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Repair of DNA double strand breaks and radiosensitivity: modulation of DNA repair and radiosensitivity by microRNA-335 and mtPAP

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Repair of DNA double strand breaks and radiosensitivity: modulation of DNA repair and radiosensitivity by microRNA-335 and mtPAP

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biomedical Physics

by

Nathan Thomas Martin

2014

ABSTRACT OF THE DISSERTATION

Repair of DNA double strand breaks and radiosensitivity:

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Professor Richard A. Gatti, Chair

Biologic responses to ionizing radiation are complex, and numerous cellular signaling cascades are activated with specific temporal kinetics upon exposure. Induction of DNA lesions, especially DNA double strand breaks (DSBs), are thought to be the main mechanism by which ionizing radiation kills cells and the rapid recognition and accurate repair of DSBs is a central determinant of cell survival after irradiation. DNA DSB repair is a complex and coordinated process involving numerous proteins (possibly >1000) that recognize double strand breaks, transmit the damage signals downstream, modify chromatin structure, and localize at break sites leading to the repair of breaks and the maintenance of genomic stability. Deficiencies in DNA DSB repair can cause cellular sensitivity to ionizing radiation or oncogenic transformation. Chapter 1 of this

thesis provides an introduction to the effects of ionizing radiation on cells and the chorography of DNA repair mechanisms.

Individuals with genomic instability have been described who are deficient for specific proteins involved in double strand break repair and these patients often develop malignancies at an early age. Due to an inability to adequately repair their DNA in a timely manner, these patients respond severely to radiotherapy and other cytotoxic therapies used to treat cancer. These patients are described under an umbrella syndrome characterized by x-ray sensitivity, cancer predisposition, immunodeficiency, neurologic involvement, and DNA double strand break repair defects (XCIND). Our laboratory has developed radiosensitivity testing in an effort to diagnose these patients and, thereby, avoid severe, and often lethal, reactions to radiotherapy. Radiosensitivity is determined by measuring clonogenic survival in colony forming cell lines derived from lymphocytes isolated from whole blood samples, and a radiosensitive range has been defined by studying numerous radionormal cell lines and radiosensitive cell lines derived from patients with ataxia-telangiectasia, the archetypal radiosensitivity disorder. Chapter 2 summarizes the human radiosensitivity disorders discovered to date and the current state of the field for clinical radiosensitivity testing.

Current radiosensitivity testing takes approximately 90 days to complete because establishment of a colony forming cell line is required. This relatively long turn around is not optimal for patients needing timely radiotherapy intervention for malignancies. Additionally, approximately 5-10% of routine radiotherapy patients have severe reactions to radiotherapy and would benefit from radiation sensitivity screening prior to treatment. Chapter 3 explores two promising assays: 1) post-irradiation measurement of gamma-

H2AX foci kinetics and 2) the neutral comet assay, as potential rapid surrogates for the clonogenic survival assay. I confirmed that the neutral comet assay (NCA) is the most promising surrogate assay for the clonogenic survival assay and have adapted the methodology to assay whole blood samples from patients submitted for radiosensitivity testing. This represents a test that could be completed in 2-3 days which is within the timeframe needed for use in the oncology clinic or for suspected XCIND patients requiring faster results.

The gamma-H2AX foci kinetics assay tested in Chapter 3 was not predictive of clonogenic survival, but did identify DNA repair kinetics in a radiosensitive cell line of unknown etiology that were similar to a radiosensitive cell line of known etiology, RNF168 deficiency. I postulated that the radiosensitive cell line of unknown etiology, RS73, had a defect in the same DNA repair pathway as the RNF168 deficient cell line because of their similar kinetics. This 'candidate pathway' approach led me to profile 53BP1 and BRCA1 foci kinetics, and I ultimately found a defect in the formation or retention of BRCA1 foci in RS73. However, this approach fell short of identifying the underlying genetic defect responsible for the BRCA1 foci defect and radiosensitivity observed in this cell line due to incomplete knowledge of the BRCA1/53BP1 signaling pathway, at the time of study.

This follow-up study of RS73 was an example of how the 'candidate pathway' approach can be hindered by limited current knowledge of specific DNA DSB recognition and repair signaling mechanisms. Further, the 'candidate pathway' approach is biased towards identifying defects in known DNA repair genes because the assays used for discovery are designed to test only those DDR mechanisms that are already

characterized. Thus, in Chapters 4 and 5, unbiased genome wide methodologies were utilized to profile a panel of radiosensitive cell lines of unknown etiology to identify novel molecules involved in DNA repair and the radiation response; a 'candidate gene' approach.

The study presented in Chapter 4 utilized a microRNA microarrays to profile the radiation response of ~1200 microRNA to identify those associated with radiosensitivity. MicroRNA are small, non-coding RNA which can preferentially target specific mRNA through complementary sequences and regulate protein expression by inhibiting translation of these target mRNA. MiR-335 was selected for follow-up study because it was regulated in an ATM dependent manner after irradiation, which suggested it may play a role in the DDR. Interestingly, miR-335 was also overexpressed in two radiosensitive cell lines of unknown etiology, RS7 and RS73, which further suggested a link to radiosensitivity and the DDR. I demonstrated that miR-335 modulates the DDR by targeting CtIP protein levels, a protein involved in end resection and cell checkpoint signaling, leading to disrupted BRCA1 focus formation and radiosensitization. miR-335 was not an obvious candidate for modulating the radiation response and provides an example for using an unbiased, 'candidate gene', approach to identify novel molecules and mechanisms in the DDR and in human radiosensitivity.

In Chapter 5, I describe another unbiased 'candidate gene' approach which utilized exome sequencing to associate a mutation in the mitochondrial poly-A-polymerase, *MTPAP,* with radiosensitivity in two siblings from an Amish family. *MTPAP* was also not an obvious candidate for radiosensitization and was one of many variants identified in the patients studied. I demonstrated a causal link between *MTPAP*

and radiosensitivity by rescuing the radiosensitivity and DNA DSB repair defects observed in the two siblings, RS63-3 and RS63-7, by transfecting WT *MTPAP* into the patients' cells. Further profiling of the radiation response in these cells indicated that a cellular state of oxidative stress, likely induced by mitochondrial dysfunction, resulted in increased DSBs/Gy induction and increased cell death. Reduced clonogenic survival and the DSB repair defect were abrogated by pre-treating the patients' cells with antioxidants, further indicating that oxidative stress played an important role in the radiosensitive cellular phenotype of these patients. The data presented in Chapter 5 suggested that RS63-3 and RS63-7 are an atypical presentation of XCIND and indicated that the current working model of radiosensitivity may need to be broadened to include genes outside of those directly related to recognizing and repairing DSBs (i.e. classical XCIND presentation).

Chapter 6 summarizes this thesis and presents my concluding thoughts in the context of DNA repair mechanisms, radiosensitivity testing for XCIND and routine oncology patients, and implications for the current working model for human radiosensitivity. I have shown that the neutral comet assay can be rapidly performed on whole blood samples and is an attractive option for radiosensitivity testing in the oncology clinic. The remaining hurdles for translating a functional radiosensitivity assay package to the clinic are discussed and approaches for addressing these hurdles are explored. Unbiased 'candidate gene' approaches vs. 'candidate pathway' approaches for identifying novel DDR modulating molecules are discussed in the context of the miR-335 overexpressing and *MTPAP* mutated cells, and molecular tools for following-up candidate gene studies are highlighted. The current working model for XCIND patients

assumes that human radiosensitivity stems from defects in DSB recognition or repair genes. The data presented in Chapter 4 and 5 indicate that the current working model for radiosensitization likely needs to be broadened, or a separate category of radiosensitivity patients created, for developing a working model that better describes the spectrum of patients. Chapter 6 discusses the importance and implications of these data for our understanding of the DDR in humans and in the context of the immunologic and neurologic phenotypes observed in many XCIND patients.

The dissertation of Nathan Thomas Martin is approved.

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2014

DEDICATION

This dissertation is dedicated to: my loving and supportive wife, Ciara, who challenges me to think outside the box and always keeps a smile on my face; my supportive family, Chris, Pete, and Suzanne, who have always encouraged and supported me to reach further; my maternal grandma who encouraged my inquisitive nature, helped with backyard ‘experiments’, and taught me about smart risk taking; my paternal grandparents for teaching me this is all just a dumb game, and that I might as well have some fun while I’m here; and my Uncle Dick for showing me that reward only comes after hard work.

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BIOGRAPHICAL SKETCH

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PUBLICATIONS

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* Indicates authors contributed equally to the manuscript.

ACCEPTED CONFERENCE ABSTRACTS

1. **Martin NT,** Kotoka Nakamura, Jennifer Woo, Christina Brown, Brandi Woo, Hailiang Hu, Patrick J. Concannon, Richard A. Gatti. ATW 2013, Birmingham, England. July 2013. Poster.

