A Mitotic DNA Damage Response Requiring FANCD2 Enables Mitosis with Broken

DNA

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

#### <u>ABSTRACT</u>

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#### Abstract

In order to maintain genome integrity cells employ a set of well conserved DNA damage checkpoints. DNA damage checkpoints are active during interphase and serve to prevent mitosis with broken DNA. Mitosis with broken DNA is associated with DNA segregation errors, genome instability and even cell death in resulting daughter cells. It has recently has been appreciated that cells can compensate for damaged DNA during mitosis. However, little is known about this mitotic DNA damage response.

In this work, I have utilized a genetically tractable system to study mitotic DNA damage responses in *Drosophila*. During development, *Drosophila* rectal papillar cells undergo developmentally programmed inactivation of DNA damage responses. Following inactivation, papillar cells undergo two rounds of mitosis. We find that papillar cells fail to undergo cell death or high-fidelity DNA repair prior to mitosis and instead enter mitosis with DNA double stranded breaks (DSBs). Remarkably, papillar cells segregate acentric DNA fragments into daughter cells during mitosis resulting in viable daughter cells, normal organ development and function. Proper segregation and organ formation is dependent on the FANCONI Anemia gene FANCD2. Loss of FANCD2 results in unaligned acentric fragments and mis-segregation of broken DNA resulting acentric micronuclei formation. Mis-segregation of acentric DNA results in cell death and failure to form a developmentally normal and functional organ. Thus, we have uncovered a role for FANCD2 in mitotic DNA damage responses.

Additionally, we find that single-stranded DNA (ssDNA) is present during papillar cell mitosis following DNA DSB induction. ssDNA is present on both the edge of segregating and lagging DNA as well as spanning short regions between fragments of lagging DNA. The observation that ssDNA is present suggests that while papillar cells do not initiate complete repair, some level of DNA resection must occur following DNA DSB induction. In line with this reasoning, we find a role for the DNA damage sensor complex, the MRN complex, in papillar cell survival following I-Cre induction. The MRN complex consists of three components, Mre11, Rad50 and NBS1. Loss of Mre11 or NBS1 results in reduced papillar cell survival following I-Cre induction. Furthermore, Mre11 is a nuclease. Thus, we propose that MRE11 acts at sites of DNA DSBs in papillar cells to create ssDNA. We hypothesize that formation of ssDNA is sufficient to form a DNA/protein bridge between segregating and lagging DNA to enable proper DNA segregation. Interestingly, resistance to DNA damage is also observed in many cancers. We speculate that such DNA damage resistant cancer cells may utilize similar mechanisms to compensate for DNA breaks during mitosis.

### Dedication

I dedicate this thesis to my advisor Don Fox. During my time in his lab Don has been so much more than an advisor to me. He has supported me, encouraged me, calmed me down when needed, cared about me and given me self confidence in myself as a scientist. I will leave Duke thinking of Don not only as my advisor, but as someone I hope to be friends with for life. Finally, I would like to thank Don for countless afternoon runs- he has served as an excellent running buddy.

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#### Acknowledgements

First and foremost, I would like acknowledge my advisor, Don Fox. Don has taught me more than I can even begin to put in words. During my time in his lab I have learned to conduct research, push myself, and think critically about scientific topics. Perhaps most importantly, Don has taught me to love cell biology.

I would like to also thank the other members of the Fox lab: Ruth Montague, Dr. Jess Sawyer, Benjamin Stormo, Kevin Schoenfelder, Erez Cohen, Nora Peterson, Delisa Clay and Scott Allen. They have provided an incredibly amount of insight and constructive criticism that has been valuable in many ways. As one such example, they dedicated two hours to helping me prepare for my postdoc seminar. I would like to say a special thank you to Ruth Montague who not only taught me almost all of what I know about fly genetics, but also kept the lab running smoothly making research all that much easier. A special thank you is also in order for Erez Cohen who was very generous with his knowledge of statistics and image analysis.

I would like to also thank the Department of Pharmacology and Cancer Biology. This department has provided me with a very friendly and collaborative atmosphere to do research in. In addition, Jamie Baize Smith has kept the department running smoothly on the student end and quickly taken care of any paper work for you before you even realized it needed to be done. Likewise, our grants manager, Sonya Owens was always helpful when it came to submitting fellowships and made it so that I never had to worry about things being submitted on time.

Finally, I would like to thank my family: my dad, my mom and my sister Erika. My dad is also a cell biologist whom I am very proud of. It has been fun to talk with him about science as I move through graduate school. Most importantly, I would like to thank my sister, Erika. Despite the fact she is not a scientist, she supported me in ways I don't think even she is aware of as I moved through graduate school. She was always willing to listen and always sympathetic. I am so lucky to be able to call her my sister.

# 1. Introduction: The DNA damage response: Preventing Mitosis with Broken DNA

The ability to respond to and repair DNA damage is crucial to all forms of life. Lack of a DNA damage response results in genome instability, which is associated with cell death and disease. In order to prevent genome instability, organisms have evolved well conserved DNA damage checkpoints. The discovery of checkpoints and their ability to alter cell cycle progression in the presence of DNA damage was first observed in 1980 (Painter and Young 1980). Scientists found that cells derived from patients with Ataxia telangiectasia (A-T) disease, characterized by hypersensitivity to DNA damage, failed to stop replication the way normal cells did following x-ray irradiation. In the 37 years following this discovery, much research has been focused on elucidating proteins involved in an organisms' ability to respond to DNA damage resulting in careful characterization of this crucial response. The goal of the DNA damage response is to prevent mitosis with broken DNA (Ciccia and Elledge 2010). Despite the importance of the DNA damage response, not all cells contain a fully intact DNA damage response. Yet many of these cells are able to proliferate despite broken DNA. Segregation of broken DNA during mitosis is an emerging area of interest. In this chapter, I will focus on aspects of DNA damage signaling that have proven relevant to my study of acentric DNA segregation.

## 1.1 The DNA damage response maintains genome integrity in mitotically active cells.

During the mitotic cell cycle, cells must replicate their DNA and divide it accurately between two daughter cells. DNA replication occurs during synthesis (S) phase resulting in pairs of homologous chromosomes (Takeda and Dutta 2005). Homologous chromosomes are attached by a structure known as the centromere. The centromere is crucial for homologous chromosome pairing and ensuring that each daughter cell receives a copy of each chromosome (McKinley and Cheeseman 2015). The division phase of mitosis begins with chromosomes lining up at the center of the cell and attaching to a microtubule based bipolar structure known as the spindle. The spindle is crucial for separating homologous chromosomes into daughter cells. Centromeres of homologous chromosomes attach to spindles from opposite poles so that each daughter cell will receive one chromosome from each pair of homologous chromosomes (Prosser and Pelletier 2017). Attachment occurs at a specialized structure known as the kinetochore that assembles on the centromere. Kinetochores fail to form in the absence of a centromere (McKinley and Cheeseman 2015). Following accurate attachment, the spindle pulls homologous chromosomes to opposite ends of the cell creating two identical daughter cells (Prosser and Pelletier 2017). The mitotic cell cycle must be tightly regulated as any errors in replication or division could result in loss of genetic material and genome instability.

One of the barriers to accurate mitosis is DNA damage. The most common form of damage occurring in cells is single-stranded DNA (ssDNA). ssDNA is produced regularly during the normal cell cycle from collapsed replication forks or replication errors during S-phase (Takeda and Dutta 2005). These DNA aberrations must be sensed and responded to in order to accurately replicate the genome (Brown and Baltimore 2000). In addition to responding to endogenously occurring DNA damage, cells also must respond to damage from exogenous sources. For example, x-ray irradiation results in a more severe DNA double stranded break (DSB) (Kitagawa and Kastan 2005).

A DNA DSB results in two fragments of DNA: a centromere containing fragment and a fragment that lacks a centromere. The fragment lacking a centromere, known as the acentric fragment, is unable to attach to the spindle (**Figure** 1). **Figure** 1 shows a cell preparing to divide with pairs of homologous chromosomes lined up in the middle. The blue chromosome contains a DNA DSB resulting in an unattached acentric fragment.



Figure 1: Acentric DNA fragments cannot attach to the spindle

Cell division in the presence of a DNA DSB typically results in incorrect segregation of the acentric fragment. Incorrect segregation is associated with genome instability and cell death. In order to ensure each daughter cell receives an accurate copy of each chromosome, organisms have evolved a complex network of proteins that function to prevent genome instability. This family of proteins is collectively known as DNA damage response proteins.

#### 1.2 The canonical DNA damage response

The DNA damage response is designed to inhibit cell cycle progression in the presence of damaged DNA. This allows cells to either repair damage or undergo programmed cell death, known as apoptosis, prior to re-entering the cell cycle. The DNA damage response is carried out at three levels. The first step of the DNA damage response is executed by the "sensors" or proteins designed to recognize damaged DNA (Kitagawa and Kastan 2005).

#### **DNA DSB Sensors:**

DNA DSBs are recognized by the Mre11/NBS1/Rad50 (MRN) complex (**Figure** 2). The MRN complex plays two important roles in DSB repair. First, Rad50, in complex with Mre11 and NBS1, forms a bridge connecting the two free ends of dsDNA (Hopfner et al. 2002) (**Figure** 2). This connection prevents the ends from getting separated thereby facilitating future repair. Second, the MRN complex activates the most upstream transducers in the DNA damage response (Lee and Paull 2005). These sensor proteins will be discussed further in chapter 3.

#### **DNA DSB Transducers:**

Once the sensors have recognized the damage, they must alert the cell to the presence of damage; a task carried out by a collection of transducer proteins. The

primary upstream transducer of the DSB is the PI3 kinase ATM (**Figure** 2) (Uziel et al. 2003). ATM phosphorylates several downstream targets resulting in both cell cycle inhibition and recruitment of repair factors (Kitagawa and Kastan 2005). Central to this response is phosphorylation of the histone mark H2AX (Fernandez-Capetillo et al. 2002) or H2AV in *Drosophila*. H2AX phosphorylation is crucial for immediate cell cycle arrest (Liu et al. 2007; Fernandez-Capetillo et al. 2002) and serves as a platform for recruiting repair factors (Paull et al. 2000). A second crucial target of ATM is the transducer protein checkpoint protein 2 (chk2) (**Figure** 2). Chk2 is responsible for activating effector proteins allowing for a cell-wide sustained response to DNA damage (Lukas et al. 2003).

#### **DNA DSB effectors:**

One of the best characterized targets of chk2 is the effector protein/ transcription factor p53 (**Figure** 2). p53 phosphorylation by chk2 serves to stabilize p53 and allow for nuclear translocation and expression of target genes (Hirao et al. 2000). p53 targets include genes functioning in continued cell cycle inhibition (p21), expression of repair factors and apoptotic genes. (Khoronenkova and Dianov 2015). The importance of p53 in preventing genome instability and disease is highlighted by the fact that it is the most mutated gene in cancers (Baker et al. 1989). Transducer and effector proteins will be discussed further in chapter 2.



Figure 2: Overview of the DNA damage response to DNA DSBs

#### ssDNA also activates sensor, transducer and effector proteins:

#### In chapter 3, I will discuss a role for ssDNA in acentric DNA segregation.

Much like the DNA DSB, activation of the DNA damage response to ssDNA is dependent on sensors, transducers and effectors. ssDNA is sensed by RPA ,which binds with high affinity to stretches of ssDNA as short as 20-30 nucleotides (Kim, Paulus, and Wold 1994) (**Figure** 3). The presence of RPA on ssDNA activates the most upstream transducer of the ssDNA response: ATR (Zou and Elledge 2003). Like ATM, ATR is a PI3-kinase with the ability to set off a cascade of events and even shares some targets with ATM. For example, ATR also phosphorylates the histone mark H2AX which recruits repair factors to damaged DNA (Ward and Chen 2001). ATR dependent checkpoint activation relies mainly on the downstream transducer Chk1 to initiate cell cycle inhibition (Xiao et al. 2003). Chk1 also phosphorylates the effector protein p53 to coordinate the repair process or organized cell death (**Figure** 3) (Shieh et al. 2000).



Figure 3: Overview of DNA damage response to ssDNA

#### The FANCONI Anemia family of proteins responds to a diverse set of DNA

aberrations:

As I will discuss in chapter 2, my thesis work has identified a role for Fanconi Anemia proteins in regulation of acentric DNA segregation. -The FA family is divided into two parts: the core complex and the I/D2 heterodimer. The presence of an Interstrand crosslink (ICL) activates FANCM which serves as a scaffold for the FA core complex (Singh et al. 2013). Once activated, FANCM translocates to the DNA and forms a complex with other FA core proteins (Singh et al. 2013). In mammals, the core complex contains eight FA proteins. Of these eight proteins, one of the most essential is the E3 ubiquitin ligase FANCL (Meetei et al. 2003). FANCL mono-ubiquitinates the FA heterodimer pair FANCD2 and FANCI. (Meetei et al. 2003). This ubiquitination serves to localize FANCD2/I to sites of damage and initiate the repair process (Meetei et al. 2003).

The FANCONI Anemia proteins are probably best known for their role in interstrand cross-link repair. Inter-strand crosslinks (ICLs) result when a chemical reaction occurs crosslinking two nucleotides on complimentary DNA strands (Stone et al. 2008). ICLs pose a significant threat to cells as they prevent DNA unwinding required for transcription and thus activate a unique DNA damage response. ICLs activate the FA core complex, which in turn activates FANCD2 and FANCI allowing them to initiate removal of ICLs (Andreassen, D'Andrea, and Taniguchi 2004). FANCD2 is also well known for its role in S-phase in preventing replication fork collapse and protecting ssDNA from degradation (Andreassen, D'Andrea, and Taniguchi 2004). Finally, in order to initiate repair via homologous recombination (HR), FANCD2 collaborates with the MRN complex to recruit CtIP, a co-factor required for HR (Roques et al. 2009).

#### 1.3 DNA Damage responses are well conserved in Drosophila

The ability to activate a DNA damage response to prevent mitosis with broken DNA is well conserved throughout evolution. The above provides a generalized mammalian-centric explanation of the DNA damage response. My thesis work focuses on studying DNA damage in *Drosophila*, which has a DNA damage response very similar to mammals.

*Drosophila* and mammals share the same set of sensors, transducers and effectors for DNA DSBs with a few minor variations. Recognition of DNA DSBs as well as initiation of DNA repair is dependent on the MRN complex in *Drosophila (Ciapponi et al.)*. Downstream of the MRN complex, *Drosophila* ATM (Tefu) and ATR (Mei-41) function similarly to their mammalian counterparts. Both proteins are responsible for the activation of downstream transducers Chk1 (Grps) and chk2 (Lok) (Song et al. 2004). This pathway is crucial in initiating damage-induced checkpoints as well as activating the effector protein p53 (Song et al. 2004)

While *Drosophila* contain only a subset of the FA family genes, the basic pathway and function of such genes is well conserved. In *Drosophila* the FA core complex is limited to FANCM and FANCL (Marek and Bale 2006). FANCL operates as an E3 ubiquitin ligase responsible for mono-ubiquitinating both FANCD2 (Marek and Bale 2006) and FANCI (Smogorzewska et al. 2007). Mutations in any of these proteins render animals hypersensitive to cross linking agents such as mitomycin C (Smogorzewska et al. 2007; Marek and Bale 2006). The high degree of conservation in the DNA damage response between *Drosophila*, mammals and throughout evolution highlights the crucial function of this response. Despite the importance of the interphase DNA damage response in preventing mitosis with damaged DNA, occasionally chromosomal aberrations can persist into mitosis.

## 1.4 Mechanisms for responding to broken DNA during mitosis often rely on DNA/protein bridges

The above sections describe key roles and several important regulators of a fully functioning interphase DNA damage response. Despite the rigor of these interphase DNA damage responses, some forms of DNA damage can escape the checkpoint resulting in mitotic entry without fully intact DNA. While it might be expected that such a checkpoint bypass would be lethal to the cell, it is becoming increasingly appreciated that cells can compensate for such deficiencies during mitosis. Thus far, several different chromosomal abnormalities have been described that persist into anaphase, but are resolved prior to cytokinesis (Royou et al. 2010; Chan et al. 2009; Naim and Rosselli 2009). In each case, a unique set of proteins has been identified that are required for proper resolution. Interestingly, the proteins identified appear to be serving a function outside of their canonical role. One common theme between all structures is the presence of thin DNA strands. The exact nature of these thin DNA structures remains to be determined. Additionally, in most cases it remains unclear as to whether the canonical DNA damage proteins described above are required for the formation and resolution of mitotic DNA structures. Thus, the study of the resolution of chromosomal abnormalities during mitosis represents and exciting, new emerging field. Next, I discuss the current state of the mitotic DNA damage response field.

