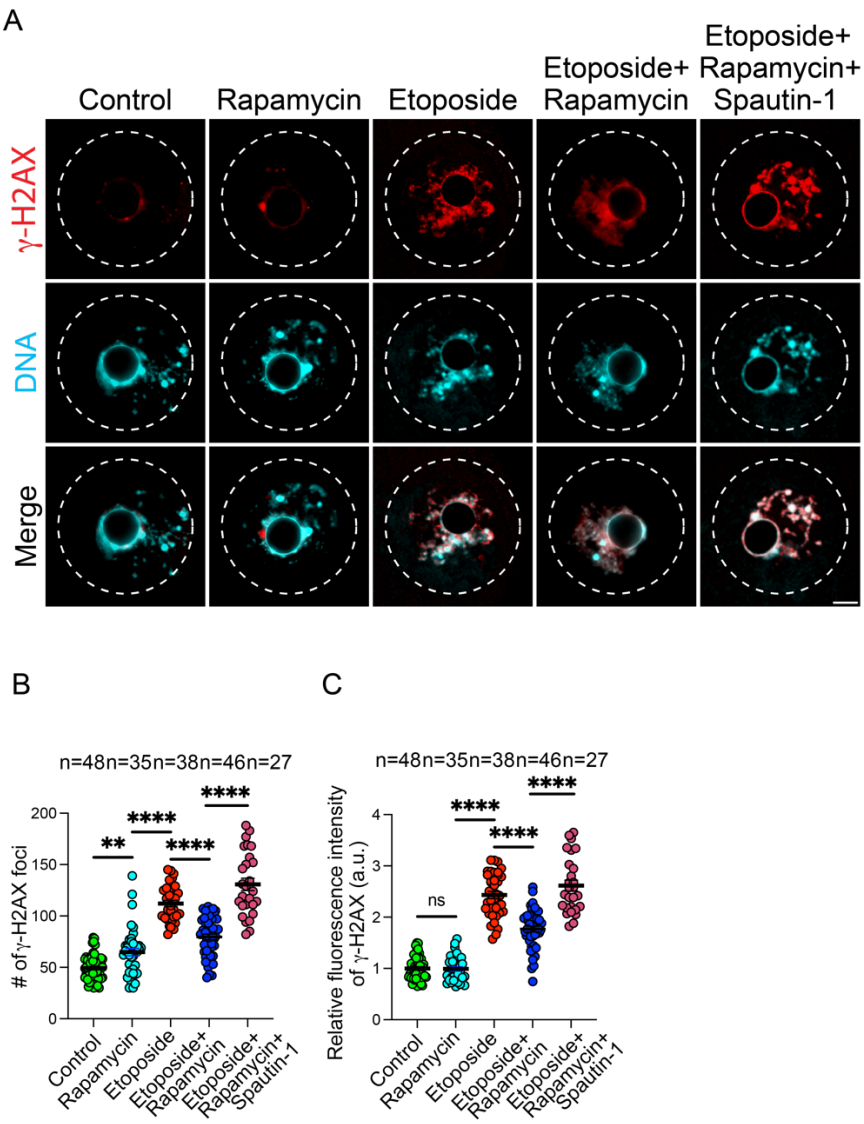

Figure 13: Autophagy induction (by rapamycin) decreases γ-H2AX in DNA-damaged oocytes (by etoposide).

(A) Fully grown prophase I-arrested (germinal vesicle) oocytes were incubated in milrinone-containing CZB medium supplemented with the indicated treatments for 3 h. Oocytes were fixed and immune-labeled with γH2AX antibody. Representative images are shown. (B) Quantification of γH2AX foci numbers in "A". (C) Quantification of γH2AX fluorescence intensity in "A". Where applicable, DNA was stained with DAPI (blue). Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, $^{**}p < 0.01$, $^{****}p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

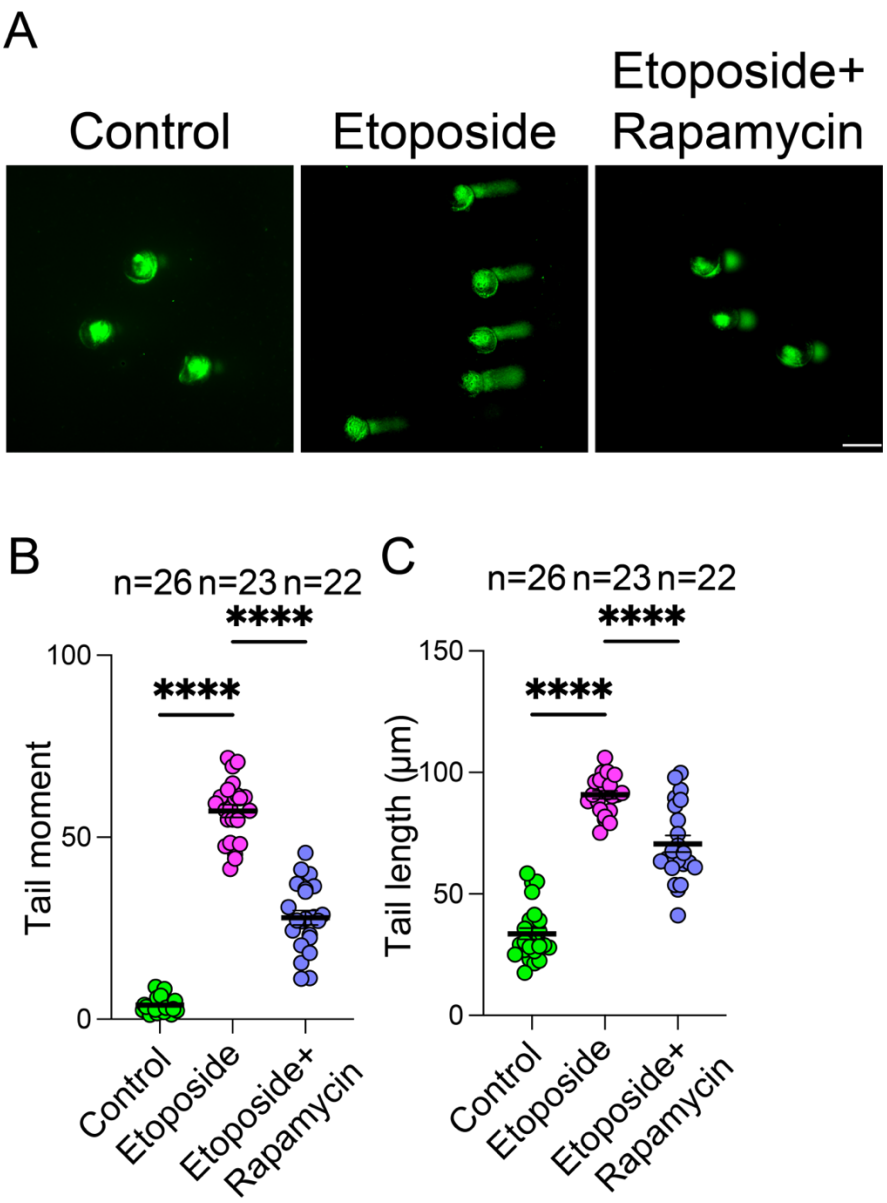

Figure 14: Autophagy induction (by rapamycin) rescues DNA damage in DNA-damaged oocytes.

(A) Fully grown prophase I-arrested oocytes were incubated in milrinone-containing CZB medium supplemented with the indicated treatments for 3 h followed by alkaline comet assay. Representative images are shown. (B) Quantification of tail moment in “A”. (C) Quantification of tail length in “A”. Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, ****$p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

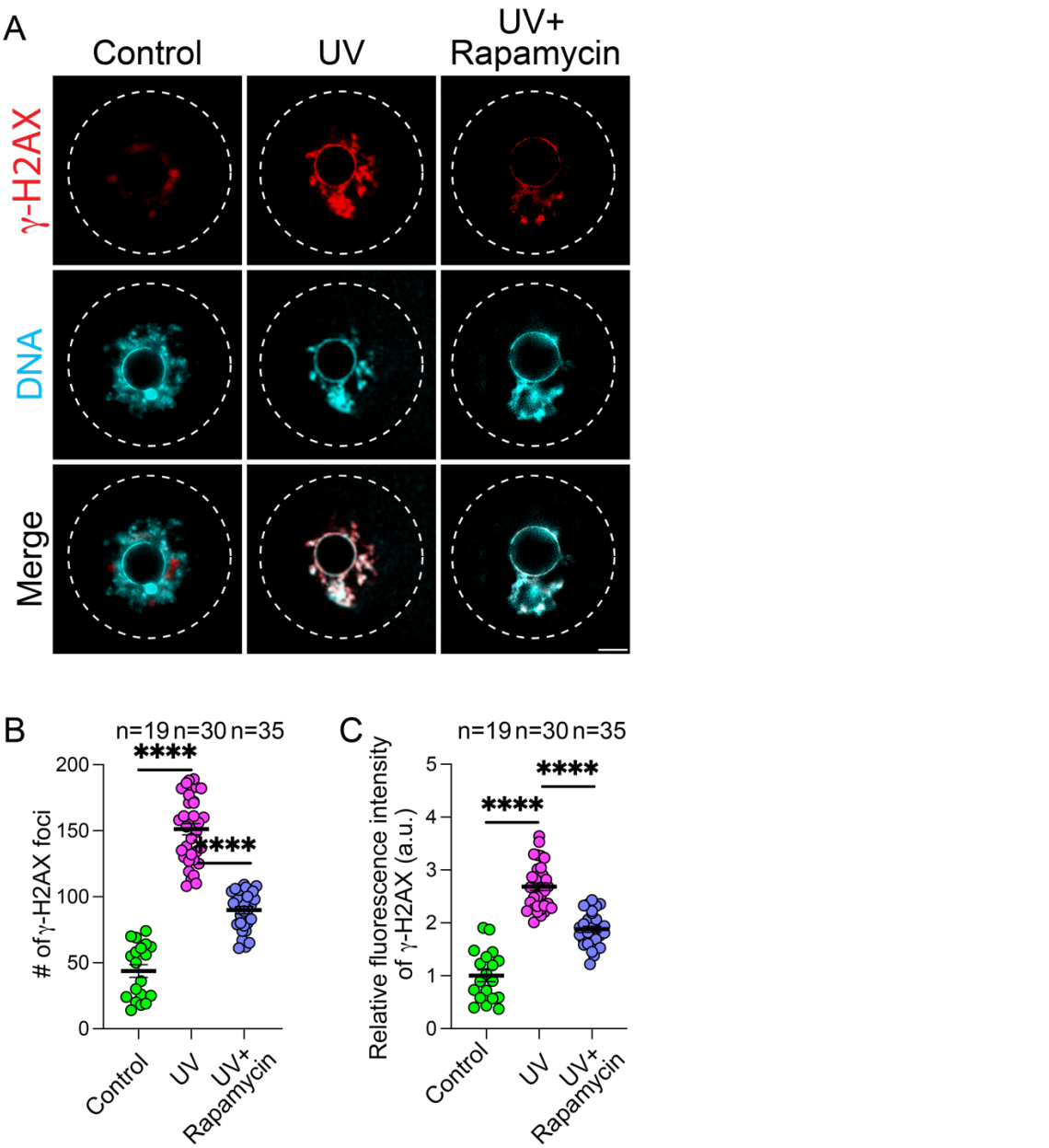

Figure 15: Autophagy induction (by rapamycin) decreases γ-H2AX in DNA-damaged oocytes (by UV).

