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Msh2 Depletion Mitigates Chromosomal Instability in Fancd2-Deficient Cells

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ABSTRACT

The Fanconi Anemia pathway is a complex DNA damage response which combines translesion synthesis and homologous recombination to repair DNA interstrand crosslinks (ICLs). If untreated, these crosslinks will stall replication forks and inhibit DNA synthesis. Mutations in any of the fifteen known FA genes, such as *Fancd2*, result in widespread chromosomal instability and the formation of radial chromosome structures. The underlying cause of the ICL-induced genomic instability in FA-deficient cells is not known. We considered the hypothesis that proteins of the mismatch repair (MMR) pathway contributed to this genomic instability since MMR proteins bind ICLs to induce DNA breaks. This MQP presents evidence that Msh2 depletion mitigates the formation of radial chromosomes in Fancd2-deficient cells. In addition, we show that Msh2 depletion reduces cellular sensitivity to crosslinking agents such as Mitomycin C, and suppresses the ATM/ATR checkpoint response.

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BACKGROUND

Fanconi Anemia

Fanconi Anemia (FA) is an autosomal recessive disease characterized by widespread chromosomal instability. Although the disease is rare and occurs in only 1/350,000 births, the symptoms are often severe (Moustacchi, 2003). The principle features of FA include bone marrow failure, congenital abnormalities, development of solid tumors, and susceptibility to leukemia (Alan and D'Andrea, 2011). The average FA patient experiences the onset of bone marrow failure at around 7 years of age, but cases have been documented in which symptoms occurred earlier, later, or not at all (Moustacchi, 2003). Bone marrow failure results in a number of hematological disorders since affected individuals are unable to produce a significant amount of blood cells. The condition is lethal if untreated, and 90% of FA patients require a bone marrow transplant by age 40 (Alan and D'Andrea, 2011).

Congenital anomalies are a highly prevalent symptom of Fanconi Anemia and are often the initial sign of the disease. Anomalies vary greatly between patients but can affect the genital, renal, skeletal, auditory, ocular, and central nervous systems (Glanz and Fraser, 1982). Since malformations vary so greatly, it is impossible for doctors to diagnose the disease based on specific developmental abnormalities. Up to 25% of patients do not exhibit any type of congenital abnormality (Glanz and Fraser, 1982). Yet, some abnormalities occur much more often than others. Mutations in skin pigmentation are the most prevalent, and are observed in 55% of patients. Café au lait spots are the most common pigment defect and are typically small and light brown in color (Alter, 2003). In addition, 51% of patients suffer from growth retardation which results in a

short stature (Alter, 2003). Two hallmark features include abnormalities in the upper limbs and male gonads. Upper limb defects usually pertain to the thumb (**Figure-1**) which is malformed or missing in over 40% of patients (Glanz and Fraser, 1982). Other common defects include hearing loss, cardiac murmur, mental retardation, and microcephaly, a brain development disorder.

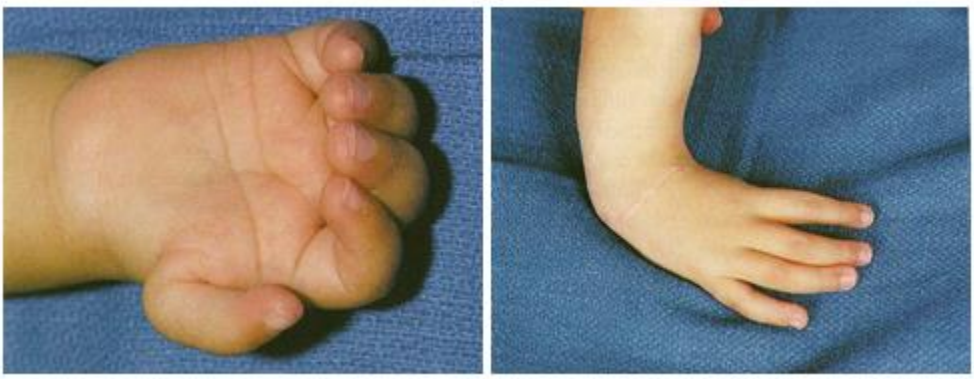

Figure 1: Common Thumb Malformations in Fanconi Anemia. Left photograph, type IV thumb deficiency where the thumb is completely disconnected from the skeleton and lacks musculature and tendons. Right photograph, type V deficiency where the thumb is entirely absent (Kozin, 2008).

Perhaps the most striking symptom of Fanconi Anemia is the patient's disposition to tumors and cancers such as leukemia. Children diagnosed with Fanconi Anemia are 15,000 times more likely to develop acute myelogenous leukemia (AML) than the general population (Auerbach and Allen, 1991). Leukemia develops extremely early in FA patients and is highly lethal. The average age of death for these children is age 15 (Auerbach and Allen, 1991). In addition to the risk of developing AML, FA patients are also extremely susceptible to the formation of solid tumors. FA patients are 50 times more likely than the general population to develop tumors (Rosenberg et al., 2003). By age 48, 1 in 3 FA patients are expected to develop a solid tumor (Rosenberg et al., 2003). FA patients are also more 700 times more likely to develop squamous cell carcinoma,

predominantly in the head and neck (Singh, 2003). FA patients are also extremely sensitive to numerous other types of cancer which can eventually lead to their death.

The FA Pathway

Fanconi Anemia is a disease characterized by widespread chromosomal instability which results from the improper repair of DNA interstrand crosslinks (ICL) (Niedernhofer et al., 2005). ICLs are covalent links that form between the two complementary strands of DNA, and can inhibit the formation of replication forks. Since the DNA is unable to unwind, both replication and transcription are effectively blocked (Moldovan and D'Andrea, 2010). ICLs are highly lethal, and crosslinking agents such as Mitomycin C and Cisplatin are commonly used as chemotherapy drugs. Normally, ICLs are repaired through a complex response known as the Fanconi Anemia pathway. This pathway is able to remove ICLs and reinitiate DNA replication. In FA patients, this process is deficient due to mutations in the Fanconi Anemia genes. FA cells are unable to correctly clear ICLs which results in the characteristic genomic instability of the disease. Often, the DNA becomes broken or fragmented due to improper repair, or large radial structures comprising multiple chromosomes are formed (Moldovan and D'Andrea, 2010).