2. **Martin NT,** Kotoka Nakamura, Christina Brown, Hailiang Hu, Richard A. Gatti. 13th International Wolfsberg Meeting on Molecular Radiation Oncology. Wolfsberg Castle, Switzerland. June 2013. Oral Presentation.

3. **Martin NT,** Kotoka Nakamura, Christina Brown, Hailiang Hu, Richard A. Gatti. 2013 UCLA Biomedical Physics Colloquium. Los Angeles, USA. May 2013. Oral Presentation.

4. **Martin NT,** Hailiang Hu, Kotoka Nakamura, Francesca Fike, Richard A. Gatti. ATW 2012. Delhi, India. February 2012. Poster Presentation.

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6. **Martin NT,** Richard A. Gatti. UCLA Biomedical Physic Colloquium. Los Angles, USA. May 2010. Poster Presentation.

DISSERTATION MOTIVATION

The XCIND syndrome (x-ray sensitivity, cancer predisposition, immunodeficiency, neurologic deficits, and DNA repair defects) broadly covers a family of diseases that arise from rare, autosomal recessive mutations in DNA repair genes(1). Mutation of DNA DSB recognition and repair genes results in genomic instability, greater oncogenic potential and hypersensitivity to ionizing radiation, one of the main treatment modalities used in cancer therapy(2). Severe reactions to radiotherapy in Ataxia-Telangiectasia (A-T) patients, the archetype XCIND disorder, prompted the development of a clinical diagnostic to identify potential XCIND patients by testing for radiosensitivity(3-5). The current diagnostic methods have aided in the diagnosis of patients with A-T and other known XCIND disorders, and are thought to have helped avoid severe reactions to ionizing radiation in XCIND patients.

The current 'gold standard' methods require generation of a cell line, however, and take approximately 90 days to complete, which is not optimal for XCIND patients requiring radiotherapy in a timely manner for growing tumors. Approximately 5-10% of all non-XCIND (i.e. routine) radiotherapy patients experience severe side effects from their radiotherapy, though less severe than XCIND patients. Thus, there is great interest to extend radiosensitivity testing methodologies to routine oncology patients to identify patients at risk for developing adverse normal tissue reactions to radiotherapy. Current methodologies, however, are prohibitively slow for broad use in the oncology clinic. The first part of this thesis will examine whether the current leading surrogate assays for the time intensive, 'gold standard', colony survival assay can be adapted to be completed in

2-3 days, while remaining predictive of radiation sensitivity in suspected XCIND patients.

The molecular study of XCIND disorders and their relationship to DNA DSB repair has helped to expand our understanding of these mechanisms and their functional relationship to immune development, neurologic development, and the radiation response in humans. A 'candidate pathway' approach is often employed to identify new XCIND disorders by probing DDR pathways in radiosensitive cells derived from patients. Disrupted pathways are identified in individual patients and further profiled to identify the underlying genetic defect. To date, this approach has led us to the understanding that XCIND disorders stem from mutations in DNA DSB recognition or repair genes. Previous mechanistic studies, though, would suggest that other mechanisms beyond DSB repair, such as oxidative stress responses, mitochondrial function, and immunologic responses after irradiation, should also play a significant role in the radiation response and might lead to radiosensitivity in humans when disrupted. However, radiosensitive patients with core defects in pathways not directly related to DSB repair have not been identified. Though, this observation may be an artifact of the 'candidate pathway' approach used to discover new XCIND disorders, an approach utilizing assays *designed* to focus on DNA DSB recognition and repair.

Thus, our laboratory sought to screen a panel of radiosensitive cell lines of unknown etiology using recent advancements in technology, expression microarrays and exome sequencing, to make unbiased associations between genes and radiosensitivity. MicroRNA expression microarrays and whole exome sequencing measure thousands of cellular dimensions in parallel and were used to rapidly identify candidate genes

predicted to be involved in radiosensitivity. The second part of my thesis will focus on two molecules associated with radiosensitivity by this 'candidate gene' approach. I will validate the bioinformatics predictions that the two candidate molecules, miR-335 and mtPAP, are responsible for the radiosensitivity observed in patients' cells. I will profile the DDR in the patients' cells to better understand the mechanisms or pathways by which miR-335 and mtPAP modulate the DDR. Interestingly, miR-335 and mtPAP are not obvious candidates for radiosensitization or XCIND and I will explore whether the cellular phenotype of these patients fits within the current working model for XCIND or whether this unbiased 'candidate gene' approach has identified radiosensitization molecules that expand, or modify, our current working model.

Specific Aim 1: Assess the diagnostic potential of γ-H2AX foci and the neutral comet assay to be rapid surrogate assays for the colony survival assay. The gamma-H2AX foci and the neutral comet assays are attractive surrogates for the CSA because they can be performed on primary lymphocytes isolated from whole blood, reducing the time for performing and interpreting the assay to 2-3 days for a radiosensitivity diagnostic. I **hypothesize** that the assays can be adapted to test DNA repair in whole blood samples for a rapid radiosensitivity diagnostic and will correlate with clonogenic survival. In this aim, I will determine if assaying γ-H2AX kinetics can be predictive of clonogenic survival in cell lines. I will adapt the best performing of the γ-H2AX foci or neutral comet assays for use on whole blood samples, and will determine the diagnostic performance on discarded whole blood from patient samples submitted for radiosensitivity testing.

Specific Aim 2: Determine if and how miR-335 overexpression sensitizes cells to ionizing radiation in two suspected XCIND patient cell lines. Elevated miR-335 levels were associated with radiosensitivity by a microarray experiment assaying microRNA levels in 20 radiosensitive cell lines of unknown etiology and two A-T cell lines. miR-335 levels were regulated in an ATM dependent manner and miR-335 was predicted to target CtIP, a protein involved in DNA DSB repair. I **hypothesize** that miR-335 overexpression sensitizes cells to ionizing radiation by suppressing CtIP protein levels. In this aim, I will determine if miR-335 radiosensitizes cells by targeting CtIP and will test if endogenous miR-335 overexpression is responsible for radiosensitizing two patient derived cell lines.

Specific Aim 3: Does biallelic mutation of mtPAP radiosensitize cells? Full exomic sequencing of two siblings with an XCIND-like phenotype associated, among many variants, *MTPAP* with radiosensitivity. The known function of *MTPAP* as the mitochondrial poly-A-polymerase made it an unlikely candidate for radiosensitization, however, bioinformatics analysis suggested that it was the best candidate gene for the clinical phenotype observed in the patients. I **hypothesize** that mutation of *MTPAP* leads to radiosensitization of cells, likely through disruption of mitochondrial function and increased levels of reactive oxygen species. In this aim, I will determine if mtPAP is, indeed, causally linked to the radiosensitivity observed in these patients. I will profile the DNA damage response in the patients' cells to determine the impact mtPAP mutation has on DNA repair kinetics and will determine if markers of mitochondrial health, such as ROS levels, apoptosis, and mitochondrial bioenergetics, are abnormal in the patients' cells.

CHAPTER 1

CELLULAR RESPONSES TO IONIZING RADIATION

Ionizing radiation and DNA damage:

Ionizing radiation:

Ionizing radiation is used widely in medicine and it's beneficial uses and harmful biologic effects have been extensively studied, characterized, and used since the discovery of this type of electromagnetic radiation by W. Roentgen in 1895. This radiation falls on the electromagnetic spectrum at energies high enough to generate ions when interacting with atoms through the photoelectric effect, Compton scattering, and, at higher energies, electron-positron pair production. Ionizing radiation can be generated by accelerating electrons at high-Z materials, which causes X-rays to be emitted as the electrons decelerate when they approach the high-Z material (i.e. Bremsstrahlung radiation), or through nuclear decay of specific isotopes to generate gamma-rays. High-energy electrons, protons, and heavy atomic nuclei can also be ionizing , however, for the purpose of this thesis, 'ionizing radiation' will refer specifically to X- and gamma-rays. Ionizing radiation is of particular interest for medical imaging applications because of the low interaction potential with matter at X- and gamma-ray energies, resulting in electromagnetic radiation that is very good at penetrating and passing through the human body, especially soft tissue. W. Roentgen first observed the novelty and usefulness of this characteristic when he produced a rough radiograph of his wife's hand, and was able to non-invasively 'see' into the body.

The ability of ionizing radiation to penetrate through many centimeter's of human tissue and other materials, compared to the limited penetrance of visible, infrared, and

ultra violet radiation, has made it popular for imaging the human body. In the early days after the discovery of X-rays, ionizing radiation was viewed as something almost magical, and was applied many different ways, including treatment of benign and malignant tumors. However, very few precautions for radiation exposure were taken in these days and soon people observed the toxic effects of ionizing radiation exposure. Since the early 1900s, many medical advancements have been made, along with radiation accidents and nuclear weapons, which continue to illustrate the powerful benefits and dangers ionizing radiation poses to humans.

