GENETICALLY DISSECTING THE MEIOTIC CHECKPOINT ACTIVE DURING PROPHASE I IN FEMALE MICE

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GENETICALLY DISSECTING THE PROPHASE I MEIOTIC CHECKPOINT IN FEMALE MICE

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Females have a non-renewable number of gametes at birth. These oocytes are extremely sensitive to environmental factors that generate DNA damage. Oocyte death due to DNA damage can result in infertility and ovarian failure. In contrast to postnatal oocytes, at earlier stages of gametogenesis these cells withstand hundreds of developmentally programmed DNA breaks (DSBs). During the first meiotic division these DSBs promote synapsis (homologous chromosomes pairing), and recombination, which are both essential for sexual reproduction and environmental fitness. However, DSB repair and synapsis need to occur in a timely manner, or the quality of the gametes becomes compromised.

The mechanisms that guarantee oocyte quality were hypothesized to operate through two independent pathways: one that surveys DNA integrity, and the other synapsis. However, I present experimental evidence that oocytes defective for either DNA repair or synapsis are eliminated by the same DNA damage response. Furthermore, through the detailed analysis of DNA repair dynamics, I provide evidence that the protein HORMAD2, which localizes to unsynapsed chromosomes, regulates DSB-repair. I hypothesize

that HORMAD2 interferes with repair by preventing broken DNA from using the sister chromatid as a repair template. This “block to sister-chromatid repair” (BSCR) assures that the homologous chromosome is the substrate of choice. Whereas BSCR guarantees homologous recombination, it also prevents unsynapsed chromosomes from fixing DSBs. Thus, failure to synapse will result in persistent DSBs. Since DNA damage causes oocyte death postnatally, unsynapsed chromosome will trigger the DNA damage checkpoint.

Through the understanding of this checkpoint, I was able to test if the transient inhibition of the DNA damage checkpoint protein (CHK2) prevents oocyte death. My finding that oocyte death was prevented, and fertility was preserved, provides evidence that chemically protecting oocyte from DNA damaging agents is a viable clinical approach. This result will hopefully translate into a treatment to delay ovarian failure. Taken together these results have implications on our current understanding of the prophase I checkpoint.

TEACHING AS RESEARCH

My interest in improving teaching strategies led me to research the qualitative outcome of using a novel teaching tool during the laboratory section of a histology course. I tested an interactive response system (IRS) as formative assessment tool. I found that IRS results in a positive experience, however my study was not able to detect quantitative difference on students’ grades was detected.

BIOGRAPHICAL SKETCH

Vera Rinaldi was born on August 13th, 1979, in Brasília- Brazil. She grew up in Brasília, where her parents nurtured her fascination towards the unknown. When she was 17 years old, she left her parents house to study at a renowned Brazilian University in São Paulo state – UNICAMP. Initially she focused her studies to applied mathematics. However, in 2001 she changed her major to Biological Sciences and Education. In Brazil there are no honors for academic achievements, nevertheless she was one of the top three students of her class.

Her first experience doing a research project was helping a PhD student by setting up cameras and a hideout post in the middle of the Mata-Atlântica rain forest. While assisting the research about nocturnal pollination of plants by bats, she started studying anatomy of the conductive system of endemic Mata-Atlântica plants. During this period she learned many histological techniques and realized she enjoyed mentoring other students.

During her junior year, she was awarded an undergraduate research fellowship to work at the Brazilian Synchrotron Light Laboratory (LNLS) – a highly competitive program - to study protein crystallography. There she worked with human oncoproteins, proteins involved in nucleic acid metabolism, and other proteins from *Xylella fastidiosa*. *Xylella* sp. is a bacterial parasite that impairs the conductive system of citrus plants, which are economically relevant to Brazil. After working two years with structural biology

and citrus parasitology at the LNLS, she was invited to work at the Brazilian Agricultural Research Corporation - Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) – the foremost Brazilian enterprise for agricultural research. The research was focused on computational experiments aimed to develop web-based tools for structural bioinformatics. Although she felt excited about learning computer programing, she missed the excitement and unpredictability of “wet” science. This was when she realized she wanted to become a researcher in biomedical sciences. However, while helping the community with stray animals, her lack of knowledge about animal physiology, anatomy and medicine made her feel helpless. This overpowering limitation could only be amended by acquiring more knowledge, thus she went to the veterinary school at the University of São Paulo, FMVZ-USP, the best and most competitive veterinary school in Brazil.

In 2008 her husband got a postdoctoral fellowship at Cornell University in the United States – in one of the best veterinary schools in the world! Vera saw this as a career opportunity, and accompanied her husband, taking a leave of absence from her veterinary studies. Once at Cornell she was mesmerized. The research conducted in Cornell’s College of Veterinary Medicine was amazing, and she wanted to be part of it. However, whereas in Brazil she was part of “the cream of the crop”, her academic achievements meant little at Cornell. Luckily, in 2009, Dr. Todhunter and his assistant Marta Castelhano offered her a non-degree graduate student position to work for the Cornell University Hospital for Animals DNA Bank (CUHA DNA Bank) for

almost two years. This position allowed her to work in close contact with animals, researchers and veterinarians. The workload was high, but she was allowed to enroll for few Cornell classes. Subsequently, once the appointment was over, she started working as a temporary technician for Dr. Gary Whittaker. Dr. Whittaker and Dr. Collins (his wife) provided the necessary training Vera needed to be competitive to apply for the PhD program at Cornell. During her work with Dr. Whittaker and Dr. Todhunter, she learned the importance of having animal models to verify the applicability of *in vitro* findings.

As a Comparative Biomedical Science PhD candidate at Cornell, Vera joined Dr. Schimenti's laboratory. There she used mice as animal model to study mammalian gametogenesis, more specifically meiosis. Under Dr. Schimenti’s supervision Vera learned how to be a scientist. Dr. Schimenti nurtured her curiosity towards biological questions and her desire to become a better mentor and improve her teaching skills.

Vera’s next step is to obtain the necessary training needed to pursue a career as a research scientist. Her ultimate goal is to find an academic position where she can do cutting edge research, in a friendly and collaborative environment. She also wants to teach undergraduate students, and mentor the next generation of scientists. She is a strong believer that scientists should be passionate about their work, and less egotistical about their career and reputation.

Ao meu marido lindo. Mozinho, sua paixão por ciência é a minha inspiração. Sua felicidade é o que me dá forças para continuar lutando por um mundo melhor.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, my boss, and my role model, Dr. John Schimenti. He welcomed me in his lab, gave me freedom do pursue crazy questions, opportunity to do cutting edge research, and showed me how to see simplicity in the most complicated subjects. With the open door policy and his casual walks by the lab, he made himself helpful and approachable. I admire and respect him, and I do fear leaving his laboratory, and never finding another place like home again.

I would also like to thank the other members of my special committee, Dr. Paula Cohen, Dr. Eric Alani, and Dr. Scott Coonrod, who contributed immensely to my scientific growth. As a graduate student I could not have asked for a better group of scientist to supervise my academic progress. Thank you for keeping me focused, believing in me even when I doubt myself, and guiding me on how to improve my communication skills.

I am grateful to all Schimenti lab members, past and present. They are my lab family. They make the lab environment comfortable and safe, a place where it is OK to make mistakes, ask questions, and challenge scientific ideas – as long as the lab does not get burned to the ground, and data does not get lost! I could not have done this without our morning, freshly brewed coffee with lots of half & half; evening birthday cake breaks; midi-fire-drill ice-creams; long walks to Biotech; mid-night beer; nerf-gun fights; summer paintball; reagent and food “sharing”; summer barbecues; Yankee swaps; burst of laughter out

of nowhere; and all those other good stuff that make you my family. Thank you all!

I would not have done half of this thesis if it weren't for Dr. Ewelina Bolcun-Filas, who has shaped my scientific development. She was, and still is, a great mentor and friend. Together with Dr. Adrian McNairn, they are whom I go to when I need help. Giving help without expecting any retribution is a virtue, especially with the "publish or perish" culture that young scientists are faced with. Therefore, I want to extend this thanks to all my colleagues, mentees, and friends who helped me with every-day experimental misfits.

Because a lab is not a lab without the special people that make things go smoothly, I have to say a special thanks to Rob Munroe. Lab manager and mouse-God (together with Chris Abratte), thank you for creating transgenic mice, performing surgeries, and teaching me a lot about cultural differences, science and animals in general. I have had many different mouse strains, some more special than others. Nevertheless, they all like to be fed, be clean and happy, and only Gina knows how to do it right! Gina thanks for keeping my mice alive, and the mouse room tidy, organized, and pleasant to work in.

I want to become a research scientist, a mentor, and also a good teacher. Therefore, thank you Dr. Nancy Lorr for the opportunity to teach a subject I like, as well as perform my research in teaching wile assisting you. It was Dr. Robin Davisson who awarded me the teaching fellowship (GRTF), for which I will be forever grateful. My teaching experience at Cornell was amazing, and all the CTE personnel are responsible for that.

I want to thank all the members of the Cohen lab. I always felt welcome at your lab. Thanks for all the help with reagents, troubleshooting, staying focused, discussing experimental design, allowing passionate scientific discussion, and making the VRT penthouse such an amazing place to be. There are also a few people from other labs that have been key for my career development, thus I want to thank the members of the Weiss lab, Lin Lab, Suarez Lab, Fortune Lab and former Davison Lab. Together with Stephen Parry from the CSCU, Becky Williams and Johanna Dela Cruz from the imaging facility, I want to express my pleasure in working with you all. Your scientific integrity and skills are inspiring! Thank you for all the help.

I only had the chance to become part of an American Ivy League University because Marta Castelhano, Dr. Rory Todhunter, Liz Corey and Linda Hunter believed I had potential. Thank you so much for the opportunity and the amazing time I had when we worked together. I was heartbroken to leave the Todhunter lab. Fortunately Dr. Ruth Collins and Dr. Gary Whittaker came to the rescue. Thank you both for encouraging me to pursue a research career. Thank you all for believing in me when no one else did. I will be forever grateful and I hope to not let you down.

From my undergraduate universities in Brazil I have to thank Dr. Paula Papa, and Dr. Denise Fantoni from FMVZ-USP and Dr. Paula Kuser Falcão from UNICAMP-EMBRAPA for encouraging me to pursue education abroad, endorsing my DVM-student status for two years, and writing good recommendation letters.

It may be tacky but without the mice, science would be ages behind; so I want to thank all the mice I used for my research. Their little lives were not taken for granted at any moment. My greatest comfort is knowing that these little creatures have a purpose. They are contributing to something much greater than themselves. Something many humans will never achieve in their lifetime.

Finally I want to thank my friends, my husband and my biological family for all the support, words of encouragement, and for reading my papers. Yes, it is mandatory! I love you, admire you, miss you and hope you come visit me wherever I go.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Various specialized cells make up the different organs and tissues of complex organisms, however they all originate from one single cell: the fertilized egg. Meiosis is the specialized cellular division responsible for generating the sperm and egg. Similar to somatic cells, meiotic cells have regulatory mechanisms that assure the transmission of genetic information from parental to daughter cells. These surveillance mechanisms are called “checkpoints” (Hartwell and Weinert 1989; Subramanian and Hochwagen 2014). During the cell cycle there are multiple checkpoints with different molecular mechanisms and a common goal: precise cellular division.

Many of the quality control mechanisms are conserved between somatic and meiotic cells. However, meiosis has specific quality control mechanisms to assure that one parental diploid cell will give rise to four daughter haploid gametes, while increasing genetic variability (MacQueen and Hochwagen 2011; Zickler and Kleckner 2015). Whereas in males the four daughter cells give rise to sperms, in female mammals only one daughter cell is viable and will give rise to the embryo upon fertilization. The understanding of meiosis specific surveillance mechanisms, checkpoints, has impact in health and disease. Besides their role in reproductive health, meiotic cells constitute a platform for the study of DNA repair and recombination. These

cells are proficient in repairing massive amounts of damaged DNA without compromising the organism's genome or environmental fitness.

1. *Introduction to meiotic division*

Sexual reproduction depends on gametogenesis (process in which cells undergo meiotic cellular divisions). During mitosis, one parental diploid (2n) cell generates two identical 2n daughter cells (Figure 1.1 A). In meiosis the 2n parental cell generates up to four daughter cells, each with half of the parental chromosome content (e.g. haploid cells). In order to generate the haploid gamete, the 2n parental cell undergoes one round of DNA duplication followed by two rounds of cellular divisions (Figure 1.1 B) (Gray and Cohen 2016).

In the first meiotic division (meiosis I) the maternal and paternal homologous chromosomes segregate (reductional division), and at the second meiotic division (meiosis II) the sister chromatids separate (equational division) (Figure 1.1 C) (Gray and Cohen 2016; Handel and Schimenti 2010). Like mitotic division, meiosis I and II are divided in five stages: prophase, metaphase, anaphase, telophase and cytokinesis. This classification was established during the early 1900s when discrete morphological differences within the cells were initially observed. Even though there is variability between fungi, plants and animals, in protein sequences and genetic regulators of meiotic division, the protein function is highly conserved. Even within mammals, species-specific differences in meiosis have been found, such as pathways that affect genetic diversity and determine recombination

hotspots (Clément and Massy 2017), but the overall molecular mechanisms are conserved. In mammals, mouse is the species in which meiosis is best studied and is the model system of this dissertation.

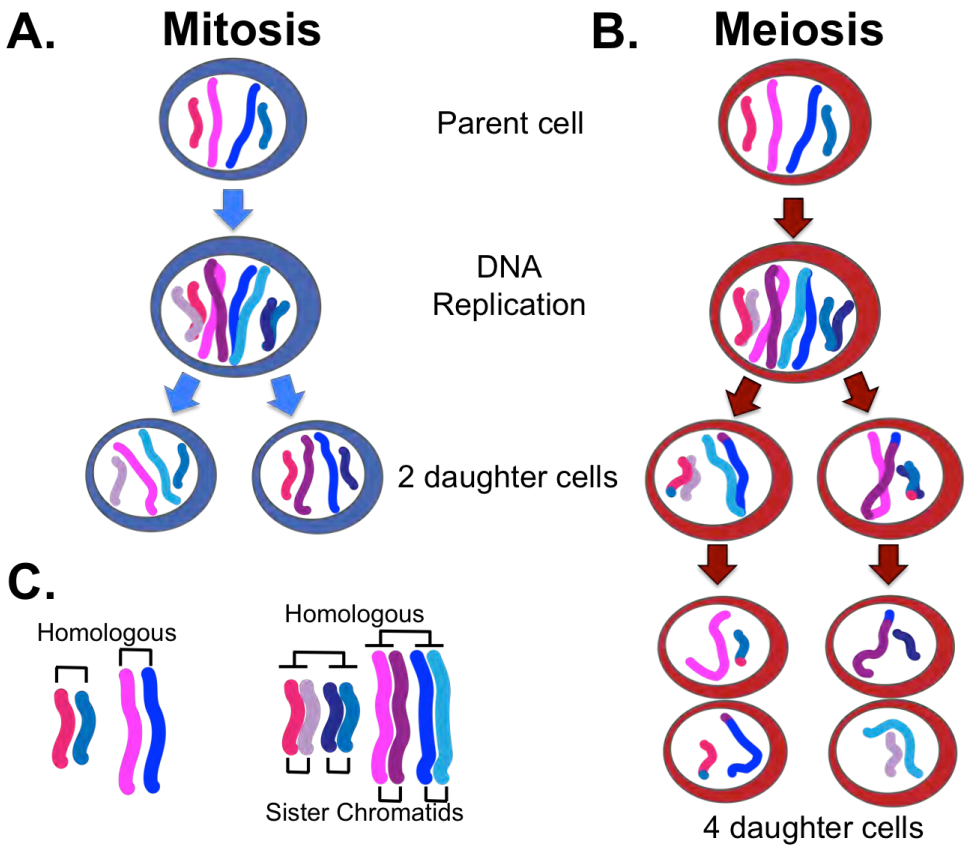

Figure 1.1) Representation of mammalian mitosis and meiosis. (A) Mitotic cell division; the blue parental cell (top left) undergoes one round of DNA replication followed by a cellular division that results in two daughter cells with comparable DNA content. (B) Meiotic cell division; the red parental cell undergoes one round of DNA replication followed by two rounds of cellular division, resulting in four daughter cells with half of the parental DNA content. (C) Schematics showing how homologous chromosomes and sister chromatids are illustrated in A and B.

Committing to meiotic division

Eutherian meiosis is a sexually dimorphic process, and gamete formation differs between males and females. In female mice meiosis begins during embryonic life, soon after the primordial germ cells reach the gonadal ridge (Figure 1.2 A). Retinoic acid (RA), a Vitamin A derivative, plays major role in meiotic entry. In mice RA is secreted by the mesonephroi at approximately 12.5 days post-coitus (dpc). Whereas female germ cells promptly respond to the RA stimuli, males at this developmental age secrete a retinoid-degrading enzyme, CYP26B1 (a member of the cytochrome P450 family) (Gray and Cohen 2016; Handel and Schimenti 2010; Niederreither and Dollé 2008), that prevents meiotic entry up to postnatal day (dpp) seven (Feng, Bowles, and Koopman 2014; McLaren and Southee 1997).

Females – only one chance to make it right

In female mice the first meiotic division is semi-synchronous with prophase I starting between 13.5 and 15.5 dpc, and arresting at birth (Bowles and Koopman 2007). The number of embryonic germ cells in the ovary is finite and non-renewable (Bowles and Koopman 2007; Zhang et al. 2014), thus leading to the unavoidable decline of germ cells throughout the lifetime of the animal. In females, meiosis I resumes upon ovulation, and progresses to meiosis II only if the egg/oocyte is fertilized. As previously mentioned, male meiosis initiates after birth when the renewable pool of spermatogonial stem cells start spermatogenesis, which will continuously produce sperm throughout the male's reproductive life. Upon completion of meiotic division, the male

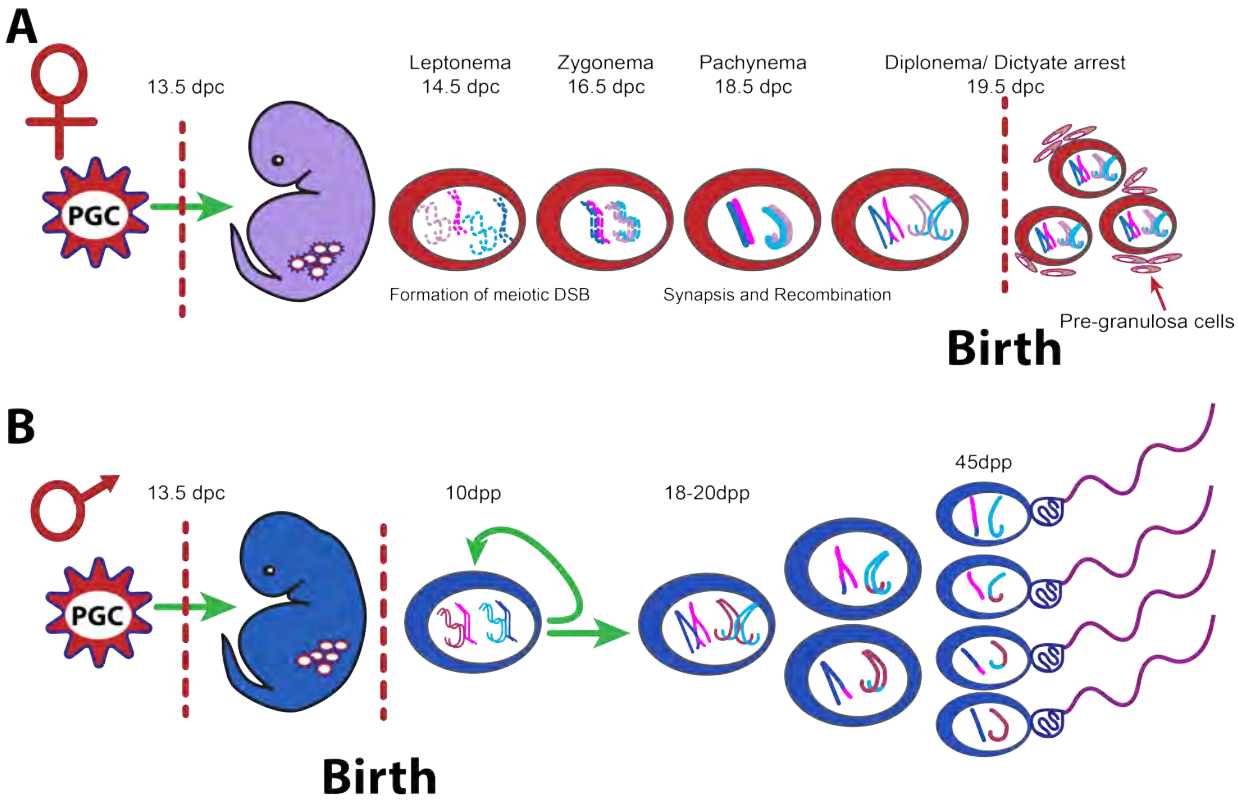

Figure 1.2) Gametogenesis is sexually dimorphic. (A) Female mammals have a finite number of oocytes that is determined during embryonic development. In female mice meiosis starts by 13.5 days post coitus (dpc) when the primordial germ cells (PGC) reach the urogenital ridge. It is a semi-synchronous cellular division that by birth results in a non-renewable, dictyate arrested, oocyte reserve. Only ovulated oocytes will finalize the first meiotic division. (B) In males, PGCs arrest during embryonic development, and only commit to gametogenesis after birth. Once the male gonad begins spermatogenesis it will constantly produce gametes (represented by the green circular arrow). In mice spermatogenesis starts around postnatal day (dpp) 13. It takes about 30 to 35 days for sperm to be fully formed.

parental germ cell will give rise to four functional gametes (Figure 1.2 B), whereas the female parental germ cell will produce only one viable oocyte and three polar bodies, smaller cells destined to degenerate (Dalton and Carroll 2013; Handel and Schimenti 2010).

2. *Prophase I – establishing connections*

The first meiotic division is unique compared to meiosis II and mitosis. Prior to meiosis I the replicated chromosomes will loosely align with their homologs, recombine, and segregate. Prophase I is the longest stage of meiosis (Cobb and Handel 1998) during which most of the aforementioned events occur. It is divided in five main sub-stages, classically defined by the morphological appearance of the chromosomes. Progressions through the subs-stages also correlate with the meiotic DNA breaks dynamics (Figure 1.3):

- Leptonema, when hundreds of programmed endogenous DNA double strand breaks (DSBs) are generated;
- Zygonema, when DSBs are repaired through recombination, which promotes the search for the homologous chromosomes (homologs) initiating the tight tethering of the homologs (synapsis);
- Pachynema, when most, if not all, the DSBs are repaired and the homologs are fully synapsed;
- Diplonema, when the proteinacious structure formed between the homologs during synapsis begins to disassemble;

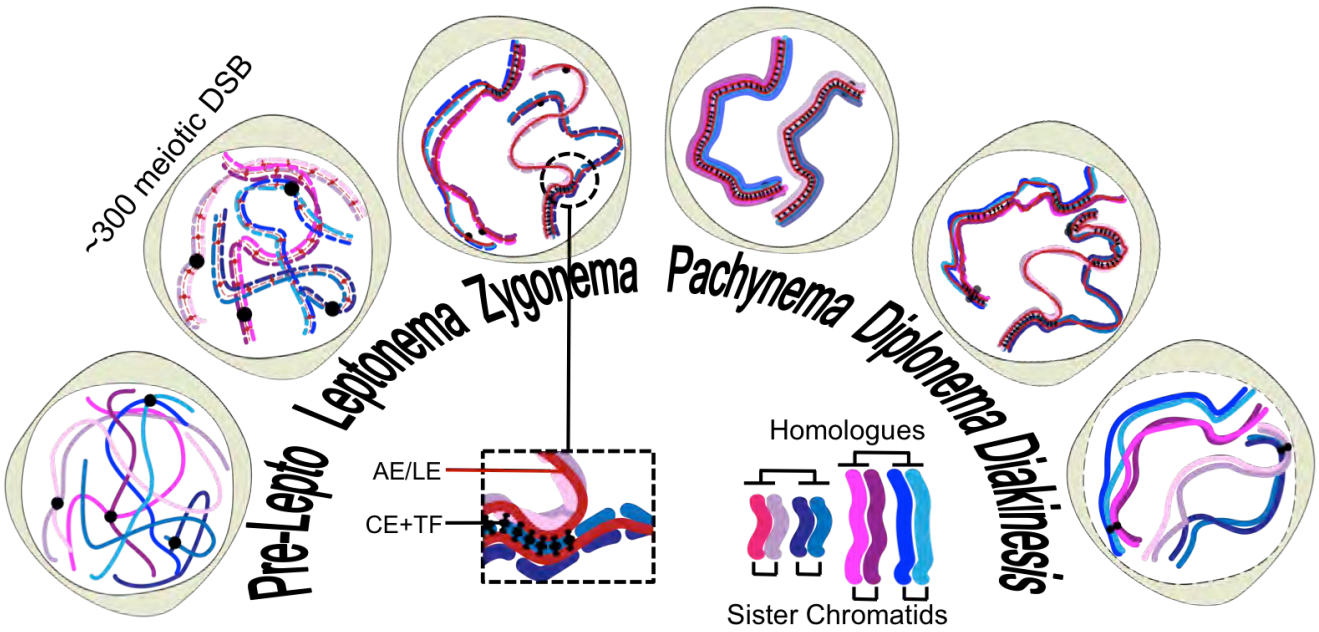

Figure 1.3) Prophase I is divided in five sub-stages. Prior to entry in meiotic division primordial germ cells undergo one last round of DNA synthesis, called pre-meiotic DNA replication, referred here as **pre-leptotene** stage. Entry into meiosis is marked by the occurrence of hundreds of endogenous programmed DNA double stranded breaks (DSBs), at **leptonema**. As the DSBs begin to be repaired by recombination, the axial elements (AE) at the chromosomes axes (in **zygonema**) become juxtaposed and polymerization of the synaptonemal complex (SC) begins. At **pachynema** most, if not all, of the DSBs are repaired and the homologs are fully synapsed. After pachynema the SC starts to de-assemble (in **diplonema**) and prophase I is finally completed at **diakinesis** when the nuclear envelop starts to disappear. AE, Axial elements; LE, lateral elements; CE, central elements; TF, transverse filaments.

- Diakinesis, when the nuclear envelop starts to disappear as the cells transition to metaphase I, and the sites of reciprocal genetic exchange between homologs (cross overs (CO)) can be visualized (chiasmata).

Sister Chromatids – cohesion mediated by cohesins

The decision to enter meiosis is made before pre-meiotic DNA replication, when the retinoic acid mediated expression of the "*stimulated by retinoic acid 8" (Stra8)* gene is initiated (Baltus et al. 2006; Handel and Schimenti 2010; Niederreither and Dollé 2008). *Stra8* expression induces pre-meiotic DNA replication, which precedes prophase I by about 15 hours (Baltus et al. 2006; Koubova et al. 2014; Spiller, Bowles, and Koopman 2013). The sister chromatids from the resulting chromosome are held together by different multi-protein cohesin complexes that mediate sister chromatid cohesion. These complexes form a ring-like structure surrounding the sister chromatids which will assure that they are held together past anaphase I.

Although the resulting sister chromatids are maintained together by cohesins, homologous chromosomes still need to find, pair, and recombine for reduction of cell ploidy upon anaphase I. Even though juxtaposition of homologous chromosome occurs independent of DSBs, the DSB mediated homology search is necessary for their stable association (Boateng et al. 2013; Ishiguro et al. 2014). There is evidence that this initial pairing of homologs is a pre-leptotene event that requires the non-catalytic function of a meiosis specific topoisomerase- like protein (SPO11- see below), and the

formation of inter-axis bridges (of about 400nm) between the loosely paired homologs (Boateng et al. 2013; Ishiguro et al. 2014; Zickler and Kleckner 2015). This proximity between homologs precedes a tighter (~200nm) (Johanna L. Syrjänen et al. 2017), side-by-side association (synapsis), and is likely important for preventing the erroneous association of non-allelic, homologous genomic regions.

Besides the pre-meiotic parallel arrangement of homolog-axes, another event that helps in the organization of the chromosomes is the positioning of the telomeres. During leptonema the telomeres become tethered to the nuclear envelope and dynamically cluster together. This disposition is referred to as “bouquet conformation” due to its cytological morphology, and has also been associated with chromosome pairing (Inagaki, Schoenmakers, and Baarends 2010; Subramanian and Hochwagen 2014). Therefore, sister chromatids and homologous chromosomes are not stochastically arranged in the meiotic nucleus.

Homologous chromosomes – pairing and repairing

The synaptonemal complex

The organization of the chromosomes within the meiotic nucleus is suggestive of its importance for synaptonemal complex (SC) assembly to take place between homologs. The SC is a proteinacious structure that forms between homologs as they synapse. It can be morphologically distinguished

as comprising of three components (Bolcun-Filas et al. 2007; Schücker et al. 2015; Johanna L. Syrjänen et al. 2017):

1- Axial elements (AE) / Lateral elements (LE): Proteinacious filaments that form along the side of chromosomes (vertical axis) where chromatin loops are held in place. Prior to synapsis the proteins associated to the chromosome axis are referred to as axial elements (AE). Components include synaptonemal complex protein 3 (SYCP3) and 2 (SYCP2).
2- Central element (CE): Proteinacious filaments that form parallel to the vertical axis, and are present at the mid-region between the two homologs (e.g. between the LE of the synapsed chromosomes). Components include synaptonemal complex central element protein 1, 2, 3 (SYCE1, SYCE2, SYCE3, respectively), and testis expressed 12 (TEX12).
3- Transverse filaments (TF): Proteins with an arrangement perpendicular to both the axial/lateral and central elements. Their horizontal organization forms a ladder-like structure, connecting LE to CE, and is composed of SYCP1.

Initiation of SC assembly correlates to DSBs, which triggers a cascade of events leading to the lateral elements associating with the transverse filaments and central elements. The resulting structure is a zipper-like proteinacious complex formed between the homologs. Once the tripartite structure is fully

assembled the chromosomes are said to be synapsed (Kleckner 2006; Page and Hawley 2004; Zickler and Kleckner 2015).

DNA damage and SC assembly

SPO11 is a meiosis specific protein with putative structural function on homolog juxtaposition (Boateng et al. 2013), and endogenous programmed nucleolytic activity (Keeney 2008). The transesterase activity of this type II DNA topoisomerase-like protein is likely responsible, but not sufficient, for the catalysis of DSBs (Keeney 2008; Lam, Mohibullah, and Keeney 2017). SPO11 is homologous to the A subunit of archaeal type II topoisomerase VI (TopoVI), which is composed of two subunits (A and B) (Nichols et al. 1999). The A subunit of TopoVI is involved in DNA interaction, and the B subunit in ATP hydrolyses, thus both subunits are required for DSB formation (Robert et al. 2016). Similar to archaeal TopoVI, catalysis of meiotic DSBs depends on the formation of a heterotetramer between SPO11 and the TOPOVIBL protein (product of the mouse *Gm960* gene) (Robert et al. 2016). Meiotic DSB formation also requires other accessory proteins (for example MEI4, MEI1, IHO1, *etc*) that have regulatory and structural roles. Besides the regulation prior to DSB formation there are also multiple complex feedback loops to assure that the appropriate number of breaks are introduced (Kauppi et al. 2013; Zickler and Kleckner 2016; Robert et al. 2016). Furthermore, there is evidence of sexual dimorphism between the two SPO11 isoforms: beta and alpha. The beta isoform is responsible for DSB formation, and the alpha is not well characterized but is expressed after synapsis initiation. While it is

dispensable for female meiosis, the alpha isoform is important for the recombination of heterologous sex chromosomes in males (Kauppi et al. 2013). Another interesting feature of meiotic DSBs is that they are more likely to occur at specific regions of the genome (hotspots), and are cytologically associated with axial element (AE) proteins (Cole et al. 2012; Kumar et al. 2015).

Formation of the filament-like aspect of the AE is concurrent with SPO11 induced DSBs. Cohesins are likely the first layer of proteins to associate along the chromosomes. As the cells enter leptonema, SYCP3 and HORMAD1 (one of the mammalian “HORMA” domain-containing proteins orthologous to the *Saccharomyces cerevisiae* Hop1p) proteins become cytologically evident, with a spotty distribution that coincides with both cohesins, and DNA damage markers. SYCP3 is a coiled-coiled protein with a N-terminus-DNA binding motif (Johanna Liinamaria Syrjänen, Pellegrini, and Davies 2014). Like other coiled-coiled SC-proteins, it easily polymerizes into polycomplex fibers in non-meiotic conditions (Johanna L. Syrjänen et al. 2017; Yuan et al. 1998). Based on recent high-resolution microscopy and structural studies (Rong et al. 2016; Johanna L. Syrjänen et al. 2017; Johanna Liinamaria Syrjänen, Pellegrini, and Davies 2014) it is not unreasonable to hypothesize that epigenetic marks (changes in chromatin structure without any DNA alteration) and cohesins have a role in guiding SYCP3 polymerization to the genomic region destined for SC axis formation. HORMAD1 is one of the two meiotic HORMA-domain containing proteins of mammals (Wojtasz et al.