(A) Fully grown prophase I oocytes were incubated in milrinone-containing CZB medium supplemented with DMSO (control) for 3 h. Another set of oocytes were exposed to UV light (302 nm for 30 seconds) and incubated with or without rapamycin for 3 h followed by immunostaining with γH2AX antibody. Representative images are shown. (B) Quantification of γH2AX foci numbers in "A". (C) Quantification of γH2AX fluorescence intensity in "A". Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, ****$p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

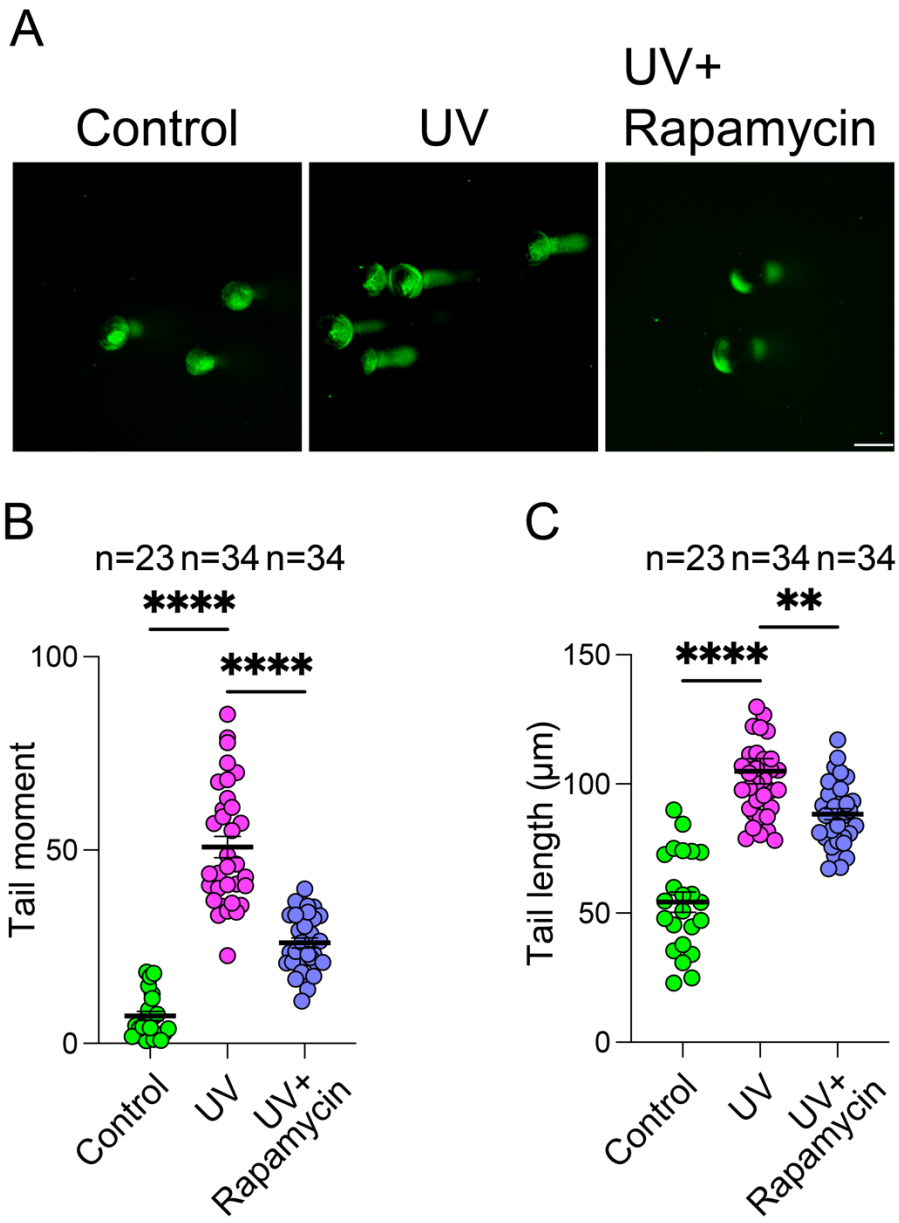

Figure 16: Autophagy induction (by rapamycin) decreases DNA damage in DNA-damaged oocytes (by UV).

(A) DMSO (control)-, UV- and UV+rapamycin-treated oocytes were assessed for DNA damage by alkaline comet assay. Representative images are shown. (B) Quantification of tail moment in "A". (C) Quantification of tail length in "A". Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, $^{**}p < 0.01$, $^{****}p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

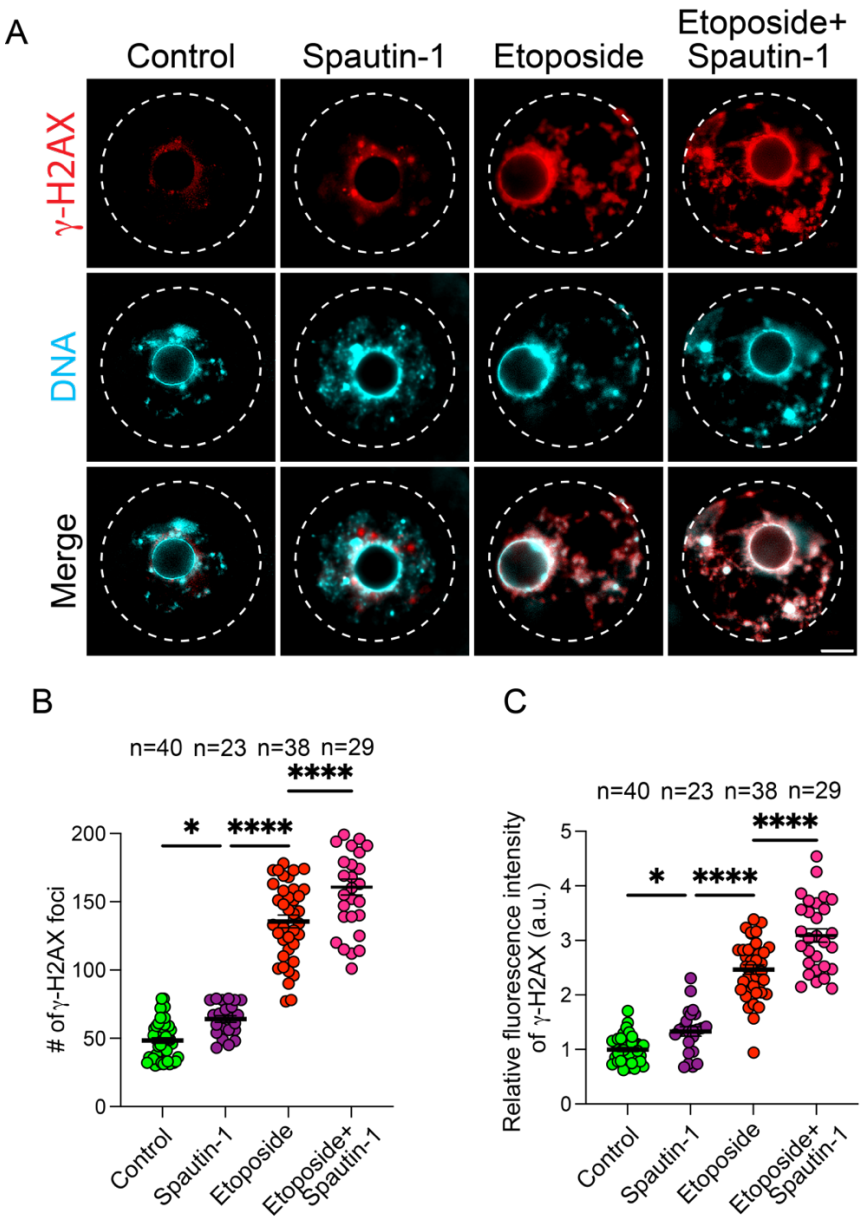

Figure 17: Autophagy inhibition (by spautin-1) decreases γ-H2AX in DNA-damaged oocytes (by etoposide).

(A) Fully grown prophase I-arrested oocytes were incubated in milrinone-containing CZB medium supplemented with the indicated treatments for 3 h. Oocytes were fixed and immune-labeled with γH2AX antibody. Representative images are shown. (B) Quantification of γH2AX foci numbers in “A”. (C) Quantification of γH2AX fluorescence intensity in “A”. Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, $*p < 0.05$, $****p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

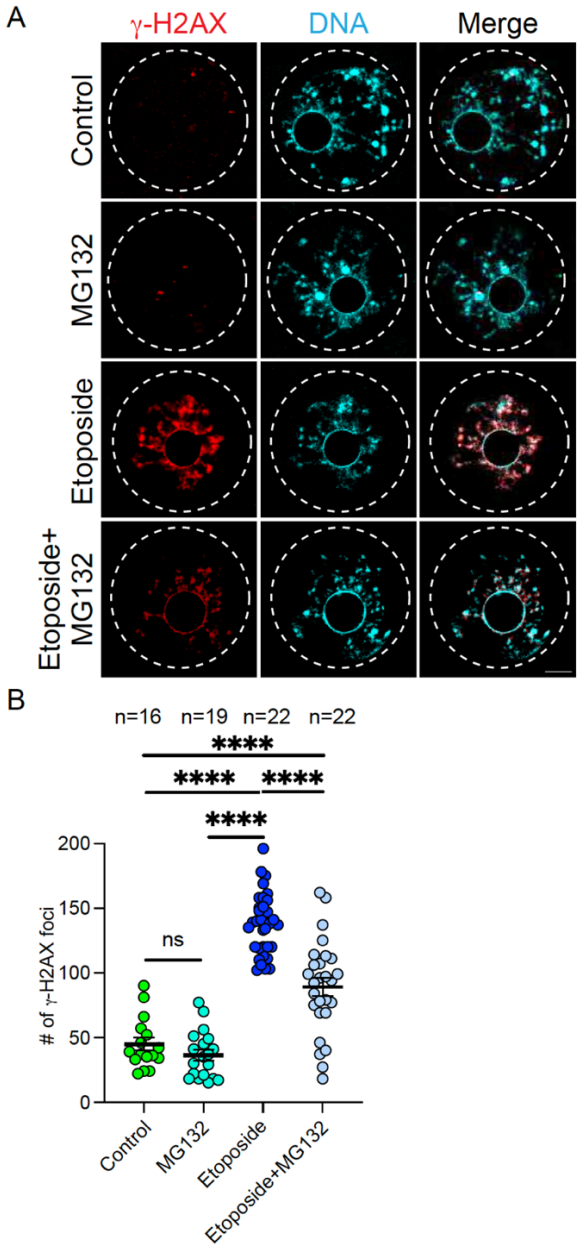

Figure 18: Autophagy induction (by MG132) decreases γ-H2AX in DNA damaged-oocytes (by Etoposide).