Ionizing radiation induced DNA damage:

Physically, ionizing radiation interacts with electrons orbiting the atomic nucleus, or the atomic nucleus itself, and these interactions can change the ionization state of atoms or compounds. These ionization events, when occurring in cells, can have significant impact on biologic functions and the direct or indirection interaction with cellular DNA, resulting in DNA breaks, is of central importance. Ionizing radiation, or ionizing radiation induced free radicals, can modify DNA bases or damage the sugar backbone resulting in the generation of single strand breaks (SSB) or double strand breaks (DSBs). SSBs occur with the highest frequency after ionizing radiation exposure and from endogenous sources, as well. SSBs are repaired easily and with high fidelity because SSB repair pathways can use the complimentary strand of DNA as a template for repair. DSBs occur at lower frequencies from both endogenous sources and ionizing radiation, but the rapid recognition and repair of DSBs is the main determinate of cellular survival(6, 7). Interestingly, the ability of ionizing radiation to penetrate tissue and effectively generate DSBs makes this type of radiation ideal for killing malignant cells, in

addition to imaging, but is also the main source of toxicity and mutagenesis in healthy tissue. Thus, the use of ionizing radiation in medicine is a balancing act between it's beneficial characteristics and it's mutagenic and cytotoxic potential.

DNA Single strand break repair:

Single strand break repair pathways:

SSBs can occur from many endogenous and exogenous sources. DNA adduct formation can occur when chemicals covalently bind to DNA molecules, base mismatches can occur during replication which result in SSBs, and DNA crosslinks can occur when DNA lesions occur in two different positions along the DNA molecule and become linked together. The nucleotide excision repair (NER) pathway is utilized for repairing most DNA adducts and crosslink lesions and the mismatch repair (MMR) pathway is activated to resolve base mismatches(8, 9). These pathways function with high efficiency and fidelity and are not significant sources of DNA breaks or repair defects after exposure to ionizing radiation. As such, these pathways will not be reviewed in detail in this thesis. DNA crosslinks, however, can result in stalled replication and DSB repair pathways can become involved and will be reviewed in the DSB repair pathway section(10). Indeed, a number of anti-tumor drugs have been designed to induce cross-liked DNA damage. DNA base modification, such as oxidation, alkylation, deamination and hydrolysis, are commonly generated by ionizing radiation and are repaired by the base excision repair pathway(11, 12).

Base excision repair:

The base excision repair pathway is summarized in **Figure 1**(13). For DNA lesions resulting from base modification (oxidation, alkylation, or deamination), the first

step for BER is the removal of the base lesion by a DNA glycosylase (such as NEIL1, NEIL2, OGG1, or NTH1), which generates a SSB(12, 14, 15). For SSBs lesions resulting from a break in the DNA backbone, BER is initiated when PARP1 senses the DNA break and is recruited to the sites of damage along with XRCC1(12). The ends of the DNA SSB are then processed and cleaned by PNKP or APE1. For short-patch BER, DNA Pol-beta and XRCC1 are recruited to the break for further end processing and repair is finished when DNA Ligase III ligates the broken DNA back together resolving the SSB with the correct base inserted(12, 16). For long patch BER, which involves repair over several bases, PCNA and Pol-delta/epsilon are recruited to the break to insert the appropriate bases and FEN1 removes the leftover overhanging DNA fragment. DNA ligase 1 finalizes repair by ligating the broken ends of DNA(12, 14).

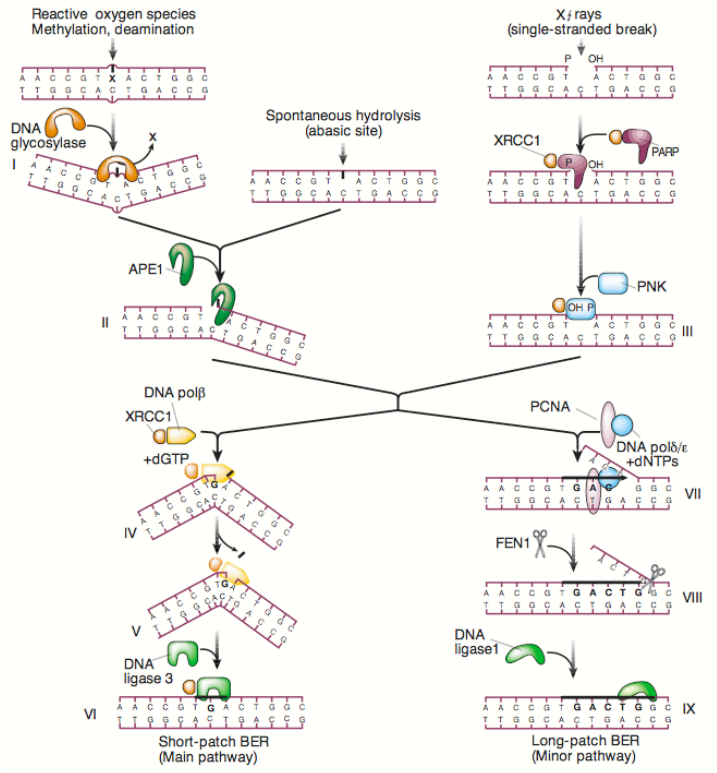

Figure 1. Outline of the base excision repair pathway adapted from Hoeijmakers et al.(13). BER is the main repair pathway utilized for ionizing radiation induced SSBs.

DNA Double strand break repair:

An introduction to double strand break repair pathways:

While many SSBs are induced by ionizing radiation, SSBs are generally repaired efficiently and without error. DSB repair, however, is a more complex process and can result in loss or modification of the genetic code during repair. Additionally, DSBs, left unrepaired, are the most deleterious DNA lesions impacting cellular survival and oncogenesis. Thus, DSB recognition and repair mechanisms are of paramount importance for cell survival after exposure to ionizing radiation and protection of genomic stability. It has been roughly estimated that dividing cells incur approximately 10 DSBs/cell/day from endogenous and exogenous agents such as DNA replication and naturally occurring sources of ionizing radiation(17). For comparison, a flight between Philadelphia and Paris induces approximately 0.05 DSBs/cell, an average computed tomography (CT) body scan induces approximately 0.3 DSBs/cell, and one standard fraction of radiotherapy (1.8-2 Gy) induces approximately 80 DSBs/cell(18). Due to the daily occurrences of DSBs, mammalian cells have adapted efficient mechanisms to repair DSBs and maintain genomic integrity and stability.

Non-homologous end joining (NHEJ) and homologous recombination (HR) repair are the main DNA DSB repair mechanisms in human cells. NHEJ is an efficient repair mechanism, but can result in errors during repair. Nevertheless, NHEJ is the main repair mechanism in human cells and is active in all phases of the cell cycle(19). HR repair is

active in the late S and G2/M phases of the cell cycle and utilizes the sister chromatid present in these phases of the cell cycle as a template for DSBs repair. HR is also activated to repair stalled replication forks and damage induced by DNA crosslinking agents(20, 21).

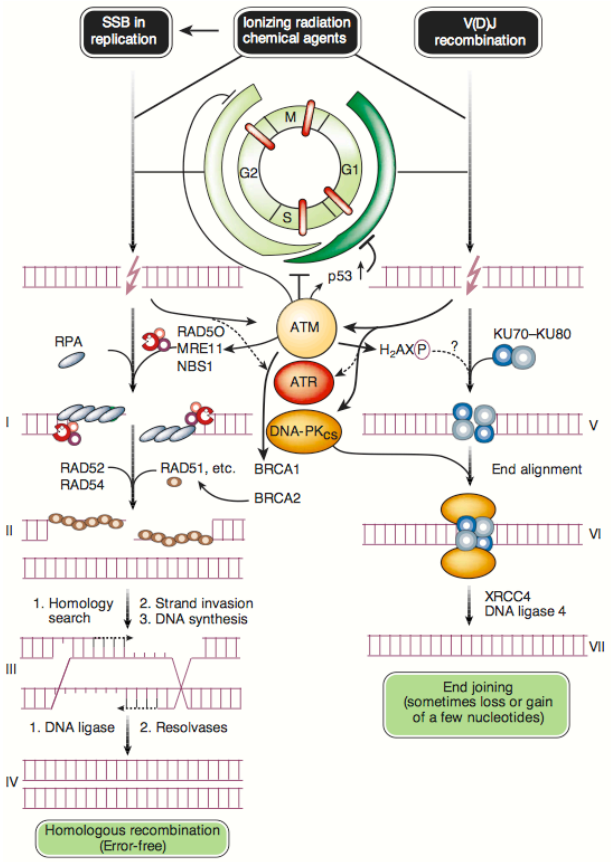

Figure 2. Core proteins involved in non-homologous end joining and homologous recombination DSB repair pathways. Adapted from (13).