2009). HORMA-domains are described as a signal-response mediator of protein-protein interactions (Rosenberg and Corbett 2015). Both HORMAD1 and HORMAD2 are involved in the meiotic recombination control, and are not present in synapsed chromosomes. SYCP3, on the other hand, remains associated with the chromosome from leptonema to diplonema. Recent studies show that HORMAD1 helps promote DSB formation on the unsynapsed chromosomes axes (Stanzione et al. 2016), and that radiation induced DSBs in *Hormad1*$^{-/-}$ knockout mice, DNA repair is expedited (Shin, McGuire, and Rajkovic 2013).

Structural and functional studies suggest that HORMAD1 binds to cohesins at the chromosome axis (Kim et al. 2014; Rosenberg and Corbett 2015). Studies of cohesin mutant mice have not yet identified the HORMAD1 interactor, but immunopreciptation studies suggest cohesin SMC3 as a likely candidate (Fukuda et al. 2010; Hopkins et al. 2014; Wojtasz et al. 2012). Since SMC3 is an essential cohesin (ubiquitously expressed and embryonic lethal if knocked out), studies of its role in meiosis are limited (Singh and Gerton 2015). Even though REC8 and RAD21L have no obvious HORMAD1 interaction (Fukuda et al. 2010; Kumar et al. 2015; Wojtasz et al. 2009), they are particularly important as meiosis-specific cohesin subunits. REC8 and RAD21L are implicated in preventing SC formation between sister chromatids and non-homologs, respectively (Ishiguro et al. 2014; Lee 2013; Xu et al. 2005). *Rec8* expression, similarly to *Stra8*, is RA-dependent (Bannister et al. 2004; Koubova et al. 2014; Xu et al. 2005). Presence of REC8 during pre-

meiotic S phase is important for sister chromatid organization prior to meiotic entry. Without REC8, the polymerization of the SC happens within the chromosome, alongside each sister chromatid (Agostinho et al. 2016; Ishiguro et al. 2014; Rong et al. 2016). Curiously, it is still unknown if in wild type meiocytes the axial element SYCP3 assembles as two separate parallel structures (one for each sister chromatid) or as only one that simultaneously incorporates both sisters (Ortiz et al. 2016). The analysis of RAD21L deficient meiocytes implicates this cohesin in meiotic recombination, presumably by placing the damaged DNA closer to its homologous chromosome (Agostinho et al. 2016; Rong et al. 2016). The spatiotemporal correlation between RAD21L and meiotic DSB formation/resolution further supports that RAD21L plays a role in the stability of allelic homolog pairing during DSB mediated homology search (Ishiguro and Watanabe 2016; Koubova et al. 2014). Thus, RAD21L might have a role in “proofreading” homologous recombination. However, non-homologous synapsis is a frequent phenotype associated with the depletion of other meiotic structural or recombination-related proteins. Nonetheless, independent from where synapsis occurs, upon synapsis HORMADs are removed form the chromosome axes in a TRIP13 dependent manner, and the proteins form the transverse filaments and central elements become evident (Wojtasz et al. 2009).

DNA damage and homologous recombination

In mice, SPO11 generates about 200 to 300 DSBs per meiotic cell. Disregarding the afore mentioned accessory proteins and TOPOVIBL for

proper meiotic DSB formation, it is well established that SPO11 remains covalently bound to the broken DNA (Robert et al. 2016; Stanzione et al. 2016). Each DNA break requires one heterotetramer that contains two SPO11-monomers, each monomer cleaving one DNA strand in a reaction that results in SPO11 covalently linked to the cleaved DNA strand. The two intermediates (SPO11 bound to the 5' DNA on both sides of the break) are removed by the nucleolytic activity of the MRN complex (Garcia et al. 2011; Gray and Cohen 2016; Keeney 2008). The MRN complex consists of three proteins (MRE11, RAD50 and NBS1) and, in addition to its role in DNA damage response, it is also essential for the initial processing of DSBs. In meiosis, MRN introduces a single strand DNA (ssDNA) break at either sides of the SPO11-DNA intermediate (Gobbini et al. 2016) which are not only essential for removal of the SPO11-DNA intermediate but also for DNA-end-resection. These nicks allow 5' to 3' exonuclease resection, which results in a 3' ssDNA tail initially coated with the replication protein A (RPA), thus forming the substrate for homologous recombination (HR). After being loaded the RPA nucleoprotein filament is gradually replaced by the recombinases RAD51 and DMC1 (the meiosis specific recombinase). Although to a lesser degree, RPA coated ssDNA is also substrate for other genome maintenance factors such as the ATR-interacting protein (ATRIP) that recruits the ATR kinase to processed DNA breaks (Duursma et al. 2013; Mermershtain and Glover 2013). The ssDNA coated with RAD51 and DMC1 forms a nucleoprotein filament capable of invading a double-stranded DNA (dsDNA) from an intact chromatid (with

predicted predilection for the homolog rather than the sister chromatid) (Subramanian et al. 2016). Strand invasion marks the search for a DNA template to be used in the repair process.

Once engaged with a homologous region, the DNA from the invading strand can be repaired, and will form either crossovers (CO) or non-crossover (NCO) products. The best-described NCO products originate from synthesis-dependent strand annealing (SDSA), in which DNA polymerase extends the invading strand that is then displaced, and will re-anneal with the other arm of the DSB. For CO formation reciprocal exchange of chromatid and associated cohesins must occur between the homologs. The most prevalent mechanism to form COs requires, after the initial single strand invasion, a second-end capture of the other DSB arm (Gray and Cohen 2016). Needless to say that the template used by the invading strand is essential for proper CO formation between homologs. Furthermore, proper segregation of homologs at anaphase I depends on the formation of at least one CO event in each chromosome pair.

DNA damage and repair template of choice

The mechanisms acting on DNA-repair template choice are not fully understood in mammals. There is evidence for a molecular mechanism that blocks the use of the sister chromatid as a repair template (Chapter 3 and Shin et al., 2013) as well as for a mechanism where the cell continuously produce DSBs on unsynapsed chromosome (Cloutier et al. 2015; Shin, McGuire, and Rajkovic 2013; Stanzione et al. 2016). These explanations are

not mutually exclusive and it is likely that both act in concert to assure that recombination occurs between homologs.

Recent findings support the hypothesis that mechanisms to control DSB load exist. For instance, the serine/threonine kinase ATM was implicated in SPO11 regulation. According to *in vivo* and *in vitro* studies ATM is recruited and activated by the MRN complex (Blackford and Jackson 2017; Gobbini et al. 2016). Besides phosphorylating the histone H2AX at serine 139 (γH2AX), ATM negatively regulates SPO11-DSB formation. In meiosis γH2AX is not solely a DSB marker but also a critical epigenetic sign for transcriptional silencing (see below). Therefore, whereas ATM prevents the creation of excessive levels of DSBs, HORMAD1 promotes DSBs formation on unsynapsed regions (Lam, Mohibullah, and Keeney 2017; Stanzione et al. 2016). This regulation of DSB load may serve as a gauge of synapsis progression. However, it does not explain how synapsis between non-homologous chromosomes is avoided.

One explanation for why synapsis preferentially occurs between homologous chromosomes is the existence of inter-axis bridges, formed prior to SC assembly (Kleckner 2006; Page and Hawley 2004; Wang et al. 2017; Zickler and Kleckner 2015). These interhomolog connections would be formed during late leptonema, when the invading RAD51/DMC1 nucleoprotein filament engages with a non-sister chromatid that is associated with axial element proteins. At this time the RAD21L cohesin mediates a wide-range chromosome “proofreading” to ensure that the invaded strand is indeed from

an allelic homologue (Agostinho et al. 2016; Ishiguro et al. 2014; Ishiguro and Watanabe 2016; Rong et al. 2016). This hypothesis unites the observations that RAD21L forms bridge like structure (~400 nm) between homologs at zygonema with the predilection for synapsis between “true” homologs (Ishiguro and Watanabe 2016; Rong et al. 2016).

In summary, there is a fine coordination between DSBs formation, synapsis between homologs, and DSB repair (Kauppi et al. 2013; Keeney, Lange, and Mohibullah 2014), in order to assure that pairing and repairing is achieved by pachynema.

3- Female meiosis

Meiosis in mammals is sexually dimorphic (Figure 1.2 A and B). Besides the differences in developmental timing of meiotic entry, number of gametes, and final number of viable cells resulting from each meiotic division, there is also difference in genomic content: males have different sex chromosomes (one X and one Y), while females have two X chromosomes. In normal male meiosis only a small region of the sex chromosomes (~700 kb) (Perry et al. 2001) will synapse, while most of the chromosomes remaining unsynapsed.

Synapsis or silence

Since the heterologous regions of the sex chromosomes are never synapsed, HORMAD1/2 persist on the unsynapsed chromosomes axes.

However, DNA-damage markers such as RAD51, DMC1, and RPA disappear as prophase I progresses, being practically absent at late pachynema (Carofiglio et al. 2013; Moens et al. 2007). In addition to the proteins from the axial element, the heterologous sex chromatin has an extra layer of proteins from the DNA-damage response pathway. These markers are involved in the repression of transcription, which progressively spreads through the chromatin loops, forming a domain surrounding the sex chromosomes. The repression of transcription of sex chromosomes is essential for meiotic progression in males, and failure to silence “toxic” sex-genes results in spermatocyte demise (Wojtasz et al. 2012; Hélène Royo et al. 2010). The meiotic sex chromosome inactivation (MSCI) is mediated by γH2AX, and can be cytologically identified by its characteristic heterochromatic appearance. This domain is called the “X Y body” or “sex body” (Cloutier and Turner 2010; Turner 2015) and is a consequence of a more general phenomena termed meiotic silencing of unsynapsed chromosomes (MSUC) (Turner 2015).

Genetic analyses show that HORMAD2 is essential for meiotic silencing. Furthermore, *Hormad2* deletion affects localization of ATR to unsynapsed axis (Kogo, Tsutsumi, Inagaki, et al. 2012; Wojtasz et al. 2012). There is convincing evidence that the phosphorylation and spreading of silencing factor γH2AX is dependent on ATR kinase and the γH2AX binding partner MDC1 (mediator of DNA damage checkpoint 1), respectively (Y. Ichijima et al. 2011; H. Royo et al. 2013). Therefore, there is a complex interdependency between these silencing factors, which produces massive

signal amplification through kinase activity and auto-phosphorylation events. Immunoprecipitation analyses suggest that the ATR kinase phosphorylates HORMAD2 at an SQ/TQ phosphorylation motif (Fukuda et al. 2012; H. Royo et al. 2013).

Oocytes are also able to form regions of transcriptional inactivation, which are often referred to as “pseudo-sex body” (Cloutier et al. 2016). The “pseudo-sex body” is a manifestation of meiotic silencing of unsynapsed chromosomes (MSUC) in oocytes, and is also characterized by accumulation of asynapsis-associated factors such as HORMAD1/2, BRCA1, ATR and γH2AX. In females MSUC can lead to cell death due to silencing of essential genes. However, MSUC is inefficient in cells with pervasive synapsis failure (more than 2-3 unsynapsed chromosomes) presumably due to limiting amounts of silencing factors such as BRCA1(Kouznetsova et al. 2009). Thus, MSUC response is limited, but has an important role in the elimination of karyotypically abnormal oocytes (either lacking or carrying an extra chromosome) (Cloutier et al. 2016).

The silencing paradox

Whereas spermatocytes have expression of lethal genes if they fail to mount a silencing response, females have to silence all copies of an unsynapsed essential gene in order for oocytes to be eliminated. Presumably due to limiting factors, an oocyte with massive asynapsis may fail to silence every copy of an essential gene, thus remaining as a constituent of the oocyte

pool. The failure to mount an effective MSUC can explain the presence of oocytes in ovaries of mice with mutations that cause massive synapsis failure (such as *Spo11*$^{-/-}$ and *Mei1*$^{-/-}$); each cell will have different silencing patterns that will not necessarily incorporate all copies of an essential gene. Thus, of the aforementioned mice, the observed small pool of oocytes present in the ovaries would represent the MSUC escapers.

Based on current knowledge, DSB formation is regulated by the presence of HORMAD1 on unsynapsed chromosomes, which works as a signal for SPO11-DSB formation (Stanzione et al. 2016). The observation that asynapsed chromosomes have DNA damage markers (e.g. RAD51, RPA, DMC1) only up to early pachynema suggests that DSBs are resolved by late pachynema, even at unsynapsed chromosomes. This argues against the existence of a block to use the sister chromatid as DNA repair template (BSCR). In this model asynapsis results in DSBs that will be resolved at late pachynema and meiocyte elimination occurs through HORMAD2 mediated MSUC of essential genes (Kogo, Tsutsumi, Inagaki, et al. 2012; Wojtasz et al. 2012). Interestingly, the findings reported in Chapter 3 argue in favor of HORMAD2 mediating the BSCR. This paradox (discussed in Chapter 4) is likely due to failure of recent studies to account for the phosphorylation status of the proteins involved in the MSUC and DSB response.

For instance, one hypothesis that requires BSCR, constant formation of DSB on asynapsed chromosomes, and MSUC takes into the spatiotemporal phosphorylation events (see Chapter 4). In such model, the formation of DSB

starts a phosphorylation cascade that is not strong enough to trigger MSUC if the DSB is resolved. However, if the DSB is not resolved this phosphorylation cascade is amplified and starts MSUC. In asynapsed chromosomes, the failure to repair the DNA damage using the sister chromatid results in persistent DSBs that will either mount MSUC or continuously form new DSBs via HORMAD1 signaling to SPO11. The presence of feedback loops and signal amplification responses add an extra layer of complexity to both MSUC and DSB repair. Nevertheless, meiosis encompasses a fine tuned cascade of events, and may be equipped with multiple surveillance mechanisms to eliminate unfit cells, with post-translational modifications being one of them.

4- Definition of checkpoint

Whereas leptotene meiocytes resist high levels of DSBs, they become highly sensitive to genetic insults after diplonema (see Chapter 2 and 3). Dictyate arrested oocytes promptly die in response to DNA damage but are refractory to synapsis and CO malformations, which is one of the reasons for aneuploidies being often traced back to the female gamete (Hassold and Hunt 2001). In 2005, Di-Giacomo and colleagues proposed a two-branch surveillance mechanism for female meiosis (Di Giacomo et al. 2005). The model suggested that oocytes have one lax quality control mechanism to survey synapsis and a stringent one to survey DNA integrity. Together, the two branches constituted the pachytene checkpoint.

Historical overview

The concept of a checkpoint pathway was introduced by Hartwell to describe the existence of control mechanisms that assured the prerequisites necessary for proper progression of cellular division had been satisfied (Hartwell and Weinert 1989). He was the first to refer to an extrinsic mechanism that did not rely on substrate formation as a limiting factor for cell cycle progression. His definition implicated the existence of a quality control mechanism that actively monitors cell cycle events and provides feed-forward signals for cellular development and tissue homeostasis (Zhou and Elledge 2000). The definition was not limited to DNA integrity, and considered all the prerequisites for a cell to be ready for division; for example, organelle load, DNA duplication and arrangement of the segregation machinery.

One possible interpretation of Hartwell's concept is that a checkpoint consists of signaling mechanisms that creates dependency between otherwise metabolically independent processes (MacQueen and Hochwagen 2011; Subramanian and Hochwagen 2014). For example, DNA integrity is metabolically independent from chromosome segregation, however, the presence of a DNA-damage checkpoint creates dependency between them: DNA integrity must be achieved before segregation is initiated. Interestingly, Subramanian and Hochwagen added to this definition the idea that the checkpoint did not evolve to survey and respond to abnormal events. For Subramanian and Hochwagen, DNA damage is an inherent part of the cell cycle, therefore is not an abnormal event *per se*. In contrast, other authors

interpret checkpoint to be a pathway that detects errors and therefore is non-essential if the cell remains free of errors (Elledge 1996; Rieder 2011). By this definition, elimination of checkpoint components would not be detrimental unless the cell is stressed or under abnormal circumstances. Consequently, it can be challenging to determine if an element or pathway that participates in multiple interacting signaling networks also acts as a checkpoint of something (Khodjakov and Rieder 2009; Rieder 2011).

Prophase I checkpoint

There are mainly three hypothesized checkpoints that survey meiocytes quality during female prophase I:

1- DNA damage – Failure to repair meiotic DSBs triggers oocyte death starting at diplonema. In female mice CHK2 is the checkpoint kinase responsible for DNA-damage dependent oocyte elimination regardless of animal age (Appendix I). This protein is constitutively expressed but its activation depends on the presence of damaged DNA.

2- Asynapsis - Failure to synapse has been correlated with oocyte elimination, but there is little evidence to support the existence of a surveillance mechanism. Nevertheless, HORMAD1 and HORMAD2 are good candidates for such a checkpoint. HORMAD1 is essential for DSB formation and synapsis thus is indispensable for meiotic progression. In contrast HORMAD2, which if deleted does not obstruct synapsis but abrogates death in synapsis-deficient oocytes, may be deemed as non-

essential and is a strong candidate to serve as a synapsis checkpoint factor. Regardless, both are alluded to as a checkpoint protein. The presence of a true synapsis checkpoint is a polemic topic (see Chapter 4 for a more detailed discussion). The finding that oocytes carrying accessory chromosomes (hemizygous chromosomes carrying only non-essential genes, for example carrying a single copy of the human chromosome 21) reach metaphase (Cloutier et al. 2015) suggests that synapsis is not under surveillance.

3- Silencing – The presence of unsynapsed chromosomes results in meiotic silencing. In cells competent for making and repairing meiotic DSBs, MSUC seems to protect extranumerary accessory chromosomes from accumulating DNA breaks at late pachynema. MSUC requires the DSB-response machinery, and there are ongoing debates about if DSBs are required to trigger it. γH2AX and its binding partner MDC1 are the hallmarks of meiotic silencing and are known DNA damage markers, yet oocyte death due to MSUC has been linked to failed transcription of germ cell expressed essential genes rather than to the DNA damage checkpoint activation. Silencing of genes can trigger cell demise through a plethora of molecular mechanisms, and should not correlate to only one stage of cell division, however, Cloutier and colleagues report loss of cells only at the pachytene/diplotene transition (Cloutier et al. 2016, 2015). There are different opinions regarding MSUC status as a checkpoint, but lack of a defined pathway and

requirement of DSB-response proteins are contentious elements of silencing as a checkpoint.

Regardless of terminology, when something goes awry with the cells, these signaling pathways are activated. These pathways have evolved the molecular mechanisms necessary for fixing or eliminating the naturally occurring unfit meiocytes. Through the use of genetic and biochemical tools, these pathways can be tested under extreme conditions to allow for better understanding of these biological processes which may generate translational tools besides basic scientific knowledge.

5- Research focus and goals of this dissertation

My research goal was to identify the surveillance mechanisms that respond to DNA damage in oocytes. I wanted to understand why oocytes are sensitive to genotoxic agents after birth, but refractory to massive amounts of DSB during female embryonic development (when hundreds of meiotic DSBs are generated). In order to achieve my goal, I used *in vitro* and *in vivo* approaches that shed light on our understanding of the quality control mechanisms functioning during oogenesis.

Preventing oocyte death

Deletion of *Chk2* effectively impairs the activation of the tumor suppressor proteins TAp63 and p53, which are responsible for oocyte death (Appendix I). The ability to preserve fertility in *Chk2*$^{-/-}$ animals submitted to

ionizing radiation (IR) implied that oocytes are able to repair damaged DNA if the checkpoint is de-activated. I tested this hypothesis by transiently inhibiting CHK2 in ovaries submitted to IR. The results presented in Chapter 2 indicate that oocytes are able to repair damaged DNA if the checkpoint response is delayed by a few days. The results not only support that oocytes are able to repair damaged DNA but also that CHK2 is a potential target to preserve fertility in premature ovarian failure paradigms.

Genetically dissecting the checkpoint

While studying the DNA-damage checkpoint we found evidence that suggested CHK2 also had a role in the presumed synapsis checkpoint. In order to explain the unexpected finding that CHK2 depletion improved *Spo11*$^{-/-}$ oocyte survival, I performed a thorough genetic analysis of compound mutant mice for meiotic and "checkpoint" related genes. Lack of CHK2 does not affect pseudo-sex body formation (no interfere with MSUC response) in synaptic mutants and CHK2 does not interfere with HORMADs dynamics. Radiation experiments and quantification of non-meiotic DSBs, show that *Spo11*$^{-/-}$ meiocytes have lower DSB repair rates. The presence of HORMADs on the axis of both *Spo11*$^{-/-}$ synaptic mutant, and on the axes of synapsis proficient, DSB-repair deficient, *Trip13*$^{Gt/Gt}$ oocytes, led me to hypothesize that HORMADs were blocking DNA repair. The results supporting the BSCR are presented in Chapter 3 and further discussion of how these results fit with the current understanding of MSUC are in Chapter 4.

Research in teaching

With the goal of improving learning and teaching experiences, I performed a research project as a graduate research and teaching fellow (GRTF). The project consisted of evaluating a technology supported interactive response system during the laboratory section of Cornell's upper-level histology course. The results suggested that both teachers and students had positive experiences using the technology for providing feedback and assessing learning outcomes. This was a small study not designed to detect quantitative improvements in learning outcome, however the results suggest that such a tool had qualitative benefits. The published manuscript was incorporated into my dissertation as Appendix II.

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CHAPTER 2

PHARMACOLOGICAL INHIBITION OF THE DNA DAMAGE CHECKPOINT PREVENTS RADIATION-INDUCES OOCYTE DEATH

***** This chapter is a reprint with minor reformatting of the manuscript: Vera D. Rinaldi, Kristin Hsieh, Robert Munroe, Ewelina M. Bolcun-Filas and John C. Schimenti. “Pharmacological Inhibition of the DNA Damage Checkpoint Prevents Radiation-Induced Oocyte Death”*.GENETICS*
DOI:10.1534/genetics.117.203455

1- Abstract

Ovarian function is directly correlated with survival of the primordial follicle reserve. Women diagnosed with cancer have a primary imperative of treating the cancer, but since the resting oocytes are hypersensitive to the DNA-damaging modalities of certain chemo- and radiotherapeutic regimens, such patients face the collateral outcome of premature loss of fertility and ovarian endocrine function. Current options for fertility preservation primarily include collection and cryopreservation of oocytes or *in vitro* fertilized oocytes, but this necessitates a delay in cancer treatment and additional assisted reproductive technology (ART) procedures. Here, we evaluated the potential of pharmacological preservation of ovarian function by inhibiting a key element of the oocyte DNA damage checkpoint response, checkpoint kinase 2 (CHK2; CHEK2). Whereas non-lethal doses of ionizing radiation (IR) eradicate

immature oocytes in wild type mice, irradiated *Chk2*$^{-/-}$ mice retain their oocytes and thus, fertility. Using an ovarian culture system, we show that transient administration of the CHK2 inhibitor 2-(4-(4-Chlorophenoxy)phenyl)-1H-benzimidazole-5-carboxamide-hydrate ("CHK2iII") blocked activation of the CHK2 targets TRP53 and TRP63 in response to sterilizing doses of IR, and preserved oocyte viability. After transfer into sterilized host females, these ovaries proved functional and readily yielded normal offspring. These results provide experimental evidence that chemical inhibition of CHK2 is a potentially effective treatment for preserving fertility and ovarian endocrine function of women exposed to DNA-damaging cancer therapies such as IR.

2- Introduction

It is of paramount importance that organisms minimize the transmission of deleterious mutations to their offspring. Accordingly, sensitive mechanisms have evolved to eliminate germ cells that have sustained certain threshold amounts of DNA damage (Heyer *et al.* 2000; Suh *et al.* 2006; Bolcun-Filas *et al.* 2014; Pacheco *et al.* 2015). Under normal circumstances, this is desirable. However, because women are born with a finite number of oocytes, environmental factors that cause DNA damage to oocytes can result in primary ovarian insufficiency (POI), sterility, and ovarian failure. This is a crucial issue for cancer patients undergoing certain types of chemotherapy or radiation therapy (Woodruff 2007). For example, POI occurs in nearly 40% of all female breast cancer survivors (Oktay *et al.* 2006). The resulting premature

ovarian failure has major impact in a women's life, both physiologically and emotionally. As the life expectancy of cancer survivors increases, so does the need to address the adverse outcomes to fertility. Therefore, the ability to inhibit oocyte death and preserve fertility, in both pre-pubertal cancer patients and premenopausal women, would have a major impact on survivors' lives.

At present, cancer patients have few options regarding fertility preservation ("oncofertility") before treatment, and most involve invasive surgical procedures such as extraction of oocytes or ovarian tissue for cryopreservation, or IVF followed by embryo cryopreservation (Redig *et al.* 2011; Salama and Mallmann 2015; Kim *et al.* 2016). Not only are these invasive, but also they necessitate a delay in cancer treatment. An alternative is to co-administer drugs that protect oocytes from chemotherapy at the time of treatment. Based upon the knowledge that activation of the "TA" isoform of the DNA damage checkpoint gene *Trp63* (*TP63* in humans, also known as *p63;* the TA isoform of the protein will be referred to as TAp63) occurs via phosphorylation, the use of kinase inhibitors was suggested as a means to prevent radiation-induced oocyte loss in mice (Suh *et al.* 2006). It was reported (Gonfloni *et al.* 2009), but later challenged (Kerr *et al.* 2012) and counter-argued (Maiani *et al.* 2012), that the tyrosine kinase inhibitor imatinib (Gleevec) is effective in protecting oocytes. Even if imatinib proves to have such activity, it is a relatively promiscuous kinase inhibitor that blocks, among other targets, the receptor tyrosine kinase KIT that functions in germline stem cells (Lee and Wang 2009).

We previously reported that mouse CHK2 is a key component of the meiotic DNA damage checkpoint, and that deletion of *Chk2* prevented irradiation-induced killing of postnatal oocytes (Bolcun-Filas *et al.* 2014). We also showed that the CHK2 kinase phosphorylates both p53 (formally TRP53; TP53 in humans) and TAp63 in oocytes to activate these proteins (and stabilize p53). Therefore, the deletion of *Chk2* effectively impairs the activation of these two downstream effectors, which are both needed to trigger efficient oocyte elimination (Bolcun-Filas *et al.* 2014). More importantly, damaged oocytes that survived in the absence of CHK2 produced healthy pups suggesting that the inflicted DNA damage was repaired (Bolcun-Filas *et al.* 2014). The resistance of *Chk2*$^{-/-}$ oocytes to otherwise lethal levels of ionizing radiation (IR) prompted us to explore whether chemical inhibition of CHK2 would be effective at preventing radiation-induced oocyte death, and thus constitute a potential option for preserving ovarian function in women undergoing cancer therapy. Here we show that transient chemical inhibition of CHK2 suppresses follicle loss and allows for the production of healthy offspring.

3- Results and Discussion

Irradiation of ovaries induces CHK2-dependent phosphorylation of TAp63 in oocytes, and this phosphorylation is essential for triggering their death (Suh *et al.* 2006; Bolcun-Filas *et al.* 2014). CHK2 is a key component of the DNA damage response pathway that responds primarily to DNA double

strand breaks (DSBs), lying downstream of the apical kinase ATM (ataxia telangiectasia mutated). Members of the ATM>CHK2>p53/p63 pathway have been implicated as potential anti-cancer drug targets sensitizing cancer cells to genotoxic therapies, and chemical inhibitors have been developed against CHK2 (Garrett and Collins 2011), which, when deleted in mice, causes only minor phenotypic consequences (Takai *et al.* 2002). We therefore tested whether a well-characterized and highly specific (Arienti *et al.* 2005; Garrett and Collins 2011) CHK2 inhibitor 2-(4-(4-Chlorophenoxy)phenyl)-1H-benzimidazole-5-carboxamide-hydrate (designated “Chk2 inhibitor II” by the manufacturer, referred to hereafter as “CHK2iII”) could mimic the oocyte-protective effect of genetic *Chk2* deletion, and if it could do so in a non-toxic manner.

We employed an organ culture paradigm to control concentrations, penetration and timing of drug delivery to the ovary. Using dose ranges based upon published data (Arienti *et al.* 2005) and the manufacturer’s recommendations, we first tested the ability of CHK2iII to block phosphorylation of TAp63 in irradiated ovaries, and to block the stabilization of p53, which is normally rapidly degraded in cells unless stabilized by DNA-damage-induced phosphorylation by proteins including uding CHK2 (Chehab *et al.* 2000; Hirao *et al.* 2000). We used ovaries from 5 days postpartum (dpp) females to ensure that oocytes were in the dictyate arrest stage of meiosis, residing within primordial follicles. Explanted ovaries were cultured for two hours in the presence of 0, 10 or 20μM CHK2iII, then subjected (or not) to 3

Gy of IR, a level that not only kills oocytes, but also causes extensive p53 stabilization and TAp63 phosphorylation (Suh *et al.* 2006; Bolcun-Filas *et al.* 2014). Ovaries were harvested three hours later for protein extraction and western blot analysis. In non-irradiated ovaries, TAp63 remained unphosphorylated and p53 was undetectable (Fig. 2.1). Irradiation in the absence of inhibitor led to robust p53 stabilization, and all TAp63 was shifted to a higher mobility, which is known to be due to phosphorylation (Suh *et al.* 2006; Livera *et al.* 2008). Addition of 10μM and 20μM CHK2iII led to partial and complete inhibition of TAp63 phosphorylation, respectively, and also progressively decreased p53 levels (Fig. 2.1). This confirms that CHK2iII treatment rapidly acts to prevent activation of two pro-apoptotic factors in the ovary.