# Replication intermediates result in ultra-fine DNA bridges (UFBs) during mitosis

One source of chromosomal abnormalities that persist into mitosis are replication intermediates. Replication intermediates can arise from centromeric regions of sister chromatids that fail to undergo complete decatenation prior to mitosis. (Chan, North, and Hickson 2007) Incomplete decatenation leads to an ultra-fine DNA bridge (UFB) that connects sister chromatids at the centromere during mitosis. Interestingly, this UFB is DAPI and Hoechst negative, suggesting that the DNA structure connecting centromeres on sister chromatids lack a major and minor groove. Such structures can be detected only when cells are cultured in BrdU to label DNA (Chan, North, and Hickson 2007). In addition to containing DNA, these bridges are coated by the Bloom Helicase (BLM) (Chan, North, and Hickson 2007). While BLM is typically involved in resolution of holiday junctions in S-phase, BLM is also required for resolution of these replication intermediates during mitosis. Cells lacking BLM show elevated rates of persistent bridges and chromosome mis-segregation (Chan, North, and Hickson 2007). In addition to linking daughter nuclei during anaphase, BLM was also found linking lagging chromatin to segregating DNA (Chan, North, and Hickson 2007). In this case, it is unclear what the source of lagging chromatin is or whether lagging chromatin is acentric. Regardless, cells lacking BLM showed an increased incidence of lagging chromatin during mitosis. Thus, BLM can operate in a non-canonical way to resolve centromeric replication intermediates and prevent lagging chromatin during anaphase. Interestingly, as many as 25% of all wild type anaphases contain BLM-DNA UFBs. However, only 55% of BLM-DNA bridges connect to centromeric regions indicating that bridges must also arise from chromosome arms.

In addition to being found on centromeric UFBs, BLM has also been found on UFBs arising from sister chromatid arms (Chan et al. 2009). Like centromeric UFBs, UFBs arising from sister chromatid arms contain DNA but are not detectable with DAPI. In addition to BLM, bridges arising from chromatid arms also co-localized with the FA heterodimeric proteins FANCD2 and FANCI. Interestingly, FANCD2 and FANCI appear to be upstream of BLM as FANCD2 foci were found in cells as early as S-phase (Chan et al. 2009). Furthermore, the presence of BLM on bridges originating from chromosome arms (but not centromeres) is dependent on the presence of FANCD2 (Chan et al. 2009).

During S and G2 phases, FANCD2 foci are found in pairs on sister chromatids. These foci persist into prophase and metaphase. Metaphase spreads indicated that foci represented points on each sister chromatid. Furthermore, foci were found in recurring locations known as common fragile sites (CFS) (Chan et al. 2009). CFSs are present throughout the genome and represent sequences that are difficult to replicate. Replication difficulties encountered at CFSs can be amplified by introducing replication stress. Replication stress, such as HU that depletes nucleotide pools, results in chromatid breaks at CFSs that co-localize with FANCD2 (Naim and Rosselli 2009). Localization of FANCD2 on mitotic chromosomes is specific to CFSs as induction of radiometric chromosomes does not alter the incidence of FANCD2 foci in mitotic cells (Naim and Rosselli 2009).

Upon entry into anaphase, approximately 10% of sister FANCD2 foci result in a BLM coated UFBs during mitosis. These bridges differed from those arising from centromeric locations due to the presence of FANCD2 foci on either end (**Figure** 4A). The ssDNA binding protein RPA was also found between FANCD2 foci in further support of the notion that a physical linkage exists between foci on sister chromatids (Chan et al. 2009). The authors hypothesized that these structures may represent replication intermediates and the fact that only 10% of foci result in bridges would indicate that the remaining 90% of intermediates were resolved prior to anaphase.

The presence of BLM on UFBs arising from chromosome arms is dependent on FANCD2 (Naim and Rosselli 2009), whereas lack of BLM did not alter the incidence of paired FANCD2 foci (Chan et al. 2009). The presence of FANCD2 foci is dependent on the core complex, suggesting that mono-ubiquitination is required (Naim and Rosselli 2009). However, formation and resolution of these structures was found to be independent of canonical DNA damage transducers ATM and ATR (Chan et al. 2009). Cells lacking either FANCD2 or BLM show elevated rates of chromosome missegregation. Chromosomal mis-segregation led to micronucleus formation. Micronuclei were found to contain sequences from CFS (Chan et al. 2009), supporting the conclusion that FANCD2 and BLM are crucial for maintaining genome integrity at such location. Again, this represents a non-canonical role for BLM and FANCD2 in maintaining chromosomal stability. The formation of micronuclei in the absence of BLM/FANCD2 underscores the importance for cells to be able to respond to chromosomal aberrations during mitosis. The role of FANCD2 and BLM for resolving naturally occurring replication intermediates may explain why individuals with mutations in FANCONI anemia proteins or BLM are predisposed to cancer. In addition to being able to respond to replication intermediates, cells must also be able to respond to DNA aberrations induced after the DNA damage checkpoint has been satisfied.

# A DNA tether system is required for proper segregation of acentric DNA induced during mitosis

Prior to entering mitosis, cells must satisfy a DNA damage checkpoint, however any damage induced after this checkpoint has been satisfied results in mitosis with broken DNA. Indeed, inducing a DNA break using the endonuclease I-Cre1 directly before mitosis results in lagging acentric chromosomes in *Drosophila* neuroblasts (**Figure** 4B) (Royou et al. 2010). While it might seem that such an event would prove lethal to a cell during mitosis, remarkably cells have the ability to segregate lagging acentric DNA fragments (Royou et al. 2010). During anaphase, acentric DNA lags behind the main body of DNA but is incorporated into daughter cells prior to cytokinesis (Royou et al. 2010). In fact, approximately 75% of cells segregate acentric fragments to the correct daughter cell. Thus, such breaks that persist into mitosis do not effect cell or organismal survival (Royou et al. 2010). The authors hypothesize that survival may be attributed to proper segregation of acentrics allowing for repair during the following interphase.

Despite DNA DSB induction, a thin strand of DNA was found connecting 89% of acentric fragments to a centric fragment. The authors term this structure a tether. However, in some cases it appeared that two acentric fragments were tethered to the same centric fragment (Royou et al. 2010) suggesting that the DNA tether may not always form at the cut site. In support of this, it was found that about half of acentric fragments segregated telomere end forward and that orientation of acentric fragments had no effect on DNA tether formation (Karg et al. 2017). This suggests that tether formation is not a result of inefficient cutting. The authors hypothesize that these structures may represent replication intermediates. However, unlike UFBs, tethers are detectable by DAPI (Royou et al. 2010). This suggests that the structure of the DNA in UFBs and tethers is different. While tethers are required for acentric DNA segregation in *Drosophila* neuroblasts, tethers alone are not sufficient for proper segregation.

Tethers connecting acentric DNA to the main body of DNA in *Drosophila* neuroblasts were found coated with the spindle assembly checkpoint protein BubR1, Polo (Plk1), Aurora B and INCENP (Royou et al. 2010) (**Figure** 4B). Live imaging of neuroblasts following I-Cre induction showed both BubR1 and Polo localized along the length of the tether with their localization becoming more punctate as the tether stretched. Disruption of either BubR1 or Polo led to segregation errors and reduced organismal survival as well as defects in acentric DNA segregation (Royou et al. 2010). However laser ablation of this tether after it had formed did not appear to effect acentric segregation (Karg et al. 2017)

BubR1 typically plays a role in the spindle assembly checkpoint, however mutants of BubR1 defective in spindle assembly checkpoint activation were still proficient in tether formation (Royou et al. 2010). While BubR1s canonical role in the spindle assembly checkpoint is dispensable for acentric DNA segregation, BubR1 does play a role in local inhibition of the anaphase promoting complex (Derive et al. 2015). BubR1 recruits cdc20 to lagging acentrics. Cdc20 is required for activation of the anaphase promoting complex. Thus, the anaphase promoting complex is locally inhibited around the lagging acentric. The authors suggest that it is this inhibition that allows for proper segregation of acentric DNA (Derive et al. 2015). In addition to relying on local inhibition of the anaphase promoting complex, accurate segregation of acentrics is also dependent on cell elongation during anaphase (Kotadia et al. 2012) and delayed nuclear envelope formation (Karg, Warecki, and Sullivan 2015). As with resolution of UFBs, resolution of I-Cre induced intermediates requires proteins operating outside of their canonical roles. It would be interesting to see if UFB resolution may also rely on temporary inhibition of the anaphase promoting complex.

Despite lacking a centromere, acentric segregation is also dependent on spindle microtubules (Karg et al. 2017). During anaphase, acentric fragments were found to be bundled with microtubules. (Karg et al. 2017). Furthermore, laser ablation of the spindle resulted in regression of acentric fragments and delayed segregation until the spindle had re-formed (Karg et al. 2017). These results suggest that acentric fragments are interacting with microtubules despite the lack of a centromere. Accordingly, the chromokinesin klp3a, which interacts with chromatin and microtubules was found to be required for proper acentric DNA segregation and organismal survival (Karg et al. 2017).



#### Figure 4: Mitotic responses to chromosomal aberrations

A) Model of DNA UFBs. UFBs form when CFSs fail to completely replicate during S phase. As a result, homologous chromosomes remain attached during anaphase by DNA ultra fine bridges (UFBs). UFBs are coated with Bloom helicase (BLM) and have FANCD2 punctae on either end. B) In *Drosophila* neuroblasts, acentric DNA induced during mitosis results in lagging acentric fragments. Fragments are segregated by a tether system. Tethers are coated with BubR1 and Polo.

#### Mitotic DNA damage responses: summary

The above examples demonstrate mechanisms for coping with abnormal DNA

structures during mitosis and shed light on a new and exciting field regarding mitotic

DNA damage responses. The common theme between these two mechanisms is that a

DNA structure of unknown composition forms during anaphase linking either daughter

nuclei or lagging chromatin to daughter nuclei. These mitotic chromosome abnormalities are not detrimental to cells if handled correctly. The authors have shown that these ultrafine structures co-localize with distinct proteins which are required for their resolution. Interestingly, these proteins seem to be serving functions outside of their canonical role. This is in stark contrast to the well described interphase DNA damage response which relies on canonical regulators discussed in the section of the DNA damage response.

## 1.5 Developmentally programmed endocycles inactivate elements of the DNA damage response

In my thesis work, I have exploited a cell cycle variant- the endocycle- as a tool to study unappreciated DNA damage responses. The endocycle is a developmentally programmed cell cycle variant in which cells alternate between G and S phases without an intervening M phase. Therefore, during endocycles, cells increase in ploidy, but not cell number. Thus, rather than resulting in an increased number of cells like the mitotic cell cycle, the endocycle results in polyploid cells. Polyploid cells contain multiple whole genome duplications and are typically larger than their counterparts. Developmentally programmed endocycles are found in a diverse collection of organisms from plants (Bourdon et al. 2011) to mammals (Ullah et al. 2008), insects (Lilly and Spradling 1996) and even bacteria (Mendell et al. 2008). In many cases the ploidy of these endocycling cells can reach over 1000C. The ability of tissues within these organisms to endocycle is crucial for proper development. For example, the blood brain barrier of the *Drosophila* larvae this relies on cell size increase resulting from endocycles for proper barrier integrity and function (Unhavaithaya and Orr-Weaver 2012). In other cases, endocycling and the corresponding increase in ploidy have been found to be required for proper organ development and function in mammals, insects and plants (Ullah et al. 2008; Schoenfelder et al. 2014; Meyer et al. 2017).

Despite the importance of the endocycle in development, DNA replication during the endocycle has been shown to be error prone. During the mitotic cell cycle, cells typically undergo an early S-phase during which mostly euchromatic regions are replicated followed by a late S-phase where mostly heterochromatic regions are replicated. However, many endocycling cells fail to initiate a late S-phase (Lilly and Spradling 1996) a phenomenon which has been termed under-replication. As a result of under replication, heterochromatic regions of the genome are transcribed at lower levels than euchromatic regions. (Lilly and Spradling 1996) Such under replication has been shown to result in 10-500kb deletions in *Drosophila* salivary glands. (Yarosh and Spradling 2014) Similarly, in mouse giant trophoblast cells, which can reach a ploidy of 368C, 47 regions of under replication resulting in deletions were observed (Hannibal et al. 2014). If endocycling cells contained an intact DNA damage response, under replication would activate DNA damage checkpoints. Endocycling cells have been shown to inactivate elements of the DNA damage response allowing for under-replication as well as rendering them resistant to DNAdamage induced cell death. In *Drosophila*, endocycling salivary gland cells and fat body cells fail to apoptose in response to DNA damage induced by re-replication (Mehrotra et al. 2008). This lack of cell death was not due to lack of DNA damage in endocycling cells, as induction of re-replication led to gamma-H2AV focus formation (Mehrotra et al. 2008). Instead, endocycling cells in *Drosophila* were found to have reduced levels of p53 and epigenetic silencing of the major apoptotic gene locus (Zhang et al. 2014).

Apoptosis is also suppressed in endocycling mouse giant trophoblast cells (Soloveva and Linzer 2004). In these cells, endocycling is crucial for function, and endocycle entry is dependent on down regulation of p53 at the protein level (Soloveva and Linzer 2004). In addition to low levels of p53, Chk1 activity is also suppressed contributing to DNA damage tolerance. (Ullah et al. 2008). Trophoblast Chk1 suppression is mediated by the CDK inhibitor p21, which is retained in the cytoplasm during endocycles (de Renty, DePamphilis, and Ullah 2014). While one may think that such inactivation results in genome instability, this does not occur. This is because most endocycled cells do not re-enter the mitotic cell cycle after endocycling. Instead they terminally differentiate or senesce (Ullah et al. 2008; Mehrotra et al. 2008). Resistance to DNA damage seen in endocycling cells suggests that the endocycle may be a method cells could use to avoid DNA damage induced cell death.

#### 1.6 The Drosophila rectal papillar cells provide an excellent model to study mitosis in the absence of a DNA damage response

During development *Drosophila* rectal papillar cells (hereafter papillar cells) undergo two distinct cell cycles (Figure 5). During larval stage, approximately 3 days after hatching, papillar cells initiate two rounds of endocycle (Fox, Gall, and Spradling 2010). During this stage one hundred papillar cells are arranged in a tube like structure and increase from a ploidy of 2N to 8N. Following endocycle, cells remain quiescent for 4-5 days until after the onset of pupation. Approximately twenty-six hours postpupation, polyploid 8N cells enter mitosis (Fox, Gall, and Spradling 2010). During this stage, each cell undergoes approximately two mitoses. These mitoses are associated with organ restructuring. Restructuring occurs first by separation of papillar cells into four ball type structures. Following mitosis these balls form four ice cream cone shaped structures in the adult animal with a total of approximately 400 cells (Fox, Gall, and Spradling 2010). The fact that papillar cells undergo post-endocycle mitosis makes papillar cells fundamentally different than other endocycled cells which fail to divide. This raised the question of whether or not papillar cells had an intact DNA damage response.


Figure 5: Rectal papillar cell development during the Drosophila life cycle

During *Drosophila* larval stages rectal papillar precursor cells undergo two endocycles increasing in ploidy from 2N to 8N. At this stage in development papillar cells are arranged in a tube of 100 cells. Four to five days later during mitosis, cells undergo two rounds of polyploid mitosis increasing in cell number from 100 to 400 cells. During mitosis, cells re-arrange themselves to form four ice cream cone shaped papillae in the adult.

The first suggestion that papillar cells may lack a DNA damage response came from looking at papillar cell divisions. Unlike diploid cells which faithfully segregate chromosomes during anaphase, lagging chromosomes and chromosome bridges were observed in papillar cells (Fox, Gall, and Spradling 2010). The presence of these chromosomal abnormalities during mitosis provided the first piece of evidence that papillar cells may lack an intact DNA damage response. Here I show that like other endocycling cells, papillar cells inactivate elements of the DNA damage response during endocycle rendering them resistant to high levels of exogenous DNA damage. Despite high levels of resulting chromosomal aberrations, papillar cells segregate broken DNA during mitosis resulting in normal organ development and function. Thus, this provides us with a developmentally tractable system to study mitotic DNA damage responses.

# 1.7 Cancerous cells often lack a fully intact DNA damage response

While my thesis work exploited the endocycle to study mitosis in the absence of a normal DNA damage response, cancer cells are another context in which such altered DNA damage responses can occur. As mentioned above, several cancer therapies, such as radiation therapy, rely on the endogenous DNA damage checkpoints to induce cell death. However, one of the main obstacles to cancer treatment is resistance to therapy. Interestingly, it has been observed that one mechanism of avoiding DNA damage induced cell death is by undergoing endocycles. Cisplatin is a common therapeutic which activates the DNA damage checkpoint by stalling DNA synthesis. While cisplatin treatment generally induces cell death, some cells are able to survive such treatments by undergoing endocycles and becoming polyploid (Shen et al. 2013).

Recent work has suggested that polyploidy is often found in cancer cells (Zack et al. 2013). In fact, 37% of solid tumors have been found to contain polyploid or near polyploid cells(Zack et al. 2013). In order for diploid cells to become polyploid, they

must escape a p53 dependent polyploidy checkpoint. Therefore, it is not surprising that p53 inactivation is found in many polyploid cells (Andreassen et al. 2001). p53 inactivation is not limited to polyploid cells. In fact, p53 is the single most mutated gene in cancers. Despite lacking an intact DNA damage checkpoint, the cancer cells can still proliferate. This suggests that cells may be able to compensate for damaged DNA during mitosis.