Non-homologous end joining:

The core NHEJ mechanism involves the recognition of the DNA DSB by the Ku70/80 complex, which winds onto the broken ends of DNA(22, 23). This action recruits the DNA-PKcs subunit to the break, which then allows the recruitment of the ligation complex (DNA Ligase IV, XRCC4, and Cernunnos) and the direct ligation of broken DNA ends to repair the DSB(24, 25). PNKP and Artemis are required for a

subset of DSBs repaired by NHEJ that require further DNA end processing to prepare the DNA ends for repair(26, 27). NHEJ is very efficient and generally repairs breaks with high fidelity, but can sometimes result in the loss or gain of ~1-20 nucleotides(13, 28). **Figure 2** summarizes the core NHEJ pathway(13).

Recently, an alternative-NHEJ mechanism has begun to be described which takes advantage of microhomology surrounding some DSBs(29, 30). It has been suggested that this repair pathway is initiated by PARP1 and MRE11 and involves small amounts of end-resection via MRE11 and CtIP(22, 23, 31-34). ATM interactions with PARP1 have also been shown to be important for initiation of alternative-NHEJ(31). Once end-resection occurs around the region of microhomology, the break is resolved by DNA ligase III and potentially DNA ligase I, however the exact mechanism remains to be elucidated(35, 36). Alternative-NHEJ remains a minor pathway in DSB repair but has been estimated to be involved in up to 10% of DSBs, and may be a source of some translocations observed in lymphoid cancers(37, 38).

Homologous recombination repair:

HR repair occurs only when a sister chromatid is present as a template for repair in the late S and G2/M phases of the cell cycle and is active in repairing a relatively small proportion of DSBs induced in human cells. Only 15-25% of breaks in the S and G2/M phases of the cell cycle are repaired by HR(39). HR is initiated when the MRN complex (MRE11, RAD50, and NBS1) is localized to DSBs along with CtIP(40, 41). The MRN complex and CtIP initiate end-resection which generates single stranded DNA (ssDNA) overhangs around the DSB(39). CDKs have been recently shown to aid in the control of this end resection mechanism(42, 43). BRCA1 is then recruited to the site of damage and

functions along with CtIP (BRCA1-C complex) to support complete elongation of end-resection(44). The RPA protein coats the single-stranded DNA overhangs to protect the exposed ssDNA(45). RPA bound to the ssDNA is then displaced by BRCA2-mediated loading of RAD51 onto the ssDNA and promotes the subsequent recruitment of RAD52 and RAD54(13, 45, 46). Recruitment of these repair factors promotes invasion into the homologous template provided by the sister chromatid, chromatin exchange, D-loop formation, and resolution of the break by DNA ligases(21, 45). HR repair also resolves stalled or collapsed replication forks during the S-phase of the cell cycle, which are recognized by ATR mediated signaling mechanisms(20, 47). HR repair is less efficient than NHEJ, but is the error free DSB repair mechanism. **Figure 2** summarizes the core HR repair pathway(13).

DSB repair pathway choice and chromatin accessibility:

DNA is packaged in the three dimensional structure of chromatin and is in a highly compact state (heterochromatin) unless relaxed during active transcription (euchromatin). Repair factors, such as those involved in the core NHEJ pathway, have easy access to DSBs in euchromatin because the DNA is relaxed and is more easily accessible. In heterochromatin, however, repair factors cannot access DSBs easily and more extensive signaling is needed to modify chromatin structure to allow access for repair factors(48, 49). The ATM kinase plays a central role in this response and is responsible for recognition of DSBs, activation of cell cycle checkpoints and activating downstream signaling to relax chromatin and recruit repair factors to breaks(48, 50, 51).

MRN and ATM activate DSB repair signaling, which act in a coordinated way to recognize DSBs and transmit the damage signal to downstream effector proteins. ATM

exists in an inactive dimer form when it autophosphorylates itself in the presence of DSBs and dissociates into an active monomer form(52, 53). This action leads to the phosphorylation of KAP1, which aids in the relaxation of chromatin, and phosphorylation of the histone variant H2AX (γ-H2AX) at the sites of DSBs(54-56). γ-H2AX can extend up to megabase distances away from the site of DSBs, resulting in large nuclear repair foci(57). ATM also phosphorylates MDC1, and MDC1 is localized to the sites of γ-H2AX around DSBs and tethers the MRN complex and ATM at the sites of breaks(58, 59). The MRN complex is not required for the initial activation of ATM by DSBs, but is necessary for sustained ATM activation, amplification of the damage signal, and the localization of ATM to DSBs(53, 58, 60).

ATM also activates HERC2, which, along with MDC1 localization to DSBs, facilitates the recruitment of RNF8 to DSBs resulting in monoubiquitination of H2AX(61, 62). Ubc13 and RNF168 are then recruited to the site of monoubiquitination and build extended poly-ubiquitin chains(63). 53BP1 and the BRCA1-A complex (BRCA1, Abraxas, and RAP80) are then recruited to the sites of poly-ubiquitination and, depending on their interaction, modulate the DSB repair pathway activated to repair a specific break(63-67). Recently, SUMO modifications have also been demonstrated to play an important role in the recruitment and retention of these important repair factors at the sites of DSBs through ubiquitin and ubiquitin-like signaling cascades(62). 53BP1 and BRCA1 compete for access to the DSB region and retained 53BP1 directs repair towards NHEJ while BRCA1 directs repair to HR(68). Recent evidence suggests that 53BP1 is localized to breaks initially and inhibits end-resection along with RAP80(44, 69, 70). To initiate HR repair, BRCA1 shuttles 53BP1 and RAP80 to the periphery of the

DSB leaving behind a hole where end-resection can be initiated via CtIP/MRN and elongated in a BRCA1-dependent manner(39, 44, 71, 72). **Figure 3** summarizes the chromatin ubiquitin ligase cascade that recruits 53BP1 and the BRCA1-A complex to DSBs(62).

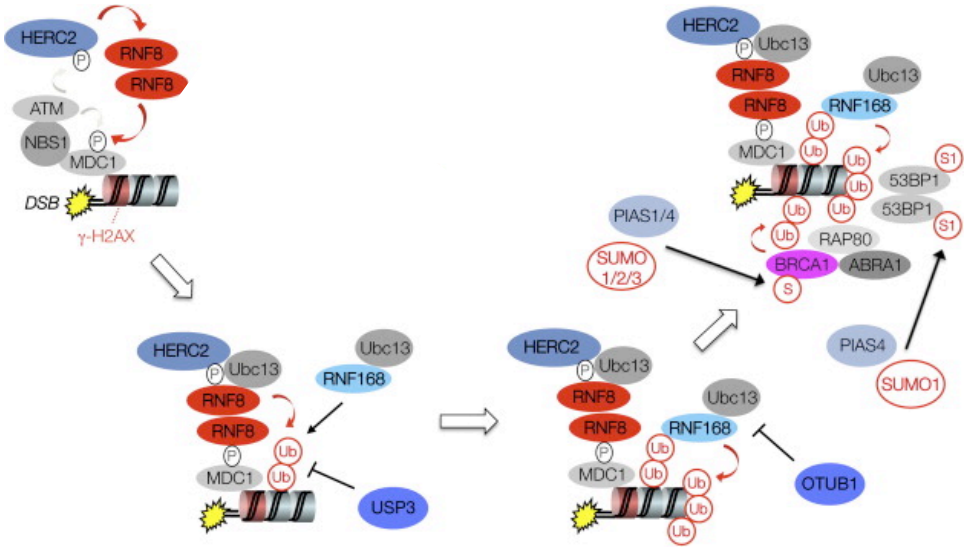

Figure 3. Summary of the chromatin ubiquitin ligase cascade, resulting in the recruitment of 53BP1 and the BRCA1-A complex to the sites of DSBs. Adapted from (62).

Programmed DSBs and DNA repair in immunologic function:

In addition to induction of random DSBs by ionizing radiation, chemicals, and cellular processes, DSBs play a role in immune development and activation. This thesis will focus mainly on DSBs induced by ionizing radiation, but the DDR's role in immune development and activation is important for patients harboring mutations in certain DSB repair genes. Therefore, I will briefly summarize the interplay between the DDR and immunologic functions.