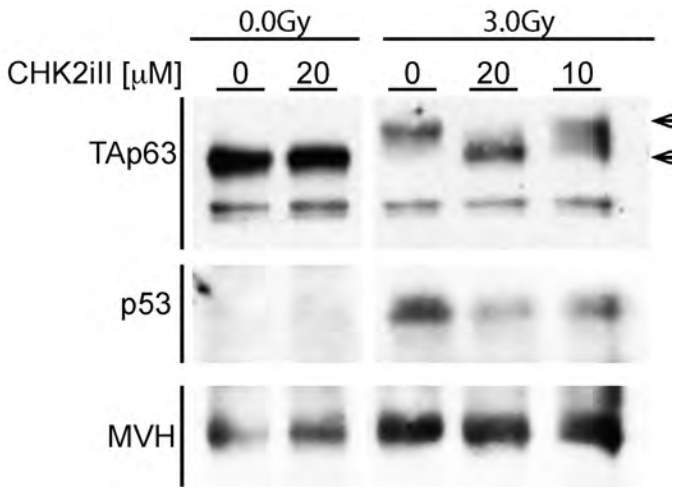
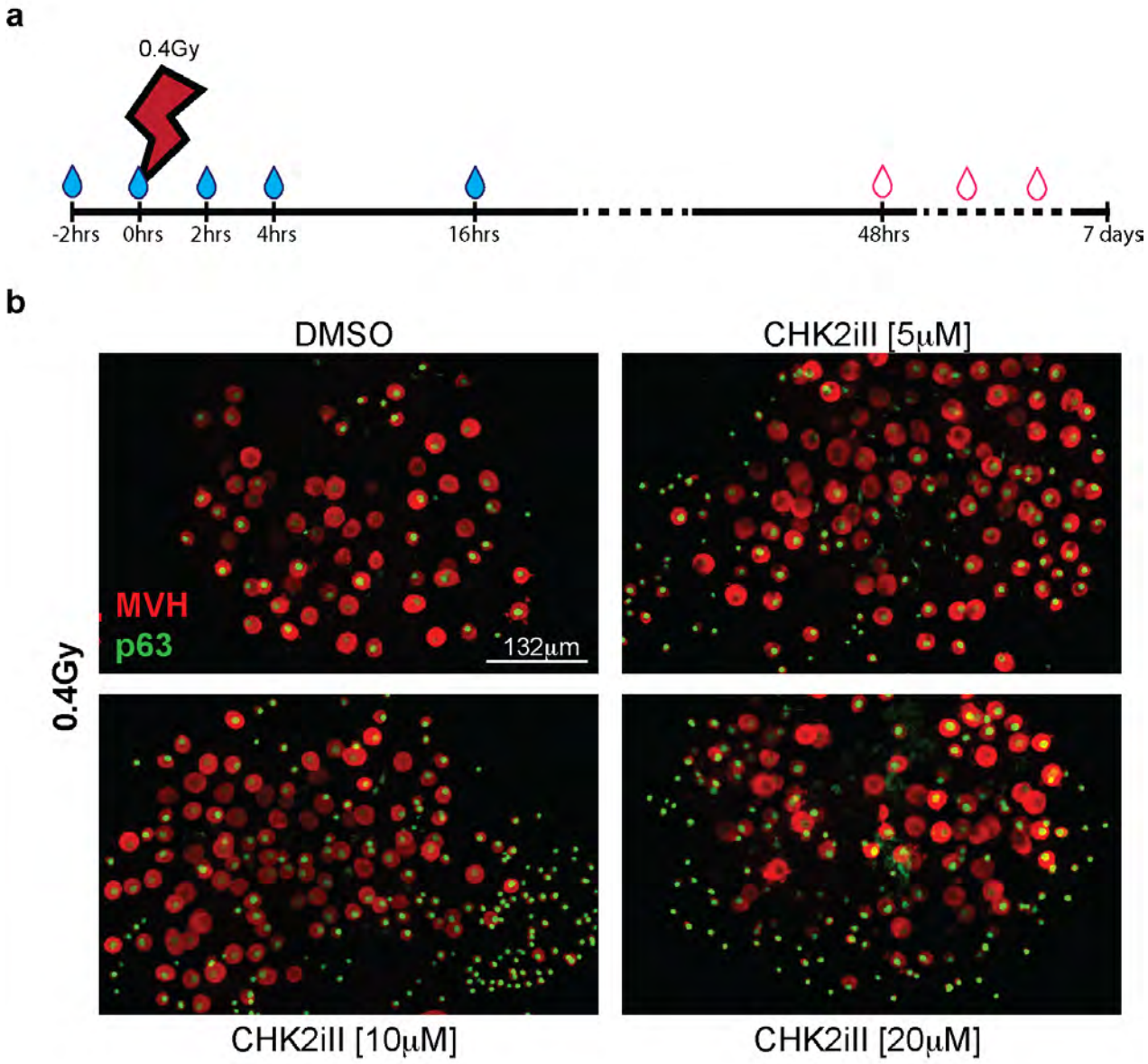


Figure 2.1) Inhibition of radiation-induced phosphorylation of p53 and TAp63 by CHK2iII. Western blot analysis of protein extracted from five dpp ovaries. Ovaries were incubated with the indicated concentrations of CHK2 inhibitor II (see Methods), and exposed or not to 3 Gy of γ-radiation. The immunoblot membrane was cut into two parts - one containing proteins >60 kDa, and the other <60kDa – and probed with anti-p63 and anti-p53, respectively. The >60 kDa portion was stripped and re-probed for the germ cell marker MVH. Arrowheads indicated the expected molecular weight of the phosphorylated form of TAp63 (upper), and non-phosphorylated TAp63 (lower).

Figure 2.2) Concentration-dependent protection of irradiated oocytes by CHK2iII. (**a**) Schematic of CHK2iII treatment regimen, beginning with placing explanted 5 dpp ovaries into culture. Blue droplets denote times at which fresh media containing CHK2iII was added/replaced. Red outlined droplets indicate changes with drug-free media. (**b**) Maximum intensity projections of immunostained ovary whole ovaries. For 3D visualization, see Supplementary movies M1, M2 and M3). The ovaries were cultured according to the timeline in "**a"** in the presence of the indicated concentrations of CHK2iII. DMSO corresponds to diluent control. MVH is a cytoplasmic germ cell protein, and p63 labels oocyte nuclei. Note that growing follicles (oocytes with larger MVH-stained cytoplasm) are relatively refractory to IR. (**c**) Quantification of follicles. Data points represent total follicle counts derived from one ovary. Horizontal hashes represent mean and standard deviation. Colors correspond to the different concentrations of inhibitors. Asterisks indicate p-value $\leqslant$ 0.0001 (Tukey HSD).


a
0.4Gy
-2hrs
0hrs
2hrs
4hrs
16hrs
48hrs
7 days
b
DMSO
CHK2iII [5μM]
MVH
p63
132μm
0.4Gy
CHK2iII [10μM]
CHK2iII [20μM]
c
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Follicles
300
250
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150
100
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20
CHK2iII[μM]

Next, we tested whether CHK2iII could permanently protect oocytes from a lower dose of radiation (0.4 Gy) that normally kills all oocytes within 2 days (Bolcun-Filas *et al.* 2014) but is far below levels (>5 Gy) lethal to whole animals. Ovaries were cultured in media supplemented with 0, 5, 10, or 20 μM of inhibitor for 2 hours before irradiation. Following IR exposure, ovaries were cultured 2 more days with media changes as delineated in Fig. 2.2a, after which the drug was removed from the medium. This protocol of media changes with drug replenishment was optimized for oocyte survival. Seven days after irradiation (and 5 days after removal of CHK2iII), oocyte survival was assessed by co-immunolabeling of histological sections (Fig. 2.3) and of whole mounts, with the cytoplasmic germ cell marker MVH and oocyte nuclear marker p63 (Figs. 2.2b, and Fig. 2.4). Under these conditions the inhibitor was well tolerated, and oocyte survival in unirradiated ovaries was not compromised (Fig. 2.4b).

Remarkably, though 10μM CHK2iII only partially inhibited TAp63 phosphorylation induced by 3Gy of IR (Fig. 2.1), it dramatically improved oocyte survival in ovaries exposed to 0.4Gy of IR, a level sufficient to trigger TAp63 phosphorylation and eliminate nearly all primordial follicles in ovaries (Fig 2.2b,c; Fig 2.5) (Bolcun-Filas *et al.* 2014). A small but significant protective effect was also observed with 5μM CHK2iII ($p=.004$, Fig. 2.2b,c).

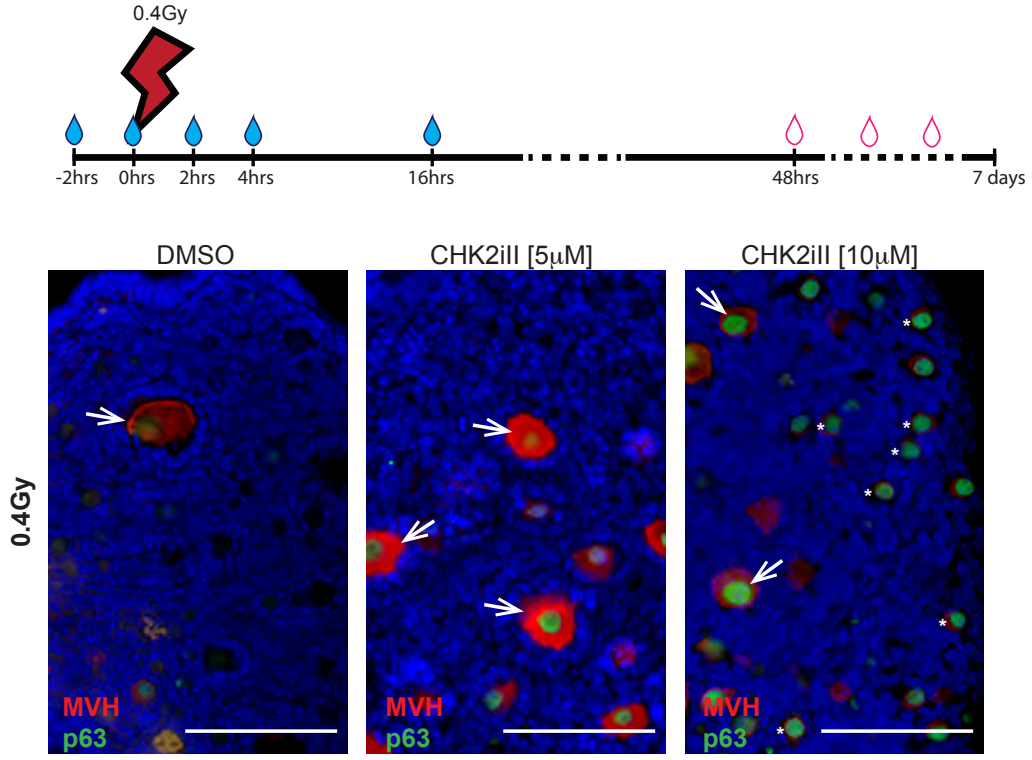

Figure 2.3) Representative immunofluorescence images of ovaries cultured according to the timeline in the presence of the indicated concentrations of CHK2iII. MVHis a cytoplasmic germ cell marker MVH, and p63 is a nuclear oocyte marker. Sections were counterstained with the chromatin-binding dye Hoechst. Asterisks indicate primordial follicles, and arrows growing follicles. The latter are generally refractory to IR. The treatment followed the same regimen as Fig 2.2a.

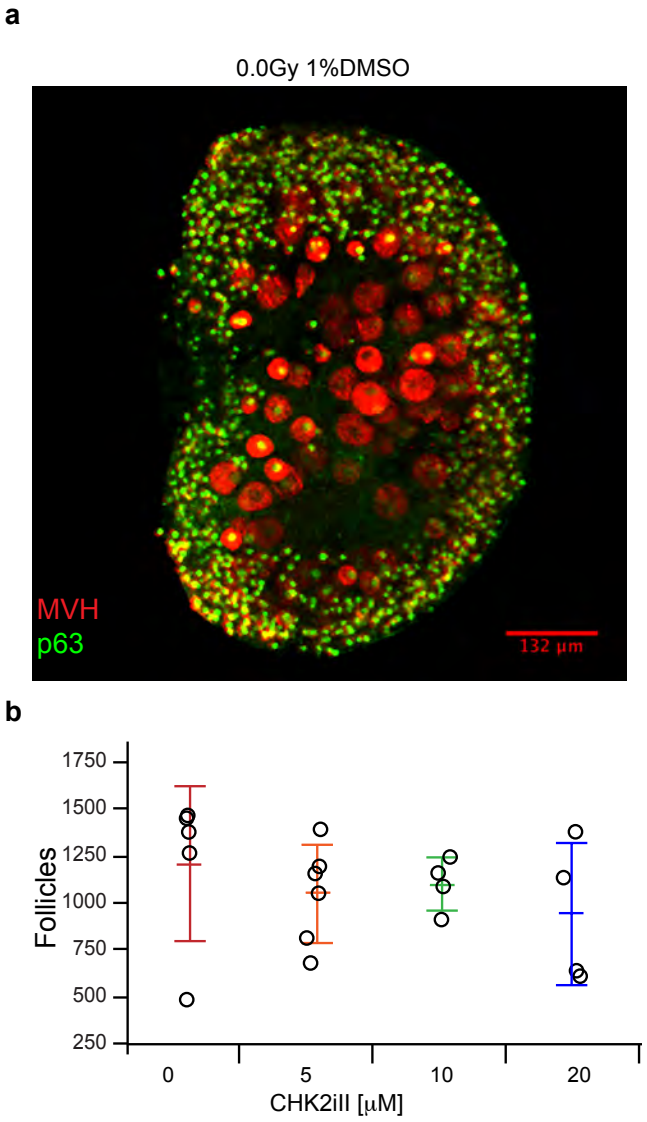

Figure 2.4) The inhibitor CHK2iII does not compromise follicle survival in non-irradiated control. (a) Whole mount maximum intensity projection of an ovary stained with MVH and p63. (b) Follicle counts of CHK2iII treated ovaries. There are no statistically significant differences between the inhibitor concentrations tested.

To assess if oocytes protected by CHK2iII from otherwise lethal levels of irradiation were capable of ovulation, fertilization, and subsequent embryonic development, we performed intrabursal transfers of irradiated ovaries (0.4 Gy) into histocompatible (strain C3H) agouti females. These recipient females were first sterilized at 7 dpp by exposure to 0.5Gy of IR. Once females were eight weeks old, sterility was verified by housing them with fertile males for at least eight weeks. The IR-induced oocyte death led to premature ovarian failure, yielding sufficient intrabursal space without the need to physically remove the vestigial ovaries before ovary transfer. These recipients were 16 weeks of age at the time of surgery. Three cultured ovaries, derived from black female animals also of strain C3H (see Methods for additional information), were placed into each bursa (total 6 ovaries per animal). Fig. 2.6a summarizes the experimental timeline. A total of 8 successful embryo transfer surgeries were completed. Three females received mock-treated (cultured in media containing 1% DMSO alone), irradiated ovaries (0.4 Gy), and five females received irradiated ovaries (also 0.4 Gy) treated with 10μM CHK2iII in media containing 1% DMSO.

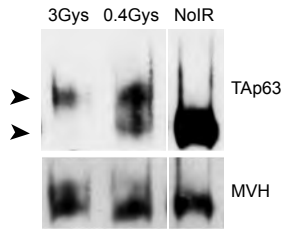

Figure 2.5) Western blot analysis of ovaries showing that 0.4Gys activates the DNA damage checkpoint.

Once the transferred donor ovary reached 8 weeks of age (with respect to the time at which it was explanted), the recipient females were mated to proven fertile C3H black (*a/a*) males for three months, and monitored for litters and the coat colors of offspring. Only females that received CHK2iII-treated ovaries delivered progeny, all of which were black, confirming that they were produced from fertilization of oocytes ovulated from donor ovaries (Fig. 2.6b and c). All offspring had no visible abnormalities that would suggest inheritance of gross chromosomal abnormalities (Bolcun-Filas *et al.* 2014). The viability of these animals indicated that even though oocytes have sensitive checkpoint mechanisms rendering them vulnerable to low levels of DNA damage, they are capable of repairing damaged DNA in a manner compatible with normal embryogenesis. Consistent with the IR-induced oocyte death in ovaries not treated with CHK2iII, and also the absence of progeny from females receiving such ovaries, postmortem inspection revealed only residual ovaries in these recipients compared to those mice that received the CHK2iII-treated ovaries (Fig. 2.7).

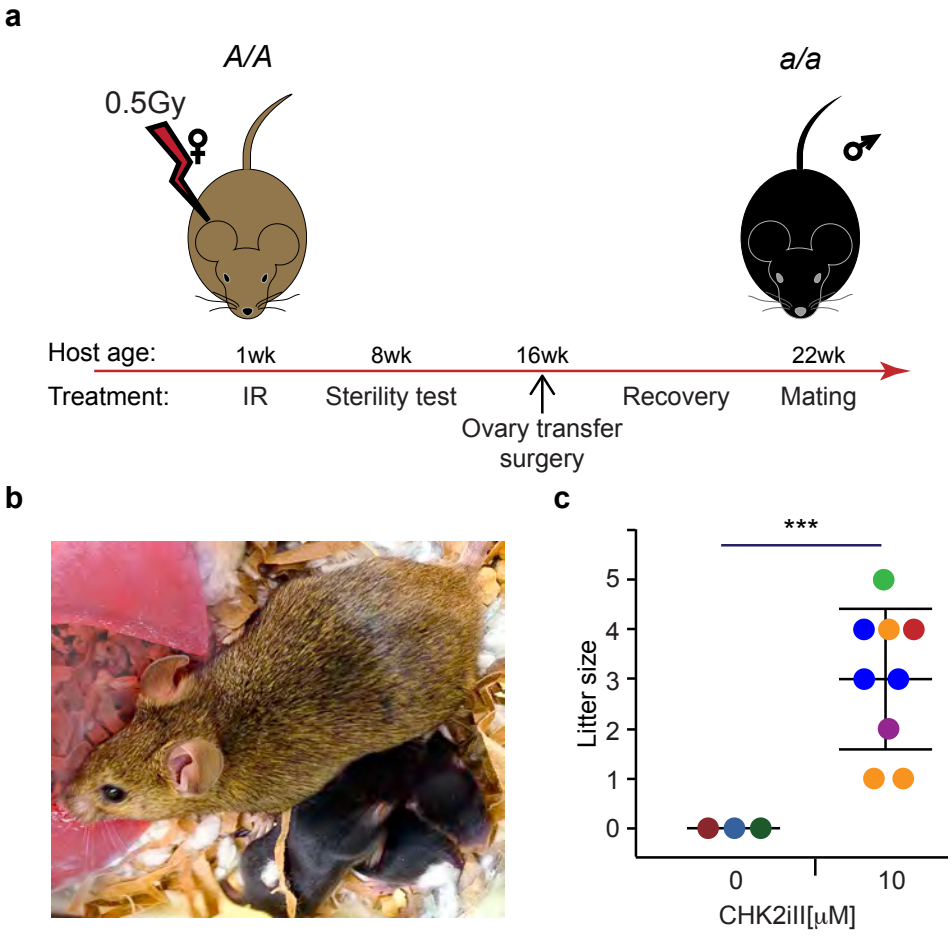

Figure 2.7) CHK2iII-rescued ovaries are fertile. (**a**) Experimental timeline. Agouti females (*A/A*) were sterilized with 0.5Gy of IR at one week of age. The transplanted ovaries were from black donor females (*a/a*) treated as outlined in Fig.2. 2a. (**b**) Agouti host females gave birth to black offspring (*a/a*) exclusively; thus, the ovulated eggs produced were from the donor ovaries. (**c**) Litter sizes of mock-treated and treated ovaries. Each circle represents a litter and the circle's color represents the female that generated that litter. The combined average litter size produced by all host females was 3.

Our results provide proof-in-principle for the strategy of targeting the CHK2-dependent DNA damage checkpoint pathway for preventing loss of the ovarian reserve - and thus ovarian failure - in cancer patients undergoing therapies that are toxic to oocytes. Importantly, checkpoint inhibitors have already been explored as potential anticancer therapies, thus substantial information is already available on members of this drug class (Antoni *et al.* 2007; Garrett and Collins 2011). Nevertheless, it remains to be seen whether systemic administration of CHK2iII or other CHK2 inhibitors can achieve similar oocyte-protective efficacy against IR- or drug-induced DSBs *in vivo*, and whether they will be effective for both pre-pubertal and adult females. Additionally, it will be important to conduct more thorough studies of potential genetic risks associated oocytes rescued from DNA damage-induced death by checkpoint inhibition.

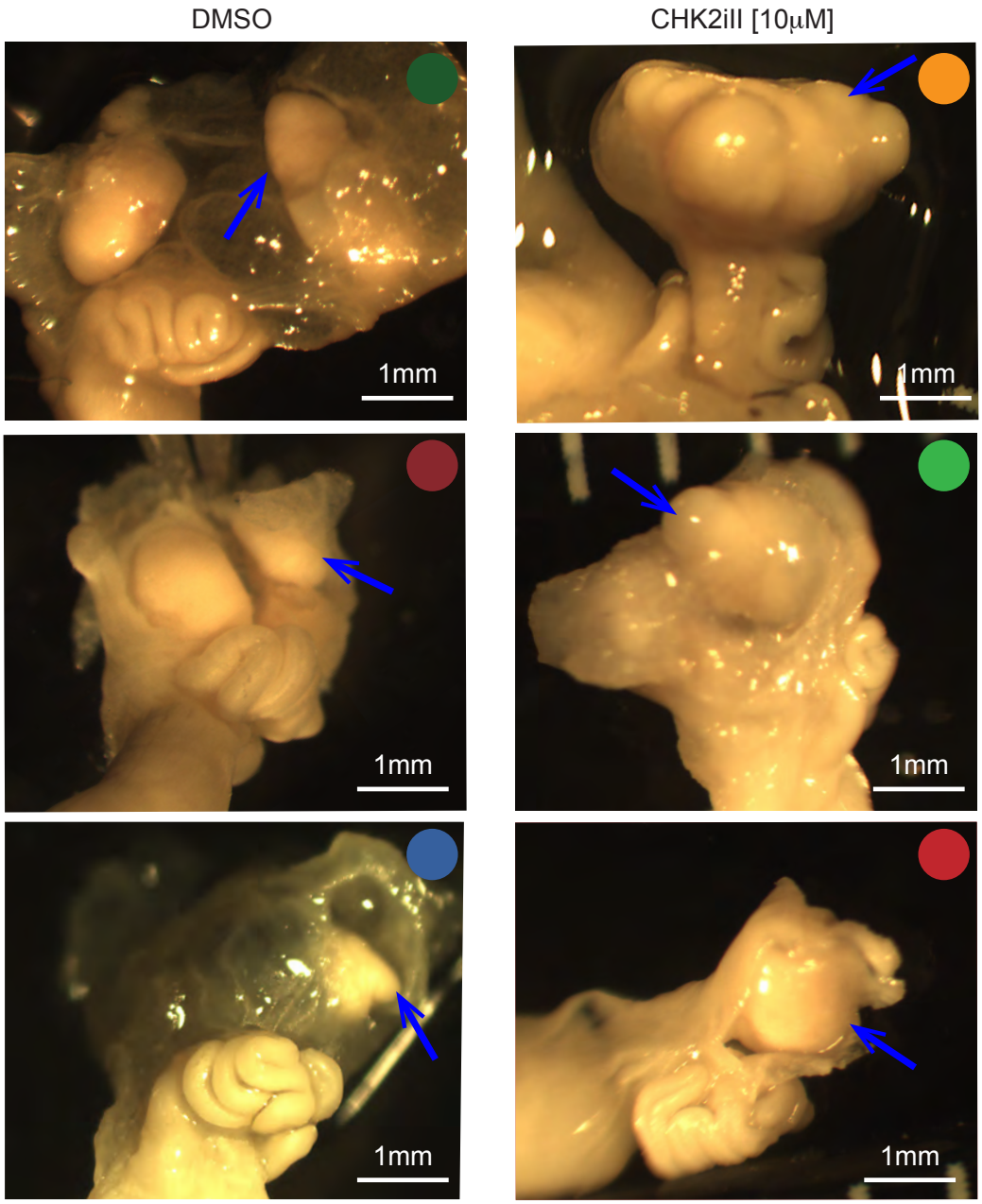

Figure 2.8) Representative images of ovaries from females that underwent ovary transfer surgery. Blue arrow points to the ovaries deemed as coming from the donor mice. Color circles correspond to females represented on figure 2.7c.

4- Material and Methods

Mice

Mice were obtained from The Jackson Laboratory, strains C3HeB/FeJ, stock # 000658 (agouti mice, homozygous dominant for coat color (*A/A*)) and C3FeLe.B6-a/J, stock # 000198 (black mice, recessive for coat color, (*a/a*)). Cornell's Animal Care and Use Committee approved all animal usage, under protocol 2004-0038 to JCS.

Organ Culture

Ovaries were cultured using an adaptation of a published method (Livera *et al.* 2008). Briefly, ovaries were collected from five day postpartum (dpp) C3FeLe.B6-a/J mice, and, following removal from the bursa, placed into cell culture inserts (Millicell; pore size: 8μm; diameter: 12mm) pre-soaked in ovary culture media: MEM supplemented with 10% FBS, 25mM HEPES pH=7.0, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizone, 1% DMSO, and CHK2 inhibitor. The inserts were placed into 24 well plates (Model MD24 ThermoFisher) with carriers for the inserts. Sufficient media was added to keep organs moist, but not completely submerged. Organs were incubated at 37°C, 5% CO_2 and atmospheric O_2.

Drug Treatment

CHK2iII (CalBiochem 220486) was prepared as 1mM and 2mM stock solutions in DMSO and kept frozen at -20°C. Media containing the desired concentration of inhibitor was prepared shortly before use, assuring that the DMSO concentration was constant (1% DMSO) throughout the different conditions. Explanted ovaries were pre-incubated for 2 hours in warm (37°C) media containing the desired concentration of inhibitor or 1% DMSO alone before being subjected to ionizing radiation in a ^{137}cesium irradiator with a rotating turntable. Figure 2.2a presents the media change regimen, with the first replacement being immediately after irradiation. The ovaries were cultured for either 3 hours before being processed for western blot analysis (to detect DNA damage responses), or 7 days followed by either fixation and immunostaining (to quantify oocyte survival), or ovary transplant surgery into sterile agouti females.

Western Blot Analyses and Antibodies

Ovary protein lysates, immunoblotting, probing and detection were conducted as described (Bolcun-Filas *et al.* 2014). Primary antibodies and dilutions used were: mouse anti-p63 (1:500, 4A4, Novus Biologicals); rabbit anti-p53 (1:300, Cell Signaling #9282); mouse anti-β-actin (1:5000, Sigma) and rabbit anti-MVH (1:1000, Abcam). Secondary antibodies used were: Immuno Pure goat anti-mouse IgG(H+L) peroxidase conjugate (1:5000, ThermoFisher) and goat anti-rabbit IgG HRP-linked antibody (1:5000, Cell Signaling).

Immunofluorescence

Cultured ovaries were fixed in 4% paraformaldehyde/PBS at 4°C overnight, and then washed and stored in 70% ethanol. Ovaries were either embedded in paraffin and sectioned at 5μm for immunostaining or subjected to whole mount immunostaining and clearing. For the standard immunofluorescence, slides were deparaffinized and re-hydrated prior to antigen retrieval using sodium citrate buffer. Slides were blocked with 5% goat serum (PBS/Tween 20), incubated at 4°C overnight with aforementioned primary antibodies (anti-p63 @ 1:500; anti-MVH @ 1:1000), and subsequently incubated with Alexa Fluor® secondary antibodies for one hour and Hoechst dye for 5 minutes. Slides were mounted with ProLong Anti-fade (Thermo-Fisher) and imaged.

Ovary transfer surgery and postmortems

Agouti females were sterilized with 0.5Gy of IR at 1 week of age. At 8 weeks of age, females were housed with males known to be fertile. Eight weeks later, and 2 days prior to ovary transfer surgery, the males were removed. Three ovaries, either treated or not with CHK2iII, were placed in the intrabursal space of each ovary (total of 6 ovaries per recipient female). The females were allowed a recovery period of 6 weeks, then housed with males. Three to four months later, upon euthanasia, dissection was performed for visual inspection of the transplantation sites.

Whole organ immunofluorescence

Ovaries cultured for seven days were fixed in freshly prepared 4%paraformaldehyde (PFA) /PBS at 4°C overnight (ON). Afterwards, tissues were washed and stored in 70% ethanol at 4°C until further processing.

Fixed ovaries were washed and left to equilibrate for a minimum of four hours in PBS before initializing whole-mount immunostaining protocol. In order to facilitate handling and tissue integrity, ovaries were kept in the culture insert throughout all the procedure. The ovaries were treated for four hours in permeabilization solution (PBS, 0.2% Polyvynal alcohol (PVA), 0.1% NaBH4-solution (Sigma) and 1.5% Triton X-100), than incubated for 24 hours in blocking solution (PBS, 0.1% Triton X-100, 0.15% Glycine pH7.4, 10% normal goat serum, 3% BSA, 0.2% sodium azide and 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Fungizone). All the immunostaining and clearing was performed at room temperature (RT) with gentle rocking. Antibodies were diluted to appropriate concentration in the blocking solution. Primary antibodies (mouse anti-p63 (1:500, 4A4, Novus Biologicals); and rabbit anti-MVH (1:600, Abcam)) were incubated for four days. Afterwards, ovaries were washes with washing solution (PBS, 0.2% PVA and 0.15% triton X-100) for 10 hours than two times of two hours. Secondary antibodies (1:1000 Alexa Fluor® secondary antibodies) were incubated for three days in a vial protected from light. Ovaries were washes with washing solution for three times of 12 hours (if needed DAPI 50ng/ml was added to the first wash).

Clearing, imaging, and oocyte quantification

Immunostained ovaries were cleared with modified, freshly-prepared ScaleS4(0) reagent (40% D-(-)-sorbitol (w/v), 10% glycerol, 4M urea, 20% dimethylsufoxide, pH8.1 (Hama *et al.* 2015), gently mixed by inversion at 50°C for 30min and degassed prior to use). Solution was refreshed twice daily until tissue became transparent (usually two days). The insert was placed on top of a glass slide, and the membrane containing the cultured ovaries was carefully removed with a fine tip scalpel and placed on the slide. Slides were imaged on an upright laser scanning Zeiss LSM880 confocal/multiphoton microscope, using a 10X NA 0.45 water immersion objective. For proper image stitching the adjacent images (tiles) were overlapped by 20%. The z-steps were set for 5μm between optical sections. Images were reconstructed, visualized and analyzed using Fiji-ImageJ (Schindelin *et al.* 2012).

Movies were made using the 3D project feature of Fiji-ImageJ (Schindelin *et al.* 2012). Oocyte quantification was performed in flattened maximum intensity projections of the Z-stacks image-series, using the "analyze particle" feature of Fiji-ImageJ.

Statistical analysis

Statistical analyses were done using JMP Pro12 software (SAS Inc., Cary, NC-USA, version 12.0.1). Fertility was analyzed using a mixed model with mother as random effect and ovary treatment as fixed effect. Least

square (LS) means difference between litter sizes derived from treated vs non-treated ovaries was performed using the Student's t test. LS mean differences between follicle counts from the different treatment groups were tested using Tukey honest significance different (HSD).

5- Authors contributions

V.D.R. performed experiments and contributed to the writing of the paper. H.K. and R.M. performed experiments. E.B.F and J.C.S. supervised the work and contributed to the writing.

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CHAPTER 3

THE DNA DAMAGE CHECKPOINT ELIMINATES MOUSE OOCYTES WITH CHROMOSOME SYNAPSIS FAILURE

* This chapter is a reprint with minor reformatting of the manuscript accepted at Mol. Cel.: Vera D. Rinaldi , Ewelina Bolcun-Filas, Hiroshi Kogo, Hiroki Kurahashi, and John C. Schimenti. “The DNA damage checkpoint eliminates mouse oocytes with chromosome synapsis failure”.

At the time of approval of this dissertation the DOI for this manuscript was not available. However, a previous version of the manuscript can be found at BioRXiv under the title “A Single Checkpoint Pathway Eliminates Mouse Oocytes With DNA Damage Or Chromosome Synapsis Failure”.

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1- Abstract

Pairing and synapsis of homologous chromosomes during meiosis is crucial for producing genetically normal gametes, and is dependent upon repair of SPO11-induced double strand breaks (DSBs) by homologous recombination. To prevent transmission of genetic defects, diverse organisms have evolved mechanisms to eliminate meiocytes containing unrepaired DSBs or unsynapsed chromosomes. Here, we show that the CHK2 (CHEK2)-dependent DNA damage checkpoint culls not only recombination-defective

mouse oocytes, but also SPO11-deficient oocytes that are severely defective in homolog synapsis. The checkpoint is triggered in those oocytes that accumulate a threshold level of spontaneous DSBs (~10) in late Prophase I, the repair of which is inhibited by presence of HORMAD1/2 on unsynapsed chromosome axes. Furthermore, *Hormad2* deletion rescued fertility of oocytes containing a synapsis-proficient, DSB repair-defective mutation in a gene (*Trip13*) required for removal of HORMADs from synapsed chromosomes, suggesting that many meiotic DSBs are normally repaired by intersister recombination in mice.

2- Introduction

Genome maintenance in germ cells is critical for fertility, prevention of birth defects, and the genetic stability of species. Throughout mammalian germ lineage development, from primordial germ cells (PGCs) through completion of meiosis, there are mechanisms that prevent transmission of gametes with genetic defects. Indeed, mutation rates in germ cells are far lower than in somatic cells (Murphey et al. 2013, Conrad et al. 2011, Stambrook and Tichy 2010). This is reflected by the exquisite sensitivity of PGCs to mutations in certain DNA repair genes (Agoulnik et al. 2002, Luo et al. 2014, Nadler and Braun 2000) (Watanabe et al. 2013), resting oocytes to clastogens such as radiation and chemotherapeutics (Perez et al. 1997,

Maltaris et al. 2007, Suh et al. 2006), and developing prophase I meiocytes to genetic anomalies including a modicum of DNA damage (Meirow and Nugent 2001, Suh et al. 2006) or the presence of a single asynapsed chromosome or even a chromosomal subregion (Burgoyne and Baker 1985, Homolka, Jansa, and Forejt 2012).