(A) Fully grown prophase I-arrested oocytes were incubated in milrinone-containing CZB medium supplemented with the indicated treatments for 3 h. Oocytes were fixed and immune-labeled with γH2AX antibody. DNA was stained by DAPI. Representative images are shown. Scale bar represents 10 μm. (B) Quantification of γH2AX foci numbers in "A". Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, ****$p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

3.4 Autophagy has both DNA damage protective and repair roles in oocytes by promoting RAD51 recruitment to the DNA

To understand how autophagy regulates DNA damage response in mouse oocytes, we first investigated whether autophagy protects against or repairs DNA damage, or both. To answer this question, we designed two separate experiments. To investigate whether autophagy protects against DNA damage, we first stimulated autophagy by treating the oocytes with rapamycin for 2 h followed by oocyte exposure to DNA damage by etoposide for an additional 1 h, in a rapamycin-free medium. Our results reveled that inducing autophagy before DNA damage significantly decreased γH2AX foci numbers and intensity when compared to non-rapamycin-pretreated oocytes (Figure 19), indicating that high levels of autophagy can protect against DNA damage in mouse oocytes. To investigate whether autophagy decreased DNA damage by stimulating DDR, we first induced DNA damage by treating the oocytes with etoposide for 1 h followed by culturing the oocytes in etoposide-free medium with or without rapamycin for additional 2 hours. Again, our results reveled that increasing autophagy after DNA damage induction significantly decreases γH2AX foci numbers and fluorescence pixel intensity when compared to non-rapamycin-treated oocytes (Figure 20). Taken together, our results indicate that autophagy plays a role in both the protection and repair mechanisms against DNA damage in mouse oocytes.

To understand the molecular mechanism of autophagy in DDR, we screened several DNA damage checkpoint and repair proteins in DNA-damaged oocytes incubated with or without rapamycin. Upon inducing DNA damage, the ATM kinase, the master regulator of DNA damage checkpoint, is activated, a necessary step for DDR initiation. When damaged DNA is repaired, ATM is inactivated (Goodarzi et al., 2004; Tang et al., 2019). Therefore, phospho-ATM (p-ATM) is considered a sensitive marker for DNA damage sensing and repair. Based on immunofluorescence pixel intensity measurements, p-ATM was significantly increased in etoposide-treated oocytes (Figure 21), suggesting that DNA damage sensing mechanism is functional in oocytes. As expected, treating the oocytes with rapamycin significantly decreased p-ATM levels in etoposide-treated oocytes (Figure 21), further confirming the rescue effect of autophagy on DNA damage. We then assessed the localization of several DDR proteins using immunocytochemistry. In somatic cells, RAD51 expression is activated in response to DNA damage and plays a critical role in DDR by its recruitment to damaged-DNA foci (Tarsounas et al., 2004; Tashiro et al., 2000). Unlike somatic cells, RAD51 failed to localize to the DNA in etoposide-treated oocytes (Figure 22). Strikingly, autophagy induction rescued the mis-localization of RAD51 in DNA-damaged oocytes (Figure 22). We did not observe a significant difference in the oocyte content or localization of major DDR proteins, BRCA1, BRCA2 and MRE11 (data not shown). These results show that autophagy promotes the recruitment of DDR protein, RAD51, to damaged DNA through a yet to be characterized mechanism.

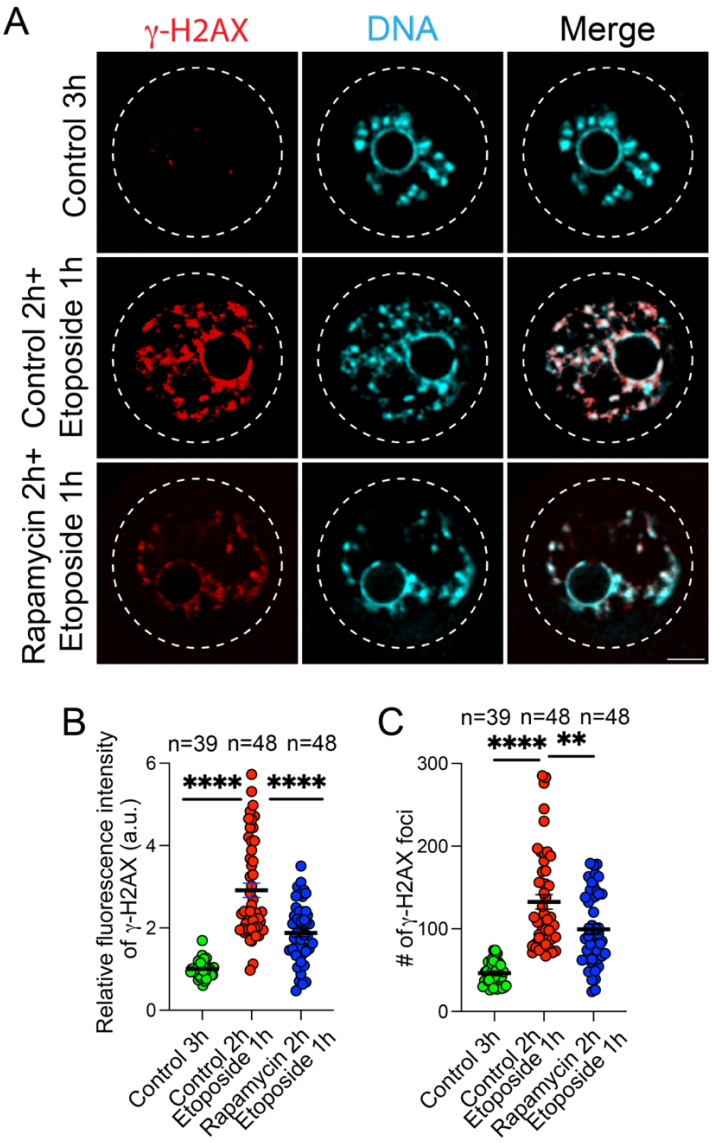

Figure 19: Autophagy plays a role in protection mechanisms against DNA damage in mouse oocytes

(A) Fully grown prophase I oocytes were incubated in milrinone-containing CZB medium supplemented with DMSO (control) or rapamycin for 3 h. After 2 h, a subset of control and rapamycin-treated oocytes were incubated in etoposide-containing medium for 1 h. All oocytes were fixed and immune-labeled with γH2AX antibody. Representative images are shown. (B) Quantification of γH2AX fluorescence intensity in “A”. (C) Quantification of γH2AX foci numbers in “A”. Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, $**p < 0.01$, $****p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

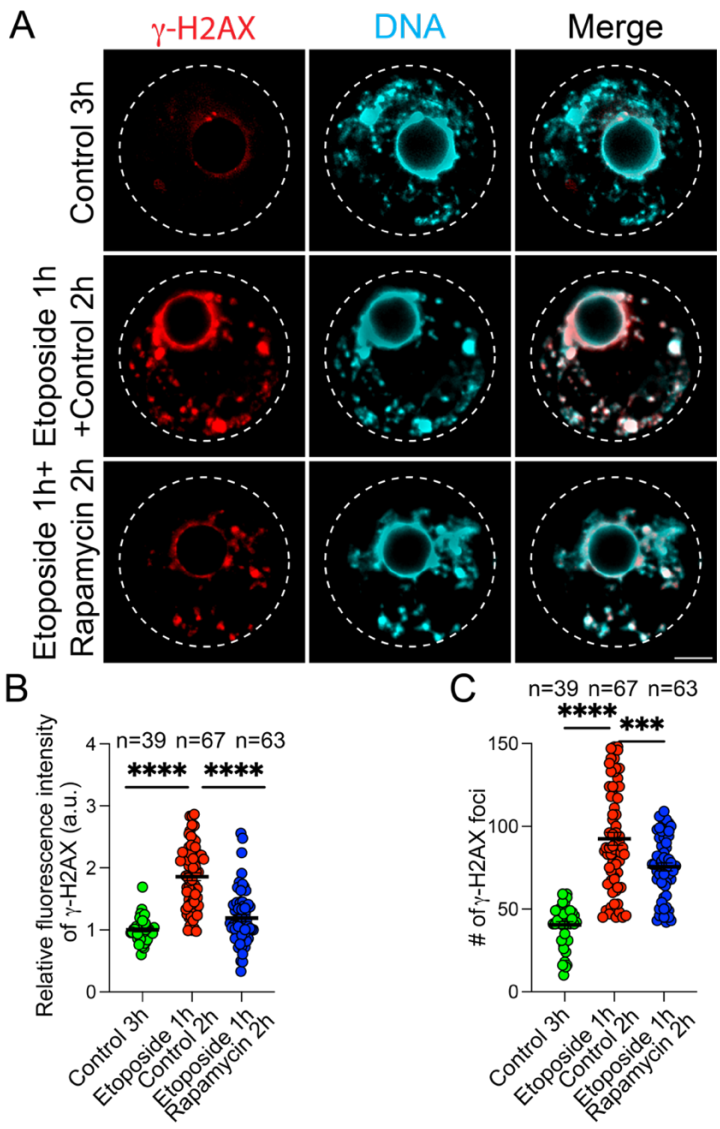

Figure 20: Autophagy plays a role in repair mechanisms against DNA damage in mouse oocytes

(A) Fully grown prophase I oocytes were incubated in milrinone-containing CZB medium supplemented with DMSO (control) for 3 h or etoposide for 1 h. The etoposide-treated oocytes were then washed and cultured in DMSO- or rapamycin-containing medium for additional 2 h. Oocytes were fixed and immune-labeled with γH2AX antibody. Representative images are shown. (B) Quantification of γH2AX fluorescence intensity in “A”. (C) Quantification of γH2AX foci numbers in “A”. Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, ***$p < 0.001$, ****$p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