The DDR has been implicated to play an important role in retroviral infection and in DSB-induced activation of immune responses. During infection, a virus attempts to insert its DNA into the host genome and DSBs arise. Integration of the viral cDNA needs DSB repair mechanisms to be successfully inserted and cells deficient in NHEJ mechanisms leave these DNA lesions unrepaired and undergo apoptosis as a result of the persistent DSBs(73, 74). Thus, repair of viral induced DSBs are necessary for the survival of the host cell and proliferation of the virus. DSBs have also been shown to activate aspects of innate immunity. The receptor NKG2D plays an important role in the activation of natural killer cells in response to infection and cellular transformation(75). Expression levels of NKG2D ligands can be induced in an ATM- and ATR-dependent manner after DSBs, providing a mechanism by which infected cells or cells with persistent genomic instability, such as tumor cells, could be eliminated through immune signaling(76). DNA damage has also been shown to modulate the interferon-regulatory factor family of transcription factors in ATM and DNA-PK dependent manners providing another link between DDR mechanisms and induction of immune signaling events(77, 78). The most striking connection, perhaps, between the DDR and immune function are the programmed DSBs that are necessary for the development and maturation of lymphocytes and their antigen receptors through V(D)J recombination and Class Switch Recombination (CSR).

V(D)J recombination is summarized in **Figure 4**(79). RAG1 and RAG2 proteins recognize recombination signal sequences (RSS) and introduce a DSB at this site. ATM, the MRN complex, and γ-H2AX recognize and localize to these RAG1/2 initiated DSBs. It has been suggested that these proteins function to provide a scaffold for DNA repair

and to arrest the cell cycle to allow time for repair to occur(80, 81). The RAG1/2 induced breaks occur in the G0 or G1 phase of the cell cycle and NHEJ is responsible for repairing the breaks to rejoin the V, D, and J fragments. Protein deficiencies in Ku, XRCC4 and DNA ligase IV show severe defects in V(D)J recombination and individuals and mice deficient for these proteins present with a SCID phenotype because of the inability to develop specific antibodies or activate immune cells(79, 82, 83). It appears that ATM is also involved in V(D)J recombination, but is not required. However, ATM null cells do present with reductions in mature lymphocytes(84). It is proposed that ATM and DNA-PKcs can act redundantly to phosphorylate Artemis to resolve hairpin structures arising at the coding ends of breaks during V(D)J recombination, resulting in a role, but not a requirement, for ATM in repair of breaks during V(D)J recombination. DNA-PKcs, however, appears to be required for V(D)J recombination through it's role in NHEJ repair(85). ATM, along with NBS1 and the MRN complex, has been demonstrated to function in a greater role to regulate cell cycle checkpoints during recombination, which allow time for repair and acts to avoid genomic instability and translocations arising from aberrant recombination events(84, 86). Artemis is involved in a subset of V(D)J recombination events, but is not required, and deficiency in Artemis results in a 'leaky' block of V(D)J recombination(83).

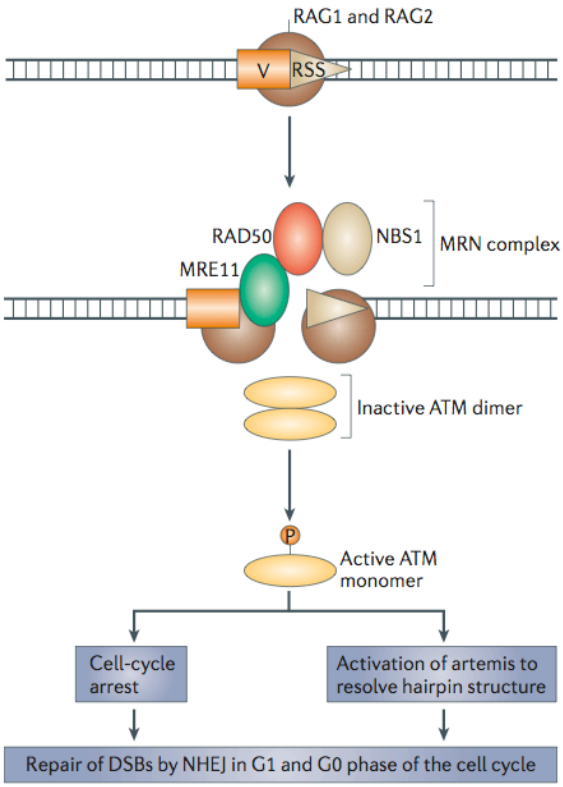

Figure 4. Description of V(D)J recombination events. Adapted from (79).

CSR is another immune mechanism whereby targeted DSBs are induced during B-cell activation to switch the constant IgH region from Cμ to a downstream C region resulting in generation of a different class of antibody(87, 88). CSR is summarized in **Figure 5**(79). DNA DSBs are induced by AID in the switch region and NBS1 and γ-H2AX foci localize to the sites of these DSBs(89). Similar to V(D)J recombination, NHEJ is the central repair mechanism for the DSBs induced by AID during CSR and deficiencies in DNA-PK abolish or impair CSR(90). CSR is also impaired in ATM deficient cells and it has been suggested that ATM functions to induce cell cycle arrest in cells during recombination and/or may be involved in synapsing distant switch regions along with γ-H2AX(91). NBS1, 53BP1 and MDC1 also co-localize to breaks along with γ-H2AX foci and are required for CSR(91-93). γ-H2AX, and these related factors, do

appear to provide a scaffold that maintains broken ends of DNA across megabase distances to avoid splitting and dissociation of the broken ends of DNA during repair, suggesting that these proteins act as a scaffold or anchor for DNA fragments during CSR in addition to their roles in cell cycle checkpoint signaling(94, 95).

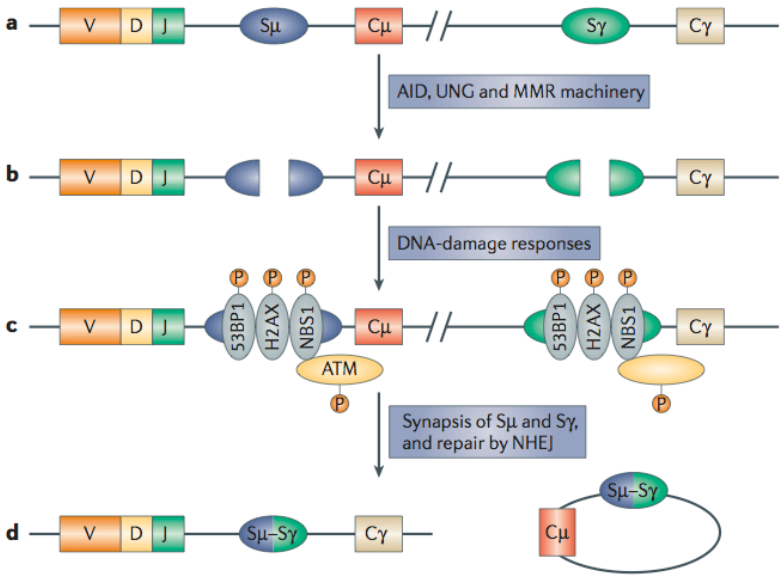

Figure 5. Summary of Class Switch Recombination and the DSB repair molecules involved. Adapted from (79).

Non-DNA repair components of the cellular radiation response:

The response of cells to DSBs after irradiation remains a central determinate of cell survival. There are several mechanisms that can act upstream, in parallel to, or downstream of DSB lesion repair that impact upon cellular survival and maintenance of genomic stability. In this section, I will briefly discuss the roles reactive oxygen species (ROS), mitochondria, apoptotic signaling, and immune response pathways have in the cellular response to ionizing radiation.

ROS are generated in cells by endogenous and exogenous sources and the mitochondria are one of the main producers of ROS, which occur during respiration.

Mitochondria are also dependent on ROS for various stages of respiration and additional cellular process require ROS for various biochemical reactions. Thus, cells must maintain a balanced redox state for cellular metabolism and signaling events(96, 97). Mitochondria can leak superoxide ions during respiration, which have a relatively short half-life and are quickly converted to hydrogen peroxide. Mitochondrial dysfunction can lead to increased release of these ROS species, which have been associated with disease, DNA lesions, increased levels of cell death, and cancer(11, 98, 99). Ionizing radiation also generates ROS species, namely the hydroxyl radical during radiolysis of H_2O, and cells must react quickly and efficiently to reduce these ROS species to protect against DNA and protein damage by irradiation. Interestingly, DNA DSB damage has also been shown recently to induce increases in ROS species(100).

Maintaining a balanced redox state is crucial to avoid increased or chronic DNA breaks and oxidative stress has been shown to lead to cell death via apoptotic pathways(101). Cellular antioxidant defenses are induced by Nrf2, a transcription factor that is located predominately in the cytosol of cells, which has a short half-life due to constant degradation by the proteasome under normal levels of ROS(102, 103). Under conditions of oxidative stress, Nrf2 is stabilized and localizes to the nucleus where it induces transcription of genes involved in the antioxidant response, such as SOD2(104-106). SOD2, and SOD2 mimetics, have been shown to render cells and mice more resistant to radiation through antioxidant properties(104, 105, 107). Nrf2 can also activate a broad range of genes to deal with oxidative stress through mitochondrial biogenesis, reduction of ROS species, and autophagy(102, 103, 108).