Interstrand crosslink repair is a highly complex process that requires multiple repair mechanisms including nucleotide excision repair (NER), translesion synthesis (TLS), and homologous recombination (HR). While most DNA lesions can be processed through multiple damage pathways, ICLs can only be repaired through the function of the FA pathway. The pathway currently contains at least fifteen known FA complementation

groups which are named FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N, P, and RAD51C (Kottemann and Smogorzewska1, 2013). The majority of these genes comprise the FA core complex. In response to DNA lesions that occur during replication, the core complex monoubiquitinates FANCD2 and FANCI which then localize to sites of DNA damage and help initiate the proper response to ICLs (Montes de Oca et al., 2005).

The most commonly mutated gene in Fanconi Anemia patients is FANCA which is responsible for 60% of all cases. It is a member of the core complex along with FANCB, C, E, F, G, L, and M (Moldovan and D'Andrea, 2010.) One function of the core complex is to monoubiquitinate FANCD2 and FANCI, and initiate the FA pathway (**Figure-2**). FANCD2 is mutated in roughly 5% of FA patients, yet it is used as the primary diagnostic tool for the disease. Since FANCD2 is relatively far downstream in the FA damage response pathway, mutations in any of the upstream FA proteins will prevent its monoubiquitination. This can be clinically measured and used to accurately diagnose an FA patient (Moldovan and D'Andrea, 2010).

As previously mentioned, interstrand crosslinks cannot be repaired by any single DNA repair method. When a DNA replication fork encounters a crosslink, helicases are unable to separate the two strands of DNA. This stalls the replication fork and repair factors are recruited to the lesion. The first response is the induction of a double strand break (DSB) (Niedernhofer et al., 2005). Once a DSB is induced on both sides of the lesion, the cross-linked base can be removed and bypassed via a TLS mechanism. This allows the replication fork to resume its progress along the DNA. Homologous recombination is then used restore the DNA to its original structure. FANCD2 is believed to hold an important but poorly understood role in mediating HR. Fancd2 has been shown

to colocalize with an important HR protein, RAD51, at sights of DNA DSBs (Nakanishi et al., 2005). In FA patients, the loss of FANCD2 activity appears to promote non-homologous end joining (NHEJ). This method does not require a DNA template to repair DSBs, but is highly inaccurate and produces frequent mutations. NHEJ is also responsible for the formation of radial chromosomes, a hallmark of cells deficient in the FA pathway. This has led to the conclusion that one of the most vital roles of the FA pathway is to promote HR instead of less accurate mechanisms of repair (Naim and Rosselli, 2009).

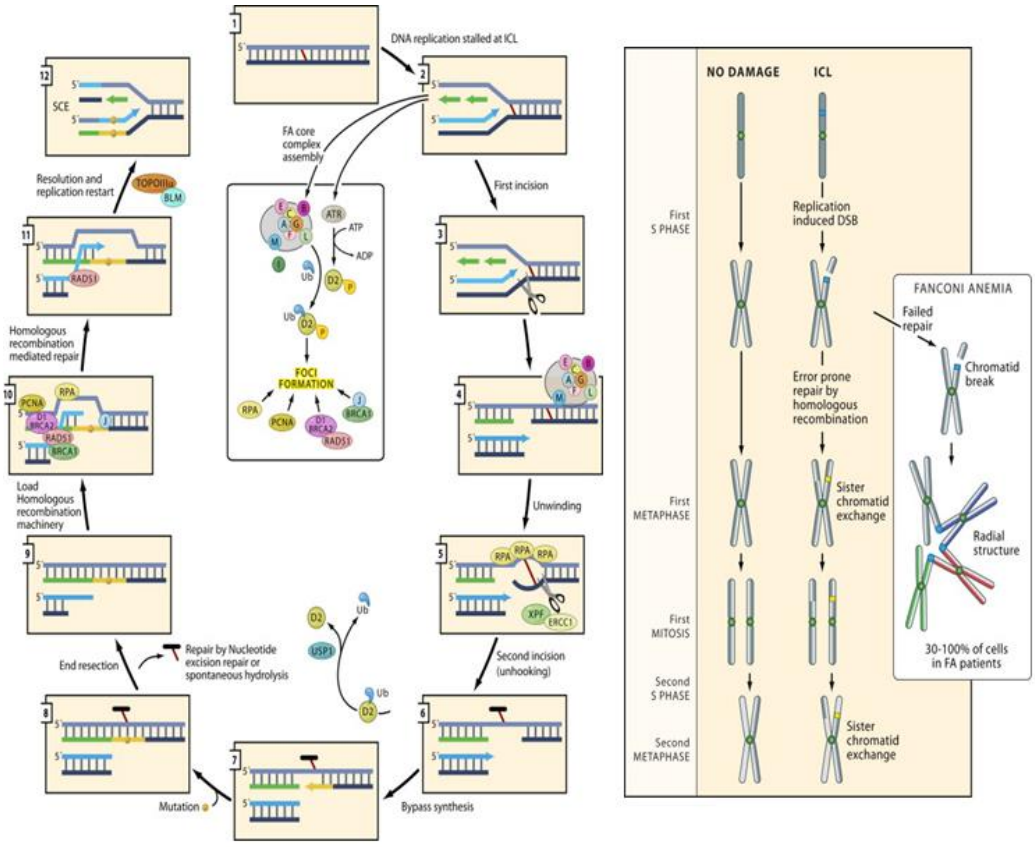

Figure 2: Repair of ICLs Through the FA Pathway and the Formation of Radial Chromosomes. When a replication fork stalls due to an ICL, the FA core complex assembles and triggers the monoubiquitination of FANCD2 with the assistance of ATR. FANCD2 then localizes to sites of damage and initiates the proper damage response. In FA deficient cells, improper repair can result in the formation of Radial chromosomes (Niedernhofer, 2005).