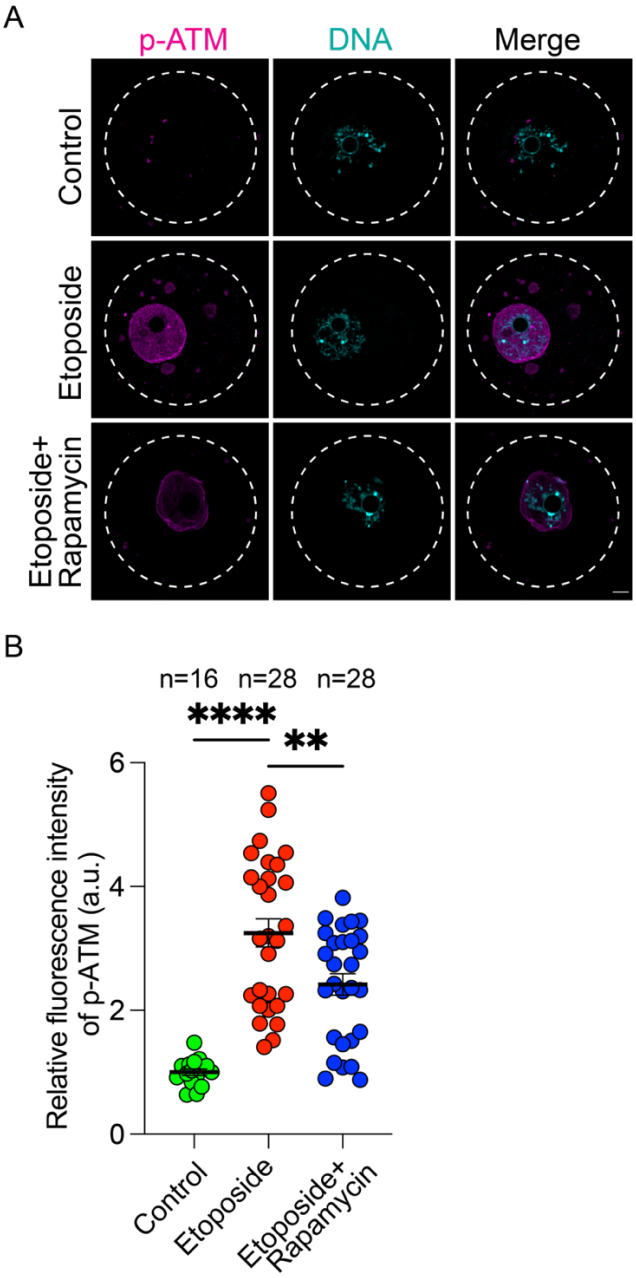

Figure 21: Autophagy induction decreases p-ATM in DNA-damaged oocytes.

(A) Fully grown prophase I-arrested oocytes were incubated in milrinone-containing CZB medium supplemented with the indicated treatments for 3 h. Oocytes were fixed and immune-labeled with p-ATM antibody. DNA was stained by DAPI. Representative images are shown. Scale bar represents 10 μm. (B) Quantification of p-ATM fluorescence intensity in "A". Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, $**p < 0.01$, $****p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

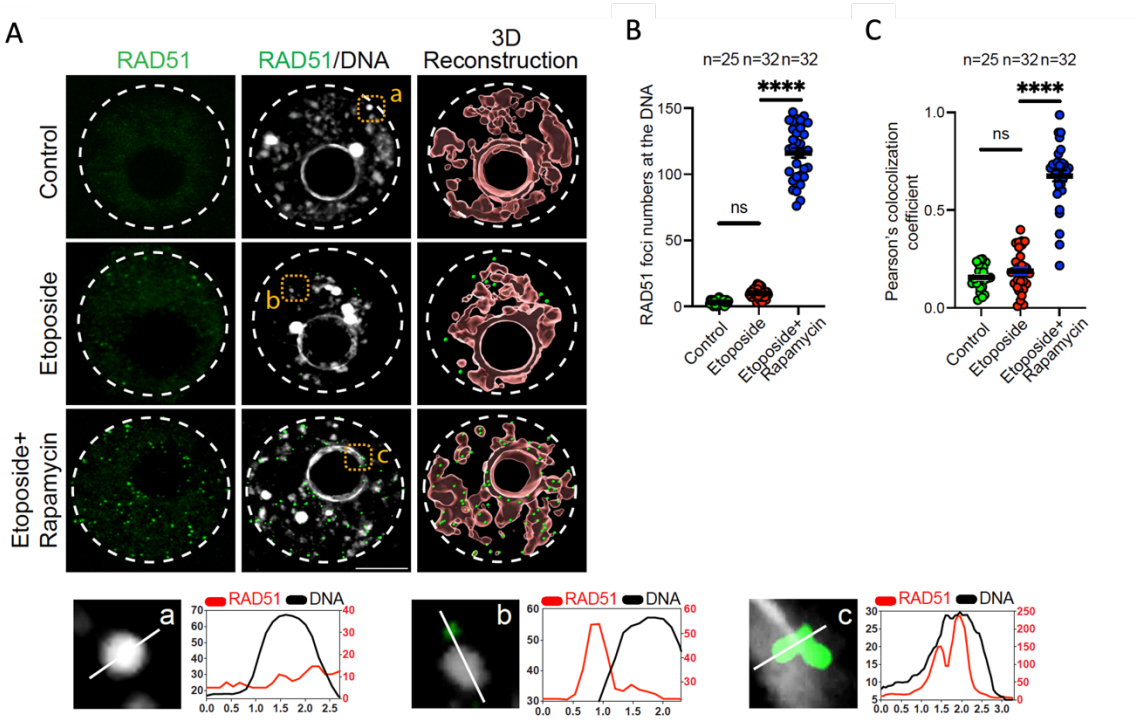

Figure 22: Autophagy induction rescues RAD51 mislocalization in DNA-damaged oocytes

(A) Fully grown prophase I-arrested oocytes were incubated in milrinone-containing CZB medium supplemented with the indicated treatments for 3 h. Oocytes were fixed and immune-labeled with RAD51 antibody. Representative images are shown. Plot profile representative images are shown (a-c). (B) Analysis of RAD51 foci numbers at the DNA in 'A'. (C) Pearson's colocolization coefficient analysis of A. Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, ****p < 0.0001. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

3.5 Autophagy regulates chromatin remodeling in DNA-damaged oocytes

Chromatin remodeling from condensed heterochromatin to open euchromatin is required during DDR to render damaged DNA accessible to DDR proteins (Ataian and Krebs, 2006; Bao, 2011; Citterio et al., 2000; Czaja et al., 2014; Dion and Gasser, 2013; House et al., 2014). In somatic cells, autophagy-deficiency decreased chromatin ubiquitination, leads to closed chromatin structure which, in turn, impedes the access of DDR machinery to damaged DNA (Wang et al., 2016). Considering the compact chromosome phenotype in DNA-damaged oocytes, we hypothesized that the failure of RAD51 to localize to the DNA in DNA-damaged oocytes is due to closed chromatin structure, and that autophagy induction promotes the access of RAD51 and its localization to the DNA by causing the chromatin to open.

To assess chromatin conformation in DNA-damaged oocytes, we employed DNase sensitivity assay (Tsompana and Buck, 2014; Weintraub and Groudine, 1976). This assay is based on the ability of DNase I enzyme to cut open DNA/chromatin faster than closed DNA/chromatin. Therefore, when the chromatin is open, the ratio between DNA fragmentation in DNase treatment vs. non-DNase treatment (DNase/non-DNase ratio) is higher than that of closed chromatin. As anticipated, culturing non-DNase-treated oocytes with etoposide increased DNA fragmentation/damage compared to control oocytes, whereas autophagy induction reduced DNA fragmentation in etoposide-treated oocytes (Figure 23A-C). Etoposide-treated oocytes showed a ~4-fold decrease in tail moment DNase/non-DNase ratio and ~2.5-fold decrease of tail length

DNase/non-DNase ratio than control oocytes (Figure 23D, E), indicating that chromatin is indeed relatively closed in DNA-damaged oocytes. Importantly, induction of autophagy in DNA-damaged oocytes by rapamycin caused the chromatin to open as shown by the significant increase of tail moment DNase/non-DNase ratio (~3-fold increase) and DNA tail length DNase/non-DNase ratio (~2-fold increase), compared to etoposide-treated oocytes (Figure 23D, E). These findings suggest that autophagy regulates DDR by modulating chromatin conformation in mouse oocytes.

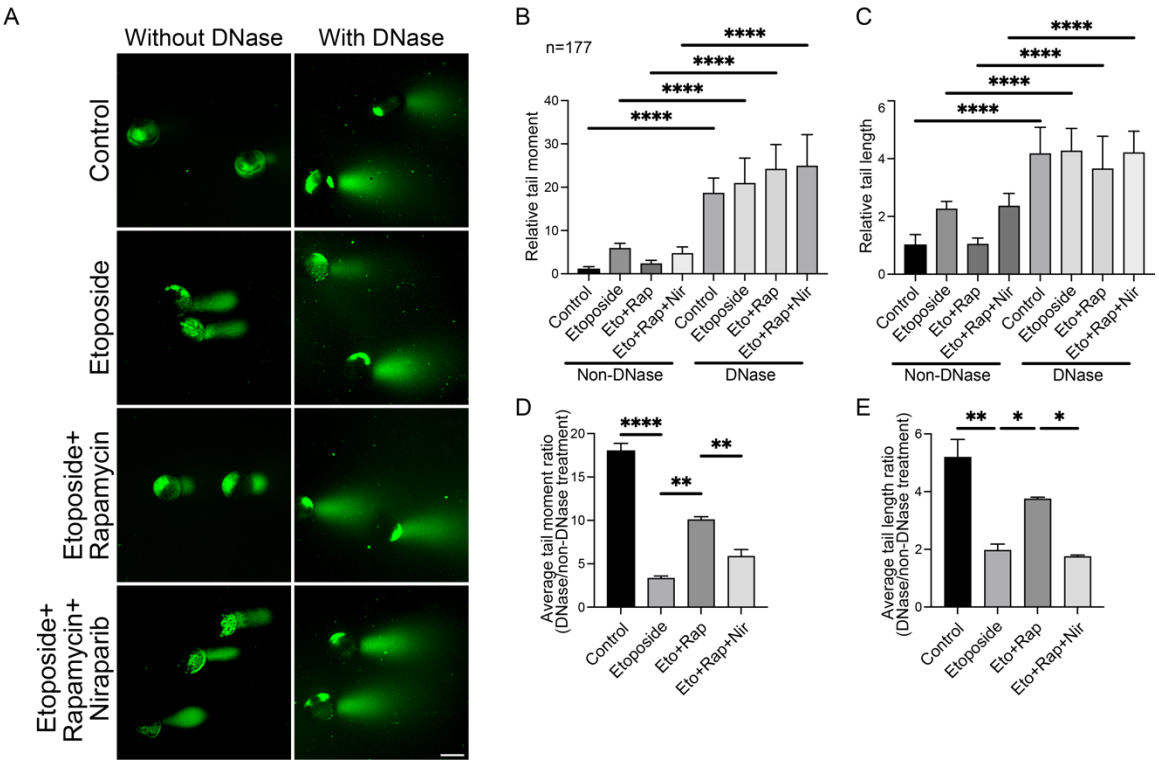

Figure 23: Autophagy induction rescues closed chromatin architecture in DNA-damaged oocytes.