#### Summary:

The above review highlights the importance of the canonical DNA damage response in responding to DNA damage in interphase cells. This response is designed to prevent mitosis with broken DNA. Mitosis with broken DNA often leads to micronucleus formation resulting from a cells inability to segregate broken DNA during mitosis. Micronucleus formation leads to genome instability and is associated with cell death. In rare cases, genome instability can give rise to pathological conditions, such as cancer. Interestingly, new data suggests cells can respond to chromosomal aberrations during mitosis. Work from other groups has identified mechanisms by which cells can compensate for replication intermediates as well as acentric DNA fragments during mitosis. This suggests that cells may have mechanisms to respond to broken DNA during mitosis. In further support of this notion, DNA damage response proteins are frequently mutated in cancer cells, yet these cells can divide at high rates even in the presence of damaged DNA. Understanding the way in which cancerous cells can divide without a DNA damage response is crucial in cancer therapy. Studying mitotic DNA damage responses is difficult due to the fact that most systems contain intact interphase DNA damage checkpoints preventing mitosis with broken DNA. Therefore, the field has relied primarily on genetic manipulation of these checkpoints or DNA damage induction at specific time points. A developmentally tractable system lacking an intact DNA damage response would be a valuable tool in which to study mitotic responses to DNA damage.

## 2. Proliferation Of Double Strand Break Resistant Polyploid Cells Requires *Drosophila* FANCD2

For my second chapter I am including my previously published first author paper of the same title. This work was originally published in the journal *Dev Cell* in 2016. The authors of this work were myself and Dr. Don Fox. Together Dr. Fox and I designed the experiments, organized the figures and wrote the manuscript. I generated all of the data for this paper with the exception **Figure** 7 E,F,I,J which were generated by Dr. Fox. All of this work was undertaken at Duke University under the supervision of Dr. Fox

### 2.1 Summary

Conserved DNA damage responses (DDRs) sense genome damage and prevent mitosis of broken chromosomes. How cells lacking DDRs cope with broken chromosomes during mitosis is poorly understood. DDRs are frequently inactivated in cells with extra genomes (polyploidy), suggesting study of polyploidy can reveal how cells with impaired DDRs/genome damage continue dividing. Here, we show continued division and normal organ development occurs in polyploid, DDR-impaired *Drosophila* papillar cells. As papillar cells become polyploid, they naturally accumulate broken acentric chromosomes, but do not apoptose/arrest the cell cycle. To survive mitosis with acentric chromosomes, papillar cells require Fanconi Anemia proteins FANCD2 and FANCI, and Blm helicase, but not canonical DDR signaling. FANCD2 acts independently of previous S-phases to promote alignment and segregation of acentric DNA produced by double-strand breaks, thus avoiding micronuclei and organ malformation. As polyploidy and impaired DDRs can promote cancer, our findings provide insight into disease-relevant DNA damage tolerance mechanisms.

### 2.2 Introduction

Conserved DNA damage responses (DDRs) prevent mitosis of cells with DNA damage by promoting cell cycle arrest, DNA repair, and apoptosis. Attenuating these responses leads to mitosis with damaged/unstable genomes, which can ultimately enable tumor progression (Hanahan and Weinberg 2011; Halazonetis et al. 2008). Thus, understanding mechanisms enabling expansion of DDR-attenuated, genomically unstable cells is of great interest.

An increasingly recognized cause of DDR attenuation is the endocycle. This modified cell cycle lacks M-phase, and repeated endocycles thus generate polyploid cells (Edgar et al. 2014; Fox and Duronio 2013). In mammalian cancer cells, endocycles can promote resistance to irradiation and cisplatin, common cancer therapies that induce high levels of DNA damage (Shen et al. 2008; 2013b). Further, endocycled mouse embryonic fibroblasts generated by transient telomere DNA breakage are tumorigenic (Davoli et al. 2010; Davoli and de Lange 2012). Thus, continued division of endocycled cells associates with DNA damage therapy resistance and tumor growth.

Developmentally programmed endocycles also induce DNA damage and DDR inactivation. DNA damage can result from under-replication in the endocycled genome (Beliaeva et al. 1998; Gall et al. 1971; Nordman et al. 2011; Yarosh and Spradling 2014; Hannibal et al. 2014). Such under-replicated DNA is prone to deletions and inversions, (Yarosh and Spradling 2014) which may result from DSBs. Indeed *Drosophila* endocycled cells accumulate the ATM/ATR phosphorylation mark γ-H2AV (Mehrotra et al. 2008), a readout of double-strand DNA breaks (DSBs). In *Drosophila*, such DSB accumulation is likely due to low p53 (a core DNA damage-responsive transcription factor) levels and chromatin silencing at p53 pro-apoptotic target genes (Mehrotra et al. 2008; Hassel et al. 2014; Zhang et al. 2014). Similarly, in mice, differentiation of endocycling trophoblast giant cells involves decreased p53 (Soloveva and Linzer 2004), and suppression of the DDR kinase Chk1 (Ullah et al. 2008;2011). Thus, in both developmental and cancerous settings, endocycles promote impaired DDRs and tolerated DNA DSBs.

However, many developmentally endocycled cells do not resume mitosis, and thus these systems cannot be used to identify responses enabling continued mitosis of genome-damaged cells. We recently developed study of *Drosophila* papillar cells as a developmentally and genetically tractable model of polyploid mitosis after endocycles. Here, using our model, we uncover mechanisms permitting these polyploid cells to undergo viable division with DNA damage. Similar to previous studies, we find endocycled papillar cells lack p53-mediated apoptosis. Further, we find papillar cells lack S-phase checkpoints and enter mitosis without undergoing high fidelity DNA repair. Despite lacking these normally crucial DDRs, both papillar mitosis and organ development are highly resistant to DNA damage by DSBs. By live imaging pupal development, we show an important part of the papillar DDR involves alignment and segregation of broken, acentric chromosome fragments. This response does not depend on p53, or core DNA damage kinases.

Instead, the Fanconi Anemia protein FANCD2, its frequent partner FANCI, and the Bloom helicase (Blm) are a crucial part of this non-canonical DDR. We show FANCD2 acts independently of S-phases prior to mitosis entry, and does not require its core complex partner FANCM to promote segregation of acentric fragments produced by DNA DSBs. This response ensures normal organ development by preventing acentric micronuclei. Our results pinpoint a mechanism enabling viable mitosis despite an impaired DDR.

### 2.3 Results

2.3.1 Lack of apoptosis and S-phase checkpoints during pre-mitotic endocycles

Previous study of endocycle-induced DDR inactivity focused on post-mitotic tissues. To understand the impact of endocycles on subsequent divisions, we turned to an accessible *in vivo* model: *Drosophila* rectal papillar cells (hereafter: papillar cells or papillar precursors).

During 2<sup>nd</sup> larval instar, papillar precursors endocycle, producing octoploid nuclei (Fox et al. 2010; Schoenfelder et al. 2014). Unlike previously studied examples of endocycled cells with an inactive DDR, papillar cells then undergo polyploid divisions. We thus asked if these mitotic endocycled cells also lack an apoptotic response to damaged DNA. It is well established that exposure to Ionizing Radiation (IR) causes DNA damage and apoptotic cell death in diploid cells. Accordingly, we find induction of pycnotic nuclei and TUNEL labeling in diploid wing imaginal tissue after 20 Gy of X-ray induced IR (**Fig6A,B,E, Fig7A,B**, Methods). In contrast, IR does not induce pycnotic nuclei or TUNEL in endocycling 2<sup>nd</sup> instar papillar precursors (**Fig6C-E, Fig7C,D**). The lack of apoptosis in papillar precursors is not due to lack of IR-induced DNA breakage, as IR causes robust γ-H2AV accumulation in endocycling papillar precursors one hour after IR (**Fig7E,F**).

To examine molecular regulation of this apoptotic inactivity, we examined the consequences of expressing p53 and its pro-apoptotic targets. Using a *UAS-p53* construct used previously in salivary glands (Mehrotra et al. 2008), we find p53 expression during papillar endocycles is insufficient to induce apoptosis (**Fig6F**), whereas the same construct

expressed with the same promoter (Methods) causes robust cell loss in the diploid hindgut pylorus (**Fig7G,H**). In contrast to p53 expression, co-expression of p53 pro-apoptotic targets *hid* and *reaper* during endocycles induces robust papillar precursor death (**Fig6G**). We conclude that as in non-mitotic endocycling cells, mitosis-capable papillar precursor cells attenuate a DNA damage-responsive, p53-dependent apoptotic response during endocycles.



Figure 6: Lack of p53-dependent apoptosis in papillar precursors

A,B.3<sup>rd</sup> instar larval wing imaginal discs. A.Nuclei in undamaged disc. B.Pycnotic nuclei (arrows) in irradiated disc, 6 hrs. post IR. C,D.2<sup>nd</sup> instar rectum (during papillar precursor endocycling). C.Nuclei in unirradiated rectum. D.No pycnotic nuclei in rectum, 6 hrs. post IR. E.% pycnotic nuclei from A-D (wing data-blue, rectum data-red). Numbers indicate mean % pycnotic nuclei/animal (N=minimum 10 animals/condition, multiple replicates). \*= significant to p<.001, two-tailed T-test. Bars=standard deviation. F.*UAS-p53* expression in rectum (using *byn-Gal4*) does not induce pycnotic nuclei. G.*UAS hid, UAS rpr* co-expression in rectum (Methods) does induce pycnotic nuclei. DAPI=DNA in all images. Scale bar=10µm..

In addition to apoptosis, cell cycle arrest is an important DDR. We thus tested if, during endocycles, papillar precursors lack DNA damage checkpoints for S-phase entry or progression. One hour after IR, there is no change in the number of papillar precursor cells in endocycle S-phases (using EdU, **Fig7I-K**). Further, by examining late S-phase patterns of EdU incorporation, we find the proportion of endocycling cells that progress to late S-phase does not decrease after IR (**Fig7L**). Thus, during pre-mitotic endocycles of papillar cells, DNA breakage fails to activate either p53-dependent apoptosis or S-phase entry/progression checkpoints.



## Figure 7: Supporting evidence of the inactive apoptotic and S-phase checkpoint responses during papillar endocycles.

**A.** Representative TUNEL staining (Green, DNA in purple) of an un-irradiated wild-type 3<sup>rd</sup> instar wing imaginal disc. **B.** Representative TUNEL staining of an irradiated wild-type 3<sup>rd</sup> instar wing imaginal disc. **C.** Representative TUNEL staining of an un-irradiated 2<sup>nd</sup> instar larval rectum. **D.** Representative TUNEL staining of an irradiated 2<sup>nd</sup> instar larval rectum. **E.** Gamma H2AV staining (Green, DNA in purple) in unirradiated 2<sup>nd</sup> instar larval rectum. Occasional foci are observed. **F.** IR causes a robust increase in gamma H2AV labeling in the 2<sup>nd</sup> instar larval rectum 1 hour after IR. **G.** Control 2<sup>nd</sup> instar larval pylorus, diploid nuclei labeled. **H.** *byn-Gal4* driving *UAS-p53* causes cell loss in the 2<sup>nd</sup> instar larval pylorus. **I.** EdU labeling in an endocycling control 2<sup>nd</sup> instar rectum. **J.** EdU labeling 1hour after IR in a 2<sup>nd</sup> instar rectum. **K.** Quantitation of # EdU+ cells in the endocycling 2<sup>nd</sup> instar larval rectum +/- IR. From N=22 control and 35 IR animals. Bars= standard deviation. **L.** Distribution of early and late S phase cells (based on EdU labeling) from the experiments in **I-K**, +/- IR. DAPI indicates DNA in all images. Scale bar= 25µm.

### 2.3.2 Acentric chromosomes accumulate but segregate during papillar mitosis

Given the inactive responses to broken DNA in endocycling papillar cells, we next examined whether such inactivity leads to unrepaired/aberrant chromosomes after endocycles. During metamorphosis (4-5 days after endocycles), papillar cells undergo 1-3 complete mitotic cycles as octoploid cells. Even in the absence of exogenous DNA damage, these divisions are frequently error-prone. While some errors are due to extra centrosomes (Schoenfelder et al. 2014), we also observed chromosome structure aberrations, which could also contribute to mitotic errors (Fox et al. 2010).

We thus examined the nature of papillar chromosome aberrations in detail, and asked if aberrations are more common in these polyploid cells relative to diploid cells. Indeed, in the absence of exogenous DNA damage, papillar cells naturally accumulate two recurring chromosome aberrations: acentric fragments (**Fig8A**) and chromosome fusions (dicentric chromosomes, **Fig9A**). We detected at least one of these aberrations in 19.8% of papillar cells, with acentric chromosomes being most common (**Fig8C,D**). In contrast, no such aberrations are found in diploid imaginal disc cells (**Fig8B-D**). Therefore, endocycled polyploid papillar cells naturally accumulate chromosomal aberrations that persist into mitosis.

To gain insight into the fate of chromosome aberrations in polyploid mitotic papillar cells, we used live imaging. To distinguish between errant chromosomes containing or lacking centromeres, we used the markers histone-GFP (to mark DNA) and CenpC-Tomato (to mark kinetochores/centromeres), as well as a fragment of Moesin-GFP (to mark cell membranes and cytokinesis). In WT, we observed normal mitotic chromosome segregation in 82% of cells (Fig8E,H, Movie S1), in general agreement with previous measurements (Fox et al. 2010; Schoenfelder et al. 2014). Our approach also enabled us to detect cells with mitotic defects, in which DNA lags in anaphase. In these aberrant mitoses, CenpC enabled us to distinguish lagging DNA lacking (Fig8F, MovieS2) or containing centromeres (Fig9B). Lagging DNA with centromeres frequently localizes in an anaphase bridge, which persists until cytokinesis (Fig9B). Such bridges could represent separation of under-replicated DNA (Unhavaithaya and Orr-Weaver 2012). Alternatively, as part of the bridge-breakage-fusion cycle (Titen and Golic 2008),

bridges are expected for cells with fused (dicentric) chromosomes, which we detect in our metaphase chromosome squashes (**Fig9A**, **C**).

Much less expected was the fate of acentric DNA fragments. In 11/92 WT papillar divisions we could clearly detect acentric fragments, in general agreement with our chromosome squash data (12.0%, **Fig8F,H** vs. 15.6%, **Fig8D**). Despite lacking detectable centromeres, these acentric fragments successfully incorporate into daughter nuclei before cytokinesis (**Fig8F,G, MovieS2**). By comparison, in 89 WT time-lapse movies of diploid imaginal disc tissue, we did not detect acentric DNA (**Fig8H**). Taken together, our data show chromosome structural aberrations arise in normal development of apoptosisdeficient papillar cells. The most common aberration is acentric DNA, which remarkably segregates into daughter cells in mitosis (**Fig8G**).



Figure 8: - Acentric chromosomes accumulate and segregate during Wild Type papillar development

A.Example papillar cell with acentric chromosome (arrow, 2X magnified inset). B.Example diploid imaginal disc cell with normal karyotype. DAPI=DNA in A,B. C.Number aberrations/cell for cells examined in A,B. D.Distribution of acentric/fused chromosomes for cells examined in A,B. A-D: data from N=96 8N and N=93 2N cells respectively, from 8 replicates. The increased incidence of aberrant karyotypes in 8N vs. 2N is significant when accounting for increased chromosomes in 8N cells (Chi square, p<.05). E,F.Time-lapses of papillar mitosis. CenpC-Tomato=kinetochores (KTs, purple), histone H2AV=DNA (green, nuclear), and Moesin-GFP=cell membranes (Memb, green). Time is in minutes relative to anaphase onset. E.Example of normal mitotic segregation. F. Example of acentric chromatids that segregate into daughter nuclei. White arrow and 2X magnified, contrast-enhanced insets highlight segregating acentric DNA. G.Diagram of fate of acentric and fused papillar chromosomes. Green=DNA, Purple=Centromeres. H.Frequency of mitotic errors in 8N papillar and 2N imaginal disc tissue. Data from N=92 (papillar) and N=89 (imaginal disc) movies (numerous replicates). Scale bar=5µm.



# Figure 9: Supporting evidence of the persistence and tolerance of chromosome aberrations during papillar development

**A.** Example papillar cell with dicentric chromosome (arrow, 2X magnified inset). **B.** Time-lapse of papillar mitosis with example of centromere-containing DNA bridge that

persists into cytokinesis. CenpC-Tomato=kinetochores (KTs, purple), histone H2AV=DNA (green, nuclear), and Moesin-GFP=cell membranes (Memb, green). Time is in minutes relative to anaphase onset. **C.** Diagram of a fused/dicentric chromosome forming a DNA bridge during anaphase. Green=DNA, Purple= Centromeres. **D.** Example of irradiated papillar cell with dicentric chromosome (yellow arrows) and acentric chromosomes (white arrows). **E.** Time-lapse of papillar mitosis from an irradiated animal, with example of centromere-containing DNA bridge that persists into cytokinesis. Labeling as in **B.** F. *AICR2*<sup>10H05</sup>-*Gal4* driving *UAS-GFP* expression in WT adult rectum. **G.** *AICR2*<sup>10H05</sup>-*Gal4* driving *UAS-GFP* expression in WT adult rectum. **G.** *AICR2*<sup>10H05</sup>-*Gal4* driving *UAS-GFP* expression in WT adult rectum after IR (induced during endocycles). DAPI indicates DNA in all images. White scale bar= 5 µm, yellow scale bar= 50µm.