Once the FANCD2-I complex has been monoubiquitinated, the specific mechanism by which it initiates repair is unknown. The complex has been shown to colocalize with vital repair proteins such as BRCA2, RAD51, and PCNA (Moldovan and D'Andrea, 2010). One theory states that ubiquitinated FANCD2 may recruit other molecules which help localize repair factors to sites of DNA damage. It is also possible that FANCD2-I may recruit essential factors for specific repair methods such as TLS. The FANCD2-I complex may also serve to stabilize repair enzymes on DNA. Whatever the mechanism, FANCD2-I localization to ICLs is crucial in initiating the proper repair cascade (Moldovan and D'Andrea, 2010).

ATR is a crucial upstream regulator of the FA pathway and is highly important in coordinating the DNA damage response during replication (Moldovan and D'Andrea, 2010). ATR is an essential component of an S-phase checkpoint that recognizes stalled replication forks, and initiates the proper response to promote fork restart and reinitialize the cell cycle (**Figure-3**) (Friedel et al., 2009). It is believed that the ATR checkpoint kinase is required to initiate FANCD2 monoubiquitination and promote the FA pathway of DNA repair (Andreassen et al., 2004). In ATR-deficient cells, MMC triggers the formation of radial chromosomes, indicating that ATR is essential for activating the FA pathway in response to crosslinks (Adreassen et al., 2004). FANCD2 has also been shown to interact with the checkpoint kinase ATM which arrests replication in response to damage. ATM phosphorylates FANCD2 which is required for proper checkpoint function (Taniguchi et al., 2002).

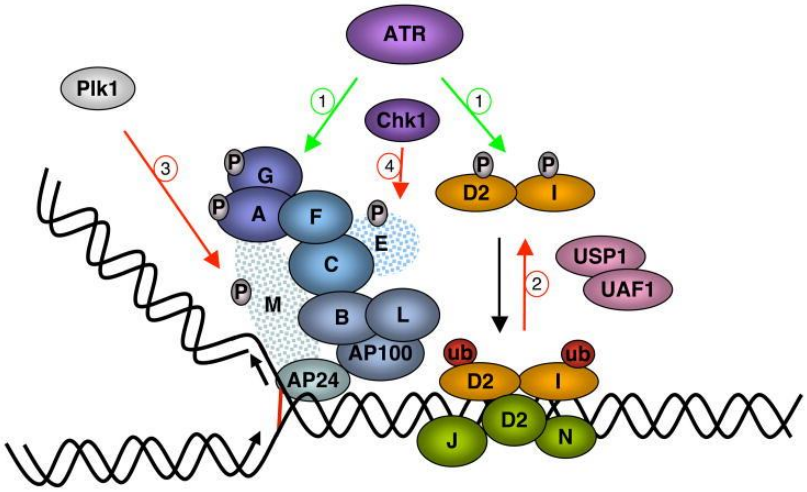

Figure 3: ATR Checkpoint Kinase Initiates the FA Pathway. In response to a stalled replication fork resulting from an ICL, ATR initiates the formation of the FA core complex and phosphorylates the FANCD2-I complex. This leads to the monoubiquitination of FANCD2-I which allows it to localize to damage and regulate the proper repair response (Maldovan and D'Andrea, 2010.)

Msh2 and DNA Mismatch Repair

DNA mismatch repair (MMR) is responsible for correcting base substitutions, insertions, and deletions that occur during DNA replication (Kunkel and Erie, 2005). Mutations occur during DNA synthesis due to nucleotide misincorporation which results in differences between the two complementary strands of DNA. These mismatches are rare and occur in only 1 in 10^{10} base pairs, yet they must be addressed to preserve genomic integrity and to prevent future replication issues (Iyer et al., 2006). Unrepaired mismatches can result in frame-shift mutations, and defects in the MMR repair system have been shown to result in cancer and tumor development (Iyer et al., 2006).

The MMR pathway is initiated when MutS homologue protein complexes recognize and localize to mispaired bases in the DNA. One vital MutS homologue is MSH2 which interacts with MLH1 and other MSH proteins at sights of base mispairs, insertions, and deletions (**Figure-4**) (Kolodner and Marsischky, 1999). Single base-base

mismatches are recognized by a MSH2/MSH6 complex known as MutSα. In addition, a MSH2/MSH3 complex, MutSβ, is responsible for recognizing insertion and deletion mismatches that contain extra nucleotides. Localization of the Mut complex at a mismatch results in the recruitment of additional repair proteins and the excision of the incorrect nucleotide. The DNA is then resynthesized by DNA polymerase δ (Larrea et al., 2010).

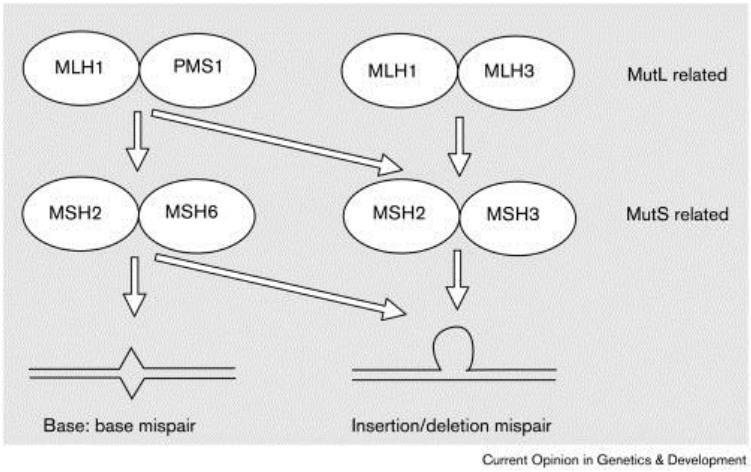

Figure 4: Msh2 is Involved in the Initiation of the MMR Pathway. MSH2 complexes with MSH6 and MSH3 to form MutSα and MutSβ, respectively. These complexes are responsible for identifying base mismatches, insertions, and deletions and triggering the MMR response (Kolodner and Marsischky, 1999).