(A) Fully grown prophase I oocytes were incubated in milrinone-containing CZB medium supplemented with indicated treatments for 3 h. The DNase-treated and non-DNase-treated oocytes were subjected to the alkaline comet assay. (B) Quantification of DNA tail moment in “A”. (C) Quantification of DNA tail length in “A”. Data were presented relative to control group. (D) Quantification of average DNA tail moment ratio (DNase/non-DNase treatment) in “B”. (E) Quantification of average DNA tail length ratio (DNase/non-DNase treatment) in “C”. Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

3.6 PARP1 activation is a downstream pathway of autophagy in mouse oocytes

In somatic cells, Poly [ADP-ribose] Polymerase 1 (PARP-1) is activated in response to DNA damage, a necessary step to synthesize a structurally complex polymer composed of ADP-ribose facilitating chromatin remodeling and the recruitment of DDR factors (Ray Chaudhuri and Nussenzweig, 2017; Sinha et al., 2021). Indeed, treating granulosa (somatic) cells with etoposide increased PARP-1 expression, compared to DMSO-treated cells (Figure 12). Again, in contrast to somatic cells, mouse oocytes failed to stimulate PARP-1 (Figure 24, 25) in response to DNA damage, a phenomenon similar to autophagy reduction in DNA-damaged oocytes. Autophagy is necessary for the maintenance of NAD(H) pool, which is necessary for PARP-1 function and activity (Sedlackova et al., 2020). To test whether the failure of PARP-1 activation in DNA-damaged oocytes is related to autophagy reduction, we assessed PARP-1 after autophagy induction in DNA-damaged oocytes. Interestingly, autophagy induction increased PARP-1 levels in DNA-damaged oocytes compared to those in etoposide-treated oocytes by using Western blot analysis (Figure 24) or immunocytochemistry (Figure 25). We then examined whether autophagy induction rescued DDR in DNA-damaged oocytes by stimulating PARP-1. Niraparib is a highly selective PARP-1 and PARP-2 inhibitor (LaFargue et al., 2019). In our mouse oocyte system, we found that niraparib at 10 and 20 μM concentrations can effectively inhibit PARP-1, compared to control oocytes (Figure 26). Accordingly, 10 μM concentration of niraparib was selected for the ensuing experiments. Inhibition of

PARP-1 by using niraparib eliminated autophagy-mediated open chromatin configuration in DNA-damaged oocytes (Figure 23). Importantly, PARP-1 inhibition abolished the rescue effect of autophagy on DDR in DNA-damaged oocytes (rapamycin+etoposide), as evidenced by increased γH2AX to a level comparable to that in etoposide-treated oocytes (Figure 27). Taken together, these data suggest that PARP-1 activation occurs downstream of autophagy in mouse oocytes.

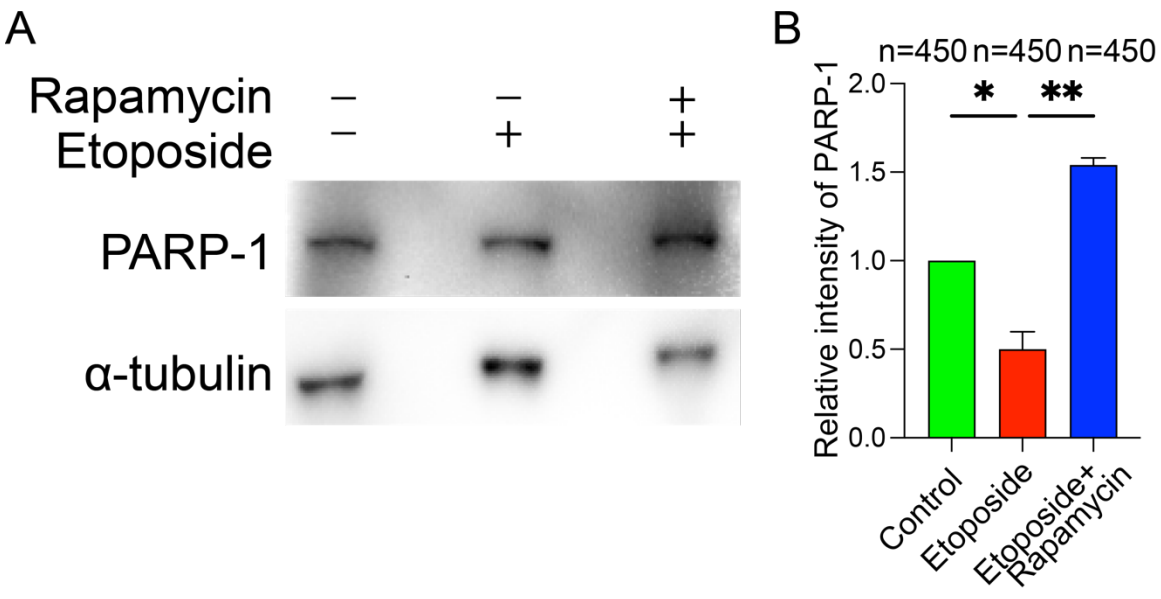

Figure 24: Autophagy regulate PARP-1 in oocytes (by western blot).

(A) Fully grown prophase I oocytes were incubated with the indicated treatments for 3 h in milrinone-containing CZB medium followed by Western blot analysis. Representative images are shown. (B) Quantification of PARP-1 in “A”. Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, $*p < 0.05$, $**p < 0.01$. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

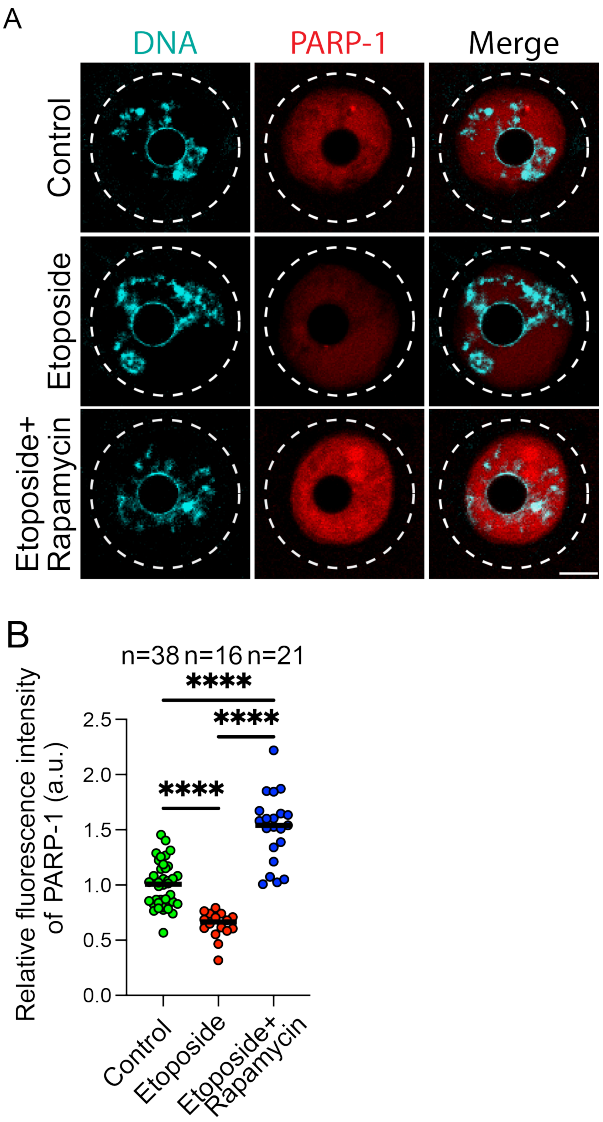

Figure 25: Autophagy regulate PARP-1 in oocytes (by immunostaining).

(A) Fully grown prophase I oocytes were incubated with the indicated treatments for 3 h in milrinone-containing CZB medium followed by immunostaining analysis. Representative images are shown. (B) Quantification of PARP-1 in “A”. Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, ****p < 0.0001. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

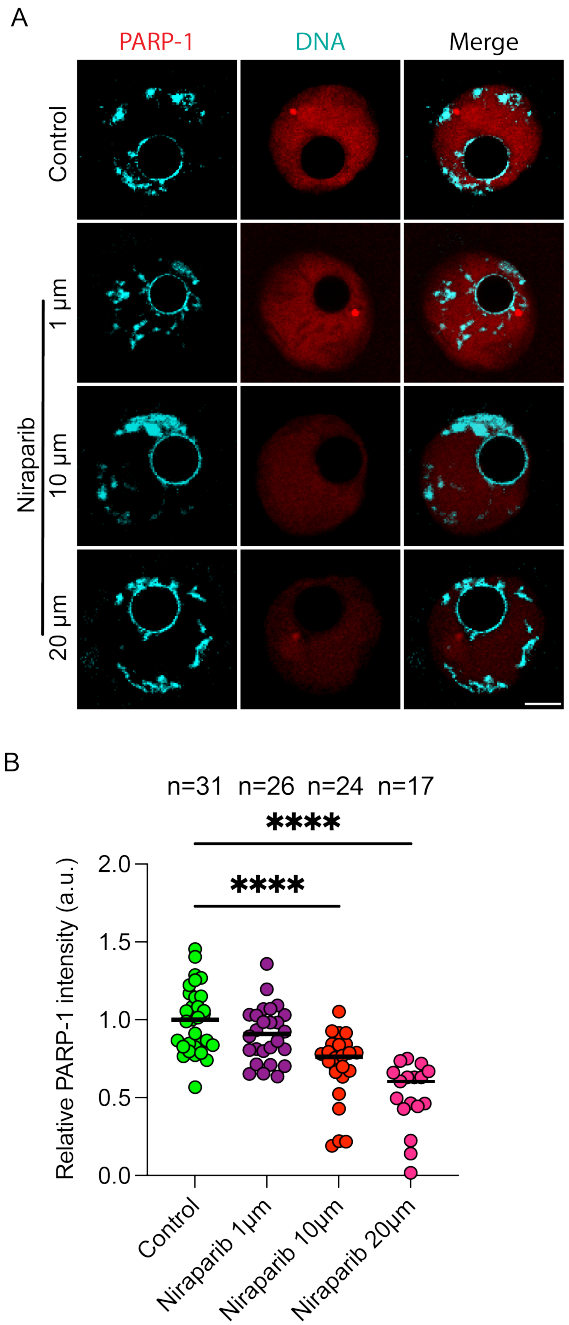

Figure 26: Effects of Niraparib on PARP-1 in oocytes

(A) Fully grown prophase I-arrested oocytes were incubated in milrinone-containing CZB medium supplemented with Niraparib at the indicated concentrations for 3 h. Oocytes were fixed and immune-labeled with PARP-1 antibody. DNA was stained by DAPI. Representative images are shown. Scale bar represents 10 µm. (B) Quantification of PARP-1 fluorescence intensity in "A". Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, ****$p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