#### **2.3.3** Papillar development is highly tolerant of DNA breakage

The purpose of canonical DDRs is to prevent mitotic expansion of genomedamaged cells. However, endocycled papillar cells lack canonical DDRs and enter mitosis with chromosome aberrations. It remained possible that the fraction of cells with such aberrations are eliminated by a non-apoptotic mechanism. We thus tested if increasing the incidence and number of papillar chromosome aberrations would cause mitotic or developmental defects. We allowed animals exposed to IR during papillar endocycles to progress through the 4-5-day period between endocycle completion and mitosis initiation. In these animals, chromosome aberrations induced during endocycles persist several days later and remain when papillar cells re-enter mitosis. This result mirrors the persistence of mitotic chromosome aberrations in papillar cells without exogenous DNA breakage. In mitotic papillar cells, IR most noticeably causes an increase in acentric (**Fig10A,C,D vs.**  **Fig8A,C,D**) and fused chromosomes (**Fig9D and Fig10A,C,D, vs. Fig9A and Fig10C,D**). Thus, IR primarily amplifies the two major classes of naturally occurring DNA damage.

The increased incidence of persistent damage days after IR suggests papillar cells lack high-fidelity DNA repair (however, the presence of chromosome fusions suggests papillar cells likely engage in limited repair). To compare the lack of high fidelity repair in papillar cells to diploid cells irradiated at the same stage, we examined genital imaginal disc cells at the same time point (5 days after IR), when they divide while enveloping forming papillae (Fox et al. 2010). Chromosome aberrations were only present in a small number of these diploid cells during metamorphosis (**Fig10B-D**) suggesting repair of damage occurs prior to re-entering mitosis or that damaged cells are cleared from the tissue. Thus, unlike diploid cells, endocycling papillar cells not only lack apoptosis and Sphase checkpoints, but also lack high-fidelity DNA repair.

Given the persistence of chromosome aberrations in mitotic papillar cells, we could next examine if elevating the level of DNA breaks alters the fate of papillar chromosome aberrations in mitosis. In animals irradiated during 2nd larval instar papillar endocycles, the number of error-prone divisions increases significantly (**Fig10F vs. Fig8H-8N**). Many of these defects are present during the first papillar division, confirming DNA damage left either un-repaired or aberrantly repaired after IR causes mitotic errors. Mitotic errors associate with a longer anaphase (**Fig10G**). As in un-irradiated animals,

centromere-containing DNA that lags forms a bridge that is resolved in cytokinesis (**Fig8E, MovieS3**). Due to co-existence of centric and acentric aberrations in some irradiated papillar cells, we could only score/follow the existence and fate of acentric DNA in a subset of irradiated papillar cells. As in un-irradiated animals, the acentric DNA that we could clearly observe segregated into daughter cells prior to cytokinesis (**Fig10E, MovieS4**). These results reinforce the model that papillar cells possess a robust response to segregate acentric DNA in mitosis.

Given that papillar cells undergo mitosis with chromosome aberrations, we next asked if increasing such aberrations affects papillar development. During development papillar cells undergo 1-3 divisions while forming four cone shaped structures of 100 cells each (**Fig10H**, Fox et al. 2010; Schoenfelder et al. 2014). Papillar development also leads to patterned gene expression, reflected in activation of enhancers such as *AICR2*<sup>10H05</sup>-*Gal4* at each papillar base (**Fig9F**). We examined *AICR2*<sup>10H05</sup>-*Gal4* expression in animals exposed to IR during endocycles. Additionally, to precisely quantify the effect of IR on papillar development, we hand-counted adult papillar cells (390+/-24 per animal without IR in WT, **Fig10J**). Strikingly, papillar development proceeds normally despite increased DNA breaks induced by IR (**Fig10H-J, Fig9G**). We detect only a minor reduction in mean adult papillar cell number after IR (352+/-43 cells/animal after IR, **Fig10J**). This suggests that even when containing a high number of chromosomal aberrations, papillar cells execute multiple rounds of division while undergoing proper morphogenesis and organ patterning. Thus, polyploid mitotic papillar cells are remarkably tolerant of acentric and fused chromosome structure aberrations.



Figure 10: Papillar development is refractory to frequent chromosome structural aberrations.

**A.**Example of chromosome structure in 8N mitotic papillar cell 5 days after IR induced during endocycles. Acentric fragments (yellow arrows, 2X magnified inset in

A') and chromosome fusions (white arrows) are indicated. B.Diploid genital imaginal disc karyotype, from animal irradiated at the same time as cells in A. C.Number aberrations/cell from irradiated wild-type octoploid (8N WT) papillar and diploid WT (2N WT) imaginal disc cells. D.Distribution of acentric/fused chromosomes from same animals as in C. Compare C,D to unirradiated data in Fig2C,D. A-D from N=70 and N=54 polyploid and diploid cells respectively. The increased incidence of aberrant karyotypes in 8N vs. 2N is significant when accounting for increased chromosomes in 8N cells (Chi square, p<.0001). E.Time-lapse of papillar mitosis after IR induced during endocycles. CenpC-Tomato=kinetochores (KTs, purple), histone H2AV=DNA (green, nuclear), and Moesin-GFP=cell membranes (Memb, green). Time is in minutes relative to anaphase onset. Example of lagging acentric DNA (white arrow, 2x magnified and contrast-enhanced insets) that segregates into daughter nuclei. Yellow arrow at 17:00 highlights contractile ring. F.Distribution of mitotic defects in 8N papillar cells after IR, scored from time-lapse data. Note- the percentage of cells with acentric DNA is likely an under-estimate, due to the co-existence of centromere-containing DNA that overlaps acentric DNA in some cases. G.Avg. time from anaphase onset to cytokinesis furrow initiation. IR divisions were binned into those with/without detectable errors. \*= significant change from No IR or IR with normal mitosis (Two-tailed T-test, p<.001). Error bars= standard deviation. Data in E-G from N=113 IR divisions, from numerous replicates. H.WT adult rectum. DNA-purple, papillae-pseudo-colored green. I.WT adult rectum in animals irradiated during endocycles. J.Adult papillar cell number in WT, from control and IR (induced during endocycles). N= 13-23 animals counted/condition from multiple replicates. Yellow bars=mean. DAPI=DNA in all images. White scale bar=5 μm, Yellow scale bar=50 μm.

# **2.3.3.** FANCD2 plays a key role in acentric chromosome segregation and papillar cell survival

To begin to understand the robust DNA breakage tolerance of papillar cells, which lack canonical DDRs, we took a candidate approach. Given that: 1)acentric chromosomes are the most prominent chromosome aberration in both naturally occurring and irradiated papillar cells, and 2)the remarkable ability of such acentric DNA to segregate, we hypothesized that genes important for segregation of acentric DNA are critical to papillar DNA damage tolerance. We thus looked for genes involved in segregating acentric DNA.

The Spindle Assembly Checkpoint gene BubR1 was recently shown to function as part of a "tether" connecting acentric DNA and its centromere when breaks are induced during *Drosophila* neuroblast mitosis (Royou et al. 2010; Karg et al. 2015; Derive et al. 2015). Using the I-Cre1 system, an established method of generating an acentric chromosome (Rong et al. 2002; Royou et al. 2010), we generated acentric X-chromosomes in diploid neuroblasts. We readily detected BubR1-positive tethers in neuroblasts (**Fig11A**). In contrast, while we clearly detected BubR1 on metaphase papillar kinetochores, no BubR1 tethers were detected in papillar anaphase cells following I-Cre1 induction (**Fig11B**) or after IR (data not shown). Further, *bubR1(KEN)* mutants, which are defective in tether formation and cause diploid cell lethality following I-Cre induction (Royou et al. 2010), have normal papillar structure/cell number after IR (**Fig11C**). Thus, BubR1 tethers do not



### Figure 11: BubR1 is not required for lagging acentric DNA segregation in papillar cells.

Example of BubR1 chromosome tether (green, arrow) in a mitotic diploid neuroblast following I-Cre1 induction. Seen in 8/8 movies (from multiple experimental replicates). Time is indicated in minutes relative to anaphase onset. B. Example of a mitotic papillar cell containing BubR1 on kinetochores, but lacking detectable BubR1 chromosome tethers following I-Cre1 induction. Tethers were seen in 0/24 cells, while kinetochore-localized BubR1 was detected in 24/24 metaphases (from multiple experimental replicates). Time is indicated in minutes relative to anaphase onset. C. Graph of adult rectal papillar cell number following IR in both WT and *BubR1-KEN* animals. There is no significant difference +/- IR (see Methods). From N=20-33 animals from multiple replicates. Yellow bars= mean. Scale bar= 5µm.

As an alternative mechanism of acentric DNA segregation, we considered a role for the Fanconi Anemia protein FANCD2. Together with FANCI and Blm helicase, FANCD2 was previously implicated in resolving "ultra-fine DNA bridges" that persist into mitosis (Chan et al. 2007; Chan and Hickson 2009; Naim and Rosselli 2009). We thus investigated if FANCD2 plays a role in the papillar DDR. To do so, we expressed either one of two fancd2 RNAi constructs (Methods, hereafter fancd2) specifically in papillar cells and the associated hindgut throughout development. In the absence of any exogenous DNA damage, fancd2 loss primarily increases acentric DNA in mitotic papillar cells (by roughly 2-fold, Fig12A,B). We thus used IR to amplify the two main naturally occurring forms of chromosome aberrations (acentric and fused chromosomes, Fig13A,B) and assayed the effect of *fancd2* depletion. IR causes papillae of reduced size and significantly decreased cell number in fancd2 relative to WT (Methods, Fig12C-F, Fig17A vs. Fig10H-J). This phenotype is not due to additional chromosome aberrations in *fancd2* after IR, as papillar chromosomes of irradiated fancd2 animals do not display novel/increased

chromosome defects after IR (**Fig13A-C**, Methods). Additionally, the mitotic index is similar between *fancd2* and WT after IR (**Fig13D**), suggesting papillar cells continue dividing without *fancd2*, but exhibit reduced survival.

To understand why fancd2 decreases cell survival after IR, we used live imaging. Similar to WT, IR causes lagging DNA with centromeres in *fancd2*, which localize in an anaphase bridge until cytokinesis (Fig13E). Therefore, it is unlikely that FANCD2 resolves centromere-containing anaphase bridges in papillar cells. In contrast, we noted two significantly enhanced mitotic abnormalities related to acentric DNA in *fancd2* after IR. First, prior to anaphase, acentric DNA frequently fails to align on the metaphase plate during *fancd2* divisions (**Fig12G,H**). Second, at anaphase, acentric DNA lags substantially and often fails to incorporate into daughter nuclei, resulting in acentric micronuclei (Fig12G,H, MovieS5). We detect unaligned acentric DNA both during the first and subsequent *fancd2* divisions (Fig13F). We also find unaligned metaphase DNA in *fancd2* mitotic cells accumulates the mitotic marker phospho-histone H3 (PH3, Fig12I). Interestingly, we see a robust increase in micronuclei in *fancd2* animals after IR, which retain PH3 even after cells exit mitosis (when the rest of the nucleus has lost the marker, Fig12J,K). This latter observation is consistent with aberrant cell cycle activity in micronuclei (Crasta et al. 2012). Taken together, lack of FANCD2 leads to unaligned acentric DNA, acentric micronuclei, and papillar cell number loss following IR (Fig11L).



Figure 12: FANCD2 promotes papillar cell viability and prevents micronucleus formation after IR.

A.Representative image of papillar chromosomes in fancd2 RNAi#1 animal. Arrows indicate acentric chromosomes. DAPI indicates DNA. B.Frequency of acentric and fused chromosomes in WT vs. fancd2 RNAi#1 papillar cells +/- IR. C.Adult fancd2RNAi#1 rectum. D.Adult fancd2RNAi#1 rectum from animal irradiated during papillar endocycles. E.Adult fancd2RNAi#2 rectum from animal irradiated during papillar endocycles. Papillae false-colored in green, DNA in purple in C-E. For comparable controls see Fig3H,I. F.Adult papillar cell number in *fancd2* +/-IR. N=8-30 animals/condition. Yellow bars=mean. \*=significant change between NoIR vs. +IR compared to WT (Methods). G.Time-lapse of fancd2RNAi#1 papillar cell after IR. CenpC-Tomato=kinetochores (KTs, purple), histone H2AV=DNA (green, nuclear), and Moesin-GFP=cell membranes (Memb, green). Time is in min. relative to anaphase onset. White arrows=unaligned DNA that form micronuclei. Yellow arrow=cytokinetic furrow. H.Frequency unaligned DNA and micronuclei +/-IR in WT vs. fancd2RNAi#1. Data from 84-113 divisions/condition, from multiple replicates. \*=significant change from no IR by Chi-squared (p<.00001, Methods). I.Unaligned PH3+ chromosomes (green labeling/ arrows, DNA in purple) in fancd2RNAi#1 after IR. J.Persistent PH3+ micronuclei (green labeling/arrows, DNA in purple) in fancd2RNAi#1 after IR. K.Frequency PH3+ micronuclei +/-IR in WT vs. fancd2RNAi#1. From N=18-27 animals/condition from multiple replicates. Yellow bars=mean. \*=significant change from no IR (Two-tailed Ttest, p<.01).L.Diagram of acentric chromosome fate in *fancd2* after IR. Green=DNA, Purple=Centromeres. Instead of segregating completely into daughter nuclei as in Fig3E, acentric DNA accumulates in micronuclei (indicated by small green circles without centromeres). DAPI=DNA in all images. White scale bar= $50\mu m$ , yellow scale bar= $5\mu m$ .





#### Figure 13: Supporting data related for fancd2 RNAi.

A. Representative image of papillar chromosomes in *fancd2 RNAi*#1 following IR (induced during endocycles). White arrows indicate acentric chromosomes, yellow arrow indicates fused chromosomes. DAPI indicates DNA. B. Distribution of acentric and fused chromosomes in fancd2 RNAi#1 papillar cells +/- IR. C. Quantitation of number of errors/ WT or fancd2 RNAi#1 papillar cell after IR. Note- for data in this graph, animals were irradiated together in the same dish (see Methods). Data in A-C from N=21-45 cells, from multiple experimental replicates. D. Number PH3+ papillar cells/animal (mitotic index) in WT and fancd2RNAi#1 after IR. Bars=std. deviation. There is no significant difference in mitotic index of WT vs. fancd2 after IR (Two-tailed T-test, p=.203). E. Time-lapse of Irradiated fancd2 RNAi#1 animal. Example of a centromere-containing DNA bridge that persists into cytokinesis. Purple marks kinetochores (CenpC Tomato) and green marks DNA (his GFP) and cell membranes (Moesin GFP). Time is expressed as minutes relative to anaphase onset. F. Quantitation of frequency of unaligned DNA in WT+IR vs. fancd2 *RNAi*#1 +IR, broken down by round of papillar division. Data are from the same movies as the graph in Fig4H. N=33-54 for 1<sup>st</sup> division and N=41-76 for 2<sup>nd</sup> division. Scale bar= 5µm.

# **2.3.4** FANCD2 responds specifically to DSBs and acts independently of previous S-phases

FANCD2 prevents genome instability in response to a host of stimuli, including inter-strand cross-links, recombination intermediates, replication stress/late replication, and lesions created by IR (Grompe 2002; Walden and Deans 2014; Moldovan and D'Andrea 2009; Pontel et al. 2015). In papillar cells, we find acentric DNA fragments are the most common aberration, and FANCD2 loss leads to inaccurate segregation of these fragments. These data suggest in genome-damaged papillar cells, FANCD2 may play an important role by responding specifically to double-strand break (DSB)-induced acentric DNA.

To test this idea, we employed the I-Cre system to generate papillar cells with acentric X-chromatids (Fig14A-C). We used I-Cre because IR causes a host of chromosome aberrations, and it was unclear which causes cell loss in *fancd*2. We optimized conditions so the number of acentric fragments/cell was similar to following IR (Fig14B-Meta vs. Fig10D-acentric, Methods). As described previously for diploid Drosophila neuroblasts, and as we observe after IR, acentric papillar DNA generated by I-Cre lags in anaphase but eventually segregates into daughter nuclei (Fig14C, MovieS6). As seen after IR, I-Cre induction during papillar endocycles causes essentially no change in adult papillar structure/cell number (Fig14D,F). In contrast, I-Cre causes significant cell number reduction in multiple fancd2 RNAi lines (Fig14E,F, Fig18B). As for IR, we detect PH3+ unaligned DNA (Fig14G) and an increase in persistent PH3+ micronuclei in multiple fancd2 RNAi lines (Fig14H,I). Thus, I-Cre largely phenocopies IR induction in fancd2. We also note that, for multiple fancd2 RNAi lines, the number of PH3+ micronuclei significantly increases without exogenous DNA breakage (Fig14I), underscoring the importance of *fancd2* in papillar cells not only following exogenous DNA damage but also under physiological conditions.