The MMR repair pathway has been shown to interact with the FA pathway and to play a role in ATM/ATR mediated checkpoint arrest (Meyers et al., 2003). MMR repair proteins, such as MSH2, respond to an ICL and induce DSBs (Zhang et al., 2007). In addition, Fancd2 is known to interact with the MMR proteins MSH2 and MLH1 (Williams et al., 2011). While the importance of this interaction is currently unknown, it is possible the MMR plays a significant role in ICL response and the FA pathway. In FA deficient cells, it is also possible that MMR repair drives chromosomal instability. MMR

proteins could promote error-prone, unproductive ICL processing pathways. In addition, MMR repair proteins could induce DSBs which are incorrectly repaired by NHEJ to create radial chromosomes.

PROJECT PURPOSE

Cells deficient in the Fanconi Anemia pathway are characterized by chromosomal instability and the formation of radial chromosomes, yet the mechanism that drives the formation of these structures is poorly understood. Mismatch repair proteins such as Msh2 have been shown to respond to interstrand crosslinks and may promote inefficient and error prone repair methods such as non-homologous end joining. This project aimed to address whether Msh2 contributes to the formation of radial chromosomes in a Fancd2 deficient background. In addition, the project focused on how Msh2 depletion affects cell survival and checkpoint activity in response to the crosslinking agent Mitomycin C.

METHODS

Cell Culture

Primary mouse embryonic fibroblasts (MEFs) were prepared from pregnant mice at between 13 and 14 days of gestation, and trypsinized with 0.25% trypsin-EDTA for 15 minutes at 37°C. Medium was added to the tube, and the tissue was minced via pipetting. MEF cells were then seeded onto 75cm^2 flasks with 1 million cells per flask. After 24 hours, cells were washed with 1X PBS and fed new medium. MEFs were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Immunoflourescence Microscopy

MEFs were seeded onto 6 well plates and incubated overnight. Cells were either untreated or treated with Mitomycin C (MMC) (250 nM) for 16 h. MEFs were then washed twice in 1X PBS and fixed with 3% paraformaldehyde/2% sucrose for 10 min at RT. After two more washes, cells were permeabilized with 0.5% Triton X-100 in 20 mM HEPES for 5 min on ice. Cells were washed thoroughly before incubation with primary and secondary antibodies. Incubation was performed with antibodies: γ-H2AX (Upstate 1:100), 53BP1 (Novus Biologicals, 1:100) or P-ATR/ATM Substrate (Cell Signaling, 1:100). Secondary antibodies were Rhodamine Red Anti-Mouse IgG (Jackson Immunoresearch, 1:200) and Fluorescein (FITC) Anti-Rabbit IgG (Jackson Immunoresearch, 1:200). Cells with greater than 10 foci per cell were scored as positive.

Survival Assays

10,000 MEFs were seeded in 6 well plates. Cells were then treated with MMC for 1 hour at dosages of 25, 50, 100, and 200 nM, and protected from light. MMC was then removed, and the cells were allowed to relax for 5 days. Cells were quantified by hand counting under a microscope.

Generation of Mice and PCR Genotyping

Msh2-deficient mice were obtained from T. Mak, University of Toronto, Toronto CA. Fancd2-deficient mice 74 were obtained from M. Grompe, Oregon Health and Sciences University, Portland OR. Mice were maintained as double heterozygotes (Msh2+/- Fancd2+/-) and were bred to obtain all genotypes studied here. Mice were housed in the same room of the IACUC-approved SPF facility at Univ. Mass. Medical School, and were bred and used under guidelines formulated by the Univ. of Massachusetts Animal Care and Use Committee (IACUC). As previously described (Reitmair et al., 1996) 50 ng of DNA was prepared from an embryo's head or mom's tail and used as a template in PCR to genotype mice. For Fancd2, forward primer MG968 (5 –TCAGCCTCACATGGAGTT -TAACG -3) and two reverse primers, MG1280 (5 – GCTACACAGCATTGCCCATA -AAG-3 and MG1008 (5 – CAGGGATGAAAGGGTCTTAC -GC-3 were used to amplify a mutant band of 459 bp or a wild-type band of 303 bp. The reaction conditions were 95C for 2 min; 36 cycles of 94°C for 25 sec, 58°C for 25 sec, and 72°C for 35 sec; and a final extension at 72°C for 2 min. For Msh2, forward primer Msh2 COM (5 –AAAGTGCACGTCATTTGGA -3) and two reverse primers, Msh2 WT (5 –GCTCACTTAGACGCCATTGT-3 and Msh2 MT (5

–GCCTTCTTGACGAGTTCTTC-3 were used to amplify a wild-type band of 174 bp or a mutant band of 460 bp. The reaction conditions were 95C for 2 min; 36 cycles of 94C for 30sec, 62C for 30 sec, and 72C for 30 sec; and a final extension at 72C for 7 min.

Metaphase Chromosome Spreads

Stable MEFs were seeded, mock treated, or treated with MMC and incubated in media containing 100 ng/ml Colcemid for 1.5 h. Cells were then harvested by trypsinization, incubated in hypotonic solution for 10 min and fixed with fixative solution (75% Methanol, 25% Acetic Acid). Cells were then spotted onto slides and stained with Giemsa. Slides were then sealed using a second slide and nail polish. Chromosome abnormalities (**Figure-5**) were scored using blind counts (**Figure-6**) based on standard guidelines (Levitt et al., 2007).