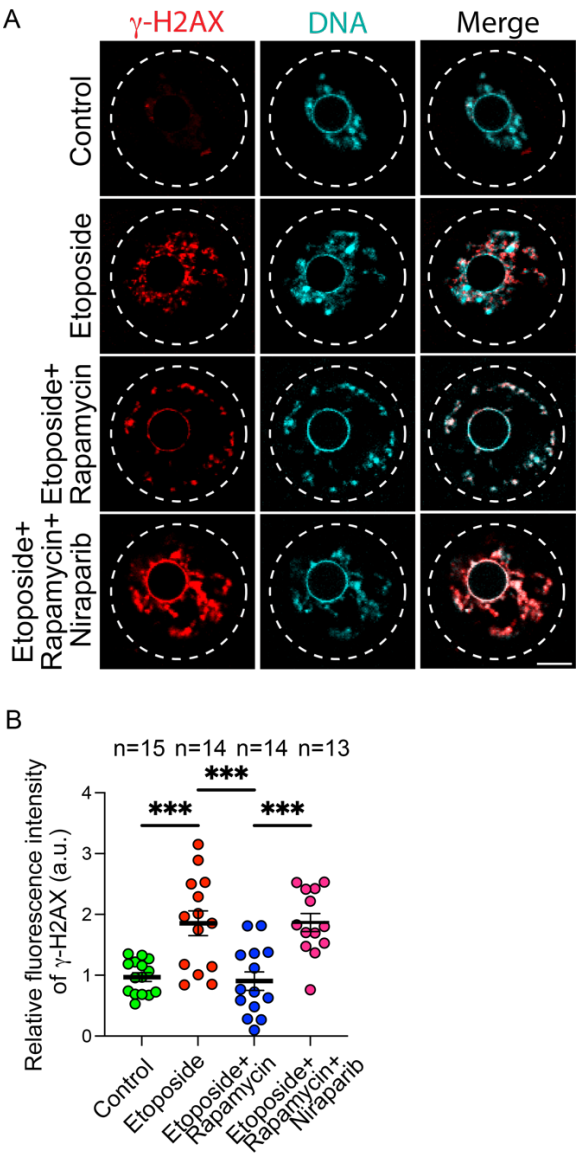

Figure 27: PARP1 inhibition abolished the rescue effect of autophagy induction on DNA damage.

(A) Fully grown prophase I oocytes were incubated in milrinone-containing CZB medium supplemented with the indicated treatments for 3 h. Oocytes were fixed and immune-labeled with γH2AX antibody. DNA was stained by DAPI. Representative images are shown. (B) Quantification of γH2AX fluorescence intensity in “A”. Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, ***$p < 0.001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above the graph.

3.7 Autophagy induction mitigates the consequences of DNA damage in mouse oocytes

The main consequences of DNA damage in mouse oocytes are decreased PBE (Figure 4A), delayed meiotic progression (Figure 4B), altered chromosome morphology (Figures 7A, B), increased chromosome fragmentation (Figures 7A, C) and the development of aneuploid gametes (Figure 5). We asked whether autophagy induction can rescue the aforementioned phenotypes. Expectedly, treating prophase I oocytes with etoposide decreased the percentage of PBE (Figure 4A), delayed oocyte meiotic progression (Figure 4B) and increased the incidence of compact chromosome phenotype (Figure 28A, B), chromosome fragmentation (Figure 28A, C) and aneuploidy (Figure 29). Autophagy induction by rapamycin greatly rescued the aforementioned phenotypes: (1) increased the percentage of PBE (Figure 4A), (2) rescued the delay in meiotic progression (Figure 4B), decreased the incidence of compact chromosome phenotype (Figure 28A, B), decreased chromosome fragmentation (Figure 28A, C) and, importantly, decreased the incidence of aneuploidy (Figure 29) in DNA-damaged oocytes. Thus, autophagy induction provides a promising approach to overcome the consequences of DNA damage in mammalian oocytes, whose efficiency to respond to DNA damage is not robust.

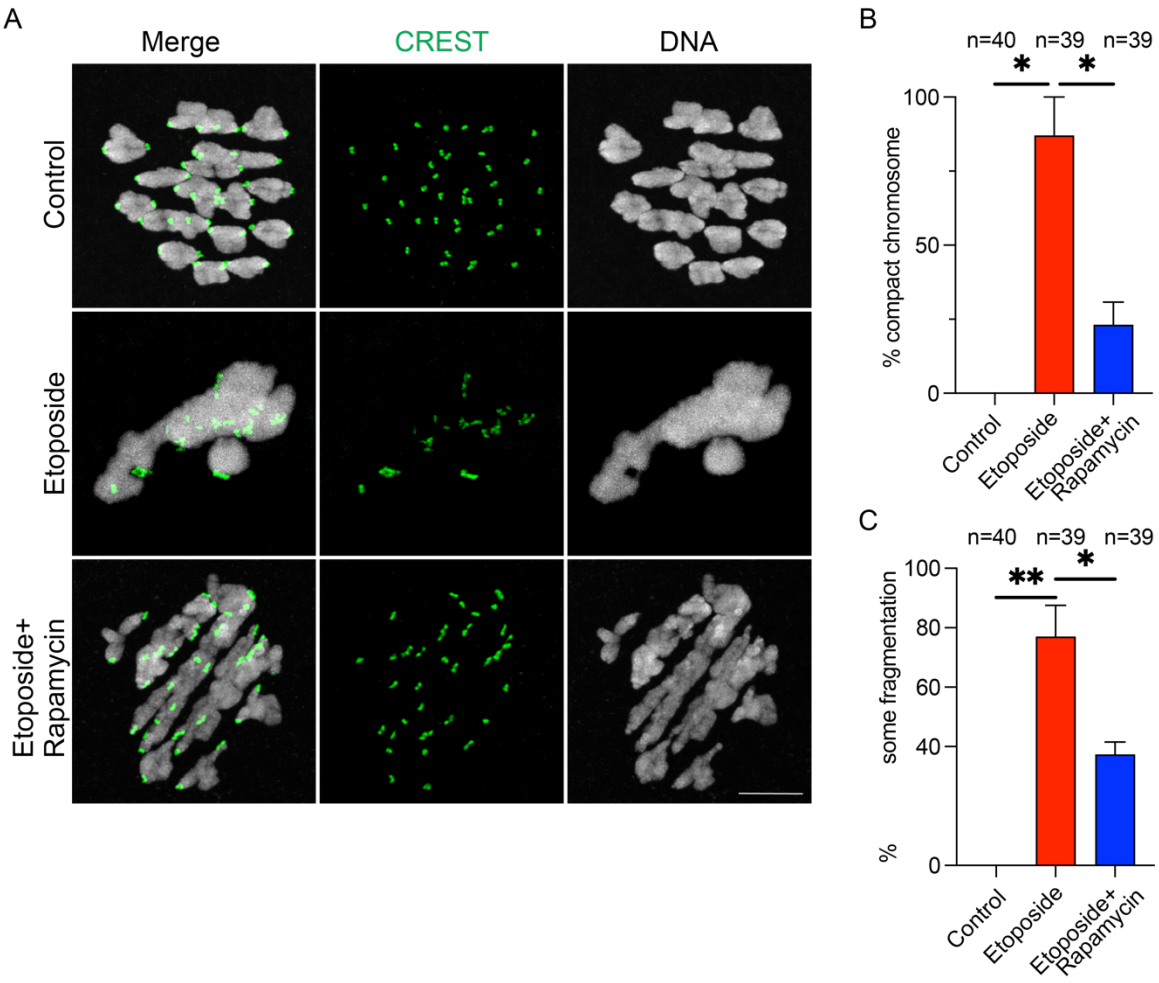

Figure 28: Autophagy induction rescues chromosome compactness and fragmentation phenotypes in DNA-damaged oocytes.

(A) Fully grown prophase I oocytes were incubated with indicated treatments for 3 h in milrinone-containing CZB medium followed by washing and in vitro maturation for 7 h (metaphase I) in CZB medium. Metaphase I oocytes were fixed and immune-labeled with CREST antibody to label kinetochores. Representative images are shown. (B) Quantification of compact chromosome phenotype in “A”. (C) Quantification of chromosome fragmentation phenotype in “A”. Scale bars represent 10 μm. DNA was stained with DAPI (blue). Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, $*p < 0.05$, $**p < 0.01$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