As a further test of the physiological importance of FANCD2 in papillar cells, we employed a test of papillar function. We previously established that animals with decreased rectal papillar cells cannot survive high levels of dietary salt, due to the role of papillar structures in salt absorption (Schoenfelder et al. 2014). To test if *fancd2* loss

impacts the physiological function of adult papillae following DSB induction, we fed WT and *fancd2* animals either control or a high-salt diet in the presence or absence of I-Cre expression. Indeed, *fancd2* loss in developing papillae renders adult flies sensitive to highsalt, a result that is enhanced by I-Cre expression (**Fig14J-M**). Together, these data show FANCD2's response to DSB-induced acentric DNA is a key aspect of the resistance of mitotic papillar cells to DNA damage.



Figure 14: FANCD2 promotes papillar cell viability and prevents micronucleus formation specifically in response to acentric chromosomes.
A.Top-Diagram of I-Cre system. One acrocentric X chromatid (green) shown before/after I-Cre induced DSB severs the connection with the centromere (purple). Bottom-Example of uncut and cut X-chromosomes in papillar cells from I-Cre expressing flies. B.Frequency of mitotic papillar cells after I-Cre (expressed during endocycles) with at least 1 aberrant X chromosome at metaphase (Meta, from chromosome preparation data) or with lagging DNA at anaphase (Ana, from live imaging data). From N=58-82 cells/condition, from multiple replicates. C.Representative time-lapse papillar DNA segregation following I-Cre (expressed during endocycles). CenpC-Tomato=kinetochores (KTs, purple), histone H2AV=DNA (green). Time in min. relative to anaphase onset. Arrow indicates lagging DNA, which segregates. D.WT adult rectum after I-Cre (expressed during endocycles). E.fancd2RNAi#1 adult rectum after I-Cre (expressed during endocycles). Papillae falsecolored green, DNA in purple in D and E. F.Avg. adult papillar cell number/animal for WT and fancd2RNAi#1 +/- I-Cre. From N=8-23 animals/condition, multiple replicates. \*=significant change between +/- I-Cre compared to WT (Methods). Yellow bars= mean. G.Unaligned PH3+ chromosomes (green labeling/white arrows, DNA in purple) in fancd2RNAi#1 after I-Cre. H.Persistent PH3+ micronuclei (green labeling/white arrows, DNA in purple) in fancd2RNAi#1 after I-Cre. I. Number PH3+ micronuclei +/- I-Cre in WT vs. fancd2RNAi#1 and *fancd2RNAi*#2. Yellow bars= mean. From N=17-23 animals/condition, multiple replicates. \*=significant difference from WT (Two-tailed Ttest, p<.005). DAPI=DNA in all images. J-M.Survival of adults of WT (blue) and fancd2 RNAi#1 (red) animals over time for the indicated I-Cre and diet conditions. Bars= standard error. Each genotype/condition represents 3 replicates with 10 animals/replicate. White scale bar=50µm, yellow scale bar=5µm.

Our data point to a role for *fancd2* in promoting acentric DNA segregation. As loss of *fancd2* can promote replicative stress (Lossaint et al. 2013; Howlett et al. 2005) it was possible that our observed role for FANCD2 was due to secondary consequences of depleting *fancd2* during endocycle DNA replication, which occurs prior to mitosis of acentric DNA. We thus tested if *fancd2*'s role in preventing acentric DNA-related defects arises during endocycle DNA replication. If so, depleting *fancd2* after endocycle S-phases (but before mitosis) should not affect the papillar response to acentric DNA. To test this idea, we exploited the changing cell cycle biology of papillar cells. A large window of time (several days) separates the last endocycle S-phase (2<sup>nd</sup> larval instar stage) from the next S-phase, the latter of which occurs after the first papillar mitosis (Pupal Day 2). We thus induced I-Cre as before during endocycles, but then used temporal control of RNAi (**Fig15A**, Methods) to only deplete *fancd2* after completion of the last endo-S-phase (as confirmed by EdU labeling, **Fig15A-C**). We then live imaged the first mitosis following this temporally restricted *fancd2* depletion. Remarkably, *fancd2* depletion in the absence of endocycle S-phase still leads to unaligned metaphase chromosomes and acentric micronuclei (**Fig15D,E**, **MovieS7**). From these data, we conclude *fancd2*'s role in preventing micronuclei in response to acentric DNA occurs independently of prior Sphases.



Figure 15: - Depletion of fancd2 in the absence of endocycle S-phases does not alter the acentric DNA phenotype.

A.Experimental procedure. I-Cre was induced during  $2^{nd}$  larval instar papillar endocycles. Following endocycles ( $3^{rd}$  larval instar), *fancd2* RNAi was induced. On pupal Day 2 (D2), the first papillar mitosis was examined. B.EdU labeling (green, DNA in purple), showing S-phase in the  $2^{nd}$  instar larval rectum (area to right of white line). C.EdU/DNA labeling as in B, now showing endocycles are complete in the rectum by  $3^{rd}$  instar stage (note-endocycles continue in the ileum, left of the white line). D.Time-lapse of I-Cre/*fancd2 RNAi#1* animal cultured under conditions shown in A. Example unaligned acentric DNA fragment (white arrow) that ends up in a micronucleus following cytokinesis (yellow arrow). Purple-kinetochores (KTs, CenpC Tomato) green-DNA (DNA, his GFP) and cell membranes (Memb, Moesin GFP). Time in minutes relative to anaphase onset. E.Graph of incidence of unaligned metaphase acentric DNA and micronuclei after anaphase in WT or *fancd2 RNAi#1* following I-Cre expression, as in panel A. From N= 30-32 cells/condition. \*=significant change by Chi-squared, p<.001. White scale bar=25 µm, Yellow scale bar=5µm.

#### **2.3.5** FANCI and Bloom also facilitate papillar DSB survival

Having found a role for FANCD2 in response to acentric DNA, we next asked if other *Drosophila* Fanconi Anemia proteins play similar roles. We first examined FANCM, a conserved helicase and member of the Fanconi core complex that recognizes DNA damage and recruits additional FANC proteins during cross-link repair (Kuo et al. 2014). As for FANCD2, we examined papillar survival in *fancm* mutants (Methods) following IR and I-Cre. *fancm* mutants are mildly sensitive to IR during papillar development (**Fig16A**, **Fig18C**). In contrast, *fancm* mutants do not exhibit a significantly increased sensitivity to DNA damage specifically caused by I-Cre-induced acentric DNA (**Fig16B,D**). Thus, FANCM responds to IR in papillar cells, but unlike FANCD2 is dispensable for the papillar response to acentric DNA. This result is consistent with previous reports of FANCD2 functions independent of the FANCM-containing core complex (Yeo et al 2014, Raghunandan et al 2015)

We next examined FANCI, a DSB-responsive nuclease known to function in a heterodimer with FANCD2 (Kondo and Perrimon 2011; Moldovan and D'Andrea 2009). fanci loss decreases adult papillar cell number not only after IR, but also after I-Cre (Fig16A,B,C,E, Fig18D-F). Further, following I-Cre induction, PH3+ micronuclei accumulate in *fanci* animals at increased frequencies relative to WT (Fig16G), and *fanci* loss leads to salt-stress sensitivity (**Fig16H,I**). We also note that, as for *fancd2*, the level of PH3+ micronuclei without exogenous DNA damage is significantly higher in *fanci* than in WT (Fig16G vs. Fig14I). These data suggest that like FANCD2, FANCI is required for the papillar acentric DNA response. We then asked if Blm helicase is active in papillar cells following DSB induction. We examined Blm because of its role in FANCD2-mediated segregation of ultra-find DNA bridges (Chan et al. 2007; Naim and Rosselli 2009). Much like FANCD2 and FANCI, Blm loss causes cell loss not only after IR, but also after I-Cre (Fig16A,B,F, Fig18G-J). We were unable to examine micronuclei in *Blm*-deficient animals due to additional, I-Cre-independent mitotic defects, which have been described previously (Fig18K, McVey et al. 2007). Taken together, our data implicate FANCD2, FANCI, and Blm as regulators of an acentric DNA response that ensures survival of DSBresistant endocycled cells.



### Figure 16: FANCI and Blm also facilitate papillar DSB survival

**A.**Avg. adult papillar cell number/animal for WT and indicated FANCD2 network mutants +/-IR. From N=7-25 animals/condition, multiple replicates. \*=significant change +/-IR compared to WT (Methods). Yellow bars=mean. **B.**Avg. adult papillar cell number/animal for WT and indicated mutants +/-I-Cre. From N=9-11 animals/condition, multiple replicates. \*= significant change +/-I-Cre compared to WT (Methods). Yellow bars=mean. **C.***fanci RNAi*#1 adult rectum after IR. **D.***fancm* adult rectum after I-Cre. **E.***fanci* 

*RNAi*#1 adult rectum after I-Cre. **F**.*blm RNAi*#1 adult rectum after I-Cre. Papillae falsecolored green, DNA in purple in **C-F. G.**Number PH3+ micronuclei +/- I-Cre in WT vs. *fanciRNAi*#1 and *fanciRNAi*#2. Yellow bars= mean. From N=9-27 animals/condition, multiple replicates. **H**,**I**.Survival of *fanci RNAi*#1 adults without (blue) or with (red) I-Cre expression, plotted over time for the indicated diet conditions. Bars= standard error. Each genotype/condition represents 3 replicates with 10 animals/replicate. DAPI=DNA in all images. White scale bar=50µm, yellow scale bar=5µm.

### **2.3.6** Core DDR components are not required for papillar DSB survival

Finally, we asked if our identified FANCD2-dependent response to acentric DNA requires core DDR signaling. The well-known DDR kinases ATM and ATR have both been implicated in FANCD2-dependent repair in specific contexts (Andreassen et al. 2004; Sobeck et al. 2006; Taniguchi et al. 2002). We first induced IR during endocycles in *ATM* (*Drosophila tefu*, hereafter *ATM*) or *ATR* (*Drosophila mei-41*, hereafter *ATR*) mutants (Methods). Both *ATM* and *ATR* animals have mild IR-specific decreases in adult papillar cell number (**Fig17A**, **Fig18L**,**M**), implicating these upstream kinases in a papillar response to IR. In contrast, null mutations in one or both of the canonical ATM/ATR downstream kinases *Chk1* (*Drosophila grp*) or *chk2* (*Drosophila lok*) have no effect on papillar cell number after IR (**Fig17A**,**C**). Similarly, *p53* null animals have no impact on post-IR papillar cell survival (**Fig17A**,**D**). Thus, papillar cells retain an IR-responsive role for ATM and ATR, but not their common downstream effectors Chk1, Chk2, or p53.

Given our demonstrated role for ATM and ATR after IR in papillar cells, we examined if, as for FANCD2, FANCI, and Blm, these kinases are required for papillar cell

viability in response to DSB-induced acentric DNA. Both ATM and ATR are dispensable for cell survival following I-Cre mediated induction of acentric chromosomes (**Fig17B,E,F**). These results differ from the clear requirement for FANCD2, FANCI, and Blm after I-Cre and suggest ATM/ATR play an IR-responsive role independent from responding to acentric DNA in papillar cells. Taken together, we conclude our newly identified response to DSB-induced acentric DNA does not require the core DDR regulators ATM, ATR, Chk1, Chk2, or p53.



# Figure 17: Canonical DNA damage regulators are not required for papillar cell survival in response to acentric chromosomes.

A-B.Avg. adult papillar cell number/animal for WT and indicated mutants +/- IR (A) or I-Cre (B). From N=7-17 animals/condition, multiple replicates. \*=significant change +/- IR or I-Cre compared to WT (Methods). Yellow bars=mean. C.*grp*, *lok* adult rectum after IR. D.*p53* adult rectum after IR. E.*tefuRNAi*#1 adult rectum after I-Cre. F.*mei*-41 adult rectum after I-Cre. DNA (labeled with DAPI) in purple and papillae pseudo-colored in green in all images. Scale bar=50µm.



Figure 18: Supporting data for IR-and I-Cre-induced phenotypes.

A. Representative *fancd2 RNAi*#2 adult rectum. B. Representative *fancd2 RNAi*#2 adult rectum following I-Cre expression. C. Representative *fancm* adult rectum following IR. D. Representative *fanci RNAi*#1 adult rectum. E. Representative *fanci RNAi*#2 adult rectum. F. Representative *fanci RNAi*#2 adult rectum following I-Cre expression. G. Representative *Blm RNAi*#1 adult rectum. H. Representative *Blm RNAi*#2 adult rectum. I. Representative *Blm RNAi*#1 adult rectum following IR. J. Representative *Blm RNAi*#1 adult rectum following IR. DNA (DAPI, Purple) and Mitotic chromosomes (Green, Phospho-Histone H3) L. Representative *tefu RNAi*#1 adult rectum following IR. M. Representative *mei-41* adult rectum following IR. DNA (indicated with DAPI) in purple and papillae pseudo-colored in green in all adult rectum images in this figure. White scale bar= 50 µm, yellow scale bar= 5 µm.

# 2.4 Discussion

Through activation of apoptosis, cell cycle checkpoints, and high fidelity DNA repair, canonical DDRs prevent cells from entering mitosis with broken chromosomes. In this study, we find naturally endocycling *Drosophila* papillar cells fail to apoptose, arrest the endocycle, or accomplish accurate repair, leading to mitosis with broken chromosomes. Despite this, papillar development is not hindered by chromosome breakage. In these damage-resistant polyploid cells, DNA fragments lacking centromeres align and segregate during mitosis. This segregation is mediated by FANCD2, FANCI, and Blm, and serves to prevent micronucleus formation and promote cell viability and proper organ function. As endocycling cells can also acquire resistance to cancer therapies that induce DNA breaks, further study in this area is likely to shed light on cancer-relevant

biology.

### **2.4.1** The endocycle is a recurrent source of DNA damage response attenuation

Previous work in post-mitotic *Drosophila* and mouse cells identified the endocycle as a source of DDR inactivation. Similar to previous work in non-mitotic endocycling cells and in mouse trophoblasts (Soloveva and Linzer 2004; Mehrotra et al. 2008; Zhang et al. 2014), we find papillar cells do not rely on/activate p53 in response to DNA damage. We recently showed papillar cells undergo a distinct endocycle: the pre-mitotic endocycle (Schoenfelder et al. 2014). Thus, a lack of a requirement for p53 activity appears common in diverse endocycling cells.

In mice, another DDR regulator- Chk1- is inactive in endocycling trophoblast cells (Ullah et al. 2011; 2008). As a potential indicator that this inactivity may be conserved, we find Chk1 (and also Chk2) are not required for the papillar acentric DNA response. In the *Drosophila* embryo, a Chk1-dependent checkpoint eliminates cells with replication errors (Fogarty et al. 1997; Sibon et al. 1997; 2000; Takada et al. 2007). Given that endocycles frequently cause replication-induced DSBs (Yarosh and Spradling 2014; Mehrotra et al. 2008; Nordman et al. 2011; Hannibal et al. 2014), we speculate a conserved property of endocycles is inactivity of this Chk1 replication checkpoint. This inactivity would explain how cells progress through what are often error-prone endo-S phases and accumulate DNA breaks. Such Chk1-independency may be reversible, as endocycled *Drosophila*  follicle cells require Chk1 for replication fork elongation during gene amplification (Alexander et al. 2015).

Beyond endocycles, polyploidy also inactivates the DDR in other ways. In mammalian cells, cytokinesis failure arrests the cell cycle. Continued division of resulting polyploid cells requires p53 inactivity (Ganem et al. 2014; Wong and Stearns 2005; Fujiwara et al. 2005). Polyploid cancer cells also re-wire canonical DDRs (Zheng et al. 2012). These examples and others (Schoenfelder and Fox 2015) highlight numerous connections between polyploidy and DDR inactivation. However, to this point little was known about how polyploid cells proliferate without functional DDR components.

### 2.4.2 FANCD2 as an emergency DNA damage regulator

Emerging evidence suggests in the absence of canonical DDRs, emergency DDRs make mitosis compatible with genome damage. In addition to FANCD2, BubR1, Polo, and Aurora B perform a similar function in response to broken chromosomes in *Drosophila* neuroblasts (Royou et al. 2010; Karg et al. 2015; Derive et al. 2015). Aurora B and INCENP also form heterochromatic DNA threads that segregate achiasmate chromosomes during *Drosophila* meiosis (Hughes et al. 2009). Little is known about these newly identified emergency DDRs.

We argue a key role of emergency DDRs is prevention of micronuclei. We previously showed papillar cells are highly tolerant of whole chromosome aneuploidy (Schoenfelder et al. 2014). In contrast to whole chromosome aneuploidy, we find papillar cells are intolerant of mis-segregated acentric DNA fragments and micronuclei. Recent work implicated micronuclei as catalysts in chromothripsis, a genome-shattering event linked to cell death and cancer (Hatch and Hetzer 2015; Zhang et al. 2015). Interestingly, polyploid cancer cells were recently shown to be more likely to undergo chromothripsis (Mardin et al. 2015). Further, Fanconi Anemia and Bloom syndrome patients are cancerprone, and cells from these patients accumulate micronuclei (Naim and Rosselli 2009). Our data suggest polyploid papillar cells are a useful model to identify other factors like FANCD2 that act in mitotic cells lacking canonical DDRs.