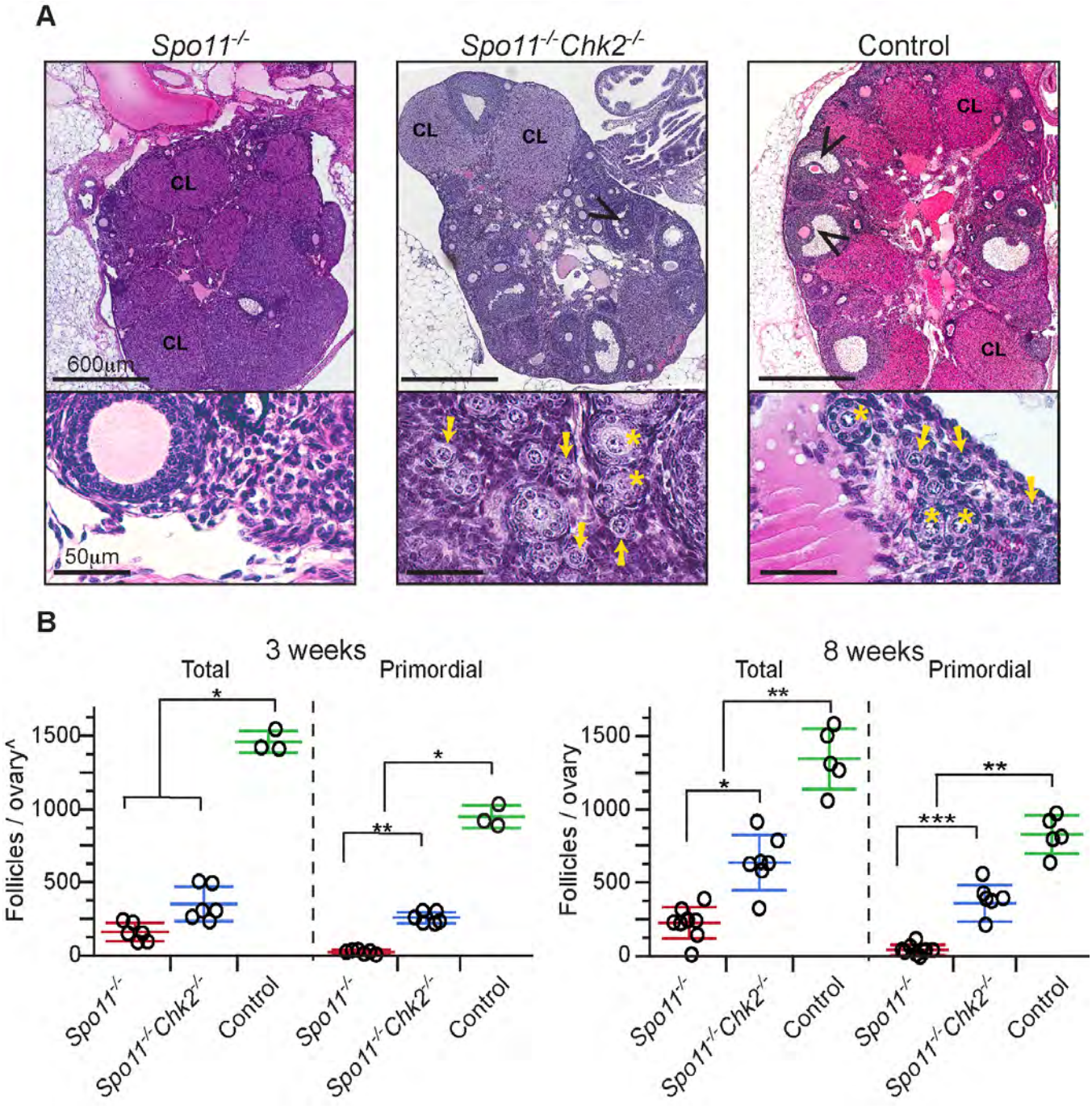
Genetic and developmental analyses of mouse mutants have suggested there are at least two distinct checkpoints during meiotic prophase I in oocytes, one that monitors DSB repair, and another that monitors synapsis. Oocytes defective for either synapsis or DSB repair are eliminated with different dynamics and severity. Females with mutations causing pervasive asynapsis alone (e.g. *Spo11*$^{-/-}$) are born with a grossly reduced oocyte pool. The surviving oocytes undergo folliculogenesis but are reproductively inviable, becoming exhausted within a few weeks by atresia and ovulation (Di Giacomo et al. 2005). Oocytes defective in DSB repair alone (*Trip13*$^{Gt/Gt}$), or defective in both synapsis and meiotic DSB repair (e.g. *Dmc1*$^{-/-}$; *Msh5*$^{-/-}$), are virtually completely eliminated between late gestation and weaning age by the action of a DNA damage checkpoint (Li and Schimenti 2007, Di Giacomo et al. 2005). Furthermore, genetic ablation of meiotic DSB formation confers a *Spo11*$^{-/-}$ -like phenotype to such DSB repair mutants, consistent with the existence of separate DNA damage and synapsis checkpoints (Li and Schimenti 2007, Di Giacomo et al. 2005, Finsterbusch et al. 2016, Reinholdt

and Schimenti 2005). For DSB repair, CHK2 (checkpoint kinase 2) signaling to TRP53/TAp63 is crucial for eliminating *Trip13*$^{Gt/Gt}$ mutant oocytes that exhibit full chromosome synapsis but have unrepaired SPO11-induced DSBs (Bolcun-Filas et al. 2014). Interestingly, *Chk2* deficiency imparted a *Spo11* null-like phenotype upon *Dmc1*$^{-/-}$ ovaries, consistent with separate, sequentially-acting checkpoints (Bolcun-Filas et al. 2014). Genetic evidence for a distinct synapsis checkpoint came from studies of mice lacking HORMAD1 or HORMAD2, proteins which load onto axes of meiotic chromosomes throughout early prophase I, but are removed upon synapsis (Wojtasz et al. 2009). Ablation of either HORMADs in mice prevented loss of SPO11-deficient oocytes, resulting in the persistence of a [nonfertile] primordial follicle reserve in adults (Daniel et al. 2011, Wojtasz et al. 2012, Kogo, Tsutsumi, Inagaki, et al. 2012). These data suggested that the HORMADs are components of a synapsis checkpoint pathway. Another mechanism for elimination of oocytes is related to the phenomenon of MSUC (meiotic silencing of unsynapsed chromatin). Though not formally a checkpoint, the transcriptional inactivation of a chromosome containing genes essential for oocyte survival and development can block progression past diplonema (Cloutier et al. 2015).

Whereas these lines of evidence support the existence of separate checkpoints monitoring DNA damage and synapsis, studies in non-

mammalian organisms indicate that the “pachytene checkpoint” – a term referring to delayed progression of meiosis or death of meiocytes triggered by genetic aberrations present in late pachynema – is more complex, consisting of both distinct and overlapping signaling pathways that also impact DNA repair modalities such as choice of recombination partner for the repair of meiotic DSBs (e.g. sister chromatid vs. homolog) (Roeder and Bailis 2000, Subramanian and Hochwagen 2014, MacQueen and Hochwagen 2011, Joshi et al. 2015). Here, we report the results of a series of experiments designed to discriminate whether the pachytene checkpoint in mouse oocytes indeed consists of distinct pathways responding to different signals, or if the responses are integrated into a single checkpoint pathway. Using a variety of mouse mutants, we show that most oocytes which are highly defective for chromosome synapsis accumulate spontaneous DSBs at a level that can trigger the CHK2-dependent DNA damage signaling pathway, leading to their elimination. Additionally, we present evidence that the reason asynaptic *Spo11*$^{-/-}$ oocytes can be rescued by HORMAD1/2 deficiency is that their absence disrupts the so-called barrier to sister chromatid recombination (BSCR), enabling intersister (IS) repair of those spontaneous DSBs. Taken together, we propose that the “pachytene checkpoint” consists primarily of a canonical damage signaling pathway, and that extensive asynapsis leads to

Figure 3.1) CHK2 is required for efficient elimination of asynaptic *Spo11*$^{-/-}$ mouse oocytes. (A) H&E stained histological sections of 8 weeks old ovaries. Black arrowheads indicate antral follicles. CL= Corpus Luteum; the presence of corpora lutea are indicate of prior rounds of ovulation. The lower portion of each panel contains a higher magnification image of an ovarian cortical region, where primordial follicles primarily reside. Yellow arrows and stars indicate primordial and primary follicles, respectively. **(B)** Follicle counts from ovaries of indicated genotypes at 3 and 8 weeks postpartum, respectively. Each data point is from a single ovary, each being from a different animal. Total = all follicle types. Horizontal hashes denote mean and standard deviation. Littermate controls included animals with the following genotypes: *Spo11*$^{+/+}$*Chk2*$^{+/+}$, *Spo11*$^{+/-}$ *Chk2*$^{+/-}$, *Spo11*$^{+/-}$ *Chk2*$^{+/-}$ and *Spo11*$^{+/+}$*Chk2*$^{-/-}$. The values obtained for the 3 weeks follicles/ovaries counts are not comparable to the 8 weeks (see methods). Asterisks indicate p-values: (*) 0.005 ≤ p-values ≤ 0.05, (**)0.001 ≤ p-values ≤ 0.005 and (***) p-values ≤ 0.001 derived from a non-parametric, one-way ANOVA test (Kruskal-Wallis).


A
Spo11-/-
Spo11-/-Chk2-/-
Control
CL
CL
CL
CL
CL
600µm
50µm
B
3 weeks
8 weeks
Total
Primordial
Follicles / ovary^
Follicles / ovary
1500
1000
500
0
Spo11-/-
Spo11-/-Chk2-/-
Control

oocyte loss by inhibiting IS repair rather than triggering a distinct "synapsis checkpoint."

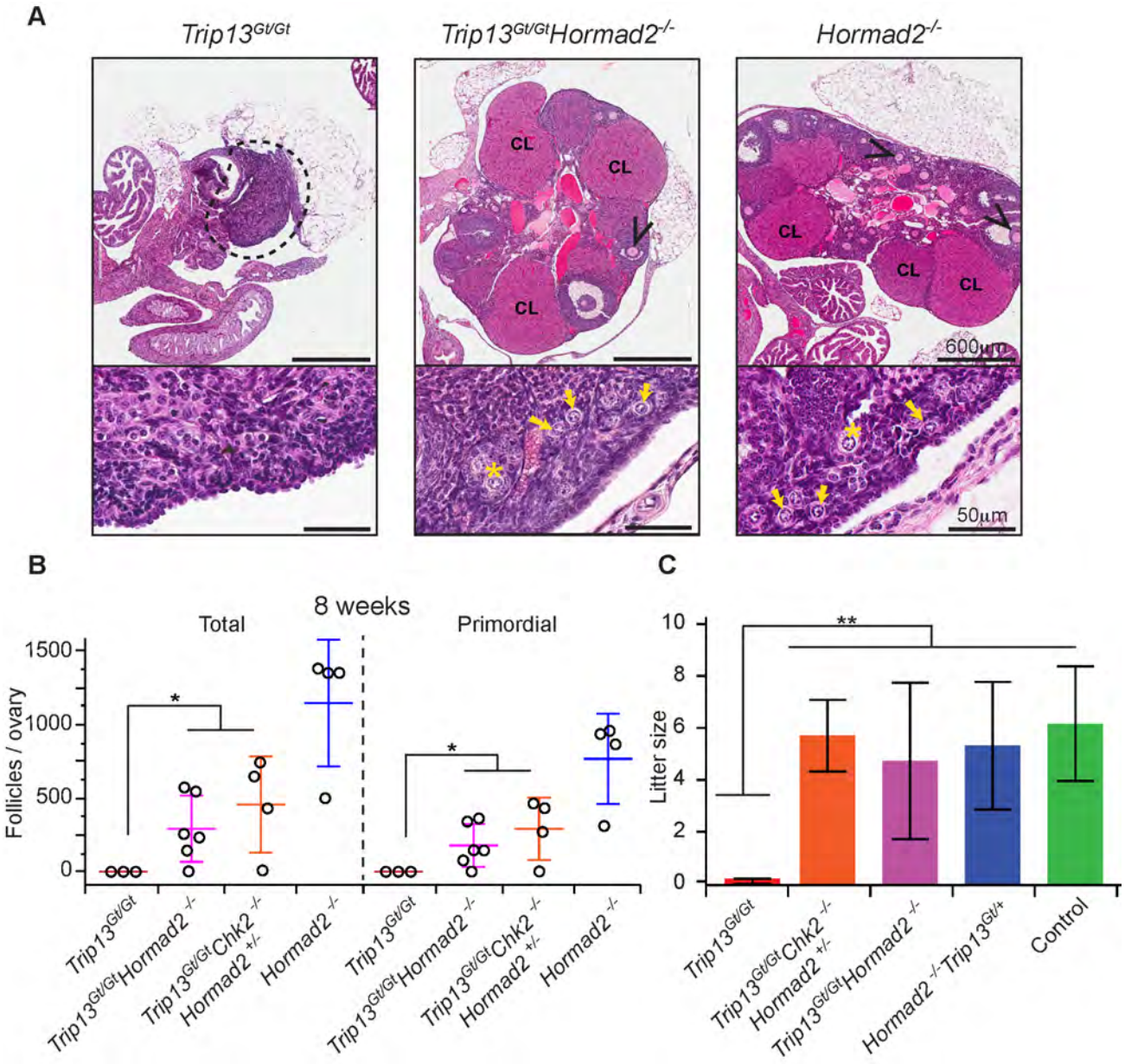
3- Results

CHK2 is involved in the elimination of *Spo11*$^{-/-}$ oocytes

To investigate potential overlap in the meiotic DSB repair and synapsis checkpoint pathways in mice, we tested whether CHK2, a well-defined DSB signal transducer, contributes to the elimination of *Spo11*$^{-/-}$ oocytes that are asynaptic due to lack of programmed meiotic DSBs needed for recombination-driven homolog pairing. Consistent with prior reports (Baudat et al. 2000, Di Giacomo et al. 2005), we observed a greatly reduced number of total follicles in 3 week postpartum (pp) *Spo11*$^{-/-}$ ovaries compared to WT, and in particular, the oocyte reserve (pool of primordial resting follicles) was almost completely exhausted by 8 weeks of age (Fig. 3.1). Surprisingly, *Chk2* deletion rescued the oocyte reserve (Fig. 3.1A,B), albeit not to WT levels. The rescued follicles in double mutant females persisted robustly at least until 6 months pp (in one case, 554 total follicles in a single ovary).

HORMAD2 deficiency prevents elimination of *Trip13* mutant oocytes that have complete synapsis but unrepaired meiotic DSBs, restoring female fertility

Figure 3.2) Synapsis-competent *Trip13*$^{Gt/Gt}$ oocytes are eliminated in a HORMAD2-dependent manner. (**A**) H&E stained histological sections of 8 week old ovaries of indicated genotypes. Black arrowheads indicate antral follicles. CL= Corpus Luteum. The lower half of each panel shows a higher magnification of cortical regions of ovaries. Yellow arrows and stars indicate primordial and primary follicles, respectively. (**B**) Follicle quantification of 8 week old ovaries. Each data point is from a single ovary, each being from a different animal. “Total” = all follicle types. Horizontal hashes denote mean and standard deviation. The statistic used was Kruskal-Wallis. * indicates p-value = 0.002. (**C**) Graphed are mean litter sizes. N ≥ 3 females tested for fertility per genotypic group. Control matings were between mice with the genotypes *Trip13*$^{Gt/+}$ and *Trip13*$^{Gt/+}$ *Hormad2*$^{+/-}$. Error bars represent standard deviation and ** indicates p-value ≤ 0.005 derived from the Kruskal-Wallis test.


A
Trip13Gt/Gt
Trip13Gt/GtHormad2-/-
Hormad2-/-
CL
CL
CL
CL
CL
CL
600μm
50μm
B
8 weeks
Total
Primordial
Follicles / ovary
1500
1000
500
0
*
*
Trip13Gt/Gt
Trip13Gt/GtHormad2-/-
Trip13Gt/GtChk2-/-Hormad2+/-
Hormad2-/-
C
Litter size
10
8
6
4
2
0
**
Trip13Gt/Gt
Trip13Gt/GtChk2-/-Hormad2+/-
Trip13Gt/GtHormad2-/-
Hormad2-/-Trip13Gt/+
Control

Taken alone, the rescue of *Spo11*$^{-/-}$ oocytes by *Chk2* deletion suggests that severe asynapsis leads to CHK2 activation and signaling to mediate oocyte elimination. This led us to postulate that either: 1) CHK2 is a common component of otherwise distinct synapsis and DNA damage checkpoints, or 2) that there is a single linear checkpoint pathway that responds to both asynapsis and DNA damage, and that DNA damage activates the checkpoint pathway more robustly or sooner in prophase I (thus accounting for the different patterns of oocyte elimination in asynaptic vs. DSB repair-deficient oocytes mentioned above (Di Giacomo et al. 2005)).

We reasoned that if there is a single linear checkpoint pathway, then putative synapsis checkpoint genes required to eliminate *Spo11*$^{-/-}$ oocytes would also be required to eliminate *Trip13*$^{Gt/Gt}$ oocytes. *Trip13*$^{Gt/Gt}$ meiocytes have synapsed chromosomes and persistent SPO11-dependent DSBs, which leads to neonatal depletion of follicles in a CHK2>TRP53/TAp63 pathway-dependent manner (Fig. 3.2A)(Li and Schimenti 2007, Bolcun-Filas et al. 2014). To test this, we determined whether deficiency of HORMAD2, a putative synapsis checkpoint protein, could rescue *Trip13*$^{Gt/Gt}$ oocytes. HORMAD2 and its paralog HORMAD1 are “HORMA” (Hop1, Rev7 and Mad2) domain-containing proteins orthologous to the *Saccharomyces cerevisiae* synaptonemal complex (SC) axial element protein Hop1p, and deletion of either prevents elimination of *Spo11*$^{-/-}$ oocytes (Daniel et al. 2011, Wojtasz et

al. 2012, Kogo, Tsutsumi, Inagaki, et al. 2012). We used a mutant of *Hormad2* rather than *Hormad1*, because deletion of the latter disrupts recombination and homolog synapsis (Daniel et al. 2011, Kogo, Tsutsumi, Ohye, et al. 2012, Shin et al. 2010). Remarkably, not only did ovaries of 2 month old *Trip13*$^{Gt/Gt}$ *Hormad2*$^{-/-}$ mice retain a substantial primordial follicle pool (Fig. 3.2A,B), but also these females were fertile (Fig. 3.2C). The rescued fertility of these oocytes suggested either that these DSBs were compatible with further oocyte maturation, or that they were eventually repaired as in the case of *Trip13*$^{Gt/Gt}$ females whose fertility was restored by *Chk2* ablation (Bolcun-Filas et al. 2014). The dynamics of DSB repair are addressed below.

Since TRIP13 is required for removal of the HORMADs from chromosome axes upon synapsis (Wojtasz et al. 2009), and persistence of HORMADs on unsynapsed chromosomes correlates with MSUC-mediated silencing of essential genes (Wojtasz et al. 2012, Cloutier et al. 2015), the question arises as to whether *Trip13*$^{Gt/Gt}$ oocytes are eliminated not because of unrepaired DSBs, but rather by transcriptional silencing. However, this is unlikely for the following reasons. First, *Trip13*$^{Gt/Gt}$ oocytes are depleted with a temporal pattern and degree consistent with mutants defective in DSB repair, not asynapsis (Li and Schimenti 2007, Di Giacomo et al. 2005). Second, *Spo11* is epistatic to *Trip13*, in that *Trip13*$^{Gt/Gt}$ *Spo11*$^{-/-}$ ovaries resemble *Spo11* single mutants in their pattern of oocyte elimination (Li and Schimenti

2007), demonstrating that unrepaired meiotic DSBs drive early culling of *Trip13* mutant oocytes. Third, HORMAD persistence on synapsed *Trip13*$^{Gt/Gt}$ or unsynapsed *Spo11*$^{-/-}$ meiotic chromosome axes is not affected by *Chk2* deletion (Fig. 3.3), which might be predicted if CHK2 was rescuing either mutant class by disrupting the ability of HORMADs to signal asynapsis. The latter is further supported by the fact that CHK2 depletion does not interfere with MSCI (meiotic sex chromosome inactivation, which is mechanistically similar or identical to MSUC) in males (Pacheco et al. 2015), and that *Chk2*$^{-/-}$ mice are fertile unlike *Hormad1*$^{-/-}$ animals (Daniel et al. 2011, Shin, McGuire, and Rajkovic 2013, Kogo, Tsutsumi, Ohye, et al. 2012)

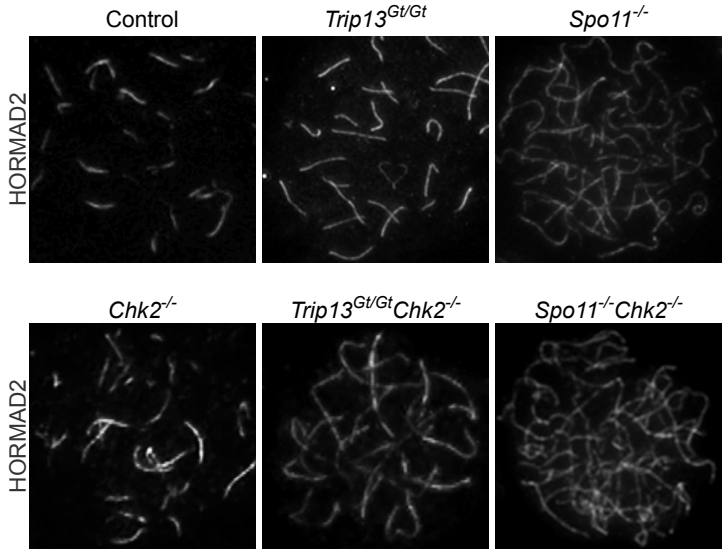


Figure 3.3) CHK2 is not required for HORMAD localization to meiotic chromosomes axes of *Spo11*$^{-/-}$ or *Trip13*$^{-Gt/Gt}$ oocytes. Meiotic spreads of 18.5dpc oocytes from different mutants showing presence of HORMAD2 on unsynapsed axis.

HORMAD2 inhibits DSB repair in prophase I oocytes

That HORMAD2 deficiency could rescue both *Trip13*$^{Gt/Gt}$ and *Spo11*$^{-/-}$ oocytes is consistent with a single checkpoint capable of detecting both damaged DNA and asynapsed chromosomes. If there is indeed a single checkpoint pathway, then combined deficiency for CHK2 and HORMAD2 should rescue asynaptic and DSB repair-defective *Dmc1*$^{-/-}$ oocytes to the same degree as deficiency for either one alone. However, *Dmc1*$^{-/-}$ *Chk2*$^{-/-}$ *Hormad2*$^{-/-}$ females had ≥3 fold increase in primordial and total follicles compared to *Dmc1*$^{-/-}$ *Hormad2*$^{-/-}$ or *Dmc1*$^{-/-}$ *Chk2*$^{-/-}$ ovaries (Fig. 3.4A,B). This lack of epistasis indicates that HORMAD2 and CHK2 are not functioning solely as members of a single linear checkpoint pathway sensing either or both asynapsis and DNA damage.

We therefore considered two alternative explanations for why *Hormad2* deficiency rescues *Trip13*$^{Gt/Gt}$ oocytes: 1) HORMAD2 deficiency reduces the number of SPO11-induced DSBs to a level sufficient for synapsis, but below the threshold for checkpoint activation; and/or 2) it facilitates DSB repair. Studies of related proteins support both explanations. Absence of the budding yeast ortholog Hop1p not only decreases meiotic DSB formation, but also increases use of the sister chromatid as a template for HR repair

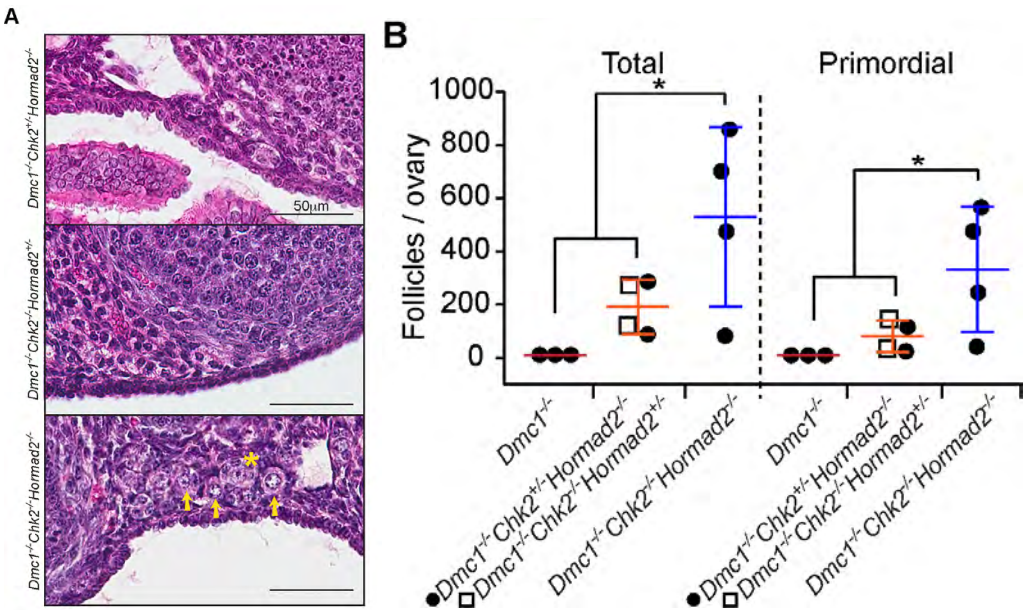
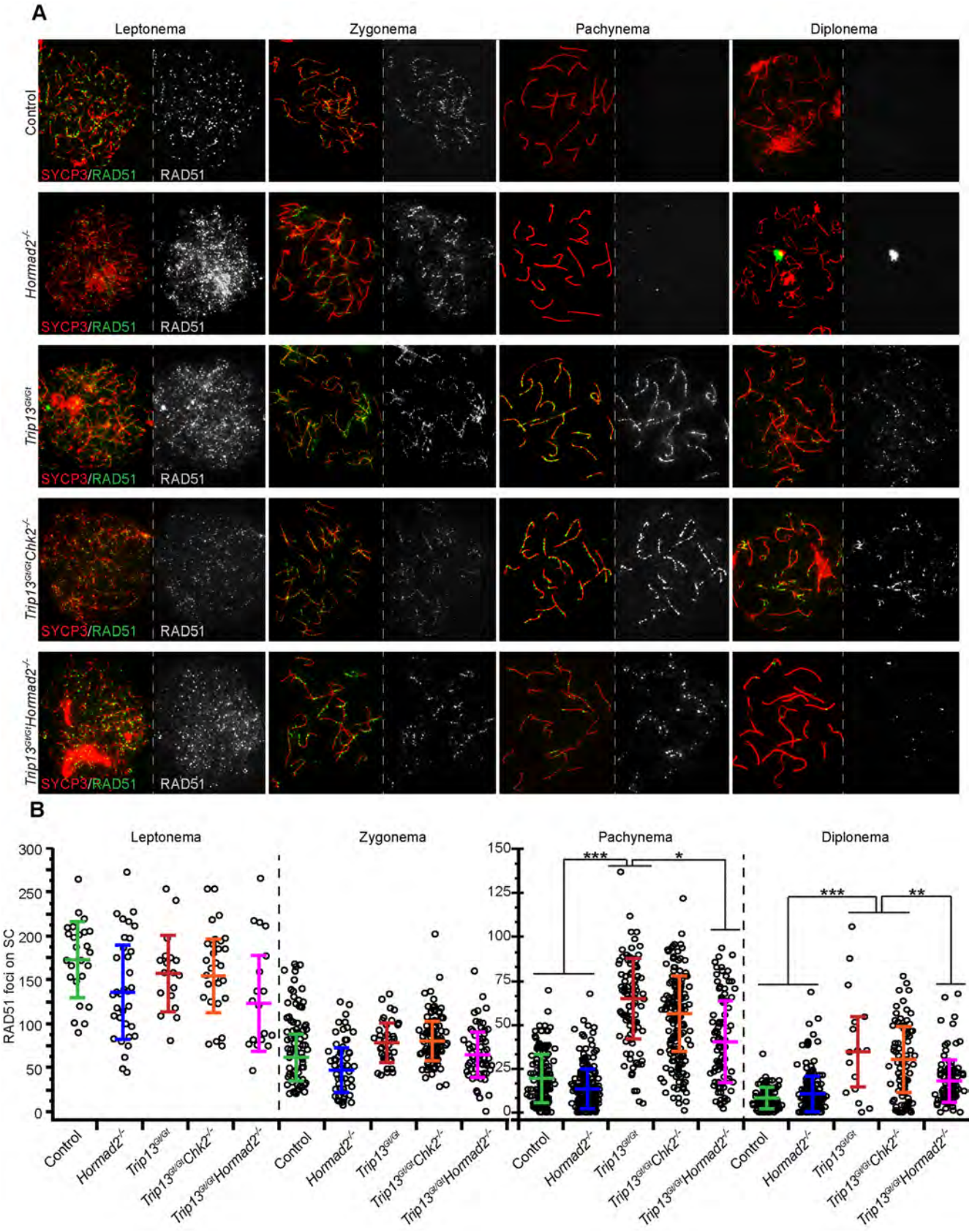


Figure 3.4) HORMAD2 and CHK2 are not in the same checkpoint pathway. (**A**) H&E stained histological sections of cortical regions of 8 week old mutant mouse ovaries, where primordial follicles are concentrated. Primordial follicles, which constitute the oocyte reserve, are indicated by yellow arrows, and a primary follicle by a star. Residual *Dmc1*$^{-/-}$ ovaries are not represented because they are completely devoid of oocytes (Pittman et al. 1998). (**B**) Follicle counts from ovaries of indicated genotypes at 8 weeks of age. "Total" = all types of follicles. Data points represent follicle counts derived from one ovary, each ovary originating from a different animal. Asterisk indicates p-value ≤ 0.05 (Kruskal-Wallis test).

(Mao-Draayer et al. 1996, Carballo et al. 2008, Lam and Keeney 2014, Schwacha and Kleckner 1997, Niu et al. 2005, Latypov et al. 2010). Mouse HORMAD1 is required for loading HORMAD2 onto unsynapsed axes, proper SC formation (Daniel et al. 2011), and normal levels of meiotic DSBs (Daniel et al. 2011, Stanzione et al. 2016). Whereas *Dmc1*$^{-/-}$ *Hormad1*$^{-/-}$ or irradiated *Hormad1*$^{-/-}$ oocytes exhibit fewer DSB markers than oocytes containing HORMAD1 (Shin et al. 2010, Daniel et al. 2011), this can be attributable largely to enhanced repair (Shin, McGuire, and Rajkovic 2013). Intersister (IS) HR repair of DSBs in *S. cerevisiae* is substantial and it increases in *hop1* mutants (Goldfarb and Lichten 2010). Moreover, disruption of SC axes in mice (by deletion of *Sycp2* or *Sycp3*) appears to alter recombination partner choice in favor of the sister chromatid, decreasing persistent DSBs in *Trip13*$^{Gt/Gt}$ oocytes to a degree that diminishes their elimination (Li, Bolcun-Filas, and Schimenti 2011). These data led us to hypothesize that the rescue of *Trip13* mutant oocytes by *Hormad2* deficiency was due to increased DSB repair, possibly by diminishing the BSCR.

To test this, we quantified levels and rates of meiotic DSB repair in various genotypes of prophase I oocytes. Whereas the number of leptotene and zygotene stage RAD51 foci was not significantly different in *Trip13*$^{Gt/Gt}$ *Hormad2*$^{-/-}$ oocytes compared to *Trip13*$^{Gt/Gt}$ or other control and mutant genotypes (Fig. 3.5A,B), there were significantly fewer compared to *Trip13*$^{Gt/Gt}$

Figure 3.5) Depletion of HORMAD2 accelerates DSB-repair during early stages of meiotic prophase I. (**A**) Representative images of meiotic chromosome spreads from oocytes at substages of meiotic prophase I, probed with antibodies for SYCP3 (SC axis protein) and the DSB marker RAD51. Oocytes were isolated from female embryos ranging from 15.5 dpc to newborns. (**B**) Numbers of RAD51 foci in meiotic prophase I substages of mutants. Only RAD51 foci present on SYCP3 stained axes were scored. Each data point represents one cell. In each genotypic group, at each stage, the counts are derived from at least three animals. Horizontal hashes in summary statistic plots denote mean and standard deviation from a mixed model calculation (Table S1). Colors correspond to genotypes. Asterisks indicate statistical significant differences between groups in terms of the least square means of RAD51 foci. p-values: *** $p \leq 0.001$; ** $p \leq 0.005$; * $p \leq 0.05$ (Tukey HSD).