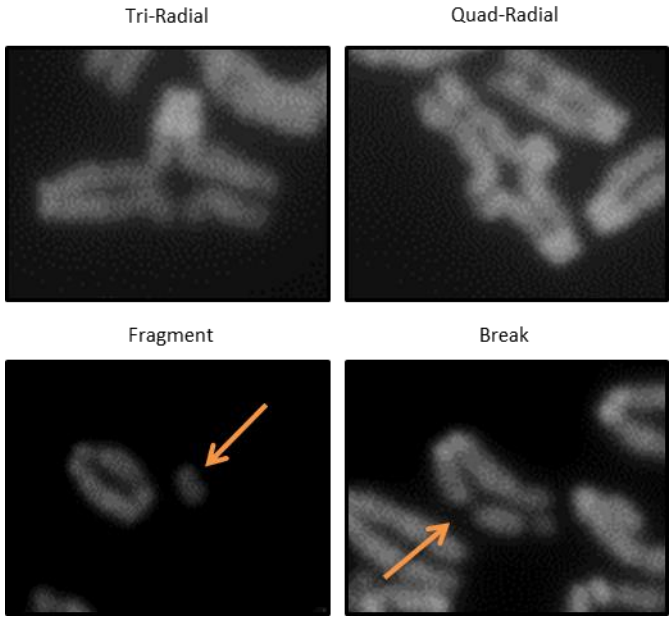

Figure 5: Classifications of Chromosomal Aberrations. Aberrations were classified into three major subgroups (radials, fragments, and breaks) according to standard guidelines.

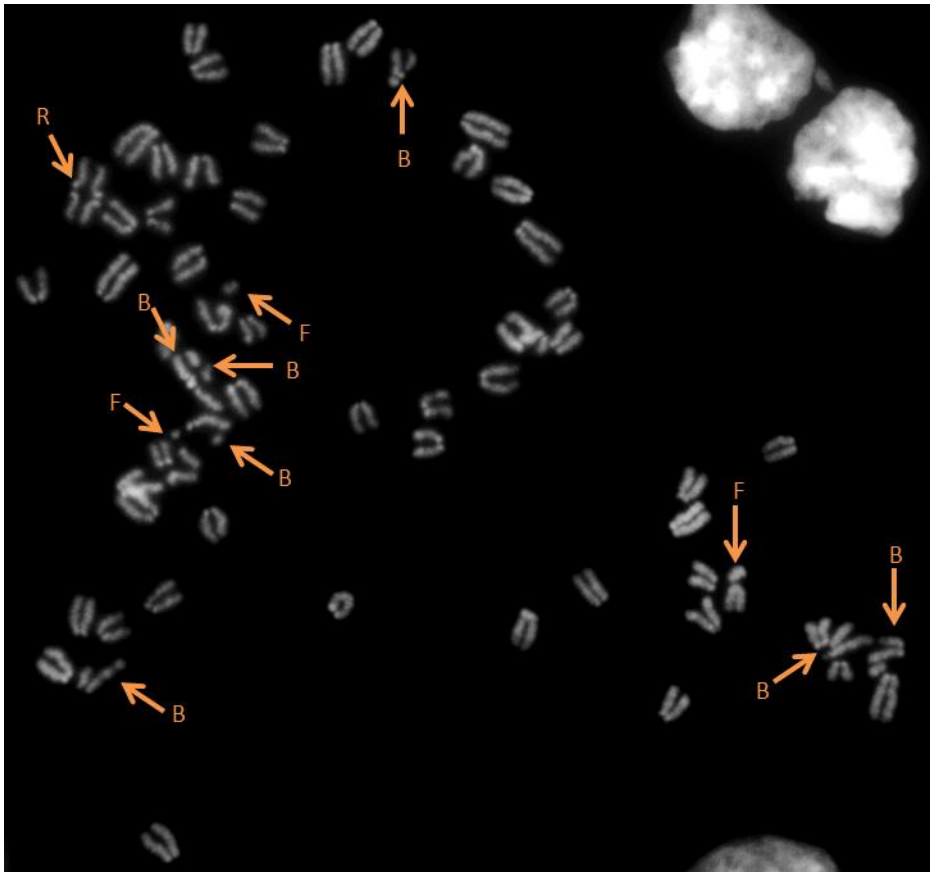

Figure 6: Spread Count Example. Spreads were observed and quantified by eye at 200x. This spread depicts 7 breaks (B), 3 fragments (F), and 1 radial (R).

RESULTS

The Fanconi Anemia pathway is a complex DNA damage response which combines translesion synthesis and homologous recombination to address interstrand crosslinks (ICLs). If untreated, these crosslinks will trigger stalled replication forks that inhibit DNA synthesis. Mutations to any of the FA proteins, such as Fancd2, result in widespread chromosomal instability and the formation of radial chromosome structures. The purpose of this project was to address the role of the DNA mismatch repair pathway in promoting error-prone and ineffective repair methods in response to ICLs. This project focused on one MMR gene, Msh2, which is important for identifying mismatches, insertions, and deletions and initiating mismatch repair.

To test whether Msh2 contributes to an abnormal DNA damage response, Msh2 was depleted in Fancd2-null MEFs. This was done by breeding two mice that were heterozygous for both Msh2 and Fancd2. The embryos were harvested 13 to 14 days after conception, and PCR was used to obtain their genotypes. Three genotypes were used in this study (**Figure-7**): WT (Fancd2+/+, Msh2+/+), Knockout (Fancd2-/-, Msh2+/+), and Double knockout (Fancd2-/-, Msh2-/-).