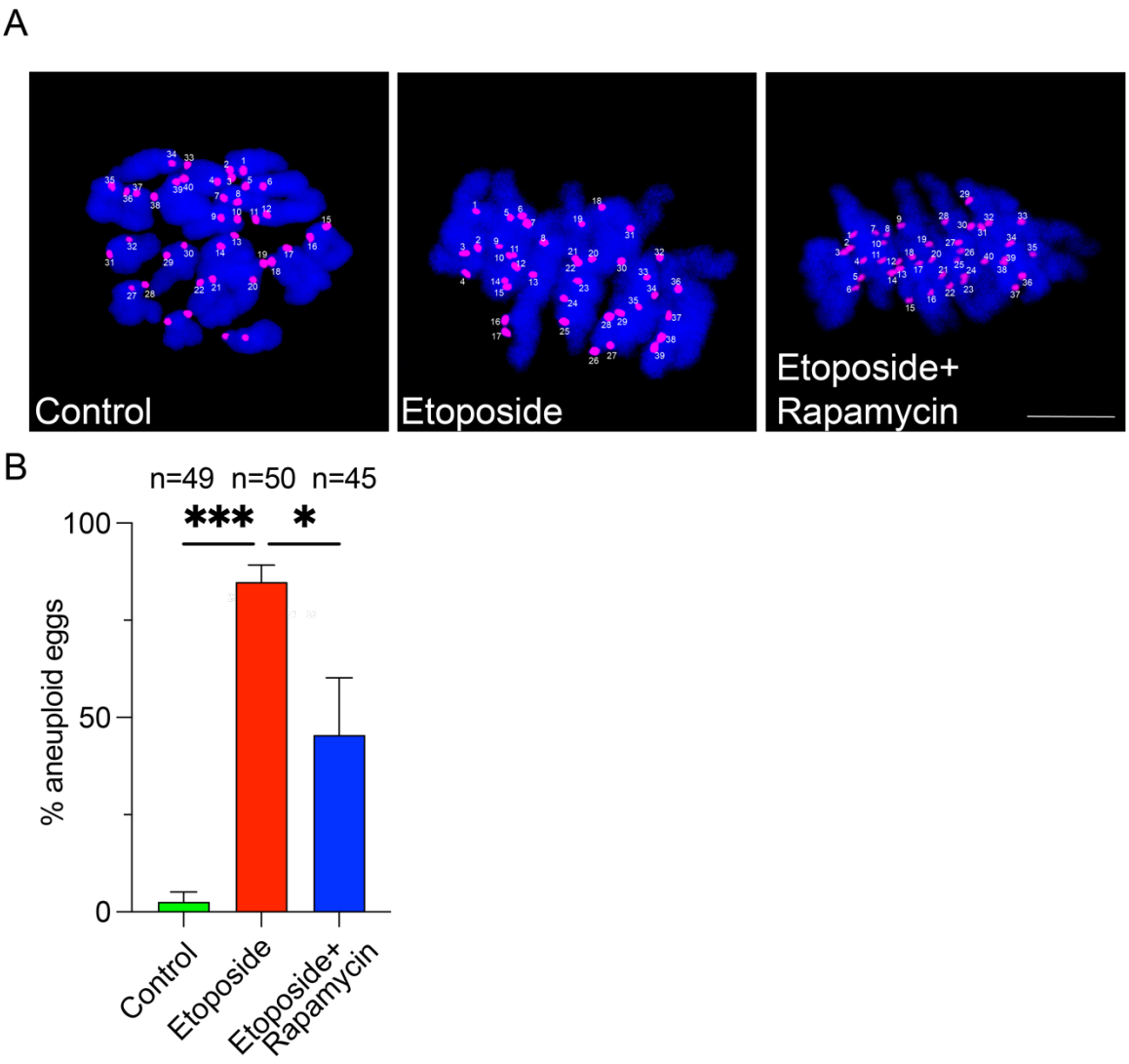

Figure 29: Autophagy induction in DNA-damaged oocytes partially rescued the incidence of aneuploidy.

(A) Fully grown prophase I oocytes were incubated with indicated treatments for 3 h in milrinone-containing CZB medium followed by washing and in vitro maturation for 14 h (metaphase II). Oocytes were treated with monastrol for 2 h, fixed and immune-labeled with CREST antibody to label kinetochores. Oocytes were scored either as euploid (containing 40 kinetochores) or aneuploid (containing ± 40 kinetochores). Representative images are shown. (B) Quantification of aneuploidy percentage in "A". Scale bars represent 10 μm. DNA was stained with DAPI (blue). Data are expressed as mean ± SEM. One-way ANOVA was used to analyze the data. Values with asterisks differ significantly, $*p < 0.05$, $***p < 0.001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

3.8 Reduced autophagy is the likely cause, at least in part, of weakened DNA damage response in oocytes from reproductively aged mice

Although DNA damage response is not robust in mammalian oocytes, including humans and mice, it is further weakened in oocytes from females of advanced reproductive age due to poorly understood reasons (Marangos and Carroll, 2012; Rémillard-Labrosse et al., 2020; Titus et al., 2013). Autophagy reduction is a hallmark of aging as it decreases gradually with the advancement of age, leading to the development of a wide variety of disorders including neurodegenerative and metabolic diseases, and cancer (Barbosa et al., 2018). Our results (discussed above) indicate that autophagy plays an important role in DDR of mouse oocytes. Taken together, we hypothesized that reduced autophagy contributes to weakened DDR in oocytes from aged mice. To this end, we assessed the autophagic activity by using Cyto-ID autophagy detection kit in prophase I oocytes collected from young (6-8-week-old) and aged mice (12-14-month-old). Compared to young oocytes, the autophagic activity was significantly decreased (~3-fold, $p<0.001$) in oocytes collected from aged mice vs. young mice (Figure 30), raising the question whether autophagy induction can rescue DNA damage response in aged mouse oocytes. Consistent with previous reports (Marangos et al., 2015; Titus et al., 2013), oocytes from aged mice exhibited higher levels of DNA damage (increased γH2AX foci numbers and intensity) compared to those in young mouse oocytes (Figure 31). Importantly, inducing autophagy in oocytes from aged mice significantly decreased DNA damage (reduced γH2AX foci numbers and intensity) to a level comparable to that of

young mouse oocytes (Figure 31). Taken together, our results suggest that reduced autophagy is the likely cause, at least in part, of weakened DNA damage response in oocytes from aged mice. Thus, autophagy induction represents a promising approach to ameliorating DNA damage-induced alterations in oocytes from aged females.

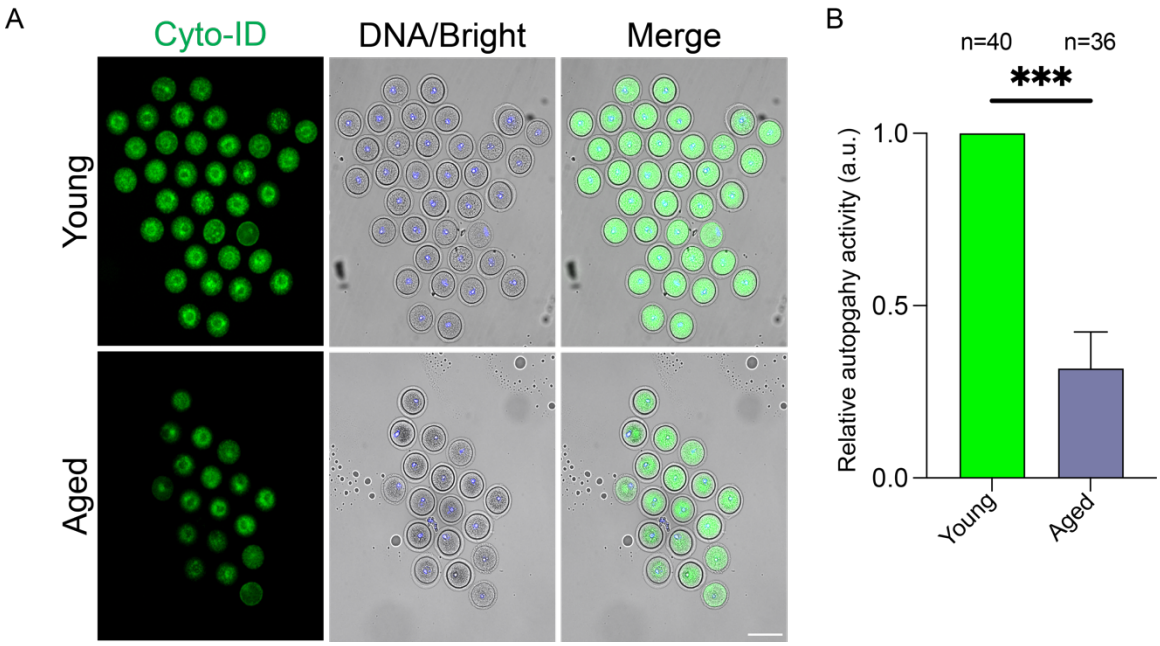

Figure 30: Autophagy activity is decreased in oocytes from aged mice.

(A) Fully grown prophase I oocytes from young mice (6-8-week-old) and aged mice (12-14-month-old) were assessed for autophagic activity by using Cyto-ID autophagy detection kit. Representative images are shown. Scale bar represents 100 µm. (B) Quantification of autophagy activity in “A”. Data are expressed as mean ± SEM. Student-t-test were used to analyze the data. Values with asterisks differ significantly, ***$p < 0.001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