Increased levels of oxidative stress and DNA damage also induce inflammatory pathways, which can have broad impacts on the response to radiation through intracellular signaling and release of cytokines to nearby cells(109). Interestingly, chronic inflammation and certain cytokines can also induce DNA damage and activate DNA damage pathways(110, 111). While, there remain many unanswered questions for the role of cytokine and inflammatory signaling after irradiation, activation of inflammatory responses are believed to play a role in the normal tissue response to irradiation through induction of both pro- and anti-apoptotic signals(103, 109, 112, 113). While an in-depth review of inflammatory cytokine signaling is beyond the scope of this thesis, I will briefly discuss the role of inflammatory signaling, along with DNA damage signaling, in apoptotic signaling pathways.

Apoptosis is a mechanism by which damaged cells can be removed, and dysfunction of apoptotic pathways play a role in cancer progression and tissue responses to irradiation(101, 114-116). While the exact details of the induction of apoptosis are still being elucidated, two predominate apoptotic pathways have been identified and are distinguished as the extrinsic and intrinsic apoptotic pathways. The extrinsic apoptotic pathway is induced by cell death receptor signaling via the tumor necrosis factor (TNF) family of proteins, tying this pathway to immune signaling events and cytokine ligand binding(110, 117). The extrinsic pathway is carried out without mitochondrial involvement and plays an important role in the development and control of the immune system and the development of some cancers. After receptor activation in the extrinsic apoptotic pathway, assembly of the DIS (death-induced signaling) complex containing FADD and caspase-8 and -10 is assembled which activates a caspase cascade, resulting in

caspase-3 activation, digestion of nuclear DNA and cellular proteins, and the final execution of apoptosis(47). The intrinsic apoptotic pathway is of particular interest for oxidative stress and irradiation induced apoptosis. A complex balance of pro- and anti-apoptotic signals via mitochondria mediates this pathway. The intrinsic apoptosis pathway is initiated by mitochondrial release of cytochrome c, which aids in the assembly of the apoptosome consisting of pro-caspase-9, Apaf-1, and cytochrome c(118). Analogous to the DIS complex in the extrinsic pathway of apoptosis, assembly of the apoptosome results in the activation of a caspase cascade culminating in caspase-3 cleavage and activation, digestion of cellular DNA and proteins, and completion of apoptosis by the cell(119). The release of cytochrome c from the mitochondria depends on the relative balance of pro-apoptotic proteins, such as Bax and Bak, and anti-apoptotic proteins, such as Bcl-2 and Bcl-xl, at the mitochondria(120, 121).

Apoptosis pathways are modulated by DNA repair and DNA damage response proteins through complex signaling cascades that are not well understood. For example, ATM can induce NF-kB nuclear localization, which induces transcription of *both* anti- and pro-apoptotic genes(122, 123). Similarly, ATM and ATR can stabilize p53 after DNA damage, resulting in the transcription and activation of *both* anti- and pro-apoptotic signals(114, 116). p53 also plays a role in permanent cell cycle arrest following irradiation (i.e. cellular senescence) which is another mechanism by which cells can loose clonogenicity following DNA damage or chronic oxidative stress(124). While the exact mechanism(s) that tune the balance between pro- and anti-apoptotic signals or senescence are not know, it is clear that a shift towards apoptosis plays an important role in the

radiation response, response to oxidative stress, and response to cells with oncogenic DNA transformations(125-130).

MicroRNA and the cellular response to radiation:

MicroRNA (miRNA) are small non-coding RNA fragments, which have recently been shown to play a role in modulating the DDR. These small RNA fragments are located throughout the genome, and can be regulated under their own promoter regions or can be co-transcribed with host genes when they are located within intronic regions of these genes. Interestingly, the transcription and processing of miRNA into their mature ~19-25 nucleotide fragments can be modulated by DNA damage proteins, such as ATM and BRCA1(131-134). The unique sequence of each mature miRNA can bind to consensus sequences in mRNA and this association leads to the degradation of the target mRNA or inhibition of translation into protein. Perfect sequence homology is not required for miRNA binding and, thus, it is possible for a single miRNA to target many genes with variable affinity, largely by binding in the 3' untranslated region of the target mRNA. Thus, miRNA have emerged as regulators of protein levels and can induce cellular phenotypes similar to genetic mutation and loss of function of a particular gene, but with no mutations in the specific gene(131, 135, 136).

miRNA are of particular interest for cancer development, control of metastasis and tumor re-initiation. A number of microarray studies have associated various miRNA and their predicted gene targets with modulation of tumor development or sensitivity to ionizing radiation and other genotoxic agents(132, 137-140). The p53 mediated DDR pathway and miRNA has also been extensively studied and, for example, the miR-34 family of miRNA (miR-34a-c), are induced by p53 responses to DNA damage. This p53-

inducible miR-34 family of miRNA has been shown to modulate cell cycle regulation, apoptosis, and maintenance of genomic stability(137, 141-143). Of particular interest for our lab and this thesis is the ATM kinase and ATM protein levels are targeted by miR-421 and miR-101(131, 144). ATM protein reductions due to miRNA targeting sensitizes cells to ionizing radiation and induces a cellular phenotype remindful of A-T cells, but lack ATM mutations(131, 135, 144). MiRNA-335 has also generated some interest recently for it's role in sensitizing cells to ionizing radiation (demonstrated in this thesis), suppression of tumor metastasis, and tumor re-initiation(136, 139, 140, 145-148). Thus, miRNA are emerging as potentially powerful regulators of the DRR and tumor development/progression through suppression of proteins required for normal responses to DNA damage and cellular survival after genotoxic insult.

CHAPTER 2

HUMAN RADIOSENSITIVITY DISORDERS

Cells are constantly bombarded with DNA damage and the repair mechanisms outlined in Chapter 1 are instrumental for the cellular response to both exogenous and endogenous genotoxic stimuli. Each cell type in the human body has to effectively respond to a broad array of genotoxic insults and the specific environment each cell type is in gives rise to a unique array of stimuli. Cells in the gut, for instance, are exposed to high levels of DNA damaging agents through food and digestion on a daily basis. This constant bombardment of cells by genotoxic insults, cells in the gut are turned over with relatively high frequency through repopulation by protected stem cell niches. Loss of cells in the gut, with the exception of the stem cell niche, has minimal long-term impact because cell populations can be repopulated in a relatively short period of time. Cells in

the brain, on the other hand, can be somewhat resistant to genotoxic agents because the majority of these cells are post-mitotic and only need to maintain genome fidelity for key genes involved in their day-to-day function. These cells also exist in an environment protected from many sources of DNA damage through the blood brain barrier. Unlike the gut, which has adapted to high cell turn over, loss of cells in the brain can have substantial impact on cognitive abilities because the lost cells, and associated neural signaling connections, are not easily replaced, if at all.

Individuals harboring genetic defects in DNA DSB repair genes highlight these dichotomies of cell type specific responses to genotoxic insults, and the differential impacts of DSBs across tissues. Patients with loss-of-function mutations in DSB repair genes present with hypersensitivity to ionizing radiation and radiomimetic compounds. These patients also have increased risk of developing cancer, which is presumed to arise from genomic instability after insufficient DNA repair, and tumors are observed often in childhood. One of the most striking examples of the differential impact of DSB repair defects on various tissues in the body, perhaps, is the added presence of neurologic deficits and immunodeficiency in these patients. The XCIND(x-ray sensitivity, cancer predisposition, immunodeficiency, neurologic involvement, and DNA repair defects) syndrome summarizes patients with deficiencies responding to DSBs and was meant to group these radiosensitive patients with shared clinical presentations under an umbrella syndrome(1). The study of XCIND patients has provided valuable insights into DNA repair mechanisms, the DDR, and the impact of DNA damage and repair proteins on cellular functions throughout the body(1, 149). The following sections will provide an overview of the XCIND disorders discovered to date, will briefly summarize the clinical

presentation of these individuals, and will introduce current efforts to diagnose XCIND individuals.

DNA repair disorders (XCIND):

Ataxia-telangiectasia (A-T) is, perhaps, the archetypal XCIND disorder and occurs with a frequency between 1 per 40,000-100,000 live births(150). A-T was first described in 1926(151), with a subsequent case report in 1941 by Louis-Bar of a 9 year old boy with A-T and introduction of the term 'ataxia-telangiectasia' to describe the disorder in 1958 by Boder and Sedgwick. In 1975, Taylor et al. observed an adverse response to radiotherapy in an A-T patient which indicated that the ATM gene, which was later localized and cloned, may be involved in the radiation response(5). The clinical phenotype of A-T patients is described by telangiectasia occurring initially in the eyes, radiosensitivity, progressive cerebellar degeneration, ataxia, immunodeficiencies in some patients, elevated alpha fetoprotein levels that increase with age, and hypogonadism(152, 153). A-T patients also have a predisposition for developing cancers, especially leukemia and lymphoid tumors, and carriers of the ATM gene have an increased risk of developing breast cancer, which is exacerbated by smoking(4, 154, 155).