### **2.4.3** FANCD2 responds to acentric DNA

Our data suggest FANCD2, Blm, and FANCI can recognize and respond to DSBinduced acentric DNA, and may act to tether acentric DNA to its centromere-containing fragment. What FANCD2 is specifically recognizing in cells with acentric DNA remains to be determined. In mammalian cells, FANCD2, FANCI, and Blm localize to a specific class of mitotic ultra-fine bridges (UFBs) between daughter nuclei (Chan et al. 2009; Naim and Rosselli 2009). The exact nature and various classes of these bridges is still being determined, although at least some of these structures contain DNA that fails to replicate until cells enter mitosis (Minocherhomji et al 2015). It is possible that in our experiments, acentric DNA may not be completely severed from its centromere, or limited DNA repair may join the centromere with its acentric fragment. If so, acentric fragments may be connected by structures that resemble late replication intermediates, that are then recognized by FANCD2. Similarly, during papillar development, under-replication may naturally occur, as it does during many endocycles which fail to initiate late replication (Lily and Spradling 1996; Nordman et al. 2011; Yarosh and Spradling 2014). If so, such un-replicated DNA may be recognized by FANCD2 early in the first mitosis. However, we previously showed that papillar cells are distinct from other endocycled cells in that they do initiate late replication and achieve (at least very close to) full genome duplications (Fox et al. 2010). Regardless, an advance of our present work is the finding that FANCD2 specifically responds to acentric DNA and is required for its segregation.

While future work is needed to determine the exact function of FANCD2 in cells with acentric DNA, our data here provide several key clues. First, FANCM, a component of the Fanconi core complex, is not required for the acentric DNA response. A previously established function of this core complex is to ubiquitinate FANCD2, which is important for specific repair events such as repair of DNA cross-links (Alpi et al. 2008; Marek and Bale 2006). Second, ATM is also not required for the acentric response, suggesting that ATM-mediated FANCD2 phosphorylation, which is important for FANCD2's previously established response to IR, is also not involved (Sobeck et al. 2006; Taniguchi et al. 2002). Third, FANCD2 acts independently of DNA replication prior to mitotic entry to prevent acentric micronuclei. Fourth, we show that papillar cells are a useful model of the *in vivo* consequences of FANCD2's response to DSBs and acentric DNA.

Finally, in a clinical setting, combining current cancer therapies with FANCD2 deficiency has recently emerged as a promising strategy in treating human tumors (Shen et al. 2013a; Burdak-Rothkamm et al. 2015; Liu et al. 2015). Our work may argue that such therapies would be effective in radio-resistant cancers exhibiting either polyploidy or lack of canonical DDR functions.

# 2.5 Experimental Procedures

Drosophila stocks and genetics. Unless indicated, WT was brachyenteron (byn) Gal4, UAS Moesin-GFP. byn-Gal4 expressed all UAS transgenes except in **Fig9F,G**. All experiments were at 22 °C except **Fig6G**, where animals were kept at 18 °C before shifting to 29 °C just prior to 2<sup>nd</sup> larval instar, to inactivate *Tub-Gal80(ts)*, and **Fig15**, where animals were similarly kept at 18 °C until the indicated time period. Salt stress was performed as in Schoenfelder et al. (2014). flybase.org lists the full genotypes for all stocks used. Alleles and transgenes used in this study: *byn-Gal4*, *Tub-Gal80(ts)*, *UAS-p53* (*P Gus p53 2.1*), *UAS-hid*, *UAS-rpr*, *CenpC-Tomato*, *UAS-Moesin-GFP*, *hisH2AV-GFP*, *hisH2AV- RFP*, *hs-I-Cre1* (1A), *hs-I-Cre1* (2A), *UAS tefu RNAi* (v108074, referred to as *tefu*  RNAi #1), UAS tefu RNAi (JF0142,2 referred to as tefu RNAi #2), mei-41 (29D), mei-41 (RT1), grp (Z2150), lok (30), p53 (5A 1-4), UAS fancd2 dsRNA (referred to as fancd2 RNAi #1), UAS fancd2 RNAi (v45433, referred to as fancd2 RNAi #2 in figure 5 and figure 6), UAS fancd2 RNAi (HMC03558, referred to as fancd2 RNAi #2 in figure 4), UAS fanci RNAi (HMS00769, referred to as fanci RNAi#1), UAS fanci RNAi (v24655, referred to as fanci RNAi#1), UAS fanci RNAi (v24655, referred to as fanci RNAi#2), fancm (Del), Df 3R (ED6058)-a fancm-spanning Deficiency, UAS Blm RNAi (v13309, referred to as Blm RNAi #1), UAS Blm RNAi (v13310, referred to as Blm RNAi #2), AICR-Gal4 (10H05), UAS-GFP, UAS-RFP, BubR1-GFP, UAS Moesin-mCherry, and BubR1 (KEN).

*DNA damage*. IR was performed in 2<sup>nd</sup> instar larvae aged to the stage of papillar precursor endocycles. Animals in 60 or 100mm petri dishes with a thin layer of standard *Drosophila* food were placed in an X-RAD 160 PXI precision X-ray Irradiator (calibrated by a Dosimetrist) at 20 Gy. To compare the number of aberrant chromosomes between WT and *fancd2* under the same IR conditions, GFP-marked WT larvae were mixed with RFP-marked *fancd2* larvae in the same dish, which was then irradiated. For I-Cre, animals were heat shocked in a 37 °C water bath in vials for 90 min. All experiments represent at least 2 separate IR/I-Cre treatments.

*Tissue Preparation/Microscopy.* Fixation, chromosome preparations, and live imaging were as in Schoenfelder et al. 2014. Antibodies: Mouse Phospho-Histone H3 Ser10, (1:1000, Cell Signaling), Mouse *Drosophila* Gamma H2AV, (1:2500, Lake et al. 2013),

Rabbit GFP (1:1000, Life Technologies). Nuclear labeling in all fixed images: DAPI. For EdU (Invitrogen) labeling, tissue was pulsed with EdU for 15 min., and detection was according to manufacturer's instructions. TUNEL labeling was as in Schoenfelder et al 2014. Fixed images were acquired using a Zeiss AxioImager M.2 with Apotome processing (20X, 40X or 63X). Live imaging used an Andor XD Spinning Disk Confocal Microscope (60X silicon or 100X oil).

*Image analysis.* Z-projections were assembled using ImageJ. Movies were assembled using MetaMorph (Molecular Devices). Adobe Photoshop was used to adjust brightness/contrast. ImageJ's "cell counter" was used to count cell number.

Statistical analyses of adult cell numbers. To determine if papillar cells of a given mutant led to significant cell number decreases after IR/I-Cre relative to WT, the distribution of adult papillar cell number before and after IR/I-Cre was compared for each genotype using a Z-test. Resulting p values determined if any decrease in adult papillar cell number after IR/I-Cre was significantly different than WT. Significant differences (p values ranged from p<.05 to p<1X 10<sup>-9</sup>) are noted in the figures with \*.

### 2.6 Author Contributions

DF and HB designed and conducted the experiments, made figures and wrote the manuscript.

# 2.6 Acknowledgements

The following kindly provided reagents: Bloomington and Vienna Stock Centers, Allen Bale, Roger Karess, Dan Kiehart, Christian Lehner, Jeff Sekelsky (who provided the *fancm* deletion stock before publication), Tin-Tin Su (who also provided technical advice on IR), William Sullivan (who also provided technical advice on imaging tethers), and Will Wood. We thank David Kirsch, Daniel Lew, and members of the Fox and MacAlpine laboratories for valuable comments on the manuscript. D.F. is supported by a Pew Scholar Award and NIH grant GM118447. H.B. is supported by NIH grant CA186545.

# 3. Single stranded DNA is present in papillar cells following I-Cre induction

This work represents ongoing studies in the lab that we are developing for publication. All of the experiments were designed by Dr. Don Fox and myself. I conducted all the experiments and designed the figures. The text was written in consultation with Dr. Fox.

## 3.1 Introduction

During mitosis, cells rely on a microtubule based spindle system to segregate DNA between two daughter cells. The spindle attaches to the DNA at a specialized location known as the centromere. Except for organisms with holocentric chromosomes, each chromosome contains a single centromere. Therefore, a DNA DSB creates a fragment lacking a centromere, known as an acentric fragment. Acentric fragments cannot attach to the spindle, and therefore during mitosis it is thought that such broken DNA is mis-segregated, often ending up in a micronucleus. Micronuclei are associated with genome instability and cell death (Countryman and Heddle 1976; Jagetia and Adiga 2000; Jagetia and Aruna 2000). Therefore, mitosis in the presence of broken DNA is usually catastrophic for cells. Previously (see Chapter 2), we showed that *Drosophila* rectal papillar cells lack an intact DNA damage checkpoint due to pre-mitotic endocycles. Thus, papillar cells readily enter mitosis with acentric DNA fragments (Bretscher and Fox 2016). Our laboratory has found that these acentric fragments initially lag on the metaphase plate but are ultimately fully incorporated into daughter cells (Bretscher and Fox 2016). Proper acentric DNA incorporation is dependent on the Fanconi Anemia proteins FANCD2 and FANCI as well as the BLM helicase (Bretscher and Fox 2016). While lack of FANCD2 results in micronucleus formation, the exact mechanism by which acentric fragments are incorporated into daughter nuclei remains unknown.

Given the observation that acentric fragments are incorporated into the main body of DNA, we reasoned that there was likely some form of connection between acentric DNA and segregating DNA. FANCD2 and BLM have previously been shown to bind and resolve ssDNA replication intermediates during mitosis in mammalian cells (Naim and Rosselli 2009; Chan et al. 2009). These ssDNA intermediates were not visible with a DAPI stain consistent with our failure to observe DAPI linking acentric DNA to segregating DNA. For additional information on these replication intermediates please see Chapter 1. Therefore, we wondered whether DAPI negative ssDNA may connect acentric DNA to daughter cell nuclei.

FANCD2- and BLM- associated ssDNA in mammalian systems results from replication intermediates, however, in our previous studies we have generated a DNA DSB. Therefore, it seems unlikely that ssDNA replication intermediates would connect acentric DNA to segregating DNA. One possibility is the connection may be formed by a ssDNA repair intermediate. We have observed that papillar cells fail to complete repair, however, it is possible that repair is initiated. Indeed, in other systems it has been observed that during mitosis, complete repair may be inhibited, allowing for only initiation of repair (Mahajan et al. 2002; Terasawa, Shinohara, and Shinohara 2014). In Mphase *Xenopus* egg extracts, this initiation of repair results in Mre11 and CtIP dependent 5' end resection. Mre11 is a member of the MRN complex required for sensing DNA DSB. CtIP serves as a cofactor for the MRN complex and serves to initiate the process of HR.. Together Mre11 and CtIP initiate repair by 5' end-resection. However, in M-phase extracts repair fails to proceed beyond this step (Peterson et al. 2011). Therefore, we wondered whether papillar cells may also initiate repair.

Here, we present evidence that ssDNA is present during mitosis of papillar cells following DNA DSB induction. Furthermore, we find that while acentric DNA segregation is independent of canonical DNA damage transducers and effectors, the DNA DSB sensor complex, the MRN complex, is required in papillar cells following DNA DSB induction. Thus, like other cell types, papillar cells may initiate DNA repair, but leave the process incomplete resulting in a requirement of non-canonical mechanisms to ensure acentric DNA segregation during mitosis.

# 3.2 Results

#### **3.2.1** Acentric DNA induction results in RPA3 coated single stranded DNA

Given that FANCD2 and BLM have previously been implicated in resolution of ssDNA structures during mitosis (Chan, North, and Hickson 2007; Chan et al. 2009; Naim and Rosselli 2009), we were interested in whether acentric DNA induction results in single stranded DNA in our system. To induce acentric DNA, we made use of an inducible I-Cre endonuclease, which cuts only at the *Drosophila* rDNA repeats on chromosomes X and Y. To ask whether single stranded DNA forms in our system, we took advantage of flies expressing RPA3-GFP (Anne Royou, unpublished). RPA3 is a subunit of the RPA ssDNA binding protein, and is the most upstream sensor of ssDNA. In the absence of exogenous damage, we found that 9.2% of endocycling papillar cells contain RPA3 foci (**Figure** 19A,C). This suggests that single stranded DNA is found in endocycling cells.

Previous work in our lab has shown that the papillar endocycles result in a low level of DNA breaks, and we speculate that these breaks are the source of RPA3 foci. (Bretscher and Fox 2016; Fox, Gall, and Spradling 2010). The number of cells containing RPA3 foci increased significantly one hour post I-Cre induction, suggesting that ssDNA results from I-Cre induction in our system (**Figure** 19B). I-Cre induction resulted in 95% of cells with RPA3 foci (**Figure** 19C), with 91% having 4 or more foci (**Figure** 19D). Furthermore, these foci were consistently found in the same location in the nucleus: between the DAPI-light region and the DAPI-bright nucleolus (**Figure** 19B). Since I-Cre is known to cut at the rDNA locus, we used a FISH probe recognizing the rDNA to determine whether rDNA is located in this region. We found that the FISH probe to the rDNA localizes to the same region as we see generation of RPA3 foci (**Figure** 19E arrow), indicating that these foci are found in the rDNA. Thus, I-Cre induction appears to result in RPA3-coated ssDNA in endocycling papillar cells. Furthermore, this suggests that while endocycling papillar cells inactivate elements of the DNA damage response, this inactivation occurs downstream of their ability to sense ssDNA.



Figure 19: RPA3 foci are induced in endocycling cells following I-Cre induction

A) Endocycling 2nd larval instar papillar cells in the absence of I-Cre expression. B) Endocycling 2nd larval instar papillar cells 1 hour post I-Cre induction. RPA3 foci form in area adjacent to nucleolus. C) Quantification of percent of cells with at least one RPA3 foci from N=10 animals for each condition. D) Quantification of number of RPA3 foci per cell from same animals as C, N=100 cells for undamaged, 120 for 1 hr post I-Cre. E) Endocycling 2nd larval instar papillar cells showing localization of rDNA locus, using a FISH probe to the rDNA. E' DAPI from E showing that rDNA is found in the area adjacent to the nucleolus, in the same region we see foci induction in B.

#### **3.2.2** RPA foci persist into mitosis

Previous work in our laboratory has shown that I-Cre induced DNA damage does not get repaired prior to mitotic onset, with the exception of fusion events that generate aberrant dicentric chromosomes (Bretscher and Fox 2016). However, whether RPA3 coated ssDNA persists into mitosis remained unclear. We began by looking at cells in the first mitotic division that occurs post-endocycling. In the absence of exogenously induced damage, we found that RPA3 foci were present in 12.5% of cells at mitotic onset (prophase or metaphase as indicated by Phospho-Histone H3 (PH3) staining) (**Figure** 20A, E). This number is very close to the number of RPA3 foci observed during the endocycle, suggesting that endogenous RPA3 foci generated during endocycles may also persist into mitosis. Interestingly, the number of cells containing foci prior to anaphase is roughly consistent with our previous observation that around 13% of papillar cells enter mitosis with acentric DNA fragments (Bretscher and Fox 2016). Together, these data suggest that RPA3 coated ssDNA persists from the papillar endocycle into the first mitotic division.

We next induced I-Cre to study the effect of induced acentric DNA on RPA3 localization. I-Cre induction during endocycles resulted in a robust increase in RPA3 foci at mitotic onset (**Figure** 20B), with 76.9% showing at least one DNA associated RPA3 foci (**Figure** 20E). Therefore, I-Cre induced RPA-coated ssDNA persists from endocycle until mitotic onset.

Given that RPA3 foci are present at mitotic onset, we next asked whether these foci persist into anaphase during the first mitotic division. In the absence of exogenous damage, we did not observe any anaphases with RPA3 foci, however this may be a result of a small number of anaphases examined (**Figure** 20C, E). Again, I-Cre led to a robust increase in the number of animals with RPA3 foci during anaphase (**Figure** 20D). Following endocycle-induced I-Cre, we found that 88.9% of first mitotic anaphases had at least one RPA3 focus. Foci were typically located on the edge of the main body of DNA (**Figure** 20C, white arrows) or on the lagging DNA. Interestingly, we also observed foci between the two segregating daughter cells (**Figure** 20D, yellow arrow). This suggests that single stranded DNA is present in anaphase, in first mitotic division cells. The localization of the RPA3 foci suggests that ssDNA may be present at the edge of segregating DNA as well as on regions lacking DAPI staining, as for mammalian UFB DNA (Chan, North, and Hickson 2007; Chan et al. 2009). Papillar cells undergo two mitotic divisions within a 24 hour time period and we are able to distinguish between cells in the first vs. second mitotic division (Stormo and Fox 2016). Thus, we were interested in whether RPA3 was present during the second mitotic division as well as the first, or whether the damage may get repaired during the intervening interphase. We found that the frequency of RPA3 foci in second mitotic divisions was much lower than in first mitotic divisions (**Figure** 20E vs 20F). However, to our surprise, we found that lagging DNA was still present during second mitotic divisions (data not shown). This suggests that papillar cells do not undergo accurate repair of acentric DNA. It remains unclear whether the lack of RPA3 results from repair of ssDNA or whether RPA3 is simply no longer retained on DNA after the first mitotic division.