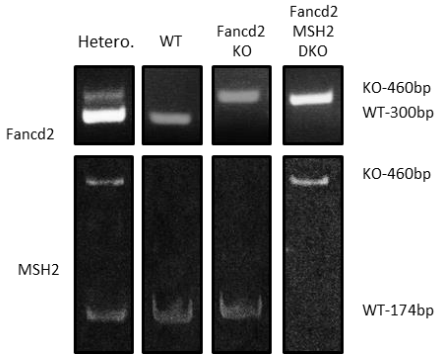

Figure 7: Genotypes of Mouse Embryonic Fibroblasts. The heterozygous parent (Lane-1) shows two bands for both Fancd2 and Msh2. Homozygous dominant individuals (WT) (Lane-2) exhibit a single band for Fancd2, and Msh2 of 300 bp and 174 bp, respectively. Fancd2 deficient individuals (Lane-3) demonstrate bands at 460bp for Fancd2 and 174bp for Msh2. Fancd2/Msh2 recessive individuals (Lane-4) demonstrate bands of 460 bp for both Fancd2 and Mss2.

Mitomycin C (MMC) is a chemotherapeutic drug that is extremely effective at producing ICLs. FA-deficient cells cannot process crosslinks, and demonstrate extreme sensitivity to MMC. WT cells are able to respond to ICLs through the FA pathway and show reduced genomic stress. To assess how Msh2 depletion affects the Fancd2-deficient cells, MEFs were subjected to a survival assay. MEFs were seeded at equal concentrations in 6 well plates and subjected four treatments of MMC (25, 50, 100, and 200 nM) for 1 hour. Cells were then allowed to relax for five days, and surviving cells were counted under the microscope (**Figure-8**).

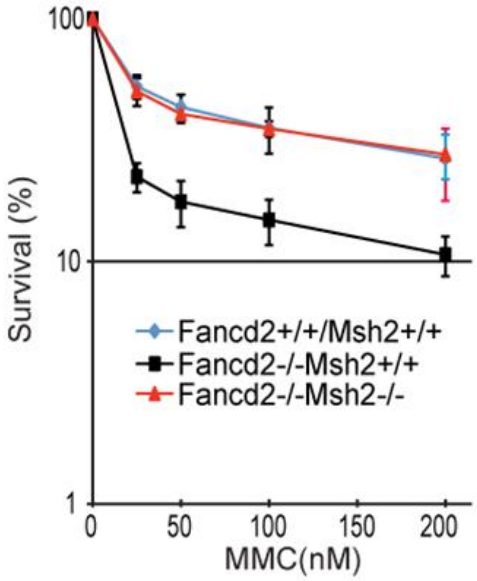

Figure 8: Msh2 Depletion Reduces Mitomycin C Sensitivity in Fancd2-Deficient MEFs. Cells were treated for 1 hour with MMC and allowed to relax for 5 days. Fancd2 deficient cells demonstrated severe sensitivity relative to WT. When Msh2 was depleted in addition to Fancd2, sensitivity was suppressed and cells showed survival rates similar to WT.

In response to MMC treatment, Fancd2-deficient MEFs (black curve) demonstrated severe sensitivity in relation to both the WT and the Fancd2/Msh2-null MEFs. At all dosages, Fancd2-deficient cells were highly susceptible to the drug, and showed poor survival rates. In contrast, the Fancd2/Msh-null cells (red curve) showed

similar survival rates to the WT cells (blue curve). Both of these cell lines demonstrated a survival rate 3 times greater than the Fancd2-deficient cells.

To further investigate this result, chromosome spreads were used to quantify chromosomal aberrations (**Figure-9**). To perform the spreads, cells were treated with MMC and incubated in media containing 100 ng/ml Colcemid for 1.5 h. Cells were then harvested by trypsinization, incubated in hypotonic solution for 10 min and fixed with fixative solution (75% Methanol, 25% Acetic Acid) before being dyed and applied drop-wise to microscope slides. Chromosomal aberrations were then counted by eye under the microscope, and recorded as a percentage based on the number of aberrations per total chromosomes. Spreads were counted in a blind manner so that bias would not be introduced.

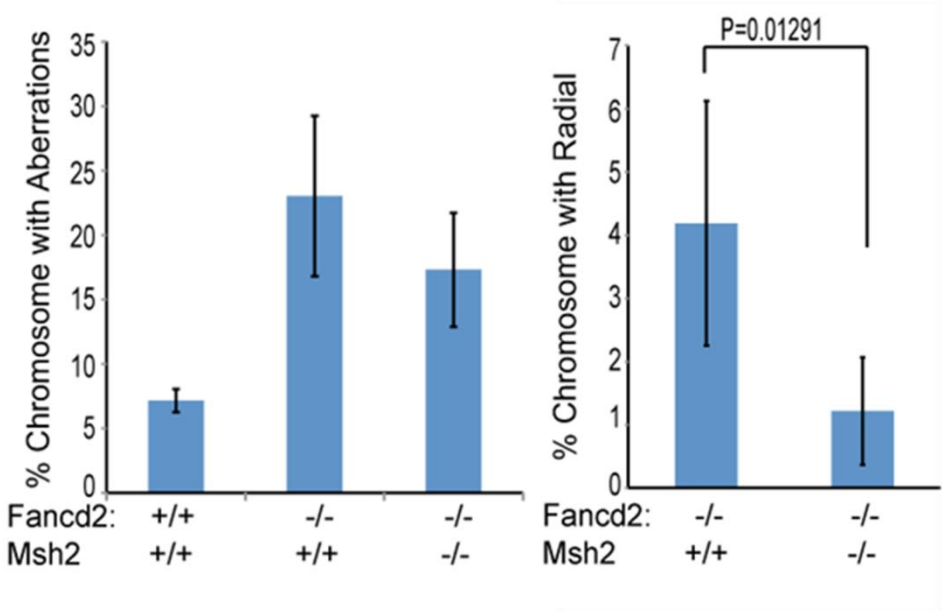

Figure 9: Fancd2/Msh2 Deficient MEFs Show Fewer Radial Chromosomes Than Fancd2 Deficient MEFs. After treatment with MMC, both KO and DKO MEFs demonstrated a higher frequency of chromosomal aberrations than WT cells. No significant difference was observed between KO and DKO cells in total aberrations. When aberrations were classified by type however, Fancd2/Msh2 deficient cells were found to have fewer radial chromosomes than Fancd2 deficient cells.