A
Leptonema
Zygonema
Pachynema
Diplonema
Control
Hormad2-/-
Trip13Gt/Gt
Trip13Gt/GtChk2-/-
Trip13Gt/GtHormad2-/-
SYCP3/RAD51
RAD51
B
RAD51 foci on SC
Leptonema
Zygonema
Pachynema
Diplonema
***
*
***
**
Control
Hormad2-/-
Trip13Gt/Gt
Trip13Gt/GtChk2-/-
Trip13Gt/GtHormad2-/-

by pachynema and diplonema ($p = 0.02$ and 0.03, respectively, using Tukey HSD in a mixed model). RAD51 levels in *Trip13*$^{Gt/Gt}$ and *Trip13*$^{Gt/Gt}$ *Chk2*$^{-/-}$ newborn oocytes remained high in diplonema compared to all other genotypes (Fig. 3.5A,B), presumably reflecting a relative deficiency in DSB repair. Furthermore, we found that RAD51 foci induced by 2Gy of ionizing radiation (IR) disappeared more rapidly in *Spo11*$^{-/-}$ *Hormad2*$^{-/-}$ oocytes than either *Spo11*$^{-/-}$ or *Spo11*$^{-/-}$ *Chk2*$^{-/-}$ oocytes, as assessed 8 hours after treatment (Fig. 3.6). Overall, the data indicate that HORMAD2 on the axes of either asynapsed (*Spo11*$^{-/-}$) or synapsed (*Trip13*$^{Gt/Gt}$) (Wojtasz et al. 2009) meiotic chromosomes inhibits DSB repair, most likely by promoting IS recombination.

Evidence that CHK2-mediated elimination of asynaptic oocytes is driven by accumulation of SPO11-independent DSBs

If indeed *Hormad2* deletion rescues DSB-containing oocytes by weakening or eliminating the BSCR, this raises the question as to why HORMAD2 deficiency rescues *Spo11*$^{-/-}$ oocytes that don't make meiotic DSBs. A clue comes from the surprising observation that *Spo11*$^{-/-}$ oocytes sustain DSBs of unknown origin (but possibly from LINE-1 retrotransposon activation) during early pachynema (Malki et al. 2014, Carofiglio et al. 2013). We hypothesized that these DSBs occur at levels sufficient to trigger the CHK2-dependent checkpoint in *Spo11*$^{-/-}$ oocytes, but that in the absence of SC axis-

bound HORMAD2, there is sufficient DSB repair to prevent checkpoint activation. To test this, we determined the threshold number of DSBs that kills WT and *Chk2*$^{-/-}$ oocytes by exposing explanted newborn ovaries to a range of IR. RAD51 foci on chromosome axes accumulated roughly linearly in oocytes exposed to 0.5 - 9Gy (Fig. 3.7A), and *Chk2*$^{-/-}$ oocytes withstood up to 7Gy (Fig. 3.7B), a dosage that induces 73.3 RAD51 foci (Fig. 3.7A). In contrast, as little as 0.3Gy (10.3 foci by linear regression) abolished the entire primordial follicle pool of WT ovaries. Consistent with our hypothesis that HORMAD2 prevents DSB repair, the SC axes of *Spo11*$^{-/-}$ zygotene/pachytene-like chromosomes in newborn oocytes contained far more discrete RAD51 foci (raw average of 39.8; likely an underestimate, see Fig. 3.8) than in *Spo11*$^{-/-}$ *Hormad2*$^{-/-}$ oocytes (avg. 7.3 foci), the latter being almost identical to WT or *Chk2*$^{-/-}$ oocytes (7.5 and 7.3 respectively; Fig. 3.7C) in which HORMAD2 has been removed from synapsed chromosomes. These data indicate that the majority of *Spo11*$^{-/-}$ oocytes (60.8%) bear a level of DSBs (>10.3 foci) sufficient to trigger their elimination by the CHK2-dependent DNA damage checkpoint, while most WT oocytes (71%) are below this threshold.

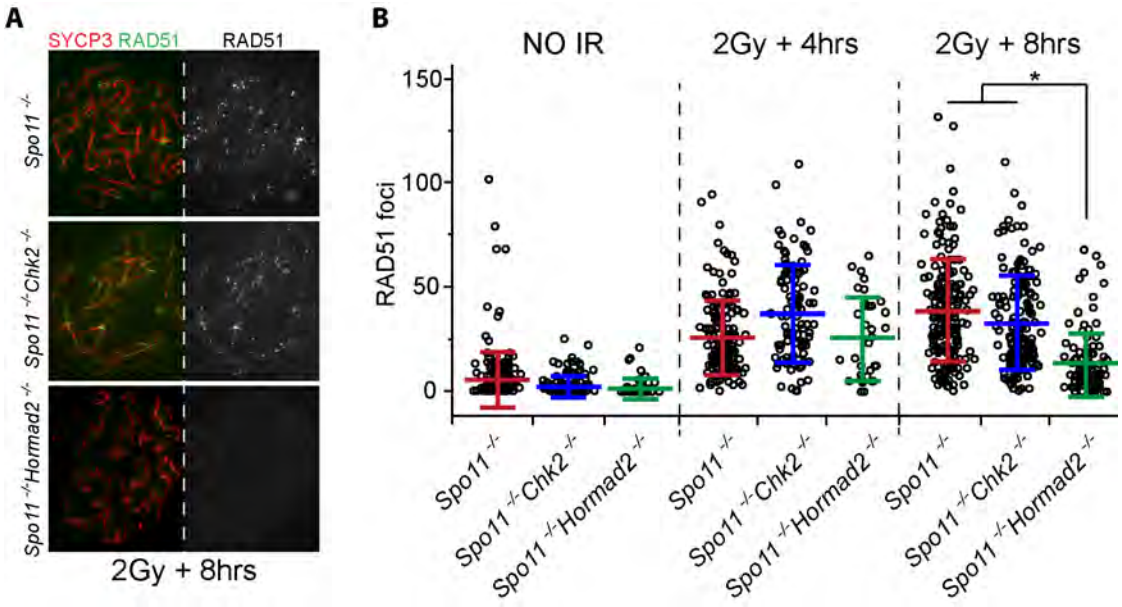


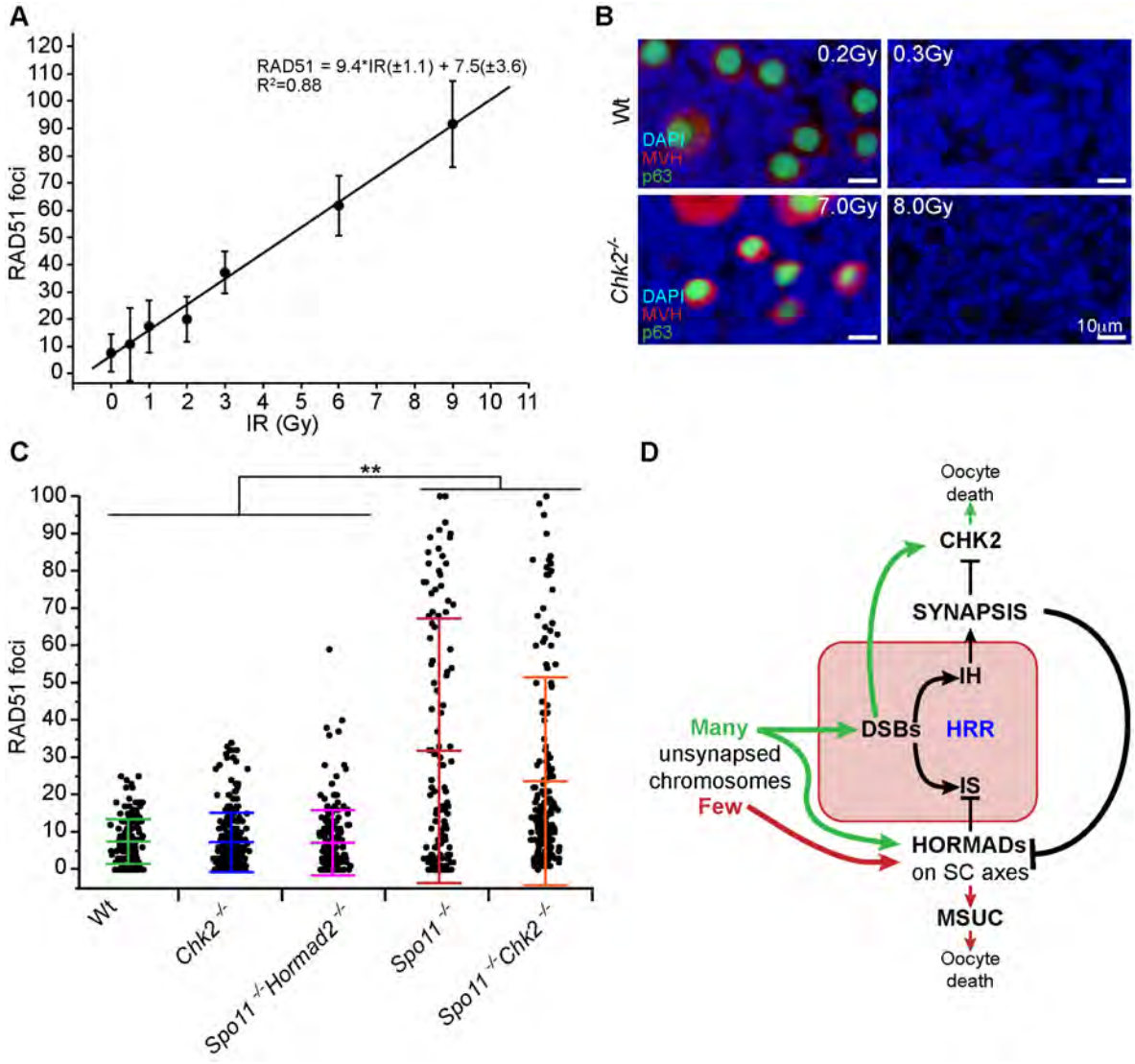
Figure 3.6) Depletion of HORMAD2 accelerates repair of induced DSBs in oocytes. Fetal ovaries were collected at 15.5dpc, cultured 24 hours, exposed to 2 Gy of ionizing radiation (IR), then cultured for an additional 4-8 hours. (**A**) Immunolabeling of surface spread chromosomes from oocytes collected 8hrs after IR. (**B**) Quantification of RAD51 foci. Each data point represents one oocyte. The graphs include mean and standard deviation, and are color coded according to genotypic group. The 4 and 8 hr unirradiated samples were combined. Data were derived from at least two different animals per condition.

4- Discussion

Meiocytes have genetic quality control mechanisms that respond to their unique developmental circumstances, chromosome biology and cell cycle. For example, the pachytene/prophase I checkpoint is active only at a point in prophase I at which DSBs have normally been repaired, but not during the time between programmed DSB formation and HR repair. While the oocyte "pachytene checkpoint" is distinct with respect to its cell cycle timing and its ability to monitor an event (chromosome synapsis) unique to meiosis, our current and prior (Bolcun-Filas et al. 2014) work indicate that for circumstances involving extensive asynapsis and DNA damage, this checkpoint in oocytes involves a DNA damage response (DDR) common to somatic cells. Our surprising finding that the DDR is involved in culling of *Spo11*$^{-/-}$ oocytes raises the question of how SPO11-independent DSBs - first reported by Carofiglio *et al* (Carofiglio et al. 2013) and confirmed here - arise on unsynapsed chromosomes. One possible source is LINE-1 retrotransposon activation, which has been correlated with natural oocyte attrition (Malki et al. 2014). However, transposon expression normally occurs only transiently at the onset of meiosis before epigenetic silencing (van der Heijden and Bortvin 2009). It is possible that the extensive asynapsis in *Spo11*$^{-/-}$ oocytes *per se*, or disruption of the meiotic program including the normal course of DSB induction and repair, interferes with transposon silencing. Another possibility is that

Figure 3.7) DNA damage threshold required to trigger oocyte death, and evidence for HORMAD-mediated inhibition of IS repair . (**A**) Linear regression for conversion of radiation dosages to RAD51 focus counts. Meiotic surface spreads were made from WT neonatal ovaries 2.5 hrs after IR. Plotted are means with standard deviations. Each IR dose has focus counts from ~25 oocytes derived from each of a total of 18 animals. (**B**) *Chk2*$^{-/-}$ oocytes are highly IR resistant. Shown are immunofluorescence images of ovarian sections labeled with nuclear and cytoplasmic germ cell markers (p63 and MVH, respectively). (**C**) RAD51 focus foci counts from newborn oocyte spreads regardless of stage. Only oocytes with discrete patterns of RAD51 foci were scored, as defined in Fig. 3.8. Data points represent individual oocytes, derived from at least five different animals from each genotypic group. Horizontal hashes denote means and standard deviations calculated using a mixed model with individual animals as a variable. Asterisks indicate statistically significant differences between groups with p-values: *** $p \leq 0.001$; ** $p \leq 0.005$; * $p \leq 0.05$ (Tukey HSD). See Table S3 for raw data and statistical calculations. (**D**) Model for pachytene checkpoint activation in mouse oocytes. Oocytes with many unsynapsed chromosomes (green) ultimately accumulate DSBs, which cannot be repaired due to block to IS recombination imposed by HORMADs on asynapsed axes. Failure of DSB repair leads to activation of CHK2 and downstream effector proteins (p53/TAp63) that trigger

apoptosis. Few asynapsed chromosomes (red) lead to inactivation of essential genes by MSUC thereby causing oocyte death. HRR - Homologous Recombination Repair; IH - Interhomolog; IS - Intersister; MSUC - Meiotic Silencing of Unsynapsed Chromatin.



unsynapsed chromosomes are more susceptible to spontaneous breakage. These outcomes could be exacerbated by extended retention of HORMADs on unsynapsed axes, inhibiting repair of these breaks. An intriguing question is whether the production of these SPO11-independent DSBs, whatever their origin, evolved as a contributory mechanism for genetic quality control. It is also conceivable that the extended presence of HORMADs themselves contributes to spontaneous DSB formation, possibly as a "last ditch" mechanism to drive pairing or synapsis in chromosomes devoid of sufficient interhomolog recombination events.

The late appearance and highly variable number (Fig. 3.7C) of SPO11-independent DSBs in *Spo11*$^{-/-}$ oocytes may explain the differences in timing and extent of oocyte elimination in exclusively asynaptic *vs.* DSB repair-deficient (e.g. *Dmc1*, *Trip13*) mutants. As reported by Di Giacomo and colleagues (Di Giacomo et al. 2005), whereas *Dmc1*$^{-/-}$ oocytes were completely eliminated before dictyate arrest and follicle formation, *Spo11*$^{-/-}$ ovaries contained ~15-20% of WT numbers of follicles (including 27 fold less primordial follicles by 4 days pp); this reduced oocyte reserve was depleted by 2-3 months of age by subsequent cycles of recruitment and maturation. Additionally, *Dmc1*$^{-/-}$ oocytes degenerate before *Spo11*$^{-/-}$ oocytes, suggesting that an earlier-acting mechanism was triggering *Dmc1*$^{-/-}$ oocyte death. These

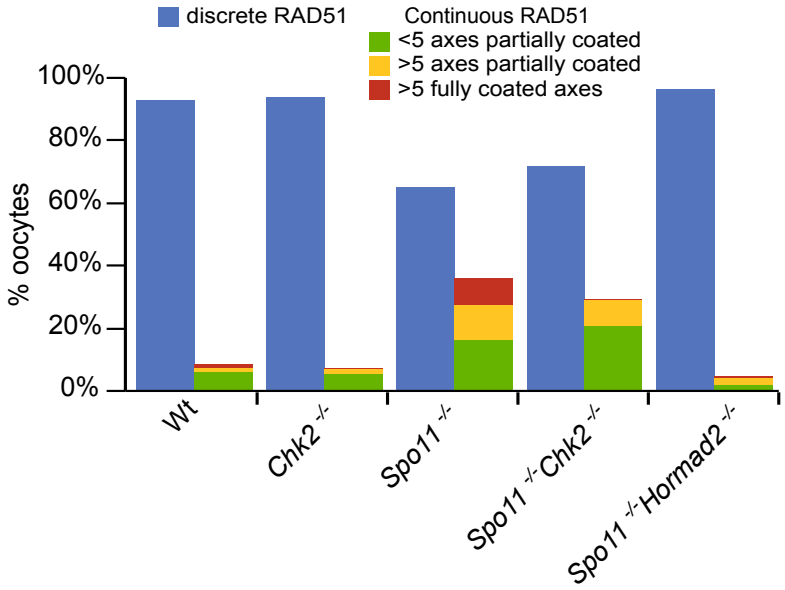
distinctions, in conjunction with epistasis analysis of mutants doubly deficient for *Spo11* and DSB repair mutations, led to the conclusion that there are DSB-dependent and -independent mechanisms to eliminate defective oocytes. We suggest that the difference in timing of oocyte elimination, at least in part, may be related to the DSB load. The abundant SPO11 DSBs formed early in prophase I may trigger the checkpoint sooner and more uniformly in recombination mutants that fail to reduce DSB levels in a timely manner. According to this scenario, spontaneous DSBs that don't arise until later stages of [abnormal] prophase I in *Spo11*$^{-/-}$ oocytes would trigger the DNA damage checkpoint at a later point. Based on our data (Fig. 3.7A), we suggest that those oocytes with below-threshold DSB levels escape the DNA damage checkpoint, and are either eliminated by other mechanisms (see below) or survive to constitute the reduced follicular reserve in *Spo11* mutants.

While the CHK2-dependent checkpoint is of central importance to genetic quality control in oocytes, our observations that *Chk2* deletion does not fully restore oocyte numbers to WT levels in mutants indicates that it is not absolutely required for eliminating all oocytes with unrepaired DSBs. Rather, the fraction of oocytes rescued is inversely related to the burden of unrepaired meiotic DSBs. For example, whereas *Chk2* deficiency rescued nearly 1/3 of *Trip13*$^{Gt/Gt}$ oocytes (which are partially proficient for DSB repair and which harbor 35±4 and 63±4.7 persistent RAD51 foci in diplonema and pachynema,

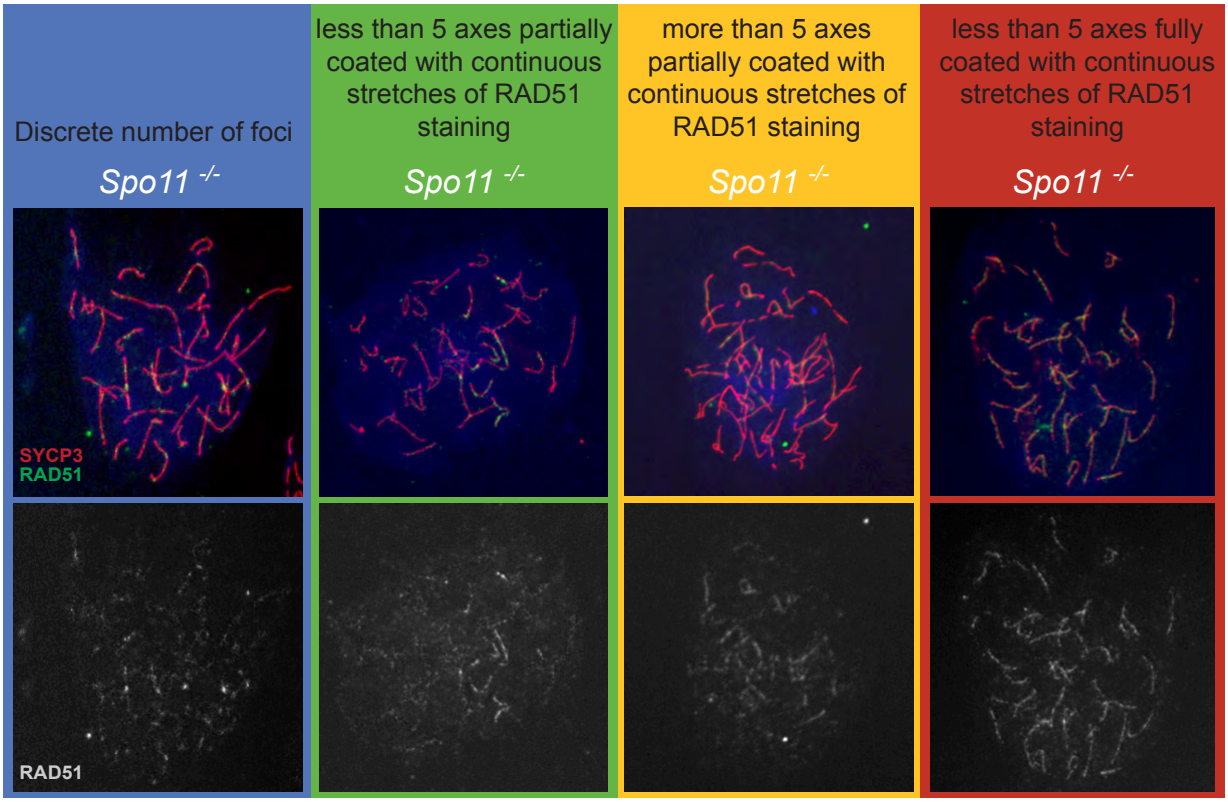
respectively; Fig. 3.5B), it rescued only a small fraction (~5%) of profoundly recombination-deficient *Dmc1*$^{-/-}$ oocytes (harboring an average of ~150 RAD51 foci (Li, Bolcun-Filas, and Schimenti 2011)). We posit that the oocytes that fail to be rescued in these mutants are eliminated either by a separate or a complementary checkpoint pathway (for example, ATR-CHK1 (Smith et al. 2010)), or succumb from catastrophic levels of DNA damage. It is informative that deletion of *Hormad1*, but not *Hormad2*, rescues *Dmc1*$^{-/-}$ oocytes to a greater extent than *Chk2* deletion. As discussed earlier, the rescued *Dmc1*$^{-/-}$ *Hormad1*$^{-/-}$ oocytes had a marked reduction in DSBs (Shin, McGuire, and Rajkovic 2013, Wojtasz et al. 2012, Bolcun-Filas et al. 2014). Since HORMAD1 is needed to load HORMAD2 onto unsynapsed chromosome axes (not vice versa), then the impact of *Hormad1* deletion upon IS recombination constitutes the combined roles of both HORMAD proteins. However, when *Hormad2* alone is deleted, the continued presence of chromosomally-bound HORMAD1 may provide a less-effective, but still substantive, BSCR. The lower level of residual DSBs in *Spo11* and *Trip13* mutant oocytes (compared to *Dmc1*$^{-/-}$) may render them responsive to a weaker BSCR such as when *Hormad2* is deleted. We postulate that because of its involvement in stimulating SPO11 activity (Daniel et al. 2011), *Hormad1* deletion is very effective in rescuing a DSB repair mutant like *Dmc1* because not only are fewer DSBs formed, but also IS recombination is more active.

Figure 3.8) Patterns of RAD51 staining on oocyte meiotic chromosomes of various genotypes. (A) The plots show the percentage of oocytes from the specified genotypes, color-coded for either discrete foci or varying levels of continuous staining patterns. (B) Classification of the different levels of continuous RAD51 immunostaining. All chromosome spreads are derived from newborn mice. RAD51 quantification was performed in images derived from an objective with 0.45μm resolving power. At this resolution, the RAD51 signals could be classified as discrete or continuous (coating AEs and/or SCs). It is likely that these continuous staining regions consist of numerous distinct foci. Because these were not enumerated in the calculation of discrete SPO11-independent DSBs, the actual number of SPO11-independent DSBs is probably higher than reported here.

A


discrete RAD51
Continuous RAD51
<5 axes partially coated
>5 axes partially coated
>5 fully coated axes
100%
80%
60%
40%
20%
0%
% oocytes
Wt
Chk2 -/-
Spo11 -/-
Spo11 -/- Chk2 -/-
Spo11 -/- Hormad2 -/-

B


Discrete number of foci
Spo11 -/-
less than 5 axes partially coated with continuous stretches of RAD51 staining
Spo11 -/-
more than 5 axes partially coated with continuous stretches of RAD51 staining
Spo11 -/-
less than 5 axes fully coated with continuous stretches of RAD51 staining
Spo11 -/-
SYCP3
RAD51
RAD51

Our results add to increasing evidence that IS recombination is important in mammalian meiosis. As discussed in the text, the HORMADs and SC axial element structure appear to inhibit IS repair of meiotic DSBs preferentially, thus allowing IH recombination to drive homolog pairing and synapsis. However, as synapsis progresses and the SC is formed, the HORMADs are removed and presumably both IS and IH recombination can occur readily as in yeast (Subramanian et al. 2016). Since not all RAD51 foci disappear by pachynema when synapsis is complete (for example, see Fig. 3.5B), it is possible that a substantial fraction of these DSBs are normally repaired by IS recombination. We speculate that the persistent unrepaired DSBs on synapsed chromosomes of *Trip13* mutants, which retain HORMADs on their SCs, may actually constitute a substantial fraction of SPO11-induced DSBs (an average of ~65/oocyte nucleus of the 200-300 induced; Fig. 3.5) that would normally be repaired by IS recombination. However, we cannot rule out the possibility that the “persistent” DSBs on synapsed *Trip13*$^{Gt/Gt}$ chromosomes actually arise from continued SPO11 cleavage signaled by continued presence of SC-bound HORMADs (Kauppi et al. 2013).

In trying to decipher the quality-control mechanisms functioning during meiosis, it is important to recognize that experimental studies such as those performed here employ mutants with pervasive, non-physiological levels of

defects. Meiocytes in wild-type individuals would have less extreme genetic defects. In oocytes bearing a small number (1-3) of unsynapsed chromosomes, the unsynapsed chromosomes underwent transcriptional silencing (MSUC) during pachynema, causing elimination at the diplotene stage (Kouznetsova et al. 2009, Cloutier et al. 2015) from lack of essential gene products encoded by these chromosomes (Cloutier et al. 2015). However, oocytes with more than 2-3 unsynapsed chromosomes impairs MSUC, presumably due to a limiting amount of BRCA1 (Kouznetsova et al. 2009). Nevertheless, *Spo11*$^{-/-}$ meiocytes typically exhibit "pseudo sex bodies," named as such because they resemble the XY (sex) body, involving a small number of asynapsed autosomes (Bellani et al. 2005). Formation of pseudo sex bodies in *Spo11*$^{-/-}$ oocytes is dependent upon HORMADs (Daniel et al. 2011, Kogo, Tsutsumi, Ohye, et al. 2012), leading to the proposal that these are responsible for oocyte elimination (Kogo, Tsutsumi, Inagaki, et al. 2012). This may be the case in a subset of oocytes where the pseudo sex body impacts either a chromosomal region containing haploinsufficient loci, or both alleles of a locus needed for meiotic progression or oocyte survival. Since CHK2 deficiency can rescue *Spo11*$^{-/-}$ oocytes while not abolishing HORMAD localization (Fig. 3.3) or pseudo sex body formation (not shown), yet does not rescue all *Spo11* oocytes, it is likely that neither MSUC nor CHK2 alone is entirely responsible for elimination of all oocytes with pervasive asynapsis.

Finally, because MSUC involves many components of the DNA damage response (Ichijima et al. 2011, Turner et al. 2004, Fernandez-Capetillo, Celeste, and Nussenzweig 2003), it is conceivable that asynapsis leading to MSUC would activate effector elements of the DNA damage checkpoint pathway, including CHK2. However, this does not appear to be the case, because silenced supernumerary chromosomes do not eliminate oocytes (Cloutier et al. 2015), MSCI (meiotic sex chromosome inactivation) does not kill spermatocytes, and asynaptic oocytes are not eliminated in a pattern typical of DNA repair mutants.

The “pachytene checkpoint” has commonly been thought to consist of separate DNA damage and synapsis checkpoints in multiple organisms. However, the finding that MSUC can cause death of oocytes led to the suggestion that there is only 1 formal cell cycle checkpoint in mouse oocytes - the DNA damage checkpoint (Cloutier et al. 2015) – is consistent with this idea, and our data provides mechanistic evidence in support of it. Current information supports a model (Fig. 3.7D) for two major mechanisms by which oocytes with synapsis defects are eliminated: 1) MSUC, for oocytes with a small number of asynapsed chromosomes that do not accumulate unrepaired DSBs above a threshold, and in which both homologs of chromosomes bearing essential genes for meiotic progression are silenced (Cloutier et al. 2015), and 2) the DNA damage checkpoint, for oocytes with multiple

asynapsed chromosomes that accumulate a sufficient number of DSBs to trigger the DNA damage checkpoint (Fig. 3.7D). These disparate mechanisms may have distinct purposes. Because oocytes with only 1 or 2 unsynapsed chromosomes may not efficiently trigger the spindle assembly checkpoint (SAC) (LeMaire-Adkins, Radke, and Hunt 1997), the MSUC pathway would safeguard against aneuploidy. Superficially, it would seem that because oocytes with extensive asynapsis would effectively trigger the SAC, that the DNA damage checkpoint mechanism is redundant. However, it is likely advantageous reproductively to eliminate such defective oocytes before they enter dictyate as constituents of the ovarian reserve, otherwise the fraction of unproductive ovulations (those terminated by the SAC) would increase, thus compromising fecundity.

5- Authors contributions

V.D.R. and E.B-F. performed the experiments and contributed to the writing of the paper. H.K. and H.K. provided the *Hormad2* mutant ESCs and provided feedback on the manuscript. J.C.S. supervised the work and wrote most of the paper.

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7- Methods

Organ Culture and Irradiation

Embryonic and postpartum explanted ovaries were cultured under conditions as we previously detailed (Rinaldi *et al.*, 2017). Ovaries were irradiated in a ^{137}cesium irradiator with a rotating turntable. Immediately after irradiation, the media was replaced, and ovaries were cultured for indicated periods of time prior to tissue processing.

Histology and Immunostaining

Ovaries were dissected and incubated in Bouin's fixative overnight at room temperature. Afterwards, tissues were washed in 70% ethanol prior to being embedded in paraffin for serial sectioning at 6mm thickness. Ovaries were stained with Harris Hematoxylin and Eosin (H&E) and follicles counted in

every fifth section except for the three-week counts reported in Figure 1B, in which every 12th section was counted. There was no correction factor applied to the values reported. Only one ovary per animal was used.

Cultured ovaries, used for histological sections followed by immunostaining, were fixed in 4% paraformaldehyde/PBS over night at 4°C. After 70% ethanol washes, ovaries were embedded in paraffin and serially sectioned at 5mm. These ovaries were immunostained using standard methods. Briefly, slides were deparaffinized and re-hydrated prior to antigen retrieval using sodium citrate buffer. Slides were blocked with 5% goat serum (PBS/Tween 20) and incubated at 4°C overnight with primary antibodies: mouse anti-p63 (1:500, 4A4, Novus Biologicals); and rabbit anti-MVH (1:1000, Abcam). Afterwards, sections were incubated with Alexa Fluor® secondary antibodies for one hour and Hoechst dye for 5 minutes. Slides were mounted with ProLong Anti-fade (Thermo-Fisher) and imaged.

Histological images were obtained from slides digitized using a Leica Scanscope CS2.

Immunofluorescence of meiotic chromosome surface spreads

Meiotic surface spreads of prophase I female meiocytes were prepared using an adaptation (Reinholdt *et al.*, 2004) of a drying-down technique (Peters *et al.*, 1997) that was described in great detail in the former reference. Meiotic

stages (leptonema-diakinesis) were determined based on SYCP3 staining patterns (Gray and Cohen, 2016). Slides were stored at -80°C until immunostained. For staining, slides were brought to room temperature (RT) and washed once with PBS+0.1% Tween-20 (PBS-T). Slides were blocked for 40 minutes at RT with PBS-T containing 5% normal goat serum (5%GS-PBS-T). Primary antibodies were diluted into 5%GS-PBS-T and incubated overnight at RT in a humidified chamber. Antibodies and dilutions used included: rabbit anti-RAD51 (1:250 Abcam 176458), mouse anti-SYCP3 (1:600 Abcam) and guinea pig anti-HORMAD2 antibody (1:1000, kind gift from Attila Toth). Secondary antibodies used were diluted 1:1000 in in 5%GS-PBS-T and included goat anti-rabbit Alexa 488/594, goat anti-mouse Alexa 488/594 and goat anti-guinea pig Alexa 488/594. Images were taken using an Olympus microscope with 40X lens or 100X immersion oil lens and CCD camera.

Focus Quantification

Foci were quantified both manually, through the visualization and annotation of individual foci, and also semi-automatically using Fiji-ImageJ (Schindelin *et al.*, 2012). Semi-automated counts were performed using binary images obtained from the RAD51-labeled channel, with the threshold set above background level. The count was obtained after performing “Watershed”, by the “Analyze Particles” functionality with size set for 1.5 to infinity. Cell counts

that displayed discrepancy of more than 20% between manual and semi-automated counts were discarded.

Fertility Test

To test if HORMAD2 deficiency was able to rescue the *Trip13*$^{Gt/Gt}$ sterility phenotype, three double mutant females were mated to wild type C3H/HeJ males proven to be fertile through previous matings. Each female provided more than 4 consecutive litters up to the time of preparation of this manuscript. All three females originated from different litters. *Trip13*$^{Gt/Gt}$ littermates were housed with fertile males and used as negative controls.