Molecularly, A-T patient's cells display sensitivity to ionizing radiation, defective recognition and repair of DSBs, and disruption of cell cycle checkpoint activation(3, 50, 156). The ataxic phenotype (i.e. neurologic phenotype) of A-T patients has been of great interest to patients, clinicians, and researchers and study of post-mortem tissue from A-T patients and mouse models suggest that the cerebellar degeneration is due to loss of Purkinje cells(157, 158). However, the molecular basis for the loss of Purkinje cells is still undetermined, and whether the cell loss occurs due to defective DSB repair or a

cellular state of oxidative stress induced by loss of ATM in neural cells is debated and under investigation(127, 159, 160). Several groups are currently developing and studying interesting animal and stem cell models of A-T with the goal of elucidating the impact ATM disruption has on the various cell populations of the cerebellum(161, 162). The study of A-T patients has illuminated and will continue to unveil an interesting and complicated interplay between DNA DSBs, the response to these breaks, mechanisms of redox signaling and the differential impact these mechanisms have on neurologic development, immune development, oncogenesis, and the cellular response to ionizing radiation.

Since the early discovery and characterization of A-T, a number of other DNA repair disorders and radiosensitivity disorders have been characterized. Fanconi anemia (FA) was first described soon after A-T in 1927, and, since, has been found to arise from mutations in the Fanconi anemia complementation genes with groups A, C, and G accounting for more than 85% of the patients observed(163, 164). FA patients first present with aplastic anemia during childhood and go on to suffer from bone marrow failure, myelodysplasia, and tumors(10, 165). The FA family of proteins are involved in resolution of DNA crosslink damage, especially crosslinks that arise during replication, and FA patients are hypersensitive to cisplatin and other DNA cross linking agents. Radiosensitivity has also been observed in FA patients(2, 3).

Deficiencies in MRE11, RAD50, and NBS1, the members of the MRN complex, have also been described in 1999, 2009, and 1981, respectively(166-168). MRE11 deficiency, also known as AT-like disorder, presents similarly to A-T patients and is very uncommon, except among Saudi Arabian populations(169). Nijmegen breakage

syndrome (NBS) and RAD50 deficient patients present similarly and the first RAD50 patient was originally mistook for an NBS1 patient(166, 167). It is difficult to comment on a 'RAD50 phenotype' because only one patient has been described in the literature to date. More NBS patients have been characterized, though, and these patients present with cranial-facial features, such as a birdlike face, receding forehead, and a prominent midface. These patients also have immunodeficiency, progressive microcephaly and, unlike A-T patients, mental retardation(170). The contrasting neurologic phenotypes observed between A-T and NBS patients are intriguing, given the co-dependence of many DDR signaling mechanisms on the MRN complex and ATM. It has been postulated that, because ATM is required for some apoptotic signaling mechanisms, A-T patients have neural cells with defects that are not readily cleared away by apoptosis and result in accumulation of dysfunctional cells. NBS1 does not appear to be necessary for apoptotic signaling mechanisms, however, and it was proposed that dysfunctional neural cells are cleared away effectively by apoptotic mechanisms giving rise to the severe and progressive microcephaly observed(171).

Patients deficient for the NHEJ proteins DNA Ligase IV, Artemis, Cernunnos, and DNA-PKcs have also been described and present with severe immunodeficiency and radiosensitivity(85, 172-174). The immunodeficiency stems from severe defects during V(D)J and CSR, and the radiosensitivity results from inadequate DSB repair. These patients have high incidences of cancer, genomic instability, and various neurologic involvement such as developmental delay or microcephaly(173, 175). However, few patients have been characterized to date and more patients will need to be studied for each individual disorder to determine a clearer clinical phenotype. More recently, two

patients with mutations in the RNF168 gene, which is involved in the chromatin ubiquitin ligase cascade, were characterized by mild ataxia, small heads, immunodeficiency, short stature and radiosensitivity and constitute the RIDDLE syndrome(176, 177).

Lastly, there are several disorders that have small overlapping features with the XCIND disorders discussed above, but are not included within the umbrella of XCIND. X-linked agammaglobulinemia and adenosine deaminase deficiency both present with immunodeficiencies and cellular radiosensitivity was noted in a small cohort of patients that were available for study(178). However, little is known about the mechanism of radiosensitivity in these patients, but it is unlikely that either disorder impacts upon DNA repair mechanisms or immune development in a manner that is similar to other XCIND disorders. It is also worth noting Werner and Bloom's syndromes in the context of radiosensitivity and DNA repair, as well. The proteins deficient in these disorders, WRN and BLM, are DNA helicases and have been shown to play a role in HR repair(179-181). However, WRN or BLM deficient cells present with photosensitivity (i.e. sensitivity to single strand breaks) rather than radiosensitivity. Patients deficient for these genes do have increased risk of developing cancer and BLM deficient patients have immunodeficiency noted, indicating that there may be some overlap with XCIND, but without the radiosensitivity or severe DNA DSB repair defects.

Radiosensitivity testing and diagnosing XCIND patients:

XCIND patients are currently identified by testing for radiosensitivity in lymphoblastoid cell lines (LCLs) derived from primary lymphocytes from patient blood samples. The Gatti laboratory has adapted the classic clonogenic survival assay for use on suspension cells (LCLs) to assess clonogenic survival in patients submitted for

radiosensitivity testing(3). A study of 104 A-T LCLs and WT controls established a radiosensitive survival fraction range post-1 Gy of <21% survival. Survival fractions of >36% were deemed radionormal and the gap between the radionormal and radiosensitive range was a non-diagnostic, 'intermediate' range(3). Upon a finding of radiosensitivity in a suspected XCIND patient, an immunoblot is performed to look for deficiencies in the proteins of known XCIND disorders, thus providing a specific diagnosis for the etiology of the radiosensitivity observed. A portion of the patients tested (~15-20% of total) are found to be radiosensitive with no observable defects in known XCIND genes, and represent the radiosensitive cells of unknown etiology that will be the main focus of this thesis.

The methodology for diagnosing radiosensitivity described above is well suited for studying and diagnosing rare XCIND disorders, such as A-T. However, these methodologies, due to the requirement for generation of a cell line, are time intensive and turnaround times for the assays are 90+ days. Interestingly, approximately 5-10% of the general oncology population (i.e. not XCIND) will experience adverse reactions, or above average level of radiosensitivity, to their radiotherapy treatment. The standard doses used to treat oncology patients were established based on population averages of tissue responses to irradiation and do not reflect individual idiosyncrasies that might lead to radiosensitivity. Thus, it is of interest to determine if radiotherapy plans could be better tailored to patient specific biology, especially in cases where patients may be at risk for adverse reactions. While the clonogenic survival assay remains the gold standard for measuring various sensitivities to radiation in human cells, the time needed to complete the assay is too long for clinical implementation and usefulness in oncology

patients or the diagnosis of XCIND patients who have developed malignancies that need to be addressed immediately.

A number of groups have attempted to identify rapid surrogate assays that measure DNA DSB recognition and repair biomarkers to determine if these correlate with clinical outcomes to ionizing radiation. Many of the studies that have explored this issue contradict each other or result in non-predictive correlations, and a rapid surrogate assay that is predictive of toxicity for use in the oncology clinic remains elusive (182, 183). Our experience has shown that DNA repair assays, such as the neutral comet assay and gamma-H2AX foci kinetics, correlate reasonably well (~87%) with the CSA in *XCIND* patients of unknown etiology, and remarkably well (100%) in the patients where we know the genetic cause of the XCIND phenotype(156, 184). Both of these assays can be adapted for use on whole blood, which would result in an assay that could be performed in 3-5 days and could be implemented in the oncology clinic. Thus, this thesis will begin by exploring the use of surrogate assays for the CSA and will build upon the work begun by Dr. Shareef Nahas.

CHAPTER 3

RAPID CLONOGENIC SURVIVAL ASSAY SURROGATE: THE NEUTRAL COMET AND γ-H2AX FOCI KINETIC ASSAYS FOR RAPID RADIOSENSITIVITY TESTING

Radiosensitivity testing has been part of a diagnostic package used to identify patients belonging to a family of rare genetic disorders resulting from mutations in DNA repair genes. Expanding radiosensitivity testing, or radioresistivity testing, to enable personalization of radiotherapy or chemotherapy doses is of great interest in oncology.