Previous results in our laboratory identified a role for the FANCONI Anemia protein FANCD2 in acentric DNA segregation (Bretscher and Fox 2016). Thus, we were interested in whether FANCD2 may be required for the presence of RPA3 foci. Therefore, we used RNAi to eliminate FANCD2 from papillar cells and asked whether lack of FANCD2 altered the incidence of RPA3 foci in the first papillar cell mitosis. In the absence of exogenous DNA damage, we found that FANCD2 was present in 18.6% of pre-anaphase cells. This number robustly increased following I-Cre induction (**Figure** 20G). This result indicates that FANCD2 is not required for generation of I-Cre induced ssDNA. Furthermore, we saw no difference in the number of anaphase cells containing RPA3 foci following I-Cre expression, suggesting that persistence of RPA3 coated ssDNA into mitosis is independent of FANCD2. Of note, we did observe a higher baseline level of foci in animals lacking FANCD2 (**Figure** 20E vs G). This is consistent with previous work in our laboratory which has shown that lack of FANCD2 alone results in an increase in acentric DNA. (Bretscher and Fox 2016).

Given the limitations of fixed imaging, we were unable to follow RPA3 throughout mitosis using fixed imaging. Therefore, we sought to better understand the dynamics of RPA3 foci during both first and second mitotic divisions.









Figure 20: RPA3 foci persist into mitosis

A) Mitotic, pre-anaphase cell B) Mitotic pre-anaphase cell after I-Cre expression showing RPA3 foci (white arrows) D) Mitotic anaphase cell E) Mitotic anaphase cell after I-Cre expression showing RPA3 foci at the edge of segregating DNA (white arrows) and in the area between the two bodies of segregating DNA (yellow arrows). D) Quantification of number of 1st mitotic division pre-anaphase cells and anaphase cells showing RPA3 foci from N=11 undamaged animals and N=15 after I-Cre expression. Increase in RPA3 foci is significant based on chi squared test p<.05. E) Quantification of number of 2nd mitotic division pre-anaphase and anaphase cells showing RPA3 foci from N=15 undamaged animals and N=12 following I-Cre expression. The number of pre-anaphase and anaphase foci observed in 1st vs 2nd division following I-Cre is significant based on a chi squared test p<.05. G) Quantification of number of 1st mitotic division pre anaphase and anaphase cells expressing FANCD2 RNAi from N=8 undamaged an N=5 after I-Cre expression. Increase in number of foci is significant for both based on a chi squared test p<.05

# **3.2.3** RPA foci segregate during anaphase and can be discrete from conventional DNA markers

In order to determine the dynamics of RPA3 during mitosis, we took a live imaging approach. We found that in the absence of exogenously induced DNA damage, the majority of papillar cells were RPA3 negative during mitosis (**Figure** 21A, D). Immediately following anaphase, RPA3 localized to DNA, but was not found in discrete foci (**Figure** 21A). Consistent with what we observed with fixed staining of RPA3, we found that the incidence of RPA3 foci in prophase/metaphase cells robustly increased in the first mitotic division following I-Cre induction (**Figure** 21 B, C, D). RPA3 foci were visible at mitotic onset (**Figure** 21B 0:00) and aligned at the metaphase plate along with the rest of the DNA prior to anaphase onset (**Figure** 21B 17:00).

During anaphase, we found that RPA3 foci segregated either with or adjacent to the DNA. RPA3 foci were found both on (or adjacent to) lagging DNA (white arrows) as well as the main segregating body of DNA (yellow arrows) (Figure 21B, C,I). In addition, we occasionally were able to observe RPA3 foci that appeared to link two pieces of lagging DNA (blue arrows). In the example shown in **Figure** 21B at 22:00 RPA3 is present in a distinct focus at the edge of the lagging DNA. As the DNA continues to separate at 25:00 we see that the RPA3 foci is found linking the two segments of lagging DNA. In order to confirm and quantify out observations, we created fluorescent intensity line profiles along a manually determined axis of DNA segregation. Since acentric DNA segregation does not occur in a perfectly straight line it was necessary to hand trace the lagging DNA segments to create accurate profiles. These profiles confirmed our observation that at 25:00 RPA3 is located in a histone negative region between two histone positive regions DNA (Figure 21B'). In the example shown in **Figure** 21C RPA3 is again localized between two histone positive fragments at both 3:00 and 4:00. In **Figure** 21C' a fluorescent line intensity plot shows that RPA3 is located in a histone negative region. These data suggest that RPA3, and therefore likely ssDNA, is present during first mitotic anaphase and connects lagging DNA to the main segregating body of DNA.

Having seen using fixed imaging that second mitotic divisions generally lacked RPA3 foci, we sought to observe the dynamics of acentric DNA in real-time using live imaging. Again, we saw many fewer RPA3 foci during 2<sup>nd</sup> mitotic divisions (**Figure** 21D vs. 3G). Interestingly, the incidence of lagging DNA was not altered between first and second mitotic divisions (**Figure** 21 H). Furthermore, DNA segregated properly during second mitotic divisions that lacked RPA3 foci (data not shown). Therefore, it appears that while RPA3 is largely absent in second mitotic divisions, papillar cells do not undergo accurate repair during interphase. One possible explanation for these results is that a ligation event occurs during interphase creating dicentric chromosomes. These chromosomes may initially lag, and then eventually segregate through spindle attachments and therefore not require the presence of RPA3 coated ssDNA. In future work we hope to examine the incidence of lagging centric DNA in the first vs second mitotic divisions.

When we first began looking at RPA3 we envisioned detecting long strands of RPA3 linking lagging DNA to segregating DNA. Instead we see RPA3 localized in foci on the edge of segregating DNA and lagging DNA. When we do see RPA3 localized to histone negative regions between fragments of lagging DNA, it only spans a short distance. One possibility is that we are unable to detect all RPA3 coated single stranded DNA in our system. As an alternative possibility, RPA3 may only bind at either end of ssDNA linkages. It is possible that RPA3 cannot bind the structure of DNA found in the linkages. Finally, it is possible that ssDNA is only found at the edges of DAPI/histone positive DNA. If this is the case, it does not eliminate the possibility that a linkage does exist between acentric and segregating DNA. The linkage could be composed of RNA or and oligomerized protein. In order to parse out these two possibilities, we will need another tool to detect the presence of absence of DNA.



Figure 21: RPA3 foci associate with both lagging and segregating DNA during anaphase.

A) Time lapse imaging of papillar mitosis. RPA3-GFP (green) and His-RFP (red). Time relative to first frame. B-C) Time lapse imaging of papillary mitosis after I-Cre induced during endocycles. RPA3-GFP (green) and His-RFP (red). Time relative to first frame. . B') Inset from B at 25:00 (E') showing position of RPA3 relative to lagging DNA. Corresponding line profiles along line of segregating DNA of intensity of red vs. green. (C'), Inset from 3:00 showing position of RPA3 relative to lagging DNA. Corresponding line profiles along line of segregating DNA of intensity of red vs. green. RPA3 foci lie between lagging DNA .D) Percent of cells in first mitotic division with RPA3 foci from N=25 undamaged cells and N=27 cells after endocycle papillar I-Cre induction. Statistically significant increase in RPA3 foci following I-Cre induction in both pre-anaphase and anaphase p<.05 F) Percent of cells in second mitotic division with RPA3 foci from N=29 undamaged cells and N=48 cells after endocycle papillar I-Cre induction. There is no statistically significant increase in RPA3 foci following I-Cre induction, p=.01H) Quantification of frequency of lagging DNA in cells from D + H. There is no statistically significant change between frequency of lagging DNA in 1st vs 2nd division following I-Cre expression chi squared test p=.05. I) Quantification of location of RPA3 foci

# **3.2.4** rDNA FISH signal provides additional evidence for presence of DAPI negative ssDNA

Having seen that RPA3, a single stranded DNA binding protein, was present during mitosis of papillar cells treated with I-Cre expression during endocycles, we sought a second method to detect DAPI negative ssDNA. Fluorescence in situ hybridization (FISH) has previously been used to detect DAPI-negative heterochromatic threads during meiosis in *Drosophila* (Hughes et al. 2009). We created a FISH probe to recognize the rDNA locus where I-Cre is known to cut. In papillar cells in which I-Cre was not induced, we found that the rDNA segregated along with the main body of DNA and did not lag (**Figure 22A**). Again, we created fluorescent intensity line profiles along the axis of DNA segregation to confirm our results (**Figure** 22A'). Following I-Cre expression, we found that rDNA was found in the lagging DNA (**Figure** 22B, yellow arrow). Upon closer inspection, in a few examples we found that rDNA signal was also found in regions that lacked DAPI signal (**Figure** 22B green arrow). We confirmed this observation using a fluorescent line intensity blot (**Figure** 22B'- arrows show rDNA signal peaks that correspond to associated staining). Thus, our FISH experiments provide additional evidence that DAPI negative DNA containing an rDNA sequence is found in areas between the main body of DNA and the lagging DNA.


Figure 22: rDNA FISH signal is present in both lagging DNA and DAPI negative regions

A)Image from undamaged cell with FISH probes to rDNA. A') fluorescence intensity plot from A showing that rDNA probe segregates with DNA. B) Image

from cell following I-Cre expression with FISH probes to rDNA. rDNA is found in segregating DNA as well as lagging DNA (yellow arrows) as well as a DAPI negative region, green arrows. B') Fluorescence intensity plot from B. Arrows link rDNA peaks to rDNA spots.

# **3.2.5** MRN complex members are required for papillar cell survival following I-Cre induction

The above data suggests that RPA3 coated ssDNA is present in papillar cells following I-Cre induction. Therefore, we next sought to understand how an endonuclease might generate ssDNA. I-Cre cuts leaving a leaving a 4 nucleotide overhang, but RPA3 requires an 8 nucleotide ssDNA fragment to bind (Cai et al. 2007). Thus, additional nuclease activity would be required to create ssDNA that RPA could bind to. Previous work in our laboratory has shown that survival of papillar cells is independent of canonical DNA DSB transducer and effectors (Bretscher and Fox 2016), however we never looked to see whether the DNA DSB sensor complex was required for papillar cell survival after I-Cre induction. MRN complex member MRE11 has 3' endonuclease activity when in complex with NBS1 and RAD50 (Anand et al. 2016), and therefore it remains possible that MRE11 may play a role in papillar cell survival and ssDNA generation. We thus tested the role of MRE11 in acentric DNA segregation.

While lack of MRN complex members did not affect papillar cell survival in the absence of I-Cre induction (**Figure** 23A, C, G), we found that lack of MRE11 (**Figure** 23B) or NBS1 (**Figure** 23D) led to papillar cell death following I-Cre induction. Furthermore,

loss of the MRN cofactor and endonuclease CtIP also resulted in papillar cell death following I-Cre induction (**Figure** 23 E vs F, G). These data suggest that the MRN complex is required following acentric DNA generation in papillar cells. Potentially, this complex functions to generate RPA3 coated ssDNA linkages between centric and lagging acentric DNA. It is worth noting that MRE11 typically only creates short ssDNA overhangs, and thus the possibility remains that additional nucleases may be required. Future work will be needed to test this model.

Finally, having seen a requirement for MRE11, NBS1 and CtIP, we wondered whether papillar cells may be initiating homologous recombination to ligate acentric and centric DNA. The first step of homologous recombination is MRE11 dependent ssDNA generation and subsequent RPA binding to ssDNA. RPA is then removed from ssDNA to allow for Spn-A (RAD51) to bind an enable homology search. Okra (RAD54) serves as a catalyst for this RPA-SpnA switch. We found that loss of okra or Spn-A had no effect on papillar cell survival following I-Cre induction (data not shown). Therefore, while the MRN complex and co-factor CtIP are required for papillar cell survival following I-Cre induction, papillar cells do not complete homologous recombination.



# Figure 23: The MRN complex and CtIP are required for papillar cell survival following I-Cre

A) WT adult rectum B) MRE11 adult rectum C) NBS1 RNAi adult rectum D) CtiP 1 RNAi adult rectum E) WT adult rectum after I-Cre F) MRE11 adult rectum after I-Cre G) NBS1 RNAi adult rectum after I-Cre H) CtiP 1 RNAi adult rectum after I-Cre I) Avg. adult papillar cell number/animal for WT and indicated genotypes +/-I-Cre. From N=7-11 animals/condition. \*=significant change +/-I-Cre compared to WT (Methods).

### 3.3 Discussion

## **3.3.1** Model: a ssDNA intermediate is critical in papillar cell acentric DNA segregation

Previous work in our lab identified a requirement for FANCD2, FANCI and BLM in papillar cells following acentric DNA induction (Bretscher and Fox 2016). Based on work from others that found FANCD2, FANCI and BLM have a role in resolution of ssDNA during mitosis (Chan et al. 2009), we wondered whether ssDNA may be present during papillar cell mitosis. We find that RPA3 coated ssDNA is present in papillar cells following DNA DSB induction. During anaphase, RPA3 typically localizes to either the edge of segregating DNA or the edge of the lagging DNA. Additionally, RPA3 was detected connecting pieces of lagging DNA in close proximity. These results indicate that ssDNA is present in papillar cells and suggests that ssDNA may be present between lagging acentric DNA fragments and centromere-containing DNA. In further support of this notion, we detect FISH signal directed against the region where we induce breaks in DAPI negative regions along the axis of segregating DNA.

Additionally, we find a requirement for the MRN complex and co-factor CtIP for papillar cell survival following exogenous DNA damage induction. Thus, we propose a model whereby following I-Cre induced DSBs in papillar cells, MRE11 and CtIP perform end resection to generate stretches of ssDNA originating from segregating DNA as well as acentric fragments (**Figure 24**). It is worth noting that MRE11 and CtIP typically only create short ssDNA overhangs, so it is likely that another nuclease may also be involved in ssDNA generation. Alternatively, BLM may be responsible for extending ssDNA overhangs. BLM is able to unwind DNA with a ssDNA overhang (Jung et al. 2014) and this unwinding could create DAPI negative ssDNA.

While ssDNA could be generated via end resection or unwinding from both the main body of DNA and the acentric DNA fragment, how these two stretches of ssDNA could be fused is unclear. Some ligases are able to anneal ssDNA without a template (Kuhn and Frank-Kamenetskii 2005). In addition, others have observed formation of DNA threads linking together two broken pieces of DNA (Royou et al. 2010). Thus, by some unknown mechanism a ssDNA linkage may form to link acentric fragments to segregating DNA (**Figure 24**).

We hypothesize that this linkage serves to hold acentric fragments in line with segregating DNA. Given that we have observed a role for FANCD2 and BLM in our

system, and that these two proteins have previously been shown to bind ssDNA(Chan et al. 2009), we speculate that linkages in our system may also be coated with BLM and FANCD2. We hypothesize that FANCD2 and BLM protect linkages between segregating and lagging DNA. Without a connection to the segregating DNA we propose that acentric DNA drifts out of alignment and fails to properly segregate (**Figure** 24). This hypothesis is consistent with our observation that in animals lacking FANCD2 acentric DNA fails to align at the metaphase plate and properly segregate into daughter cells (Bretscher and Fox 2016).

During mitosis, the spindle exerts forces within the dividing cell, and such forces could contribute to acentric DNA segregation. These forces, known as polar ejection forces, may act on lagging DNA to push it toward segregating DNA (Brouhard and Hunt 2005). While weak, these forces are strong enough to direct chromosome arms toward the poles, and thus may also be strong enough to push acentric DNA towards the segregating DNA. Furthermore, double minute chromosomes (which are acentric) have been shown to segregate by associating with centromere containing chromosomes and taking advantage of forces generated by the spindle (Kanda, Otter, and Wahl 2001).

# **3.3.2** An alternative model: protein bridges connect short ssDNA overhangs between acentric and centric DNA

In our system, we have failed to detect continuous RPA3 between acentric and centric DNA during mitosis. Instead we find RPA3 in foci mainly at the edge of segregating and lagging DNA. Thus, ssDNA may not span the distance between acentric and centric fragments. Instead, ssDNA may only be present in short fragments. It is possible instead that a protein bridge connects acentric and centric DNA.

Given that we have previously identified FANCD2 and BLM as required in papillar cells following DNA DSB induction (please see chapter 2), it is tempting to speculate that these proteins may be involved in bridge formation. Previous studies have shown that FANCD2 readily binds MRE11 generated ssDNA (Roques et al. 2009). Thus, if MRE11 were to generate even short stretches of ssDNA around the cut site, this may allow for FANCD2 binding.

In mammalian cells, FANCD2 recruits BLM to DNA UFBs (see chapter 1) (Naim and Rosselli 2009). In our system FANCD2 may recruit BLM to form a bridge connecting acentric DNA to centric DNA. Unlike most helicases, BLM exists as a circular tetramer (Karow et al.). Furthermore, BLM is known to form oligomers in the presence of complex DNA structures (Gyimesi et al. 2013). Thus, it is possible that BLM can oligomerize and link acentric DNA to segregating DNA (**Figure** 25). However, spanning the entire distance between acentric and centric DNA would require a long oligomer and thus seems unlikely.