8- Quantification and statistical analysis

Statistical analysis

Comparisons between compound mutants and controls were done using littermates or related animals. Unless otherwise noted, all experiments used at least three mice per experimental group. All statistical analyses were done using JMP Pro12 software (SAS Inc., Cary, NC-USA, version 12.0.1). Comparisons of fertility and follicle counts between genotypic groups were tested using both the Tukey honest significance different (HSD) and the non-parametric, one-way ANOVA test (Kruskal -Wallis). Both tests provided concordant results. RAD51 focus counts were analyzed using a mixed model

with animal ID as random effect and genotype as fixed effect. Least square means (LSMeans) differences were tested using Tukey HSD. The residuals from the mixed model were normally distributed.

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CHAPTER 4

DISCUSSION & CONCLUDING REMARKS

1- Overview and summary of findings

The shorter reproductive life of female mammals (as compared to males) correlates with their limited and non-renewable number of resting oocytes. These oocytes reside within primordial follicles, and they constitute the female's ovarian reserve. The size of the oocyte pool is determined by birth, and its numbers gradually decrease with time. The progressive loss of oocytes is a natural phenomenon, however it can be expedited by environmental factors that result DNA damage such as disease treatments (like the ones used in cancer treatments), stress and drug use. Under laboratory conditions, female mice submitted to low levels of DNA damage after birth show massive oocyte death that results in sterility days after exposure, and ovarian failure in a month's period. This postnatal DNA damage induced oocyte death contrasts with what happens to oocytes during embryonic development, more specifically during leptonema. At this early stage of prophase I hundreds of developmentally programmed DNA double stranded breaks (DSBs) are formed through the natural course of prophase I. This dichotomy of sensitivity to DNA damage was the driving force of my investigation.

The sensitivity to low levels of DNA damage observed after birth in dictyate-arrested oocytes can be traced back to the meiotic checkpoint that surveys DNA integrity. In female mice, prophase I starts during embryonic life, progresses up to diplonema, which coincides with parturition, at which point meiosis arrests. Therefore, after birth most oocytes are at a late-diplotene-like stage (e.g. dictyate arrest). At this resting stage a single layer of flattened cells (the pre-granulosa cells) surrounds each dictyate-arrested oocyte forming a primordial follicle. Primordial follicles constitute the ovarian reserve of a female, and the residing oocyte remains arrested at dictyate from birth until maturation/ovulation. The maturation of the primordial follicle is called folliculogenesis, and culminates with either ovulation or follicular atresia. As the female reaches reproductive maturity folliculogenesis begins and a handful of primordial follicles will be periodically recruited to grow to primary stage; when the pre-granulosa cells become cuboidal and start to mitotically divide. The follicle development continues with the granulosa cells further dividing and forming layers surrounding the residing oocyte, which also will rapidly enlarge (Fortune 2003).

Besides the natural primordial follicle decline due to ovulation and atresia, failure to repair endogenous DSBs also results in oocyte death. The DNA damage checkpoint responsible for culling oocytes is the same from conception until follicular development (results shown on Appendix I (Bolcun-Filas et al. 2014) and Chapter 2). However, once primordial follicles commit to folliculogenesis, they are no longer under the same checkpoint (based on

results reported in Chapter 2, growing follicles are refractory to the levels of DSBs I tested). Nevertheless, the observation that dictyate-arrested oocytes are under the same prophase I checkpoint, suggests that knowledge about the mechanisms governing meiosis I can translate into treatments that impact female reproductive health. For example, knowledge about the pathway responsible for oocyte death can be used to design and inform treatments for the preservation of the primordial follicle reserve. Saving primordial follicles has implications not only for fertility but also for retaining the endocrinological function of the ovary. Furthermore, if not directly through pharmacological treatments, the knowledge gained from my research may be used to aid the design of new contraceptive methods or for improving *ex-vivo* conditions used for oocyte preservation.

In addition to the presence of a surveillance mechanism that monitors DNA integrity, meiocytes were previously thought to have a "synapsis checkpoint" responsible for triggering cell death in meiotic cells that fail to find and pair homologous chromosomes (synapsis-defective oocytes). However, the data presented in Chapter 3 argues against such a checkpoint, and lends support to the possibility that accumulation of DSBs in asynapsed chromosomes triggers the DNA damage checkpoint, which results in cell death. The failure to repair DSBs in asynapsed chromosomes is likely because of a block to use the sister chromatid as a DNA repair template (BSCR). The proposed model suggests that oocytes will be eliminated if they accumulate DSBs above a putative DNA-damage threshold (in the reported

study I found that ~10 RAD51 foci, which correlates to 0.3 Gys, is the DNA damage threshold). Since synapsis-defective oocytes are not able to promptly repair the damaged DNA, they will trigger the DNA-damage checkpoint when they reach diplonema. This model explains the apparent “leakiness” of the female “synapsis checkpoint”, since DSBs below the threshold will survive even with unsynapsed chromosomes.

Based on the finding describe in this thesis, I suggest that there are mainly three ways for an oocyte to be eliminated:

1- CHK2-dependent: when there are low levels of DSBs, yet above a wild-type level (~10 RAD51 foci).
2- CHK2-independent but HORMAD2-dependent: through MSUC, therefore silencing all transcribed copies of a gene that is essential for the survival of the meiotic cell.
3- CHK2-independent and HORMAD2-independent: when cells have catastrophic levels of DNA damage (>77 RAD51 foci).

2 – *The prophase I checkpoint*

Taken together, the results presented here convey that the traditional concept of a "pachytene checkpoint" is obsolete. In 2000, based on studies in yeast, Roeder used the term “pachytene checkpoint” to refer to control mechanisms that prevent meiotic cells from exiting the pachytene stage of prophase I (Roeder and Bailis 2000). This traditional “pachytene checkpoint” consisted of two branches: DNA damage checkpoint and synapsis checkpoint.

The model was evidently inappropriate for organisms with heterologous sex chromosomes, such as humans and mice, where males have one “X” and one “Y” chromosome that will never fully synapse. If there were indeed a synapsis checkpoint, the sex chromosomes would trigger its activation, be eliminated, and the animal would never reproduce. However, there is no doubt that animals with such chromosomes are proficient in meiosis despite the presence of the asynapsed sex chromosomes. Moreover, the model did not account for cell cycle arrest that occurs at other stages of prophase I. For instance in females, asynapsis and DNA damage cause oocyte death at diplonema (Chapter 2 and 3 and Cloutier et.al. 2015).

DNA-damage checkpoint

The DNA-damage checkpoint in meiosis is similar to the classical DNA-damage checkpoint of mitotic cells. The main difference is that oocytes are much more sensitive to DNA-damaging agents than somatic cells. Combining the results presented in Chapter 2 and Chapter 3 with recent findings of the involvement of HORMAD1 in promoting DSBs on unsynapsed chromosomes (Stanzione et al. 2016), the oocyte’s sensitivity to low levels of DSBs may constitute an alternative route for the elimination of cells with synapsis defects.

In 2016, Stanzione *et.al.* described that HORMAD1 (one of the two mammalian “HORMA”-domain containing proteins, related to yeast Hop1) is required for DSB formation by SPO11 (a topoisomerase-IV-like protein) and SPO11-accessory proteins; lack of HORMAD1 results in 70 % decrease in

endogenous DSB formation (Stanzione et al. 2016). I report in Chapter 3 that HORMAD2 interferes with DSB repair in synapsis deficient oocytes (e.g. *Spo11*$^{-/-}$). The interference is observed as a delay in DSB repair in the meiotic cells with persistent HORMAD2 on chromosome axes, which supports the existence of a protein block that prevents the use of the sister chromatid as repair template (BSCR). The BSCR model suggests that unsynapsed chromosomes, which have both HORMAD1 and HORMAD2, will accumulate DSBs that, even at low levels, activate the DNA-damage checkpoint. However, like the synapsis checkpoint model, BSCR fails to explain what happens to the heterologous sex chromosomes.

Silencing of essential genes (MSUC) as a checkpoint

Studies using meiotic cells carrying heterologous chromosomes (both male “XY” meiotic cells and female “XX” carrying and additional accessory chromosome) identified that unsynapsed regions undergo transcriptional silencing during male and female meiosis in mice. The meiotic silencing of unsynapsed chromosomes (MSUC) was suggested as a separate mechanism that effectively serves as a checkpoint. The equivalent to the MSUC response in males is referred to meiotic sex chromosome inactivation (MSCI). Both MSUC and MSCI result in inactivation of transcription (see Chapter 1). MSCI is essential for survival of the male meiotic cells. Failure to invoke a MSCI response results in spermatocyte death at mid-pachynema (agreeing with Roeder’s definition of a pachytene checkpoint). However, the meiotic silencing

response is sexually dimorphic: in males, failure to silence sex-linked genes is lethal whereas in females, success in silencing essential genes triggers oocyte death (Cloutier et al. 2016).

Furthermore, in female oocytes the prophase arrest due to silencing is unpredictable, since the death response depends on whether the silenced gene is essential for meiosis; MSUC per se does result in cellular demise. In 2015, Cloutier and colleagues found that when silencing occurs in additional hemizygous accessory chromosomes, oocytes remain alive despite MSUC occurring at this extra chromosome (Cloutier et al. 2015). They further found that in females, oocyte demise through MSUC is stochastic (Cloutier et al. 2016); cell death depends on the gene and how many copies of the gene are being silenced. In pervasive synapsis deficient meiocytes, the probability of silencing all actively transcribed copies of a gene required for cell survival is low. This lack of a defined outcome when the MSUC pathway is activated does not agrees with the traditional definition of a checkpoint, and feeds the ongoing debate about whether MSUC acts as a checkpoint.

The conundrum

Studies found that HORMAD2 is essential for the silencing response (MSUC) (Wojtasz et al. 2012; Kogo, Tsutsumi, Inagaki, et al. 2012). Therefore, if HORMADs persist on the axis of a chromosome, there are two predicted outcomes. 1- accumulation of DSBs due to HORMAD1 supporting continuous

SPO11-dependent DSB formation and HORMAD2 delaying the DSB repair; 2-HORMAD2 mediated silencing response (MSUC).

Accumulation of DSBs was indeed observed in the study described in Chapter 3 that used two mice models known to have persistent HORMADs on their chromosome axes (e.g. *Spo11*$^{-/-}$ and *Trip13*$^{Gt/Gt}$) (Wojtasz et al. 2009). HORMADs are removed from the chromosomes axes upon synapsis by the protein Thyroid Hormone Receptor Interactor 13 (TRIP13) (Wojtasz et al. 2009). Mice homozygous for the hypomorphic allele (*Trip13*^{Gt}) (Li and Schimenti 2007) of this protein have persistent DSB markers and HORMAD1 and HORMAD2 along synapsed chromosome axes. In *Spo11*$^{-/-}$ oocytes HORMADs are never removed from the chromosome axes supposedly due to failure to synapse.

The MSUC response in *Spo11*$^{-/-}$ was described to correlate with the presence of HORMAD2 (Kogo, Tsutsumi, Inagaki, et al. 2012; Wojtasz et al. 2012). These reports assumed that lack of the SPO11 protein resulted in no DSBs formed during meiosis. However, presence of DSBs in *Spo11*$^{-/-}$ meiocytes was previously shown (Chicheportiche et al. 2007; Baudat et al. 2000), and Chapter 3 shows that *Spo11*$^{-/-}$ oocytes have DSBs above the wild type level for the DNA-damage threshold (>30 RAD51 foci). Although debatable, it was initially accepted that MSUC was independent of DSBs formation, even though it depends on the recruitment of proteins from the DNA-damage response to the silencing site (e.g. ATR, MDC1, γH2AX – see

Chapter 1). Nevertheless, the localization of HORMAD2 and presence of DSBs in the *Spo11*$^{-/-}$ oocytes, ties MSUC response to BSCR.

The evidence that *Spo11*$^{-/-}$ oocytes have DSBs provides support that MSUC is similar to the silencing response observed in somatic cells (Yosuke Ichijima, Sin, and Namekawa 2012). During DNA replication of somatic cells, stalled replication forks become coated with RPA, which triggers a MDC1 dependent amplification of the ATR/γH2AX phosphorylation cascade that results in silencing. However, MSUC was again described to be independent of DSB formation in oocytes carrying different types of chromosome abnormalities (Cloutier et al. 2015; Manterola et al. 2009). Cloutier *et. al.* describe that, in DSB repair proficient meiocytes with heterologous sex chromosomes or other chromosomal abnormalities, MSUC happens on the axes regardless of the presence of DSB markers. They show presence of HORMAD1/2 at asynapsed axes and claim that DSBs were either never formed or repaired prior to mounting a MSUC response. Their claim is supported by the lack of DSBs (as measured by DNA damage markers RAD51, DMC1 and RPA), which are described as being resolved by late pachynema (Cloutier et al. 2015). Another study that supports MSUC as independent of DSBs used the meiotic-DSB deficient *Iho1*$^{-/-}$ animal model (Stanzione et al. 2016). In this model, meiocytes have HORMADs at the unsynapsed axes, have no DSBs (no RPA), but mount MSUC (γH2AX cloud) similar to SPO11 deficient cells. Interestingly, on this report the authors observe complete depletion of oocytes in six weeks old *Iho1*$^{-/-}$ ovaries. The

report that HORMADs not only promote DSB but also delay DSB repair through BSCR is contradictory to the claim that MSUC occurs independently of DSBs. I envision an alternative hypothesizes where the presence of RPA, such as what is observed on asynapsed chromosomes at early pachynema, is responsible for triggering MSUC. The paradox of observing MSUC domains forming independent of DSBs in regions primed to have accumulation of DNA damage (due to the presence of HORMAD1/2) may be a result of the loss of temporal resolution when observing the histological manifestation of MSUC and DSBs. Future studies may shed light in this conundrum and determine if MSUC is indeed independent of DSBs or if this independency is just an artifact of observing a snapshot of a continuous biological process.

Spatiotemporal modifications of proteins during meiosis

The lack of temporal resolution may explain the observed lack of dependency between both MSUC and presence of DSB. Although it is not yet possible to visualize a single meiotic cell and its protein dynamics *in vivo* and at real time, studies from discrete time points of testicular development have shed some light on the phosphorylation events occurring at the chromosome axes. Proteins involved in synapsis and recombination have different posttranslational modifications such as phosphorylation, ubiquitination, and sumoylation, which likely modulate their function. The meiotic phospho-proteome has not yet been characterized. However, there are few reports that show that HORMAD1/2 have multiple ATM/ATR phosphorylation motifs that

account for at least two phosphorylation forms out of the three nuclear-bound forms found by gel shift assays (Fukuda et al. 2012; Kogo, Tsutsumi, Ohye, et al. 2012). The expression patterns between HORMAD1 and HORMAD2 are slightly different, with HORMAD2 expression delayed in relation to HORMAD1. Regarding HORMAD1 phosphorylation, there are two reports with different findings. One report implicates HORMAD1 phosphorylation as being required for an axis bound state (H1p) (Kogo, Tsutsumi, Ohye, et al. 2012) and another shows ATR-mediated phosphorylation at serine (Ser) 375 (H1p375) only upon DSB formation (Fukuda et al. 2012). One of the HORMAD2 phosphorylation sites was determined to be on serine 271, an ATR-dependent phosphorylation motif, which showed a limited localization restricted to the sex chromosomes (H. Royo et al. 2013; Fukuda et al. 2012). Based on sequence and structural analyses both HORMAD proteins have a predicted “closure motif” that specifically binds HORMAD1 (Rosenberg and Corbett 2015); in other words, HORMAD1 forms homodimers (HORMAD1-HORMAD1) and heterodimers with HORMAD2 (HORMAD1-HORMAD2). Together with the genetic studies the predicted model also suggests that HORMAD2 is dependent on HORMAD1 for recruitment to the chromosome axis. This makes the genetic analysis of *Hormad1*$^{-/-}$ confounded by the simultaneous failure of HORMAD2 to load onto the chromosome axis.

The information about the phosphorylation of axial components illustrates the need for better studies about posttranslational modifications. Phosphorylation information can be used to infer how BSCR and MSUC are

regulated by HORMAD2. In addition to the aforementioned, *Trip13*$^{Gt/Gt}$ meiocytes, which have DSBs markers and HORMADs on synapsed chromosomes, were shown to accumulate the H1p375 form of HORMAD1 at their synapsed axis. *Spo11*$^{-/-}$ meiocytes are deficient in H1p375 (Fukuda et al. 2012), but oocytes were reported to have H1p, the putative axis bound state (Kogo, Tsutsumi, Ohye, et al. 2012) on their asynapsed axis. Perhaps this is an indication that different phosphorylation forms activate different pathways.

An alternative model for MSUC initiation

The presence of "late" DSBs at early pachynema in asynapsed chromosomes together with the presence of non-meiotic DSBs in *Spo11*$^{-/-}$ meiocytes, lead me to suggest a model in which the presence of HORMAD2 at unsynapsed axis retains unrepaired DSBs. On this scenario, the delay in DSB repair results in enough RPA signaling to trigger MSUC. It may even be that lack of DSB repair results in long tracks of RPA-coated ssDNA, for example due to hyper-resection, which would explain the apparent increase in RPA foci during late Zygonema in male meiotic cells. In the synapsed autosomes, TRIP13 removes HORMADs from axis, thus removes the BSCR and expedite repair of remaining DSBs. However, if the BSCR is not lifted, thus HORMADs persists on axes, RPA coated ssDNA tracks activate ATR and starts a chain reaction of phosphorylation events that will result in MSUC. Therefore, in this model, retention of HORMAD2 will cause MSUC due to the presence of unrepaired DSB. As soon as the ATR response starts, it is rapidly amplified

through the phosphorylation of HORMADs. Phospho-HORMADs act by amplifying the ATR response, thus catalyzing the spread of the silencing factors to the chromatin loops.

This model accounts for the observed persistence of DSB marks at the unsynapsed chromosomes ("XY" and accessory hemizygous) that only disappear at late pachynema. It also explains the apparent lack of DSBs in MSUC regions. In summary, the model suggests that DSB induced phosphorylation of HORMADs enhances the ability to recruit ATR resulting in a robust yet slightly delayed response relative to the ATM-DSB response. It alludes to MSUC as a delayed ATR response that requires DSBs as nucleating factor. Because TRIP13 removes HORMADs from the axis upon synapsis, the MSUC response only occurs at unsynapsed axis. Therefore, the delayed yet amplified ATR signal from the DSBs would ensure that even small numbers of DSBs would trigger MSUC.

3 – Future directions

In order to test the proposed model, the initial step would be to determine if DSBs are indeed needed for MSUC response. This can be determined by through analysis of the onset of MSUC in *Spo11*$^{-/-}$ and *Iho1*$^{-/-}$, and how it correlates to non-meiotic DSBs. Inducing DSBs in *Iho1*$^{-/-}$, and determining if it potentiates the MSUC response can further evaluate this. If it turns out that MSUC is indeed independent of DSBs, further studies will be needed to determine why six week old *Iho1*$^{-/-}$ ovaries are devoid of oocytes,

since the present study, and Cloutier's reports, suggest that MSUC is not sufficient to explain massive oocyte attrition due to asynapsis (Cloutier et al. 2015; Stanzione et al. 2016).. Nevertheless, if MSUC is not triggered by the presence of DSBs, one explanation could be that phosphor-HORMAD2 on Ser 271 have structural similarities to RPA, triggering silencing in similar way to stalled forks in somatic cells. Another future direction is the obvious need to identify the posttranslational modification events of each meiotic stage. This can be achieved through stage specific phosphor-proteomics and generation of animals with mutations at phosphorylation sites.

The field would also benefit from the identification of the essential genes for meiosis. The latter by performing a thorough comparative analysis of RNA-seq data derived from *Spo11*$^{-/-}$*Chk2*$^{-/-}$ and *Spo11*$^{-/-}$*Hormad2*$^{-/-}$ oocytes. The less expressed genes from *Spo11*$^{-/-}$*Chk2*$^{-/-}$ oocytes (proficient in MSUC response and not eliminated due to low levels of persistent DSBs) that are highly expressed in *Spo11*$^{-/-}$*Hormad2*$^{-/-}$ oocytes (which will express all the genes) would be the candidates for essential meiotic genes. Briefly, the rationale is that there will be about a 10% reduction of the lethal genes in the *Spo11*$^{-/-}$*Chk2*$^{-/-}$ oocytes pool. The 10% reduction is based on the frequency of naturally occurring asynapsed oocytes. Another question to be addressed comes from the intriguing observation that in oocytes with chromosomal abnormalities (e.g. XO -Turner syndrome, or autosomal translocations), the silencing of the unsynapsed chromosome triggers oocyte elimination during diplonema (Cloutier et al. 2015). That is counterintuitive, because silencing of

essential genes should cause oocyte death at the stage in which the gene product is required. This observation indicates that other potential posttranslational modifications might be involved with oocyte elimination during prophase I.

Regarding the findings in Chapter2, there is still the need to verify if the animals born from the CHK2-inhibitor treated ovaries have mutations, or transgenerational epigenetic modification. The most straightforward experiment is likely through whole genome sequencing and single embryo RNA- and small RNA-seq.

Furthermore the broader field of meiosis could benefit from using high-resolution microscopy to better characterize meiotic division. An optimal approach would be with collective efforts, to gather enough material to map meiosis at a molecular level. Using super resolution microscopy and 3D imaging tools it may be possible to visualize how the SC is organized, and how HORMADs are positioned. The spatial resolution may provide the missing link on how HORMAD2 is interfering with DSB-repair. A collaborative effort would also answer major questions about recombination events, SC assembly, and overall nuclear organization.

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APPENDIX I

Title: Reversal of female infertility by mutation of Chk2 identifies the oocyte DNA damage checkpoint pathway

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Abstract: Genetic errors in meiosis can lead to birth defects and spontaneous abortions. Checkpoint mechanisms of hitherto unknown nature eliminate oocytes with unrepaired DNA damage, causing recombination-defective mutant mice to be sterile. Here, we report that checkpoint kinase 2 (*Chk2; Chek2*), is essential for culling oocytes bearing unrepaired meiotic or induced DNA double-strand breaks (DSBs). Female infertility caused by a meiotic recombination mutation or postnatal irradiation was reversed by concurrent mutation of *Chk2*. We found that both meiotically-programmed and induced DSBs trigger CHK2-dependent activation of TRP53 (p53) and TRP63 (p63) in damaged oocytes. These and other data establish CHK2 as essential for DNA damage surveillance in female meiosis, and indicate that the oocyte DSB damage response primarily involves a pathway hierarchy of ATR>CHK2>p53+p63.

One Sentence Summary: CHK2 signaling to p53 and p63 is central to the checkpoint that monitors DNA damage in both meiotic and resting oocytes.

Main Text: Fertility, health of offspring, and species success depends on production of gametes with intact genomes. Particularly crucial is the proper segregation of homologous chromosomes at the first meiotic division. This requires homologs to pair, synapse, and (in most organisms) form chiasmata (crossovers, or COs) that link homologs and enable disjunction to opposite poles during anaphase. These chromosome behaviors are driven by homologous recombination (HR), a high-fidelity DSB repair process. Meiocytes fulfill the requirement for HR by producing proteins (namely SPO11) that create DSBs, thereby driving HR-mediated repair. In mice, ~10% of the >200 DSBs are repaired as COs, and the rest by non-crossover (NCO) recombination (*1*).

Aberrations in homolog synapsis or DSB repair are potentially deleterious, but checkpoints monitor these processes and eliminate defective meiocytes (*2-4*). Mouse spermatocytes are sensitive to both unrepaired DSBs and the presence of asynapsed chromosomes, either of which trigger their apoptotic elimination at mid-pachynema of meiotic prophase I (*5, 6*). In contrast, the stage at which oocytes are completely culled depends upon the type of meiotic defect(s); those defective for both DSB repair and synapsis (e.g. *Msh5, Dmc1* mutants) occurs earlier (within a few days after birth) than in mutants defective in synapsis alone (~2 months postpartum), suggesting that

mammalian oocytes have distinct DNA damage and synapsis checkpoints (*2, 7*) (Fig.1A). Additionally, mutations preventing DSB formation (*Spo11, Mei1*) are epistatic to those affecting DSB repair (*2, 7*). The DNA damage checkpoint acts around the time oocytes enter meiotic arrest (dictyate, or resting stage) and presumably persists, since resting primordial follicles are highly sensitive to ionizing radiation (IR)-induced DNA damage (*8*).

We focused on CHK2 kinase as a candidate component of the meiotic DNA damage checkpoint. It is a canonical downstream effector of the ATM kinase that responds primarily to DSBs, and can also be activated by the ATR kinase that responds primarily to ssDNA (*9, 10*). Unlike *Atm* and *Atr, Chk2* is dispensable for fertility and viability. If *Chk2* is required for the meiotic DNA damage checkpoint, then its ablation should rescue a DSB repair mutation that otherwise causes meiocyte death. The RecA homolog *Dmc1* is required for meiotic DSB repair *via* interhomolog (IH) recombination (*11*). Its deficiency also prevents synapsis, which is HR-dependent in mice. Whereas 3 week postnatal WT or $Chk2^{-/-}$ ovaries contain primordial through antral follicles (Figs.1B,C; S1), $Dmc1^{-/-}$ ovaries are devoid of follicles (Fig.1E). Deletion of *Chk2* enabled survival of developing oocytes in DMC1-deficient 3-week old ovaries (Figs.1F,G). Primordial follicles were absent, however, leading to a nearly complete depletion of oocytes by 2 months of age (Figs.S1,S2). This pattern and timing of oocyte loss resembles that of *Spo11* or $Spo11^{-/-}$ $Dmc1^{-/-}$ mice (Fig.1A; (*2*)), suggesting that *Chk2* ablation compromises the DSB repair checkpoint, but not the synapsis checkpoint.

To test this, we took advantage of a hypomorphic allele of *Trip13* ($Trip13^{Gt}$) that causes male and female meiotic failure. $Trip13^{Gt/Gt}$ meiocytes are proficient in chromosome synapsis and CO formation, but fail to complete NCO DSB repair (*12*), causing elimination of the entire primordial follicle pool and all but a few developing oocytes by three weeks of age (Fig.1H) (*12, 13*). $Trip13^{Gt/Gt}$ oocyte elimination occurs at the DNA damage checkpoint (*12, 13*) (Fig.1A). Strikingly, $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ ovaries had a large oocyte pool at 3 weeks postpartum (Figs.1I,J; S1), and they retained similarly high numbers of primordial follicles and exhibited all stages of follicle development after two months (Fig.S2), indicating that the rescue of surviving oocytes from checkpoint elimination was permanent or nearly so (see below). We considered the possibility that the rescue of $Trip13^{Gt/Gt}$ oocytes by CHK2 deficiency might be due to repair of DSBs by an alternative pathway during pachynema, since the *Chk2* budding yeast ortholog *MEK1* is involved in DSB repair pathway choice (*14*). However, all dictyate $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ oocytes (n=54), like $Trip13^{Gt/Gt}$ oocytes, exhibited abundant γH2AX staining, indicative of persistent unrepaired DSBs (*vs.* 7% of $Chk2^{-/-}$ dictyate oocytes; n=45) (Fig.2A,B).

To test whether the rescued oocytes were functional despite the presence of DSBs into late meiotic Prophase I, we evaluated the fertility of $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ adult females. Remarkably, all produced multiple litters in matings to fertile males (Fig.2C). Litter sizes were smaller compared to control females (Fig.2D), attributable to fewer ovulated oocytes and implanted embryos (Fig.S3) as expected from the smaller primordial follicle pool. $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ females sustained fertility for many months, yielding 4-7 litters each (Fig.2C) and over 160 pups collectively. Those progeny

maintained to adulthood (n=28) showed no visible abnormalities up to 1 year of age. The fact that these rescued oocytes were functional and yielded healthy offspring suggests that all or most DSBs persisting into late meiosis were eventually repaired. Indeed, there was no evidence of persistent DNA damage (as indicated by γH2AX) in 2 month old primordial, growing, or germinal vesicle (GV) stage preovulatory *Chk2*$^{-/-}$ *Trip13*$^{Gt/Gt}$ oocytes compared to controls (Fig.S4). Thus, repair of DSBs occurred after birth by unknown mechanisms.

Next, we sought to identify the downstream target of CHK2 that mediates apoptosis in DSB repair-defective oocytes. Canonically, CHK2 signals to p53 in mitotic cells. In *Drosophila melanogaster,* CHK2-dependent p53 activation occurs in response to SPO11-induced breaks (*3*). We threefore tested whether p53 deficiency could rescue the elimination of *Trip13*$^{Gt/Gt}$ oocytes. Three week old *p53*$^{-/-}$ *Trip13*$^{Gt/Gt}$ ovaries had significantly more oocytes than *Trip13*$^{Gt/Gt}$ single mutants (Figs.3B,C; S1), however, the rescue was not as dramatic as that enabled by *Chk2* deletion (Fig.1D). The *p53*$^{-/-}$ *Trip13*$^{Gt/Gt}$ ovaries contained far fewer primordial follicles at 3 weeks postpartum, and these were progressively depleted such that by 2 months of age almost no oocytes remained (Fig.S2). Therefore, CHK2-mediated elimination of these DSB repair-defective oocytes does not occur exclusively *via* signaling to p53, indicating the existence of another downstream effector(s) that acts perinatally in primordial follicles.

One candidate is *p63*, a *p53* paralog. A predominant isoform called TAp63 appears perinatally in late pachytene and diplotene oocytes, approximately coinciding with DNA damage checkpoint activation. Since TAp63 was implicated in the elimination of dictyate oocytes subjected postnatally to DSB-causing IR (*15, 16*), and it contains a CHK2 consensus substrate motif LxRxxS (*17*), we speculated that CHK2 might activate TAp63 in response to DSBs. Indeed, whereas IR induces phosphorylation in WT ovaries (*15, 16*), TAp63 remained unphosphorylated in CHK2-deficient ovaries (Fig.3D). Moreover, mutating serine to alanine in the CHK2 phosphorylation motif in p63 also prevented IR-induced TAp63phosphorylation in cultured cells (Fig.3E). We next tested if CHK2 is required for elimination of DSB-bearing dictyate oocytes, presumably *via* TAp63 activation. Strikingly, whereas the entire primordial follicle pool was eradicated one week after IR-treatment of WT ovaries, CHK2 deficiency prevented oocyte elimination despite the presence of p63 protein (Fig.3F). Furthermore, irradiated *Chk2*$^{-/-}$ females remained fertile with average litter size (6.3± 1.8, n=7) similar to unirradiated controls (6±2.3, n=3). If this rescue of fertility was due entirely to abolition of TAp63 activation, then deletion of TAp63 should also restore fertility of irradiated females. Previous studies (*15, 16*) found that *p63*$^{-/-}$ and *TAp63*$^{-/-}$ oocytes survived 5 days after 0.45-5Gy of IR, but longer term survival was not evaluated. Surprisingly, we found that 0.45Gy IR completely eradicated primordial oocytes after 7 days in females homozygous for a viable, TA domain-specific deletion allele of *p63* (*TAp63*$^{-}$)(*18, 19*), identical to WT (Fig.4A,B).

These results suggested that in response to IR-induced DSBs (and perhaps meiotic DSBs as well), CHK2 signals to a protein(s) in addition to TAp63. Suspecting p53, we found that whereas irradiated *p53*$^{-/-}$ ovaries were essentially devoid of oocytes (Fig.4C) (*15, 16*), *p53*$^{-/-}$ *TAp63*$^{-/-}$ oocytes (including those in primordial follicles) were

rescued (Fig.4D) to a degree similar to *Chk2* mutants (Fig.3F). Interestingly, we observed partial rescue of irradiated $p53^{+/-}$ $TAp63^{-/-}$ (Fig.4E) but not $p53^{-/-}$ $TAp63^{+/-}$ oocytes. These data indicate that CHK2 signals to both p53 and p63 in resting oocytes, and that they act in a partially redundant fashion to eliminate DSB-bearing oocytes. The marked effects of p53 haploinsufficiency, and the possible inconsistencies with earlier reports showing that deletion of p63 alone could rescue primordial follicles from IR over the short term, indicate that checkpoint responses may be sensitive to quantitative variation such as that influenced by genetic background.