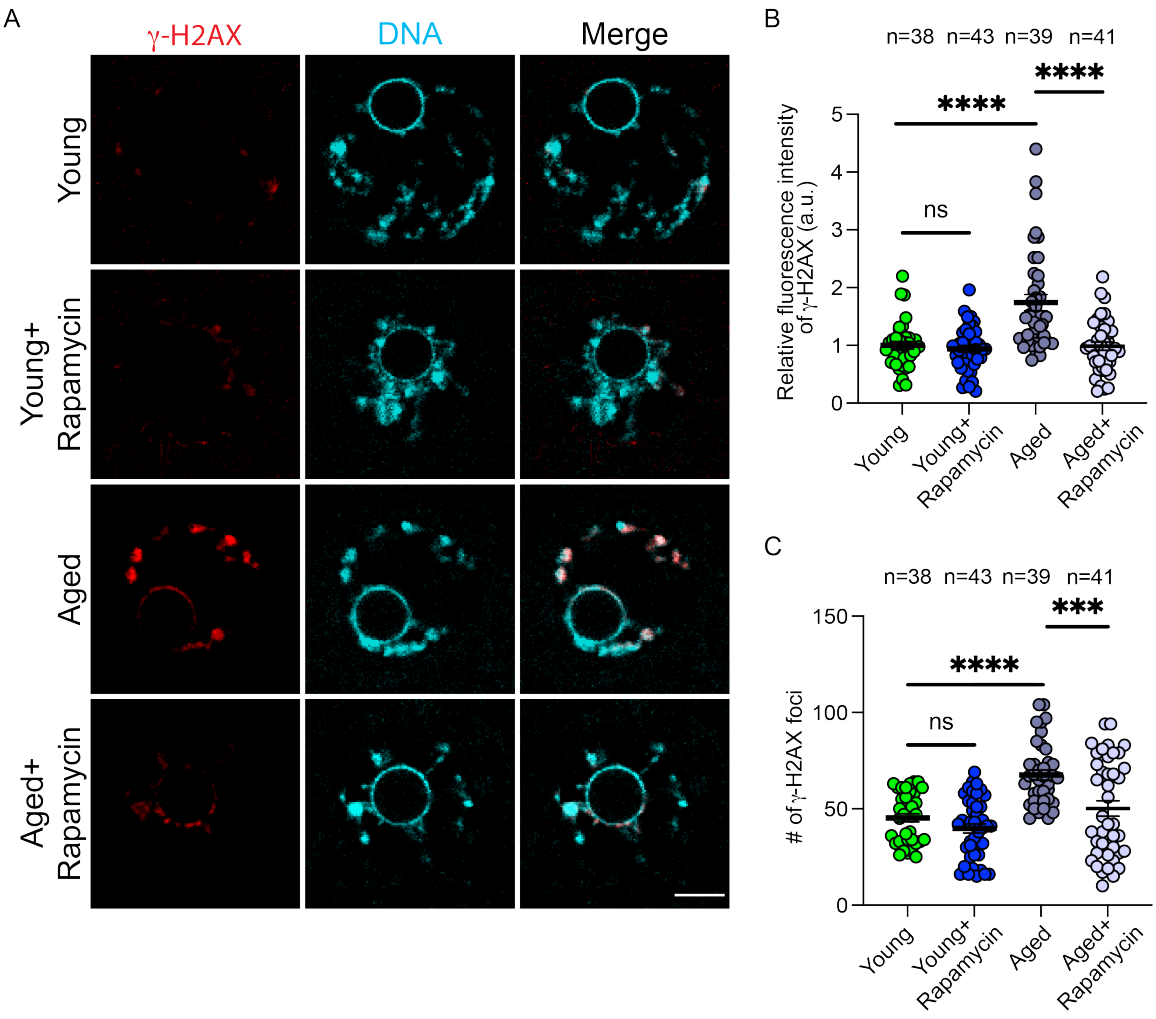

Figure 31: Autophagy induction rescues DNA damage in oocytes from aged mice.

(A) Fully grown prophase I oocytes from young mice (6-8-week-old) and aged mice (12-14-month-old) were incubated for 3 h in CZB medium supplemented with DMSO (control) or rapamycin. Milrinone was added to the medium to prevent meiotic resumption. Oocytes were fixed and immune-labeled with γH2AX antibody. DNA was stained by DAPI. Representative images are shown. Scale bar represents 10 μm. (B) Quantification of γH2AX fluorescence intensity in "A". (C) Quantification of γH2AX foci numbers in "A". Data are expressed as mean ± SEM. One-way ANOVA were used to analyze the data. Values with asterisks differ significantly, ***$p < 0.001$, ****$p < 0.0001$. The total number of analyzed oocytes (from at least three independent replicates) is specified above each graph.

4. Discussion

Aneuploidy is the leading genetic cause of miscarriage in women. Why aneuploidy occurs frequently in mammalian oocytes and correlates with maternal age is largely unknown. Here we reveal that DNA damage is a major cause of aneuploidy in murine oocytes. In somatic cells, autophagy is activated and plays an important role in DDR. Our data show that, unlike somatic cells, oocytes fail to activate autophagy in response to DNA damage. The lack of autophagy activation in oocytes is the cause, at least in part, of reduced PARP-1 activation and altered chromatin conformation in DNA-damaged oocytes, leading to the failure of RAD51 DDR protein recruitment to DNA-damaged sites. Induction of autophagy rescues altered chromatin conformation, DDR protein localization, chromosome fragmentation and aneuploidy in DNA-damaged oocytes (Figure 32). Although our data reveal that autophagy promotes DDR function by regulating PARP-1, we do not exclude the possibility of additional PARP-1-independent pathways. Importantly, we also show that autophagy is decreased in oocytes from reproductively aged mice (compared to oocytes from young mice) which is the cause, at least partially, of the increased severity of DNA damage in maternally aged oocytes.

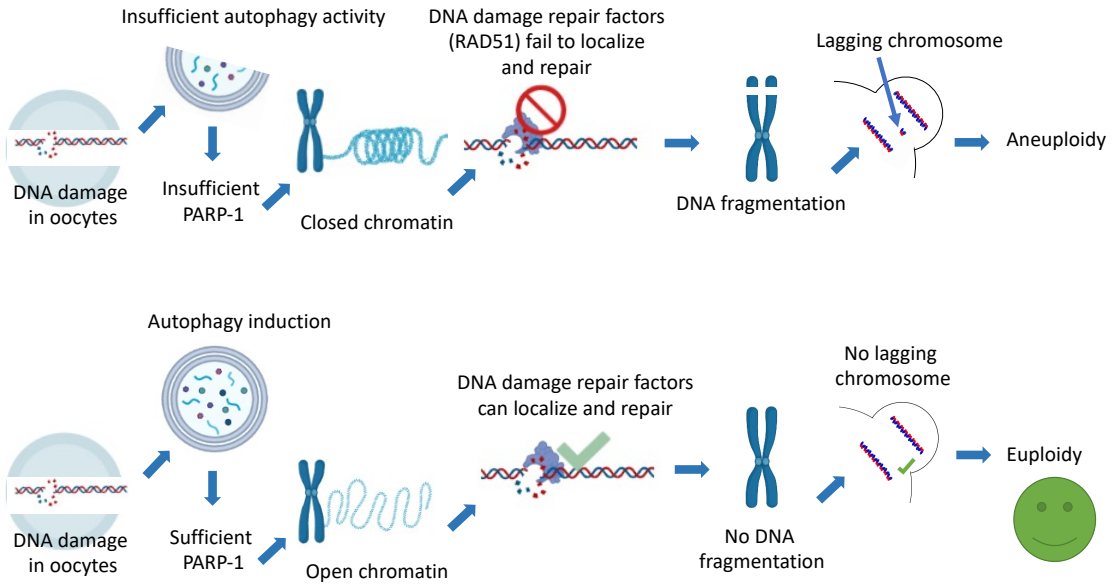

Figure 32: Schematic model summarizing the impact of DNA damage on oocyte meiosis I and the role of autophagy in DNA damage repair.

Exposure of somatic cells to mild DNA damage is sufficient to induce a cell cycle arrest (by activating G2/M checkpoint), providing sufficient time for repairing DNA damage (Marangos and Carroll, 2012). If DNA damage is not repaired, the cell activates the p53-dependent apoptotic pathway. For not fully understood reasons, DNA damage response is not robust and p53 seems indispensable in fully grown oocytes (Suh et al., 2006). Only the exposure to severe DNA damage, but not to mild or moderate DNA damage, can prevent meiotic resumption (equivalent to G2/M transition in somatic cells) in fully grown oocytes (Marangos and Carroll, 2012), increasing the risk of developing aneuploid gametes. Hence, fully grown oocytes employ an alternative mechanism to hinder meiotic progression following DNA damage by activating another surveillance mechanism, the spindle assembly checkpoint (SAC), leading to an arrest at Met I stage (Fig. 1E and Collins et al., 2015; Marangos et al., 2015). This is surprising because the SAC is known to be weak in oocytes, compared to somatic cells (Gui and Homer, 2012; Kolano et al., 2012; Kyogoku and Kitajima, 2017). Therefore, even though the SAC is activated in response to DNA damage, a considerable percentage of mouse oocytes can progress through meiosis I and reach Met II stage. Indeed, similar to a previous report (Leem et al., 2019), we find that ~40% of etoposide-treated oocytes extruded the PB and reached Met II. Similarly, human oocytes can progress through MI while harboring damaged DNA (Rémillard-Labrosse et al., 2020), suggesting that not only DNA damage-induced cell cycle arrest mechanism is weak, but DDR mechanism is also inefficient. We find that autophagy induction rescues chromatin conformation,

allowing RAD51 (a key DDR factor) localization to the DNA and reduces γH2AX level in DNA-damaged oocytes independently of inducing a cell cycle arrest. By contrast, autophagy induction increases the percentage and accelerates the timing of PBE in DNA-damaged oocytes. Thus, reduced autophagy contributes to the weakened DDR mechanism in DNA-damaged oocytes, but not to inefficient cell cycle arrest initiation.

Quiescent (nongrowing) and growing prophase I oocytes (from prepubertal mice) can efficiently repair severe DNA damage in apoptosis-inhibited mice by activating ATM, phosphorylating histone H2AX, and translocated RAD51 to the DNA-damaged sites (Stringer et al., 2020). This contrasts with fully grown oocytes. Our data reveal that although fully grown oocytes (from sexually mature mice) can activate ATM and phosphorylate histone H2AX, they are inefficient in recruiting RAD51 to the DNA and to repair DNA damage, even after spending more than 16 h arrested at prophase I (recovery after DNA damage exposure). It is noteworthy that oocytes can efficiently repair damaged DNA at the early stages of development, but not at later stages, suggesting the contribution of age-related decline in DDR machinery. Consistent with the observation that autophagy level is low in unfertilized metaphase II oocytes and is only activated after fertilization (Tsukamoto et al., 2008), we find that, unlike somatic cells, fully grown oocytes are inefficient to stimulate autophagy in response to DNA damage. Because oocytes, around the time of birth, undergo a lengthy arrest (up to decades in women) at the dictyate stage of meiotic prophase I before being

able to resume meiosis and because autophagy reduction is a hallmark of aging, our data provide an insight into age-related autophagy reduction as an explanation for why DDR is not robust in fully grown mammalian oocytes.

Although DNA damage is a fundamental problem for life and its underlying mechanism has been thoroughly investigated in somatic cells, its consequences on oocyte meiosis remain poorly understood. Our data provide evidence that DNA damage is a cause of aneuploidy in oocytes. We reveal that inefficient autophagy activation is the likely cause of weakened DNA damage response in oocytes, specifically those from females of advanced reproductive age. We also show that autophagy induction in DNA-damaged oocytes rescues DNA damage response in both young and maternally aged oocytes. This is particularly important because female gametes, specifically those from women with advanced reproductive age, are notoriously prone to reduced quality, including aneuploidy. Indeed, aneuploidy is at least 10 times higher in oocytes than that in spermatozoa and at least 20 times higher in oocytes from older (40s) vs. younger (early 20s) women (Gruhn et al., 2019; Hassold and Hunt, 2001). At least two major causes of aneuploidy in maternally aged oocytes have been demonstrated: loss of centromere cohesion and defective kinetochore-microtubule attachments (Chiang et al., 2010; Duncan et al., 2012; Shomper et al., 2014). Although much work has aimed to improve oocyte quality of women with maternally advanced age, success has been limited, suggesting the presence of additional unknown contributing factors. Our data shed new light on autophagy deficiency as another

contributing factor to increased aneuploidy in mammalian oocytes, including in those from females with advanced maternal age. Thus, autophagy induction provides a promising approach to overcome the consequences of DNA damage in mammalian oocytes, whose efficiency to respond to DNA damage is not robust.

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