Since we are inducing DNA DSBs in a repetitive sequence (the rDNA) it is possible we are cutting the DNA more than once, creating many small dsDNA fragments. If BLM were able to unwind short fragments and bind to resulting ssDNA, this ssDNA may serve as building blocks to form a BLM bridge (**Figure 25**)

In order to parse out these possible models, it would be useful to have two new tools. First, a fluorescently tagged BLM construct would allow us to visualize the location of BLM during papillar cell mitosis. Even with such a construct we would not be able to determine whether there was DNA in these bridges. Thus, the second tool we would need is a more sensitive means for detecting DNA. It is possible that the structure of DNA found in linkages is not amenable to RPA3 binding, and thus RPA3 is not a good tool to detect connections. In support of this idea, in yeast continuous BLM bridges have been detected, but RPA has been found only in discrete puncta on these bridges (Germann et al. 2014). In future work, we hope to utilize BrdU and or EdU as a more sensitive way to detect possible DNA connections.



Figure 24: Model for ssDNA generation and acentric DNA segregation



Figure 25: Alternative model for acentric DNA segregation

### 3.3.3 Evidence for a conserved role for FANCD2 and Blm in mitosis

In mammalian cells, resolution of UFBs during mitosis depends on FANCD2 and BLM. The absence of either FANCD2 or BLM results in persistent bridging and micronucleus formation. Our work has extended the role of FANCD2 and BLM to ensuring cell survival following acentric DNA induction. Furthermore, we show that lack of FANCD2 results in micronucleus formation suggesting a conserved role for FANCD2 in maintaining genome instability by preventing micronuclei formation.

### **3.3.4** FANCD2/BLM UFBs require a nuclease and polymerase to be resolved

In addition to requiring FANCD2 and BLM for resolution, mammalian UFBs also require the Mus81/EME nucleases as well as the DNA polymerase PolD3. Final resolution of UFBs depends on Mus81/EME cutting and repair by PolD3 (Minocherhomji et al. 2015). For additional information on this process please refer to chapter 1. PolD3 activity is associated with mitotic EdU incorporation. In our system, we have been unable to detect any evidence of EdU during mitosis. Thus, it seems unlikely that ssDNA is being repaired during mitosis. In further support of this, if accurate repair were occurring during mitosis we would expect to see a decrease in lagging DNA in second mitotic divisions. However, we see no difference in the frequency of lagging DNA in second mitotic divisions (**Figure 21F**).

While most of our work has focused on studying DNA aberrations resulting from endonuclease induction, it is worth noting that we see chromosomal aberrations and RPA3 foci even in the absence of exogenous damage. It is likely that these aberrations result from the endocycle and thus may represent replication intermediates. It would be interesting to see whether these intermediates may be resolved in a similar way to mammalian UFBs.

### 3.3.1 Canonical DNA damage sensors are active in papillar cell mitosis

We have detected RPA3 foci in papillar cell mitosis following DNA DSB induction. This suggests that while the canonical DNA damage response is inactivated, cells can still sense the presence of ssDNA. Furthermore, we have identified a role for the MRN complex members MRE11 and NBS1 in papillar cell survival following DNA DSB induction. This suggests that the most upstream canonical DNA damage proteins are active in papillar cells. It would be interesting to see if this holds true in other systems.

While UFB resolution is independent of ATR and chk1, it remains unknown whether DNA damage sensors (RPA or the MRN complex) may be involved. RPA has been seen bound between sister FANCD2 foci during S-phase, however the presence of RPA during mitosis has not been examined. Additionally, it remains unknown whether the MRN complex may also be required for UFB resolution in mammalian cells.

### Inactivation of DNA damage responses during mitosis

In our model, we propose that papillar cells initiate the first steps in repair but fail to complete repair. Interestingly, common DNA repair pathways are often inactivated during mitosis. During mitosis, NHEJ is suppressed by phosphorylation of the lig4 regulatory subunit XRCC4. Lack of inhibitory phosphorylation results in increased anaphase bridges, suggesting that undergoing repair during mitosis can result in increased genome instability (Terasawa, Shinohara, and Shinohara 2014). Conversely, knockdown of CtIP resulted in increased anaphase bridging, suggesting that CtIP may have some yet undefined role in preventing genome instability during mitosis (Terasawa, Shinohara, and Shinohara 2014). In addition to inactivating NHEJ, cells do not complete HR during mitosis.

As mentioned previously, it has been found that *Xenopus* M-phase egg extracts require the MRN complex and CtIP for end resection of DNA DSBs. However, when in M-phase activation of the MRN complex and CtIP fails to activate ATR or Chk1 (Peterson et al. 2011). The M-phase specific activity of the MRN complex and CtIP in egg extracts is interesting in the context of our data in papillar cells. We have identified the MRN complex, and CtIP as required for papillar cell survival and have observed RPA3 binding following DNA DSBs. Likewise, previous results have found that ATR and Chk1 are not required in our system. Like other systems, papillar cells may initiate repair, but stop the process prior to completion. Initiation of repair could result in repair intermediates that facilitate acentric DNA segregation.

The idea that cells can compensate for DNA aberrations during mitosis represents an emerging new field of study. A better understanding of mechanisms by which cells can divide with damaged DNA may be of clinical relevance. Many cancers contain mutations in DNA damage response proteins. One of the main barriers to cancer treatment is resistance to therapies such as radiation therapy. Resistance and proliferation despite administration of DNA damaging agents suggests that cell may be able to compensate for broken DNA. We speculate that compensation may rely on some of the same mechanism observed in our system and others.

### 4. Conclusion: Perspectives

It has recently been appreciated that cells can respond to damaged or incompletely replicated DNA during mitosis. One of the barriers to studying mitotic DNA damage responses, is the interphase DNA damage response, which prevents mitosis with broken DNA. However, recent advances have made study of mitotic DNA damage responses a new and emerging field. Thus far there have been three main classes of DNA aberrations during mitosis that have been studied, including our studies on papillar cells. While these have been discussed previously they are summarized in the following table for the reader's convenience.

	UFBs	Tethers	Lagging acentric
			DNA
Cell type	Mammalian cells	Drosophila	Drosophila rectal
	(In Vitro)	neuroblasts	papillar cells
Aberration	Replication	Acentric DNA	Acentric DNA
	intermediate	fragment	fragment
Source	Difficult to	DNA DSB	DNA DSB
	replicate sequences	induction during	induction during
	(CFS)	mitosis	pre-mitotic
			endocycles

DNA connection	DAPI negative	DAPI positive	Unknown- ssDNA
	ultrafine DNA	strand	exists, no DAPI +
	bridge		strand
Protein	FANCD2/FANCI,	BubR1, Polo, cdc20,	FANCD2, FANCI,
requirement	BLM, Mus81/EME1	fzy, klp3a	BLM, MRE11,
			NBS1, CtIP
Consequences for	Persistence of	Direct	Micronuclei
lack of resolution	bridges,	consequences	formation, cell
	micronucleus	unknown, results	death, organ
	formation	in death at the	malfunction
		organismal level	

# FANCD2 and BLM are critical in papillar cells and mammalian cells for resolution of mitotic intermediates

Thus far, little has been done to determine the extent of overlap between these systems. However, there are several similarities between UFBs and papillar cell's. Lack of FANCD2 results in micronuclei formation in both systems (Naim and Rosselli 2009). This suggests that the role of FANCD2 in preventing micronuclei formation may be conserved from *Drosophila* to mammals. While FANCD2 may be serving a similar role in both papillar cells and mammalian cells, the same does not hold true for the FANC- core complex. In mammalian cells, FANCD2 localization to mammalian UFBs is dependent on FANCcore complex members (Naim and Rosselli 2009). Conversely, in our system, we believe that papillar cell survival following I-Cre induction is independent of FANC core complex members. This suggest that the activation process of FANCD2 in response to mitotic DNA aberrations may differ between mammalian UFBs and *Drosophila* acentric DNA fragments. It is worth noting that the core complex is much larger in mammals than *Drosophila* and performs functions outside of ubiquitination. Thus, it is possible that the core complex in mammalian cells is not ubiquitinating FANCD2 but has another role such as in repair. The definitive experiment in both systems would be to use CRISPR to generate a non-ubiquitinatable version of FANCD2 and look at its ability to resolve replication intermediates and segregate acentric DNA

#### DAPI negative DNA is present in papillar cells and mammalian cells

In both papillar cells and mammalian cells DAPI-negative DNA is present during mitosis. In mammalian cells, this DNA has been visualized by BrdU and is coated by BLM. In papillar cells, we have observed RPA3 puncta. However, the main difference here is that we do not know if long stretches of ssDNA exist in our system. It is worth noting that BLM/DNA bridges in yeast show only punctate RPA and thus our failure to detect continuous RPA does not mean ssDNA does not exist.

In mammalian UFBs, FANCD2 binds to the DNA structure that exists at either end of the bridge. While one might assume FANCD2 would bind to several different types of DNA structures, this is not the case. For example, in mammalian cells FANCD2 does not bind radial chromosomes, or even UFBs originating from centromeres. FANCD2 selectively binds DNA breaks at CFS on chromosome arms. This suggests FANCD2 binding is very structure dependent. Based on this, if FANCD2 is binding in our system, I would speculate that the structure of the DNA at the binding site may be similar to the structure it is binding in mammalian cells. Future work is needed in both systems to better determine the structure and source of DAPI negative DNA.

Both CFSs and rDNA (where we are inducing the DNA DSB) contain repetitive sequences. Therefore, it is possible that the nature of these sequences is important in DNA segregation. Specific ssDNA structures may be able to form around repetitive regions allowing for single strand annealing or favoring binding of FANCD2. In fact, following DNA damage, heterochromatic regions have been shown to pair in mammalian cells. Furthermore, in *Drosophila* DAPI negative heterochromatic threads are crucial in proper segregation of achiasmic chromosomes during meiosis. These threads connect achiasmic chromosomes to chiasmic chromosomes allowing them to drift in and out of alignment but ensuring that they are ultimately segregated properly. In our system, if repetitive sequences from centric and acentric DNA could pair this pairing may be required to ensure proper segregation. One could argue that this is not the case since we see a role in FANCD2 following irradiation. However, irradiation may result in breaks at CFS in *Drosophila* and thus sequences there may assist in segregation.

### Nucleases and transcription in UFB resolution

Mammalian UFBs are resolved by nucleases to sever UFBs followed transcription to repair the site of damage (please refer to chapter 1). In our system, it seems like a nuclease may be the last thing we need. If there is a connection, it seems as though severing it may be a poor idea. However, one could speculate that transcription does occur in our system to re-ligate the acentric DNA to centric DNA. At this point we have no evidence to suggest this. We have not been able to detect mitotic EdU foci, nor do we see a reduction in lagging DNA during the second division. Therefore, I find it likely that the DNA intermediates that exist in UFBs and at DNA DSBs in papillar cells are similar, yet I think the similarity may stop there. Due to their difference in origin, it seems unlikely that they would be resolved in a similar fashion.

#### An argument for putting Neuroblast tethers in their own category

*Drosophila* neuroblasts challenged with DNA DSBs immediately prior to mitosis, use a tether system to properly segregate acentric DNA (Royou et al. 2010). Unlike

mammalian UFBs these tethers contain DAPI positive DNA. The source of this DNA remains mysterious given that an endonuclease is used to create the DNA DSB. Furthermore, these DNA tethers do not always form at the site of the cut. For example, tethers have been observed connecting the telomere end of the acentric fragment to the centric fragment (Karg et al. 2017). Thus, it seems that some sort of a double stranded DNA structure is being formed and then ligated onto acentric DNA to allow for segregation. Of note, these DNA tethers appear as thin strands linking acentric DNA to centric DNA. The size of these strands could reflect a lack of DNA condensation in these intermediates. The fact that this DNA structure is visible with DAPI indicates that it is of a completely different structure than other DNA intermediates discussed above. Therefore, I argue, that neuroblast tethers should be put in a category of their own. It would be interesting to see whether RPA may also be present in this system, or whether all DNA is double stranded.

In addition to DAPI positive DNA, tethers also contain several proteins including BubR1 and Polo (Royou et al. 2010). These proteins are required for proper DNA segregation. At this point, we have no evidence that either BubR1 or Polo may be localized to lagging DNA in our system. Furthermore, a genetic mutant of BubR1 known to disrupt tether formation has no effect on papillar cell survival (chapter 2). Requirement of a different set of proteins than UFBs or acentric DNA segregation is further reason to put neuroblast tethers in their own category. Micronucleus formation has been observed following improper resolution of UFBs and inaccurate segregation of acentric fragments. While evidence suggests that segregation does not occur properly in the absence of BubR1 or Polo (Royou et al. 2010), the end result of mis-segregation has not been studied. It would be interesting to see if mis-segregated acentric DNA in neuroblasts may form micronuclei similar to our observations in papillar cells. Furthermore, it would be interesting to see whether neuroblasts may also rely on FANCD2 to segregate acentric DNA.

# Moving forward in understanding mechanisms for coping to DNA aberrations during mitosis

Despite unanswered questions, this work has resulted in clear evidence in several systems that cells can compensate for DNA aberrations during mitosis. In order to advance this field further, I think that we need to better understanding the structure of DNA intermediates that exist in mitosis. It is worth pointing out that in both UFBs and neuroblast tethers, it does seem that this DNA may need to have some elastic properties in order to stretch as the cell divides. Similar abilities to stretch would also be required to segregate lagging acentric fragments in papillar cells. Yet one typically does not think of DNA as being elastic.

One possible explanation is that DNA in these linkages is breaking and proteins are bridging the gap (similar to the idea presented in **Figure 25**). In order to better

understand this, we need a method for detecting ssDNA live. This would allow us to view any breakage events and protein movements associated with these events. If breaks are occurring it is possible the proteins localized to these structures, such as BLM, may be stretching to fill in the gaps. In fact, as tethers extend in neuroblasts, localization of tether proteins becomes punctate along the length of the tether. It is possible that this re-localization of tether proteins reflects changes in DNA conformation. It would be interesting to try to measure forces on DNA in UFBs and neuroblast tethers as well as within tether proteins.

Better understanding of the nature of these DNA intermediates should shed light onto their origin. This will allow us to better understand the process by which they form. This will be especially interesting in the context of neuroblast tethers and acentric DNA fragments. Understanding these DNA intermediates will result in a clearer understanding of the events that occur following DNA DSB induction.

### Why bother with mitotic DNA damage responses?

We view these responses as a last-ditch effort to maintain genome integrity. When cells inactivate DNA damage checkpoints, or when DNA aberrations fail to activate checkpoints, cells are forced to compensate for such deficiencies during mitosis. Evidence suggests that canonical DNA damage pathways are inactivated during mitosis. This forces cells to rely on non-canonical means in attempts to maintain genome integrity. Since inactivation of canonical DNA damage pathways appears to be a common trend during mitosis, mitotic DNA damage responses may be utilized more heavily than we currently realize. It is possible that similar mechanisms may be used by all cells when canonical DNA damage checkpoints fail.

### Radiation resistance in cancer cells- a similar mechanism?

Evidence for the existence mitotic DNA damage responses also comes from disease models. One of the common treatments for cancer is radiation therapy. The goal of this therapy is to take advantage of cells endogenous DNA damage response and induce cell death. However, many patients develop radiation resistance over time. It is thought that radiation fails to kill all cells and that these cells can then give rise to new tumors. Since these cells must divide despite the induction of DNA damage suggests that they may employ mitotic DNA damage responses. Furthermore, mutations in DNA damage response genes are often found in cancers. Such lack of a DNA damage response would force cells to compensate for DNA damage during mitosis. We speculate that radiation resistant cancerous cells may utilize similar methods to compensate for broken DNA as we observe in our system.

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## **Biography**

Heidi Bretscher was born and grew up in Ithaca, NY where she attended Ithaca High School. After high school graduation, anxious to leave Ithaca, Heidi chose to attend Haverford College in Haverford, PA. At Haverford Heidi earned a BS with a major in Biology and minors in both chemistry and psychology. As an undergraduate HHMI fellow Heidi conducted her junior year research with Dr. Robert Fairman where she received a second author publication. Heidi conducted her senior year research and thesis with Dr. Stephen Emerson where she studied the differentiation of HSC to Blymphocytes in mice. While at Haverford Heidi competed all three years as a member of the Varsity Cross-Country and Track and Field teams. She served as a captain for bother her senior year. Heidi graduated from Haverford in May 2009 with awards including: Magna Cum Laude, Phi Beta Kappa and Honors in Biology.

Following graduation, Heidi accepted a position as a Research Technician in Dr. Roger Greenberg's lab in the Abramson Family Cancer Research Institute at the University of Pennsylvania. Here she began studying DNA damage and received a third author publication.

Heidi chose to attend Duke University to pursue her PhD in Molecular Cancer Biology in the Department of Pharmacology and Cancer Biology. After a very rough first year and five rotations Heidi eventually settled in the lab of Dr. Donald Fox. Here she has studied mitotic DNA damage responses in *Drosophila* rectal papillar cells. This work has led to a first author publication in *Dev Cell*. While at Haverford she has been a James B. Duke scholar and a Chancellor's Scholar. In addition, she received an honorary mention from National Science Foundation, an F31 Fellowship from the NIH, a Fitzgerald award for an outstanding paper and was selected as a speaker for the DCI retreat.

Following graduation Heidi plans to pursue a postdoc with Dr. Mike O'Connor at the University of Minnesota. She hopes she does not freeze to death.