Since *Chk2* but not *p53* deficiency reversed $Trip13^{Gt/Gt}$ female infertility, an outcome similar to the results with postnatal ovary irradiation, we hypothesized that the same DNA damage checkpoint was operative in both pachytene/diplotene and dictyate oocytes. To test this, we first examined patterns of p53 and TAp63 activation in different genotypes of ovaries, with or without IR exposure. As expected for WT, TAp63 phosphorylation and p53 stabilization/expression occurred only after exposure to IR (Fig.4F). Importantly, we observed p53 protein in unirradiated $Trip13^{Gt/Gt}$ neonatal ovaries but not WT (Fig.4F), implying a role for p53 in the elimination of mutant oocytes with unrepaired meiotic DSBs (and consistent with partial rescue of $Trip13^{Gt/Gt}$ $p53^{-/-}$ oocytes; Fig.3C). Stabilization of p53 in response to unrepaired meiotic DSBs is CHK2-dependent, since we did not detect p53 in $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ ovaries (Fig.4F). Unexpectedly, TAp63 was absent from neonatal $Trip13^{Gt/Gt}$ ovaries bearing residual oocytes (Fig.4F). Normally, *TAp63* mRNA appears in late meiotic prophase I when meiotic DSBs have been repaired, and is robustly activated in resting oocytes in response to exogenous DNA damage (*15, 16*). Nevertheless, the absence of TAp63 in $Trip13^{Gt/Gt}$ oocytes predicts that it is not responsible for their death. Indeed, no oocyte rescue was observed in wean age $TAp63^{-/-}$ $Trip13^{Gt/Gt}$ ovaries (Fig.4I). A potential explanation for TAp63 repression in $Trip13^{Gt/Gt}$ oocytes was suggested by our observation (Fig.4F) that unphosphorylated TAp63 was present in $Chk2^{-/-}$ $Trip13^{Gt/Gt}$ ovaries lacking detectable p53. These results suggest a regulatory relationship between p53 and TAp63 in the meiotic DNA damage response.

The mutual exclusivity of TAp63 and p53 in $Trip13^{Gt/Gt}$ oocytes gives insight into the failure of either single mutant to rescue fertility. We hypothesized that unrepaired DSBs that persist into late pachynema trigger CHK2-dependent p53 activation and oocyte elimination independent of TAp63, but that in the absence of p53, *TAp63* can be expressed and activated by CHK2 to drive oocyte elimination. This predicts that removal of both proteins would abolish the CHK2-dependent checkpoint. Indeed, we found that *p53* heterozygosity could rescue $TAp63^{-/-}$ $Trip13^{Gt/Gt}$ oocytes (Fig.4J). Importantly, this rescue included primordial follicles (Fig.4J, inset; note: nullizygosity for all three genes is embryonically semilethal). These and previous results with single mutants indicate that the DNA damage checkpoint pathway that monitors repair of SPO11-induced DSBs involves CHK2 signaling to both p53 and TAp63, and that this pathway also operates in postnatal resting oocytes (Fig.S5).

A remaining question concerns the upstream activator(s) of CHK2. Canonically, ATM phosphorylates CHK2 in response to DSBs, while ATR responds to single stranded DNA by activating CHK1 (*20, 21*). However, ATR and ATM have other activities in mouse meiosis. ATM negatively regulates SPO11, causing $Atm^{-/-}$ oocytes

to sustain extensive DSBs and triggering elimination by the meiotic DNA damage checkpoint (Fig.1A) (*2*, *22*). Therefore, CHK2 is likely activated by a different kinase. Indeed, *Chk2* deficiency rescued *Atm*$^{-/-}$ oocyte depletion (Fig.S6) to a degree similar to the rescue of DMC1-deficient ovaries. The facts that: a) CHK2 can trigger apoptosis in the absence of ATM in somatic cells (*9*), b) CHK2 can be activated in an ATR-dependent manner (*10*) and c) ATR localizes to sites of meiotic DSBs in mice (*23*), prompt us to propose that the DNA damage checkpoint pathway in mouse oocytes involves signaling of ATR to CHK2, which in turn signals to p53 and TAp63 (Fig.S5). Intriguingly, spermatocytes may have a distinct DNA damage response pathway; we did not observe histological evidence for rescue of DSB repair-defective/synapsis-proficient spermatocytes by deletion of *Chk2* or *p53* (Fig.S7).

Our results are of biomedical interest with respect to the primordial follicle pool depletion and premature ovarian failure that can occur following cancer radiotherapy or chemotherapy. CHK2 is an attractive target in this regard, since chemical inhibitors are available, and *Chk2* insufficiency is of minor phenotypic consequence in mice (*24*). It may also become relevant for assisted reproductive technologies that employ *in vitro* development of germ cells, for example in the assessment of how various conditions activate the pathway, or how modulating the pathway can be lead to efficient and safe outcomes.

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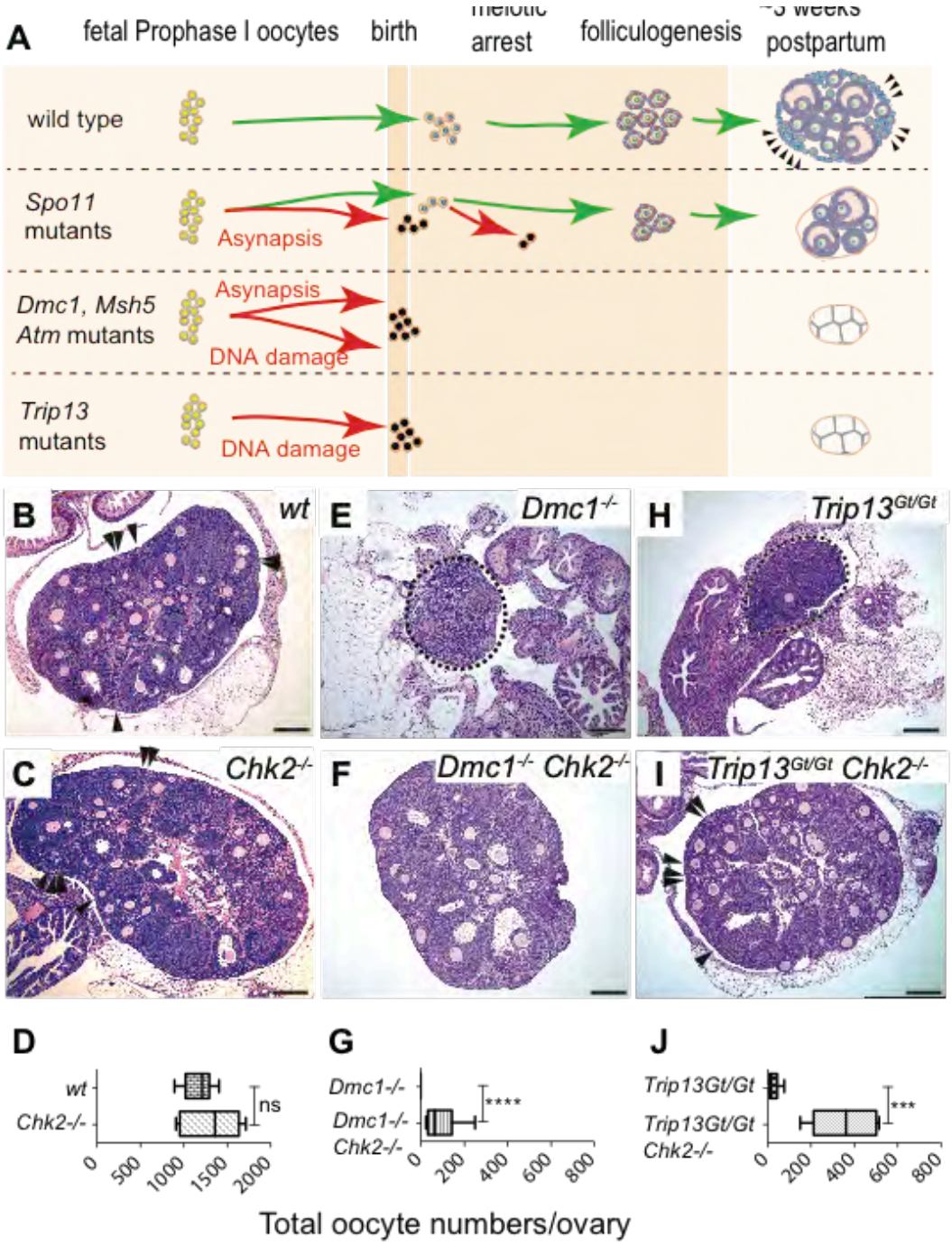

Fig. 1. **Evidence of a specific DNA damage checkpoint in mouse oocytes.** (**A**) Timing of DNA damage and asynapsis checkpoints in oocytes. (**B,C,E,F,H,I**) Histology of 3 week postpartum ovaries. Follicle-devoid ovaries are circled. **(D,G,J)** Oocyte quantification in mutants. Arrowheads (A,B,C,I) indicate primordial follicles. Scale bar = 200μm.

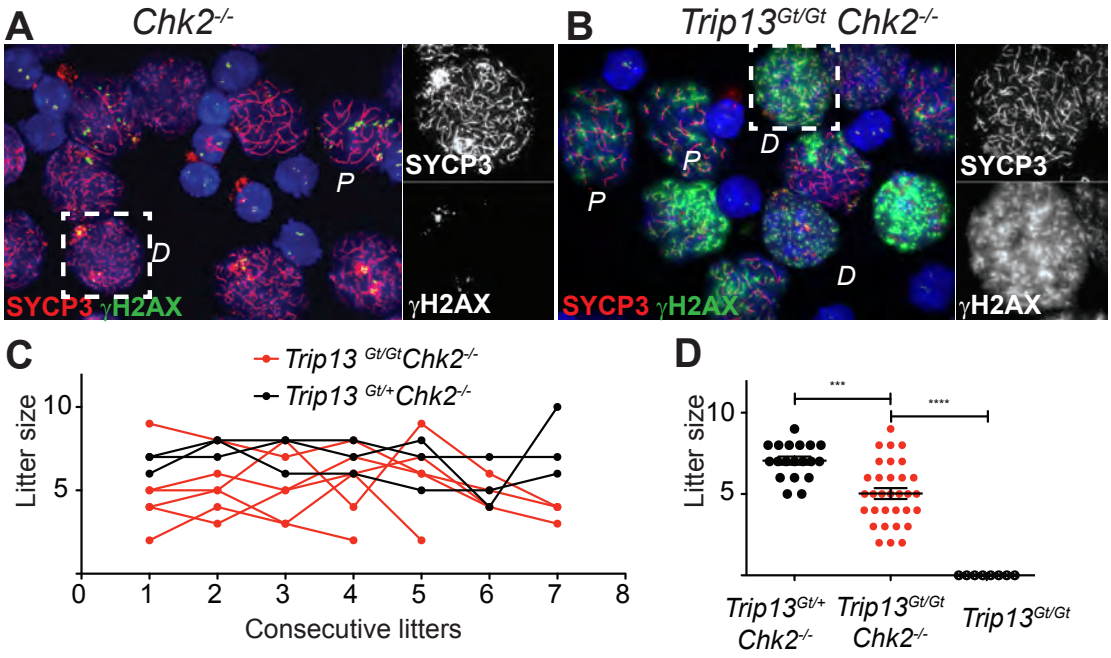

Fig. 2. DSBs in *Trip13*$^{Gt/Gt}$ *Chk2*$^{-/-}$ newborn oocytes are eventually repaired and yield offspring. **(A)** Neonatal oocytes co-immunolabeled as indicated. **(B)** *Trip13*$^{Gt/Gt}$ *Chk2*$^{-/-}$ oocytes progress to dictyate ("D") even with DSBs. P = pachytene. Boxed nuclei are magnified (inset). **(C)** Reproductive longevity and **(E)** fecundity of females.

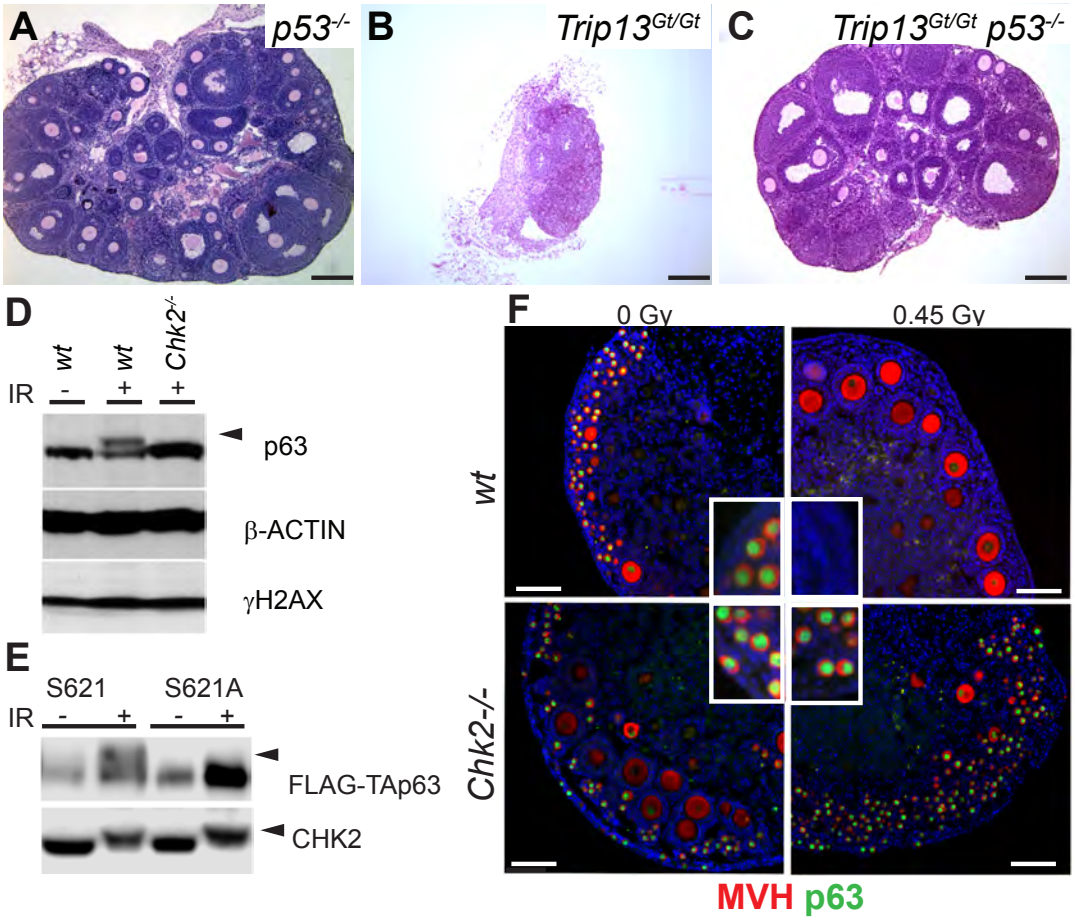

Fig. 3. Genetic and molecular analysis of the oocyte DNA damage checkpoint. (A-C) *Trip13*$^{Gt/Gt}$ oocyte depletion is only partially rescued by p53 deficiency. Scale bar = 200 μm. **(D)** DNA damage-induced TAp63 phosphorylation in newborn ovaries is CHK2-dependent. Neonatal ovaries (4) received 3Gy IR before protein extraction 2 hrs later. Note: increased p63 in *Chk2*$^{-/-}$ is likely due to increased oocytes we observe in this genotype. **(E)** p63 contains a CHK2 phosphorylation site. HeLa cells bearing FLAG-tagged TAp63 with WT (LxRxxS) or mutant (LxRxxA) CHK2 motifs. Shifted CHK2 (arrowhead) is phosphorylated. IR dose=3Gy. **(F)** Depletion of p63-positive primordial follicles by IR is CHK2-dependent. Ovaries were cultured 7 days after irradiation. Scale bar=100 μm. MVH marks oocytes. Inset: ovary cortical region containing primordial follicles.

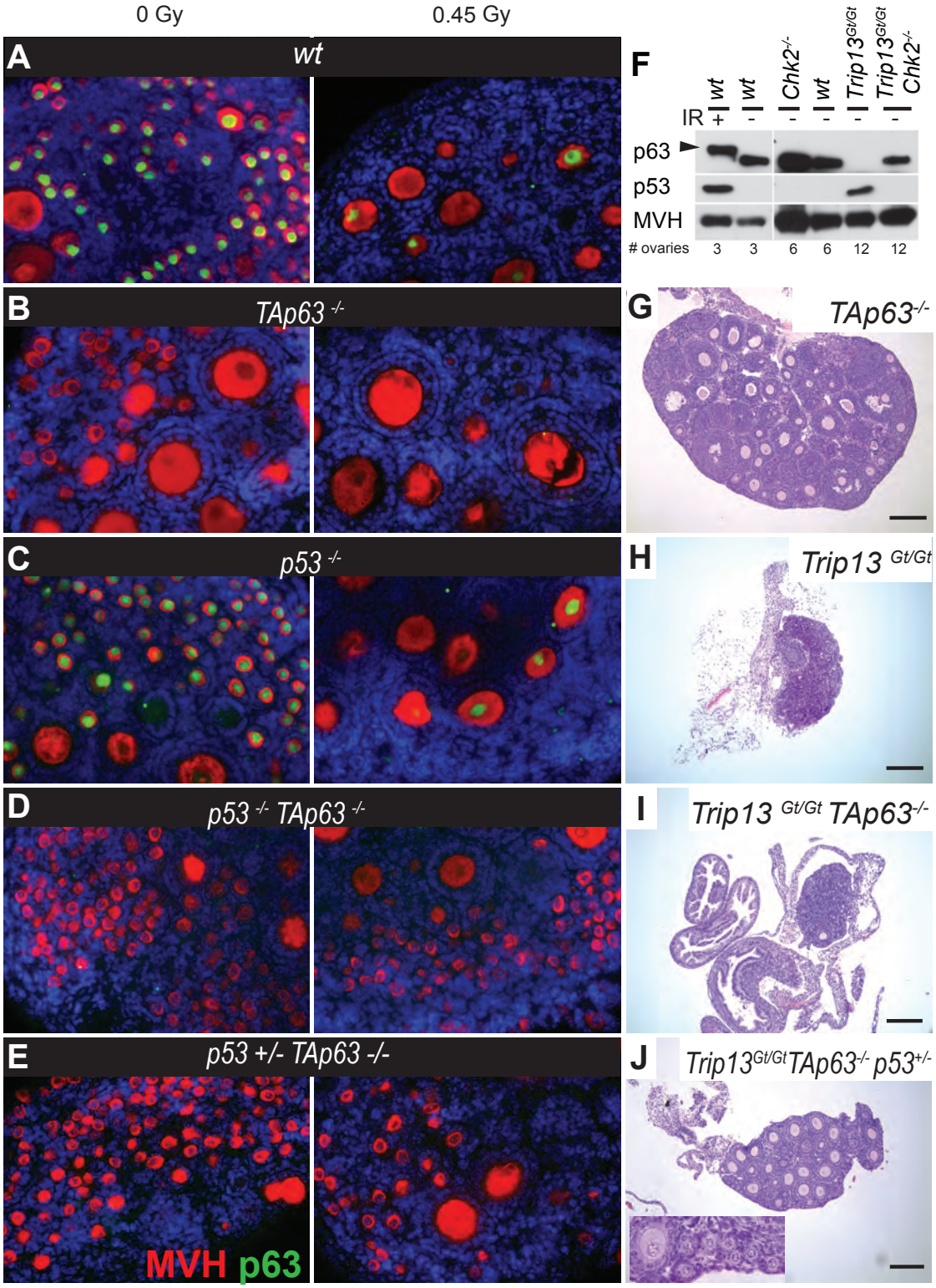

Fig. 4. CHK2 signals to both p63 and p53 in oocytes. (A-E) Depletion of primordial follicles by IR requires p53 and TAp63. One week old ovaries were irradiated then

cultured 7 days before immunohistochemistry. p63 and MVH are oocyte-specific. **(F)** Dynamic signaling to p53 and p63 in response to meiotic and induced DSBs. Shown are Western blots of neonatal ovarian protein. The irradiated sample was collected 2 hrs post-IR (3Gy). Arrowhead: phosphorylated p63 (*15, 16*). Note that *Trip13* mutants are in process of eliminating oocytes (reflected by MVH), hence use of more ovaries. **(G-J)** Both p53 and *TAp63* are required for complete elimination of DSB repair-defective oocytes. Ovaries are 3 week postpartum. Inset of J shows primordial follicles. Scale bar=200 μm.

APPENDIX II

ASE-16-0039.R1

Research Report

Evaluating a Technology Supported Interactive Response System During the Laboratory Section of a Histology Course

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Running title: Identifying misconceptions “on-the-go”

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ABSTRACT

Monitoring of student learning through systematic formative assessment is important for adjusting pedagogical strategies. However, traditional formative assessments, such as quizzes and written assignments, may not be sufficiently timely for making adjustments to a learning process. Technology supported formative assessment tools assess student knowledge, allow for immediate feedback, facilitate classroom dialogues, and have the potential to modify student learning strategies. As an attempt to integrate technology supported formative assessment in the laboratory section of an upper-level histology course, the interactive application Learning Catalytics™, a cloud-based assessment system, was used. This study conducted during the 2015 Histology courses at Cornell University concluded that this application is helpful for identifying student misconceptions “on-the-go”, engaging otherwise marginalized students, and forming a new communication venue between students and instructors. There was no overall difference between grades from topics that used the application and grades from those that did not, and students reported that it only slightly helped improve their understanding of the topic (3.8 ± 0.99 on a five point Likert scale). However, they highly recommended using it (4.2 ± 0.71). The major limitation was regarding the image display and graphical resolution of this application. Even though students embrace the use of technology, 39% reported benefits of having the traditional light microscope available. This cohort of students led instructors

to conclude that the newest tools are not always better, but rather can complement traditional instruction methods.

Keywords: Undergraduate medical education, Microscopic anatomy, Histology, Virtual microscopy, Digital morphology, E-learning, Interactive computer graphics, Formative assessment, Instant feedback assessment technique

INTRODUCTION

Most instructors strive to prevent students from making faulty interpretations of topics; but if these misinterpretations are inevitable, then identifying them early is key (Watkins and Mazur 2013). The use of any teaching tool that generates information about students' achievements and can be used by teachers and students to improve learning is a formative assessment tool (Black and Wiliam 2009; Baleni 2015). The use of formative assessment to obtain and provide continuous feedback on student's state of knowledge has been implemented in the biomedical sciences in many forms (Clynes and Raftery, 2008; Alexander et al., 2009; Trumbull and Lash, 2013; Antoniou and James, 2014). The repertoire of formative assessment tools in use at the Cornell histology course (BioAP4130/BioMS4130) encompass quizzes, laboratory reports, case study reports and laboratory activities using both light microscopy and virtual microscopy as tools. Instructors utilize these to provide constructive feedback to students and gain insight into the misinterpretations and gaps in students understanding allowing modifications in teaching strategies. For the Cornell histology course these assessments also contribute to the student's final grade, which summarizes the students' achievements for the course, i.e. the summative assessment (Nicola and Macfarlane‐Dick, 2006). Whereas formative assessments "monitors to improve" student learning, summative is an evaluation of learning. Therefore, even though all graded portions of the histology course are also summative assessments, only the final examination is not formative.

Students can benefit from formative assessments only when provided with feedback that allows them to act upon their learning (Perera et al., 2008; Trumbull and Lash, 2013). Feedback is an interactive process that provides information about a person's performance of a task, which can be used as basis for improvement (Nicola and Macfarlane‐Dick, 2006; Clynes and Raftery, 2008; Perera et al., 2008). Feedback provided through the analysis of formative assessments helps improve student's learning strategies (Nicola and Macfarlane‐Dick, 2006; Perera et al., 2008). Usually formal feedback, as those provided when students receive their graded written assignments, takes longer to reach the student and require student's motivation in order to be effective (Mullet et al., 2014). Informal feedback is mostly delivered immediately by direct interaction between students and teacher during a laboratory session (Clynes and Raftery, 2008). In laboratory settings that require instructor-student interactions, immediate informal feedback is not only important but also the most frequent. However, informal feedback may never reach shy students who are not comfortable asking questions or seeking clarification (Sinclair and Cleland, 2007) resulting in marginalization and failure to achieve full potential (Sinclair and Cleland, 2007).

The timing in which a student receives feedback is also important and has variable outcomes (Dihoff et al., 2003; Sinclair and Cleland, 2007; Clynes and

Raftery, 2008; Miller et al., 2014; Mullet et al., 2014) that inevitably depend on how students react to it (Dihoff et al., 2013). When feedback is provided while interacting with students it is said to be an immediate feedback (Shute, 2007; Mullet et al., 2014). Feedback provided days or weeks after an assessment has taken place is labeled as “delayed” (Shute, 2007; Mullet et al., 2014). Most classroom environments foster both immediate and delayed feedback. It can be generalized that students receiving immediate feedback are less likely to form memory of wrong concepts (Crouch and Mazur 2001)). In courses that progressively build new concepts upon recently acquired knowledge (e.g. concepts developed earlier in the course), the memory of a wrong concept may irreversibly impair the student’s understanding of the newer topic. Therefore, learning outcomes are at stake if a misconception is not promptly identified and corrected (Crouch and Mazur 2001); Dihoff et al., 2003). In contrast, Mullet et al. have compelling evidence of the benefits of delayed feedback that cannot be taken for granted (Mullet et al., 2014). Envisioning that no approach is singly the best, the ideal feedback should have both the immediate and the delayed component.

Histology is a detail-oriented biomedical course that requires identification and description of the cellular organization in healthy tissue (Mione, Valcke, and Cornelissen 2013). It is traditionally taught as a combination of descriptive lectures and practical laboratory sections (Bloodgood 2012; Bloodgood and Ogilvie 2006). The latter aims to develop the students’ abilities to identify

subtle morphological differences in characteristics of a tissue and integrate their knowledge of the functional aspects of an organ with their observation of a two dimensional microscopic image (Bloodgood and Ogilvie 2006; Hortsch and Mangrulkar 2015; Mione, Valcke, and Cornelissen 2013; Selvig et al. 2015). This is a complex and multistep process that is usually facilitated by interaction between learners and teachers. Therefore, an ideal formative assessment tool would nurture student teacher communication, be able to display high-resolution images that react to user interaction, provide formative assessment to instructors, and allow for formal immediate and delayed feedback to students.

With advances in technology, teachers of morphology-based courses have tools that together with virtual slides (VS) can be used to engage students and assess learning outcomes before graded examinations are performed. Such tools are often referred to as classroom response systems (CRS) (Paschal, 2002; Shell et al., 2013) or audience response systems (ARS)(Alexander et al., 2009). Examples of CRSs are “clickers” (Briggs and Keyek-Franssen 2010), the Piazza Q&A platform (“Piazza • Ask. Answer. Explore. Whenever.” 2016), internet-based voting applications (Mathiasen 2015), and the web-based Learning Catalytics™ platform (Schell et al., 2013; Mullet et al,. 2014). These teaching tools are currently used to engage students and provide immediate feedback about their state of knowledge. Even though these tools are widely used, there are few formal reports about their effectiveness and

how they are perceived by students (Karolcík et al. 2015; Selvig et al. 2015) when used during laboratory sections. Even though many excellent technology supported formative assessment tools that provide immediate feedback are available, this study was conducted using the Perason Learning Catalytics™ platform to readily identify misconceptions and provide a more inclusive and formal feedback.

Currently, the Cornell histology course uses microscopy as tool for immediate formative assessment. The course is in the form of a traditional lecture followed by an interactive laboratory session in which instructors assist students in examining histological slides; thus providing students with immediate informal feedback. The lecture component uses PowerPoint slides coupled with an educational talk. The laboratory component relies on student engagement in learning activities that refers back to concepts presented during lecture. The activities prompt students to find and correlate histological structures with organ function. Students are allowed to work in groups, formed without instructor intervention. It is during the laboratory session that all formative and summative assessments for the course take place. In the laboratory setting, students have access to multiple glass slides, computers (used to visualize the virtual slides (VS)), and traditional two head microscopes. During the laboratory section instructors make available histology books and atlases. If solicited, students receive immediate informal feedback while using virtual microscopy (VM) or light microcopy (LM) with

glass slides (GS). Unsolicited feedback is offered when instructors deem students are having difficulties with the topic (Bloodgood 2012; Collier et al. 2012). Quizzes, laboratory reports and two examinations in the middle of the semester are the formative assessments used to provide delayed formal feedback.

Objective:

The purpose of this study is to evaluate the use of an interactive cloud-based classroom response system (CRS) to identify misconceptions “on-the-go”, minimize erroneous interpretation due to contradictory or confusing informal feedback, and obtain a more inclusive teaching atmosphere (Stoltzfus, 2014). The targeted audience is undergraduate and graduate students enrolled in Cornell’s 2015 histology course. The course’s laboratory component requires student engagement through active learning and peer learning activities. As an effort to improve students’ learning outcomes, the learning activities constantly incorporate the use of new teaching tools, currently using the VM technology coupled with the LM and problem based learning activities. However, misconceptions about the topics still arise and are not detected by instructors until grading high stakes assessments (Feldman and Capobianco, 2007). The Pearson Learning Catalytics^{TM} platform is the selected CRS (Schell et al., 2013; Mullet et al,. 2014). Surveys from students and instructors provide information on their perception of this CRS. Impact on learning

outcome is assessed through self-reported experiences and through the comparison of question scores obtained on topics that did or did not use the CRS.

This research was done according to Cornell Institutional Review Board Policy # 2 and under paragraph 2 of the Department of Health and Human Services Code of Federal Regulations 45CFR46.101(b) and has protocol ID# 1503005435.

MATERIALS AND METHODS

Software:

The Pearson Learning Catalytics™, version 2015 (Pearson Education Corp., Upper Saddle River, NJ) is the Internet based teaching tool selected to provide formative assessment. The following were the reasons for making this choice: (1) Learning Catalytics™ CRS is not restricted to multiple-choice questions. Instead, examples of question types that can be created include written short or long answers, word cloud, matching pairs, identifying regions and sketching (see figure 2 and 4 for examples). (2) Students can use any web-enabled device (such as smart-phones, computer and tablets.) Thus, the existing laboratory setting did not require the purchase of any extra device. (3) It has an interactive component where students not only answer the questions in real time, but also are able to let instructors know if they understood the reasoning

behind an answer. (4) Students could submit questions without the need to raise their hands, speak out loud, or wait for all students to submit their answer. (5) Students' responses are immediately made available on instructor's device (Schell et al., 2013; Mullet et al,. 2014). (6) It is user friendly and the implementation only requires that students and instructors have access to the Internet, a web-enabled device, and a valid account. At the time of the study the student cost was US$12.00 for six months. (7) For the purpose of this study Pearson made available two instructor accounts and 50 student accounts for a period of four months free of charge.

In order to test the tool in a laboratory session, four small modules containing ten to fifteen questions each were generated using the software. The modules were delivered as 15 to 30 minute review sessions. The sessions were interactive in the sense that students and instructors actively discussed concepts. Furthermore, as each question was delivered, students articulate a response. This commitment to an answer has been shown to make students more likely to seek understanding and engage in discussion (Alexander et al., 2009; Ludvigsen et al., 2015). The notation "interactive review session" (IRS) was chosen as a descriptor since the activity was designed around previously learned topics and required meaningful learning (there was no passive delivery of information). Instructors also reminded students that the IRS was voluntary, had no impact on their grade, and used an interactive CRS platform. Placing the IRS at the end of the laboratory session was partially based on

Favero's description of review sessions, and partially as an attempt to minimize any negative impact to the course if students shunned the activity (Favero, 2011).

Study Participants:

All of the students enrolled in the spring semester of the 2015 histology course at Cornell University were encouraged to participate in the study. Every intervention and survey request provided participants with information regarding the research goal and procedures of the study. Participation was not required, and students were allowed to withdraw at any time without any penalty. The Institutional Review Board (IRB) at Cornell approved the research methods used. The Pearson's Learning Catalytics™ software was kindly made available by Pearson representatives, free of charge, for all students regardless of their choice to participate in the study.

The staff for this laboratory semester consisted of one faculty member, one postdoctoral teaching assistant (TA), one graduate TA and five undergraduate TAs. The faculty member and the graduate TA (authors of this article) envisioned and implemented the IRSs activities. The graduate TA did not participate in any survey. During the laboratory, all staff members (referred to as instructors throughout the manuscript) were encouraged to hover around the room waiting for students to call for help. Instructors had weekly meetings

in which the slides and slide descriptions were thoroughly reviewed to ensure comparable and homogeneous knowledge of the topics.

Interactive Review Session:

The IRS questions were designed around the topics: integument; gastrointestinal tract (GIT); oral cavity; and endocrine system (thyroid, liver and pancreas). There are six questions from the Oral Cavity module available as Supporting Information. Questions covering the material suggested for the week, were designed a day prior to the activity, and mirrored instructors' previous experiences about student misconceptions. Studies by Freeman et al suggest that the students are more likely to engage in an interactive exercise if they are more familiar with one another and with their instructors (Freeman et al. 2014).Therefore, these IRSs were implemented during the second half of the semester as an attempt to obtain a better participation rate once students had time to build a relationship with instructors and with each other.