The initial set of spread counts (left panel) found that both the Fancd2-null and Fancd2/Msh2-null MEFs demonstrated significantly more chromosomal aberrations than the wild-type cells. The first set of counts found WT to have 7.2% aberrations, Fancd2 KO to have 23% aberrations, and Fancd2/Msh2 DKO to have 17.3% aberrations. Unlike the survival assay, the Fancd2/Msh2 deficient MEFs did not show a significant difference from the Fancd2-null MEFs in terms of total chromosomal aberrations. To further investigate, a second round of spread counts was employed. During this count, each aberration was classified by type using standard guidelines. Fancd2/Msh2 deficient cells were found to have significantly less radial chromosomes with only 1.22% compared to 4.19% in Fancd2 deficient cells (right panel). **Figure-10** shows a comparison of aberrations classified by type for knockout and double knock out MEFs.

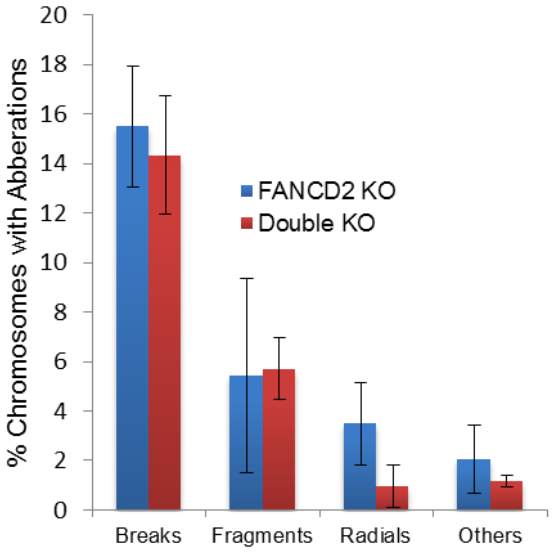

Figure 10: Prevalence of Chromosomal Aberrations by Type. Fancd2 KO (blue) and Fancd2/Msh2 DKO (red) MEFs exhibit similar percentages of breaks and fragments. The DKO genotype shows a reduction in the number of radials.

The survival assay showed that the presence of Msh2 in Fancd2 deficient MEFs severely sensitizes the cells in response to MMC, and that depleting Msh2 in Fancd2-deficient cells mitigates the sensitivity. In addition, the spread data showed that the

presence of Msh2 helped alleviate the formation of radial chromosomes, although the difference in total aberrations was not statistically significant. Given these findings, we suspected that the high mortality rates of Fancd2 MEFs could be connected to checkpoint response which leads to stalled replication and apoptosis.

ATM and ATR are kinases responsible for monitoring the FA pathway, and can halt replication in response to damage. In addition, ATR is responsible for recognizing stalled replication forks. γ-H2AX plays a role in DNA repair and is commonly used as an identifier of DSBs. To determine whether Msh2 reduces the checkpoint response in Fancd2-null cells, immunofluorescence microscopy was used to assay the levels of ATR/ATM and γ-H2AX in MMC-treated cells (**Figure-11**). Cells were seeded in 6-well plates, incubated overnight, treated with 250 nM MMC for 16 h, and then stained with antibodies to both γ-H2AX and ATM/ATR substrates.

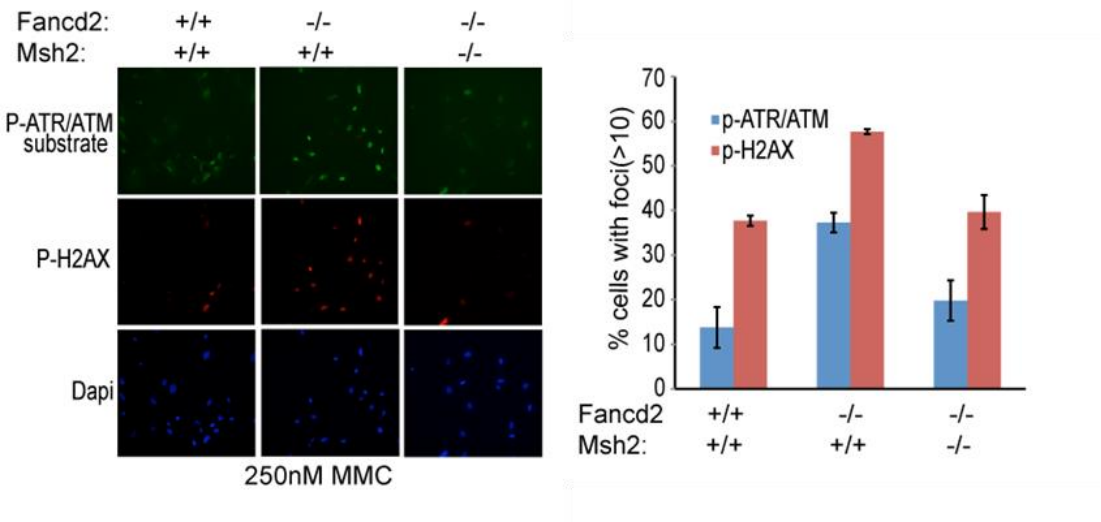

Figure 11: MMC Induces a Greater Damage Response in Fancd2-Deficient MEFs than in WT or Fancd2/Msh2-Double Deficient MEFs. Fancd2-null MEFs exhibit an elevated checkpoint response (ATM/ATR substrate) when treated with MMC. They also show elevated p-H2AX levels, indicative of DNA damage. Fancd2/Msh22-null MEFs demonstrate similar damage responses to WT cells. Cells containing greater than 10 foci were considered positive and are quantified on the right.

Phosphorylated H2AX is an indicator of DNA DSBs, and demonstrates the levels of DNA damage in each of the cells. p-γH2AX was elevated in Fancd2-null cells, but depletion of Msh2 mitigates this effect as p-γH2AX levels were similar to WT in Fancd2/Msh2-null cells. An overactive damage response is also indicated by P-ATM/ATR substrate antibody. This antibody binds to substrates that would typically trigger ATM or ATR activity. Fancd2-null cells show a two-fold increase in checkpoint activity over WT and Fancd2/Msh2 cells. This is indicative of an increase in stalled replication forks and DNA damage.

An additional immunofluorescence assay was performed without inducing ICLs to view general genomic stress (**Figure-12**). 53BP1 foci were found to be nearly 2 times more prevalent in Fancd2-deficient cells than in WT or Fancd2/Msh2-deficient cells. 53BP1 is an indicator of DNA lesions resulting from replication. In addition, p-γH2AX appears to be expressed at slightly higher levels in Fancd2-deficient cells than in the other two cell lines.

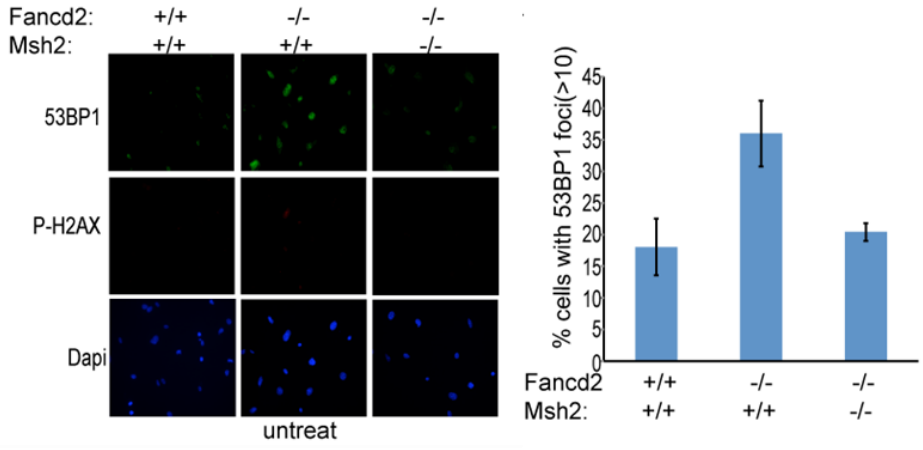

Figure 12: Msh2/Fancd2-Deficient MEFs Demonstrate Reduced Genomic Stress. 53BP1 foci are indicative of lesions resulting from issues during replication. Fand2-deficient MEFs showed increased 53BP1 foci when compared to WT. MSH2/Fancd2 deficient cells exhibit foci levels similar to WT cells. Fancd2-null cells also show an increase in p-γH2AX, a marker of DNA damage. Cells containing greater than 10 foci were considered positive and are quantified on the right.

DISCUSSION

The purpose of this project was to determine whether Msh2 function helps contribute to the formation of radial chromosomes in a Fancd2-deficient background. In addition, the project focused on how Msh2 deletion affects cell survival and checkpoint activity in response to the crosslinking agent MMC. The data obtained provide evidence that Msh2 deletion in Fancd2 deficient cells reduces the formation of radial chromosomes by a factor of four when compared to Fancd2 null cells. In WT cells, chromosomal aberrations are infrequent, and radials are rare. Normally, the FA pathway regulates homologous recombination to repair DSBs that occur during interstrand crosslink (ICL) repair. These findings suggest that when the FA pathway is deficient, the MMR pathway may respond to ICLs and promote ineffective and error-prone methods of repair such as NHEJ. Based on this, we suggest that the FA pathway could serve as an inhibitor of MMR. Thus, in the absence of the FA pathway MMR may contribute to chromosomal instability in FA-deficient cells.

Msh2 deletion in Fancd2 null cells was also shown to desensitize cells to MMC. Fancd2-null cells demonstrate significantly lower survival rates than either WT or Fancd2/Msh2-null cells. This correlates with the data collected from the spread counts, as double knockout cells should be more stable due to reduced NHEJ and radial chromosome formation. In addition, checkpoint response was found to be elevated in Fancd2 null cells, but reduced with the co-depletion of Msh2. The increased ATM/ATR checkpoint response was most likely due to high levels of damage resulting from unrestricted MMR activity. This checkpoint response was also a contributing factor behind the MMC sensitivity during the survival assay. P-γH2AX levels were also

elevated in Fancd2 null cells. This suggests an increase in DSBs which result from improper damage response to ICLs. Finally, 53BP1 activity was found to be elevated in Fancd2-null cells but was observed as compare to levels in Fancd2/Msh2-deficient cells. This finding is consistent with other findings, since 53BP1 is associated with genomic stress and instability.

Future research should further expand on these findings and continue to elaborate upon the results shown here. Previous research has shown that depletion of NHEJ alone is not sufficient to prevent the formation of radial chromosomes, and that the genome is even more unstable in its absence. On the other hand, our findings reveal the importance of the MMR pathway in triggering the formation of radials. Yet the mechanisms behind this are not well understood, and Msh2 and Fancd2 are only two components in the MMR and FA pathways. To further verify this result and determine the mechanism by which the FA and MMR pathways interact, deficiencies in other proteins in each pathway should be tested. Other FA genes could be depleted to see if this result remains consistent.

Although the initial experiments in this project focused on counting total aberrations/chromosome, this data was inconclusive and the counts were refined to quantify aberrations by type. In future experiments, this procedure could be further modified to count only radial chromosomes. This will greatly increase the efficiency of spread counts and allow for more data to be collected. Ultimately, faster spread counts should make it possible to increase the sample size of the radial counts to ensure an accurate result.

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