Measure of Impact in Learning Outcome:

In order to test if the CRS mediated feedback impacted learning outcomes, surveys and final examination question scores were utilized. Question scores derived from topics either using or not using the CRS were compared. For topics that did not use the CRS, formal delayed feedback had previously been provided through quizzes and laboratory reports. Examination scores were not

linked to students' identification, and the quantitative analysis used question scores from all students, independent of their participation on the IRS. Final examination scores did not follow a Normal distribution (both score types with a $P(W) < 0.001$ for the Shapiro-Wilk goodness-of-fit test). Therefore non-parametrical Wilcoxon comparison for each pair method was used to compare the difference between scores from IRS and non-IRS topics. A table containing the averaged scores, standard deviation (SD), and median for the 23 questions can be found in the supporting information. There was a total of 11 non-IRS and 12 IRS related questions for the final examination. All statistical analyses were done using JMP Pro, version 12.0.1 software (SAS Inc., Cary, NC). Students and instructors were asked to provide feedback about their experiences by answering voluntary and anonymous surveys. The impact on students' grades was not a focus of our study instead, the study was designed to identify misconceptions "on-the-go", minimize confusion due to informal feedback and obtain a more inclusive teaching environment.

Surveys:

In order to assess how students experienced each module of the IRS, specific internet-based surveys were designed for each module (mini-surveys). These surveys were sent to students soon after IRS ended utilizing the Cornell Qualtrics platform (a web-based survey tool available to the Cornell community). The information provided in the different surveys was used to

improve each subsequent IRS (Supporting Information). We also generated two paper-based surveys: [1] one delivered before the IRS activity began (Supporting Information); [2] and another delivered the last day of class; prior to final examination and after all IRSs (Supporting Information). All surveys had questions that used the five point Likert-type scale (1 = Strongly disagree; 2 = Disagree; 3 = Neutral; 4 = Agree; 5 = Strongly agree; 1 = “poor rating” and 5 = “excellent rating”) and open-ended questions. Kendall’s tau B and Cronbach’s alpha statistical tests were performed to assess correlation and reliability using JMP Pro12 software. Responses with correlation coefficient (r) above $r > 0.6$, Kendall’s tau p-value $p(\tau) < 0.05$ and Cronbach’s alpha $a > 0.7$ were combined to represent a measure of positive or negative students’ perception. This combined Likert is therefore a 10 points scale. All mean values provided are accompanied by respective standard deviation (SD). Survey [1], adapted from Harris et al. (2001), was designed to obtain information about the students’ perception of LM and VM, which are the two teaching tools used for immediate formative assessment and informal feedback during this histology course. Students had access to both tools in the laboratory setting (open from 8am to 5pm on weekdays) and VM at all times in their personal computers. The survey assessed student’s preference for the tools, difficulty in use and usefulness understanding the material (Supporting Information). Survey [2] was designed to provide information about how students perceived the IRS and the CRS software, and if the activity helped them understand the material. This survey was designed by the authors and

tested by six fellow graduate students to assess clarity. Answers from paper-based surveys were typed into excel spreadsheet for analysis, exactly as written by surveyed individual (Supporting Information). Sample answers found in this manuscript were transcribed exactly as originals. Authors' observations coupled with instructors' surveys (delivered using Qualtrics platform) to assess software usefulness, and effect on student performance, were used to evaluate the activity from a non-student perspective. Excel spreadsheets for all surveys are provided as supporting information.

Course Context:

The study was performed during the laboratory session of the Histology course BioAP4130/BioMS4130 taught in the spring semester of 2015 at Cornell University in Ithaca, NY. This is an upper-level undergraduate course offered by the Department of Biomedical Sciences at the College of veterinary medicine. It is a four-credit course, offered only during spring semesters; the class meets twice a week (Monday and Wednesday) for 14 weeks and the contact hours add up to 24 hours of lecture and 56 hours of laboratory. The course consists of 28 lectures during 55 minutes, followed by 28 two-hour laboratory sections. There are two laboratory sections that are used for preliminary examinations without any laboratory activity.

The classroom size for the spring 2015 semester was 39 enrolled students plus two auditing graduate students (not included in the study). The course's

prerequisite is a three-credit introductory biology course (BIOMG 1350 Introductory Biology: Cell and Developmental Biology) and students are also recommended to have taken principles of biochemistry (BIOMG 3300 or BIOMG 3310) or equivalent.

The course content covered all major organ systems. Both GS and VS from a variety of vertebrate species were available, even after course hours. Weekly packets intended to guide students through the laboratory activities contained factsheets (with brief overview of most basic concepts, together with detailed description of suggested slides) and an assignment guiding students in their learning of histology. The students used the assignments to generate laboratory reports, with five out of nine being graded. Students were recommended to hand-in the non-graded reports in order to receive formal feedback. Laboratory reports and quizzes were low stakes assessments (Gilboy et al., 2014). Grades obtained during the course length were used to assess and provide feedback on student's strength and weakness (formative assessment and formal feedback respectively) (Krasne et al. 2006; Pulfrey et al., 2013) and numerical values were used to determine the student's final grade. Even though students had access to most specimens through VS, some samples were provided only as GS with the goal of encouraging students to develop their microscopy skills. During the laboratory sessions, students were encouraged to work in pairs or small groups, but a few elected to work independently (Braun and Kearns 2008).

Assessments and Assignments

Assessments of three types were used: quizzes, written laboratory reports, and examinations. There were four quizzes, which together were worth 15% of the total grade; five graded written laboratory reports (and four non-graded and voluntary), which together were worth 15% of the total grade; three examinations (two preliminary and one final examination), which together represented the remaining 70% of the final grade. Grades are not curved. The low-risk written assignments and quizzes were designed to engage students with the subject matter and to prepare them for the examinations. The majority of the written assignments required the student to use GS to be fully answered. The impact of the use of the CRS during the IRS on learning outcome was restricted to questions derived from the final examination.

RESULTS:

Classroom Demographics

There were 39 students enrolled in the 2015 histology course, 19 females, and 20 males (table 1). From these, 28 volunteered (12 males and 16 females) to answer the first paper based survey [1] (Supporting Information) and 25 students (9 males and 16 females) answered the second paper base survey [2] (Supporting Information). These surveys provided us with information about the students' career goals, the usefulness of the teaching tools (e.g. VM, LM

and CRS) and if they recommended the tool (these are summarized in figure 1). There were always more females that answered the surveys than males, including the mini-surveys (table 1). These students are high achievers with 72% seeking to pursue a professional medical (MD) or a veterinary degree (DVM) (mean Likert scale of 4.2 ± 1.5).

Interactive Review Sessions

The IRS preferentially exposed students to images derived from unfamiliar histological samples in order to obtain information about the students' understanding. The goal of this approach was two fold: avoid responses based on memorization of particular aspects of the slide (for example histological artifacts) and help identify misconceptions on foundational concepts (for example differentiation between organ structure and cell morphology) (figure 2). In this regard, Pearson's Learning Catalytics™ software was a good tool to identify topics in which students needed more clarification. Figure 2c is an example of a composite sketch type question. This question asked students to draw a line on the interface between the dermis and the epidermis. After delivering the question it was noticeable that some students had a misconception, with seven out of 22 (32%) responses being wrong. Once students saw the composite sketch of their answers, they were asked to share the reasoning for their answer with their peers. When the question was delivered again, 96% of answers were correct. The student that

still marked the wrong answer was identified (instructors have access to individual answers) and later received an email from the instructor via the software addressing the misconception. Another example of the subtlety of misconceptions that can be identified is presented in figure 2a, a composite sketch type question. The data from students' responses allows instructors to determine that, although students could indicate the transition point, many had trouble differentiating stomach from duodenum. Figure 2a is another example taken from the endocrine IRS.

Despite the clear benefit of the application toward identifying misconceptions and promoting formal immediate feedback, for this small cohort of students, the platform did not improve final examination scores above that of the currently employed feedback methods (figure 3). The averages and median scores for individual questions can be found in the supporting information. The Wilcoxon comparison for each pair method had p-value=0.11, thus there was not strong evidence to support difference in students' performance when answering questions from topics derived or not derived from the IRS. The average score for questions derived from IRS topics was 7.4 ± 0.96, whereas the average from non-IRS topics was7.6 ± 1.07. However, these are absolute values that include all students regardless of their attendance for the IRS.

Survey [1]: Microscopes

Both LM and VM, the traditional tools used for immediate formative assessment and immediate informal feedback, were familiar to this cohort of students. There was an overall positive perception for both LM (7.1 ± 1.72) and VM (9.2 ± 1.07), as determined by combined Likert scale obtained from questions pertaining to usefulness of tool and recommendation of tool (figure 1 and Supporting Information - table 2 columns Q1-1, Q6-1 and Q1-2, Q6-2). When asked about benefits of using both VM and LM in open-ended questions, students' responses reflected appreciation for both. However some students would rather have only the VM (aperio refers to VM):

"*no, I liked Aperio and felt it was sufficient alone and convenient for me to study at home*"

There were 11 students (39%) that answered "*yes*" they benefit from having access to both methods VM and LM together and 6 (21%) answered "*no*"(Supporting Information - Table2 Q2). One student reported the LM as the favorite method of learning. The following quote best represented this cohort of students:

"*to an extent yes* [only one method is sufficient*], but both are better in tandem. The virtual is convenient, but the optical is useful on developing microscopic techniques/understanding. Both correct for each ones shortcomings"*

Survey [2]: Interactive Review Session

On average students thought the review sessions were helpful (five point Likert of 4.2± 0.91) but software only slightly improved guiding the session towards problematic topics (five point Likert of 3.8± 0.99). However, there was significant correlation between student's response when asked if "the use of an interactive software was helpful to tailor the review session to topics [they] had trouble understanding" (figure 1 and Supporting Information - table 3-Q4) and if "[they] recommend having review sessions" (figure 1 and Supporting Information - table 3-Q5). Even though the correlation was weak between the other questions, the average Likert score for all leaned towards 4, thus a good rating (figure 1 and Supporting Information - table 3). The combined Likert score of 8.0 ± 1.77 (Supporting Information - table 3- association of Q4 and Q5) suggests that students' perception of the interactive review session using the Learning Catalytics platform (IRS using CRS) was over-all positive. Furthermore, students would recommend the use of interactive software during review sessions (five point Likert of 4.2 ± 0.71) (Supporting Information - table 3- Q6). Student's self-reported experiences show that although rated as helpful, some students felt that the review modules were not well structured:

"*I like how the material is presented but it's quite time consuming. With better organization and communication, it'd be more time efficient*."

"*Learning catalytics can be more useful if the questions are improved*."

The theme about the images size and quality was common throughout the activity and is evident in the surveys:

"*Learning catalytics is great. But I wish pictures could be bigger.*"

"*Bigger pictures, pictures that showed the right answer at the end CLEARLY.*"

The ability to communicate with instructors using the CRS was re-current:

"I was able to provide anonymous question that whole class could benefit from"

"*they* [IRS using CRS] *are great to reinforce material and learn new things! I also like how you can message the instructor privately to identify material you struggle with*"

Mini-Surveys

The mini-surveys were helpful in designing and structuring the IRSs. For instance the first mini-survey (Supporting Information - table 1 – mini-survey 1 – Integument) clarified that waiting for 90% of the students to answer was not an ideal threshold for setting up the review's pace. After this feedback a time limit of 30 seconds to one minute was set for inputting the answers. Another request that aided delivery was to use a projector to go over the questions as suggested by other students:

"[have] *a set time to answer each question and then move on. If most of the class gets it wrong then explain in depth the answer but If a vast majority of the class gets it correct then move on*."

"*Maybe use the projector in the lab to put the questions up….*"

With regards to the second mini-survey (Supporting Information - table 1 – mini-survey 2 – Oral Cavity) issues with the quality and size of images were raised. Also it was noted that the IRS material was not available to students once the section ended.

"[Suggestion for the next IRS] *Bigger pictures"*

"*Yes, it was helpful, but I did not find it afterwards to aid in my study, was it posted?*"

At the third mini-survey (supplemental table 1 – mini-survey 3 – GIT) students' statements revealed familiarity with the different question types. When asked for suggestions for the next IRS students' responses reflected the types of questions they deemed helpful:

"The what is this, and what is its function- questions"

"having the images and having to sketch or circle things is very helpful!"

Only four students answered the fourth mini-survey (supplemental table 1 – mini-survey 4 – endocrine), and their response suggests that the activity was helpful:

"*It helps clarify what we are looking at in the slides.*"

Instructor Survey

A total of four out of six TAs answered the survey. From the instructors' survey, the theme about the image quality became evident one more time. When asked for their opinion about if the software was an effective method to deliver the IRS (Supporting Information - table 4 - question 7) three out of the four answers reflected the CRS's limitation:

"*I just wish that the images could be bigger. I thought the software was great because they could draw on images and I liked the way we could see which answers were the most common. The software seemed pretty good to me*."

Observations from the Authors

During the review modules the students appeared interested and engaged. Students did not limit participation in the IRS to being physically present, and three students used cell phones to access the interface while leaving the classroom. During the later sessions students were more likely to ask questions especially by sending messages using the software rather than by voicing them. For instance, two students that never requested help during the regular laboratory activity were noticeably more active and seemed comfortable texting their questions to the instructors rather than voicing them. Therefore, the Learning Catalytics was able to engage a different cohort of students that might have otherwise been marginalized (DiLullo, McGee, and Kriebel 2011) yielding in a more inclusive classroom environment.

Although the process of creating the review modules *per-se* required a lot of up-front work, the positive response of the students made the effort worthwhile. Besides the student's engagement in the activity, the CRS allowed for visualization of students' misconceptions. For instance, the example in figure 2c allowed instructors to visualize that 32% of students believed that the layers of the epidermis had a maximum depth of a couple of cell layers. While most students were able to list the layers that formed the epidermis in a short-answer type question, seven out of 22 students failed to correctly draw a line in the interface between dermis and epidermis on an unfamiliar sample (figure 2c). This interactive exercise revealed that several students resorted to the color differences, instead of conceptual knowledge, to differentiate the layers in the image. The image was from thick epidermis that had a much wider layer than the histological slide suggested by the laboratory activity handout. The example in figure 2a shows student's ability to identify the transition point between duodenum and stomach, but not differentiate which side is the duodenum. The CRS provided the ability to identify and address subtle issues like these. It also allows instructors to provide immediate feedback to all (if a prevalent misconception is identified) or individualized (if it is an isolated case). Students can benefit from the feedback immediately or delayed by revisiting previously given modules. Therefore, the CRS is an efficient immediate formative assessment tool that can be used to provide formal immediate and delayed feedback.

Every review module provided us with different observations. For instance, during the first review module, we noticed students were not expecting to actively answer questions. Even though they were informed about the need to actively participate, they seemed to be expecting a passive review in which instructors lectured about the important topics. This probably reflects previous experiences in which instructors provided a list of important concepts without the need to actively participate. However there was a shift in students' behavior for the next modules, with the majority studying the material, and preparing for the review. The students clearly did not like to answer questions incorrectly even when their identity was not disclosed. Since 75% of students were either on the preparatory track for veterinary (pre-vet) or human (pre-med) medicine our interpretation is that there was an intrinsic desire for students to excel and demonstrate their understanding of the material. Another general observation that supported this interpretation was that when questions were easy, students stopped participating, whereas if the questions were challenging, they would engage more in the activity. Therefore our observations might not represent how students would react in a less competitive environment.

DISCUSSION

CRS in the Laboratory Setting

Student's response to survey [2] suggests that this cohort of students value the IRS, independent of the use of a CRS, but would recommend implementing it if available. In order to be better suited for a laboratory setting, the CRS would need to be coupled with a microscopy method. Whereas the VM facilitates group learning (Harris et al., 2001; Braun and Kearns, 2008; Husmann et al., 2009), the LM is still the most used method outside of the classroom. For instance clinical and academic settings still require the use of the traditional microscope to expedite sample analysis or diagnosis of diseases, such as the evaluation of fine-needle aspirates and skin surface cytology (Pratt 2009).. It was unexpected to find that only 42% of the responders envision using the LM throughout their professional career (Supporting Information - table2 Q2-1) since 72% seek a medical (MD) or a veterinary degree (DVM). Although a CRS does not replace the VM or the LM as teaching tool it can be used to motivate and engage students on learning activities.

A major limitation of the CRS was image size and quality. Morphology-based biomedical courses rely on student's interpretation of images. In the same way recognition and perception of visual stimuli shifts between individuals (Partos et al., 2016), students' perceptions of histological images are variable and unpredictable (Figure 4) (Fouché 2015). It is important for teachers to

anticipate topics in which students will have difficulties and possible misinterpretations, in order to remove such obstacles as quickly and efficiently as possible. A teaching tool that allows the incorporation of an interactive live digital imaging technology would be better suited for a laboratory setting (Higazi, 2011). The results from survey [2] illustrate the importance of image size and quality in morphology-based biomedical courses. Hopefully, this result will encourage instructors and software developers to invest in integrating interactive teaching software to microscopy, in order to devise better technology-supported formative assessment tools.

CRS to identify misconception

As exemplified in figure 2 the CRS has greatly aided in the identification of subtle misconceptions. The software allows multiple types of questions (Lukoff, 2013; Schell et al., 2013; Mullet et al,. 2014) (figure2 and 4) and instructors were able to monitor responses in real-time. As soon as misconceptions were identified instructors were able to immediately guide students to correct the problem in class discussion.

Although the CRS can be used to identify students' misconceptions as they occur (e.g "on –the-go"), there are limitations for instructors to use this technology in a laboratory setting. For instance, the inability to integrate VM with the teaching software limits possible interactions to planned discussion,

since the activity has to be prepared in advance to use images. Morphology-based biomedical courses often rely on high-resolution images and benefits from students' interaction with VS. Therefore, being unable to integrate VM with the CRS is a limiting factor, especially impairing the creation of questions in real-time (Antoniou and James, 2014).

Formal Immediate Feedback

The BioAP4130/BioMS4130 laboratory setting relies on informal immediate feedback, where students have to interact with professors and TAs to clarify doubts that they may have from observing a histological sample (Bloodgood 2012; Collier et al. 2012). Besides student's personality (Sinclair and Cleland, 2007; Khalil et al., 2013), other factors that interfere with students seeking feedback and student satisfaction with feedback are inconsistencies among instructors (Warmann et al., 2016) (Supporting Information – tables 2 and 3). Furthermore, instructors have to adapt to the students' diverse learning styles and interpretations in order to provide effective feedback during every activity (DiLullo, McGee, and Kriebel 2011; Twenge 2009). In this regard, the CRS not only provided a platform for formal immediate feedback, but also allowed for a consistent yet individualized feedback. The latter can be done using the software's ability to provide written feedback that stays associated to that particular student's account (Baleni, 2015).

Inclusive Environment

The result that the CRS provided a new venue of communication with the instructors was an unpredicted positive outcome of using the CRS. Students who are shy or uncomfortable asking questions are often marginalized and may fail to achieve their full potential (Sinclair and Cleland, 2007; Khalil et al., 2013). In this regard the CRS allowed for a more inclusive environment where shy students obtained immediate feedback without the need to vocalize their questions (Chen, 2015). Both students and instructors appreciated this aspect of the CRS. The tool also minimizes erroneous interpretation due to contradictory or confusing informal feedback (Warmann et al., 2016), since the immediate feedback can be formal and homogeneous (minimizing differences in instructors' ability to guide students through the material, background knowledge and teaching experience) (Stoltzfus, 2014). Furthermore, the fact that 39% of students' report they benefit from having access to both VM and LM, one student prefers the LM (Supporting Information – table2), and 37% strongly recommend the use of the CRS, supports that having a variety of stimuli helps provide a more inclusive environment. Therefore, in training students for their professional careers, the use of different teaching tools, both old and new, results in better learning experiences. .

Limitations of this study

The findings detailed in this manuscript are dependent on self-reported experiences and student scores obtained from a small number of questions that were drawn from the course's final examination. Beyond the intrinsic difficulties in comparing student performances, the quantitative analysis included all students, regardless of their presence in the IRS, and was limited to questions from one single examination. The questions present at the final examination had different degrees of complexity that confound the analysis between IRS and non-IRS topics. Therefore, the quantitative analysis (figure 3) is limited, and cannot be taken as predictive of the impact on learning outcome when CRS-mediated feedback is compared to traditional delayed feedback. Furthermore, multiple other studies report that CRS not only engages students, but also improves learning outcomes (Alexander et al., 2009; Briggs and Keyek-franssen, 2010; Mostyn et al., 2012; Talbert 2013).

Responses for the survey [2] had weak correlation, which may indicate variability in students' interpretation of the questions or willingness to provide feedback. There were also unexpected technical difficulties reported by students during the surveys. The most common complaints were: trouble with visualization platform (Leica Biosystems' Aperio eSlideManager) from students using Macintosh Computers (Apple Corp, Culpertino CA); that instructors were inexperienced using the CRS software; and that there were significant time constraints of the IRSs activity. Although there was interaction between the participants, it relied on a planned activity. An exclusive

interactive experience, without previous planning and where the teacher is responsive to events as they arise (Antoniou and James, 2014), would require a CRS capable of incorporating the VM.

For this study, the Learning Catalytics™ software did not improve learning outcome as measured through question scores, but helped instructors gain insight about students' interpretation of fundamental concepts.

CONCLUSIONS

The use of the CRS facilitates prompt identification of misconceptions and allow for a more inclusive classroom environment. However, the fact that the questions have to be planned in advance limits its abilities as an interactive teaching tool (Antoniou and James, 2014; Warmann et al., 2016). Nevertheless, both students and instructors agreed that the use of the interactive software was beneficial. Overall the CRS improved the instructors' ability to (1) engage students in learning activities, (2) identify misconceptions that would otherwise be unnoticed, (3) provide formal immediate feedback (4) exchange information by opening a new venue of communication between students and teachers. Furthermore, this cohort of students value the availability of multiple teaching tools instead of replacement of older ones, especially when such a tool is still prevalent in the work environment. Therefore, although the CRS is not essential it is an effective teaching tool to identify misconceptions and provide feedback.

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TABLES

Table 1. Descriptive demographic showing number of respondents, sex and academic background. IRS = Interactive review session.

	Enrolled Students	Survey [1]	Survey [2]	mini-Survey IRS1	mini-Survey IRS2	mini-Survey IRS3	mini-Survey IRS4
Total	39	28	25	13	15	6	4
Male	20	12	9	5	7	0	2
Female	19	16	16	8	8	6	2
Sophomore	1	0	1	1	0	0	0
Junior	8	5	7	2	4	3	0
Senior	19	13	12	6	5	1	2
Graduate	11	7	5	3	6	2	2

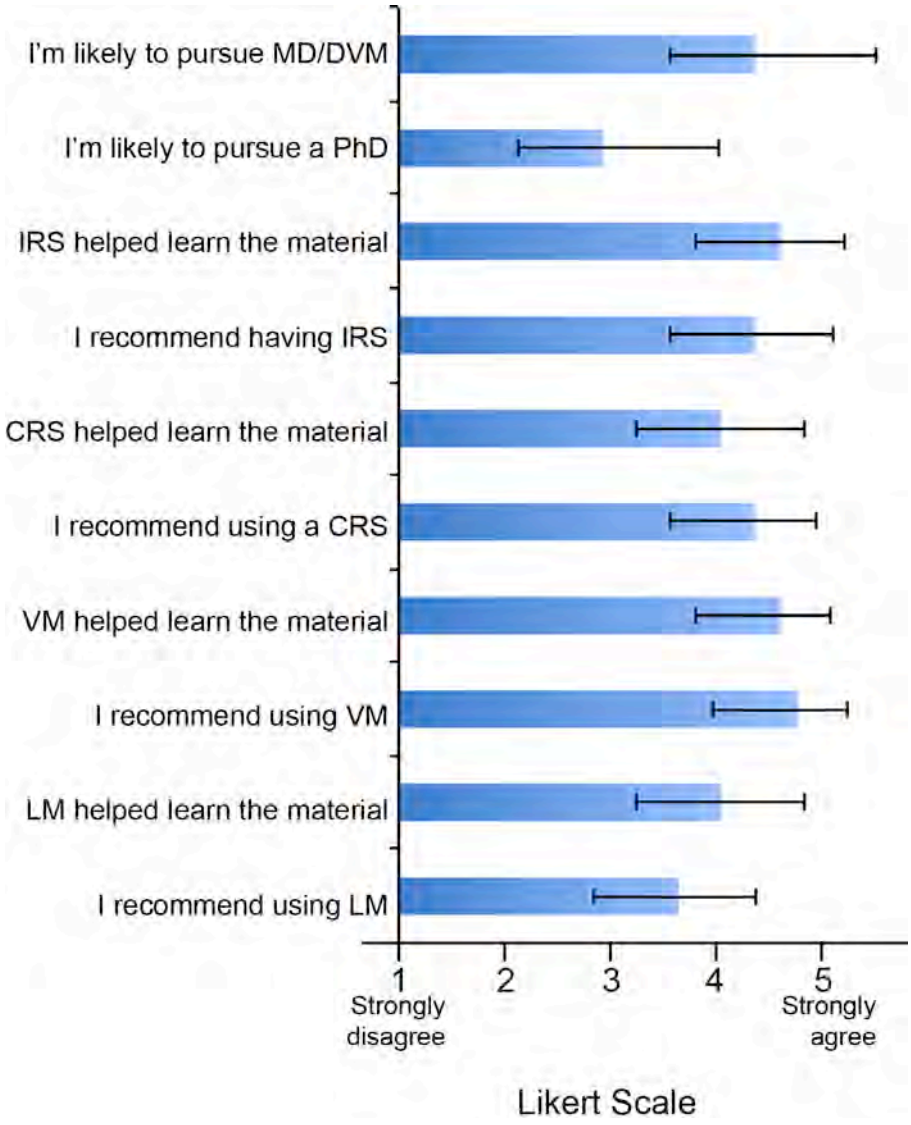
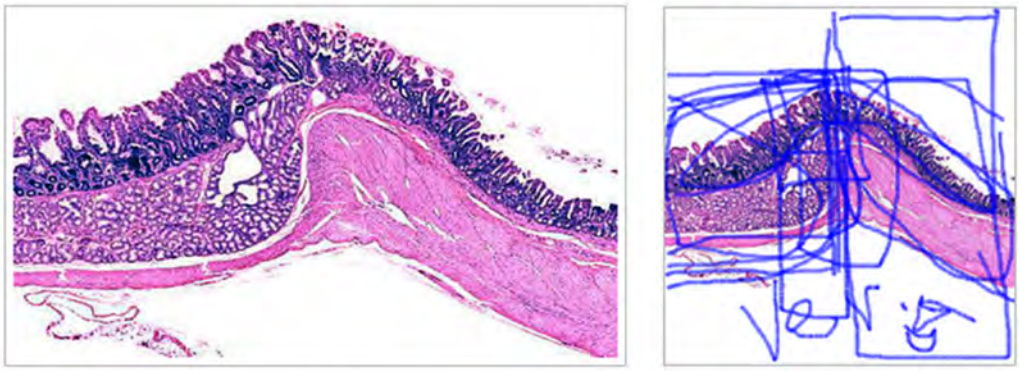


Figure 1: Summary of surveys' responses. First two bars summarize career aspirations. Following bars summarize student's perception of IRS activity, CRS used, and microscopic methods. IRS= Interactive review sessions; CRS= Classroom response system; VM= Virtual microscope; LM=Light microscope; Error bars represent standard deviation.

A

Draw a box surrounding the duodenum. Make sure that the line difining the side of your box meets the transitional region connecting the duodenum to the stomach (pyloric stomach).

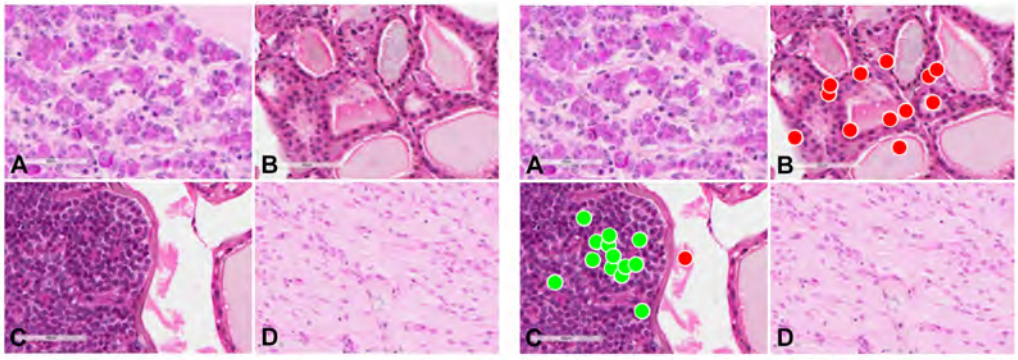
14 responses



B

Click on the region of the image that contains cells producing a hormone that would increase blood calcium level?

26 responses, 50% correct



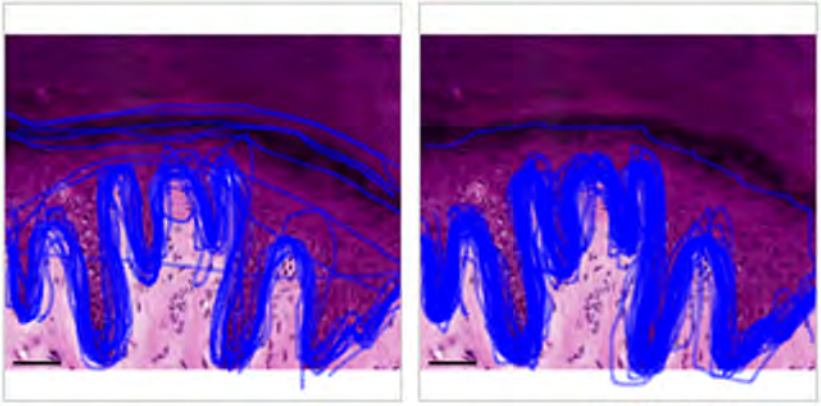
✔ 4 get it now
✖ 0 still don't get it

C

Draw a line at the interface between the dermis and the epidermis?

Round 1 ✖ 22 responses

Round 2 ✖ 28 responses



✔ 17 get it now
✖ 0 still don't get it

Figure 2: Example of Learning Catalytics question types used during review sessions. A, Example of a “composite sketch” type question. In this type of question the instructor presents the task to the student followed by an image. The students respond to the task in their own device by drawing in the image provided what he/she believes is the answer. The blue lines in the image to the right represents a superposition of all the answers; B, Example of a “regions” type question. In this type of question the student is asked to identify a specific region in the image provided. The image in the right represents a superposition of all answers. Correct answers appear as green dots and wrong answers as red dots. This particular software allows the instructors to visualize individual answers as well. C, composite sketch from students’ answers when asked to indicate the interface between dermis and epidermis on a sample image they were not familiar with. Students’ answers are in blue; the image shows an overlay of all responses. To the left are their initial answers. To the right are their revised answers after discussing the concepts and clarifying misunderstandings in the topic.

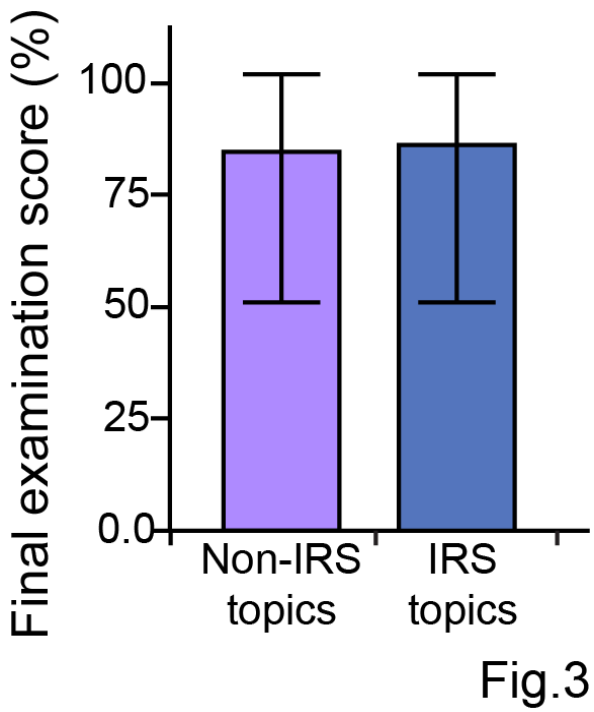


Figure 3: No improvement in final examination scores. Bar graph representing median scores of questions derived from IRS (right) and non-IRS (left) derived topics; IRS median= 8.45, non-IRS median= 8.3; p-value=0.30. Error bar represents score range.