However, current methodologies require generation of a cell line and are prohibitively time consuming for broad use in oncology for screening patients. A number of DNA damage response assays were screened on cell lines, recently, and found that the neutral comet assay was the best surrogate for current techniques. In this chapter, I determined if the neutral comet assay could be adapted to assay DNA repair in whole blood samples for 3 day turnaround radiosensitivity testing. I also utilized a version of the popular γ-H2AX irradiation induced foci assay to determine if this method could outperform the neutral comet assay or could be utilized as a test to identify repair pathway specific defects in cells scored to be radiosensitive by the rapid neutral comet assay.

Introduction:

Defects in DNA double strand break repair proteins result in varying clinical phenotypes which, thus far, can be included under the umbrella of the XCIND syndrome (X-ray irradiation sensitivity, Cancer susceptibility, Immunodeficiency, Neurological impairment, and Double strand breakage repair) (1). Patients are identified as potentially XCIND when they present with some, or all, of the clinical phenotype summarized by XCIND or have had a severe reaction to radiotherapy as pediatric oncology patients. In cases where radiotherapy or chemotherapy has not yet been administered, our lab conducts radiosensitivity testing on cell lines derived from the patient's lymphocytes to determine if they are at risk for complications during genotoxic therapies.

Once a laboratory result indicates the patient's cells are radiosensitive, the underlying DNA repair protein defect is sought. Many of the known radiosensitivity disorders resulting from DNA repair protein deficiencies, such as Ataxia-Telangiectasia (A-T), Nijmegen Breakage Syndrome (NBS), DNA LIGASE IV deficiency (LigIV),

RNF168 deficiency (RNF168), and polynucleotide kinase 3'-phosphotase (PNKP) deficiency, are now diagnosed by routine immunoblotting. The large number of potentially defective DNA repair proteins that could result in radiosensitization, however, make these techniques impractical for diagnosis of new double strand break (DSB) DNA damage response (DDR) deficiencies; radiosensitivities of unknown etiology.

The current laboratory tests employed to test for radiosensitivity, and to test for specific disorders using immunoblotting techniques, are also time intensive due to the need for a cell line to be established. While the current turnaround time is often allowable for diagnosing a patient as XCIND, the time needed to establish a cell line and to test for cellular radiosensitivity (approximately 90 days) is prohibitively slow for many patients, especially for XCIND or adult oncology patients requiring intervention for tumors that have been recently diagnosed. A rapid test to identify individuals who were at risk for severe complications due to radiotherapy would allow radiosensitivity testing to be expanded beyond diagnosing XCIND patients and enable the screening of oncology patients to reduce the 5-10% of routine oncology patients who react adversely to standard radiotherapy doses.

To address this challenge, our lab has sought to utilize *functional* DNA repair assays that can rapidly identify pathways of underlying repair deficiency and radiosensitivity. The DNA repair disorders mentioned above have been extensively studied to elucidate the role each responsible gene contributes to DSB processing. In some cases, these associations are overly simplistic and only provide working models for diagnostic evaluations. Ataxia-Telangiectasia Mutated (ATM) is activated within minutes after irradiation (IR) and is responsible for many downstream events in the DSB-

DDR (185). Nibrin plays an early role in recruiting ATM to the damage site and initiating homologous recombination (186). DNA LIGASE IV is responsible for ligation of the broken DNA in non-homologous end joining (19). RNF168 and PNKP have been characterized more recently and represent defects in the chromatin ubiquitin ligase cascade (CULC) (187-189) and 3'-5' end processing after DSBs, respectively (190, 191). Each of the proteins listed above have very different roles in the DDR, thus making identification of a single biomarker that is informative for both radiosensitivity testing and identifying underlying genetic defects challenging.

Nahas et al. compared a number of radiation response assays performed on LCLs and found that the NCA was the best surrogate for the CSA to predict radiosensitivity(184). The other assays tested in this study correlated poorly with the CSA. However, many of these assays could be used to indicate which DDR pathway was disrupted in an individual patient(184). γ-H2AX foci, in particular, are a popular biomarker of DNA damage and the diagnostic utility has generated substantial interest but, often, convoluted results in diagnostic applications(55, 156, 184, 192, 193). Frequently, γ-H2AX foci are only evaluated in cells at times early after irradiation (e.g. 30 minutes or 1 hour), to assess DNA damage levels or recognition of DSBs, or late after irradiation (e.g. 24 hours) to monitor repair of DNA damage; inferred by the resolution of foci. Measuring γ-H2AX at single time points begins to illuminate cellular phenotypes in XCIND patients, but not robustly enough to indicate a specific repair pathway defect or genetic diagnosis. Additionally, these single time point measurements are not predictive of radiosensitivity in more heterogeneous patient populations. Based on these previous studies, though, I postulated that γ-H2AX foci kinetics (i.e. measuring foci at multiple

time points post-irradiation) could give a more complete understanding of a cell line's repair kinetics and could be used to stratify patients according to unique kinetic curves, or 'fingerprint' curves, of specific XCIND disorders.

In this chapter, I will present data that demonstrate the NCA can be performed on whole blood and that this methodology remains the most promising rapid surrogate for the CSA to identify radiosensitivity in suspected XCIND patients. I will also provide a summary of data that indicate that known XCIND disorders each have unique γ-H2AX foci kinetic curves. I will further provide an example for how the characteristic curves of known XCIND disorders can be used to provide a 'candidate pathway' approach for identifying the underlying genetic defect in a radiosensitive patient cell line of unknown etiology.

Materials and Methods:

Cell panel, LCL lines, and whole blood samples

The LCLs used in this study were derived from patients with an XCIND-like disorder. Peripheral blood lymphocytes (PBLs) were isolated and transformed with EBV as previously described (3). Transformed LCLs were maintained in RPMI1640 media, 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah) and 1% penicillin/streptomycin (Gibco BRL, Grand Island, NY) at 37°C and 5% CO_2. Whole blood for the NCA was aliquoted from samples submitted for radiosensitivity testing prior to processing and immortalization.

Colony Survival Assay (CSA)

The average SF% for LCLs was assessed by CSA, using normal and radiosensitive ranges, previously defined with 29 WT and 104 A-T LCLs (3). Briefly,

cells were seeded in a 96-well plate and treated with 1 Gy γ-irradiation at a dose rate of ~4.5 Gy/min (Mark 1 Cs137 Irradiator) or mock irradiated. Plates were returned to 37°C for 10 to 13 days at which time they were stained with MTT dye (tetrazolium-based colorimetric assay, Sigma, St. Louis, MO). SF% of <21% was interpreted as RS and SF% >36% was considered radionormal (3).

Neutral comet assay for whole blood

This study utilizes the NCA protocol described by Nahas et al. but was modified for use on whole blood(184). The Comet Assay kit (Trevigen Inc, Gaithersburg MD) was used under neutral conditions according to the manufacturer's specifications. Whole blood was treated with 15 Gy or sham treated and collected at 0hr (no-IR), 30 minutes, and 8 hours post-IR. Samples were collected when indicated and placed on ice and erythrocytes were lysed on ice using Red Blood Cell Lysis solution according to the manufacturers specifications (5 Prime, Gaithersburg, MD). Once lysed, the remaining white blood cells were re-suspended in 1% low melting point agarose (Sigma Aldrich, St. Louis MO) and plated on 20-well comet assay slides. Once the agarose solidified, the slides were added to a bath of cell lysis solution (Trevigen Inc, Gaithersburg MD) containing 10% DMSO overnight at 4C. The following day, the samples (i.e., the free DNA fragments) on the slides were electrophoresed and stained using SYBR Gold (Invitrogen, Carlsbad CA). Comets were visualized using an Olympus fluorescent microscope equipped with an AxioVision camera and software. The tail moments (TM) of comets were scored using CometScore software (TriTek, Sumerduck VA). Percent repair (% repair) was determined by monitoring the return to baseline tail moment levels ($TM_{8\ hrs}/TM_{0\ hrs}$).

Immunofluorescence detection of γ-H2AX, 53BP1, BRCA1 and RAD51 foci

Immunofluorescence detection of foci has been previously described in many laboratories (55, 149). Briefly, LCLs were irradiated, spread on coverslips, and fixed with a 4% formaldehyde solution at the indicated time points. Cells were irradiated with 1 Gy for γ-H2AX and 53BP1 foci experiments and 12 Gy for BRCA1 and RAD51 foci experiments. The cells were permeabilized in 0.5% Triton-X-100, incubated with appropriate primary and secondary antibodies then mounted on slides using Vectashield with DAPI. Cells were quantified by fluorescent microscopy and nuclei containing 4 foci/cell or more were scored as positive.

G2/M Checkpoint Assay

Histone, H3, phosphorylated on serine 10 (H3pS10) has been described previously as a marker for the G2/M checkpoint (194). Briefly, LCLs were irradiated or mock irradiated and returned to 37°C for 1.5 hours. Cells were fixed in 70% ethanol and incubated with appropriate primary and secondary antibodies followed by staining with Propidum Iodide (Invitrogen, Carlsbad, CA) before analysis by flow cytometry.

Antibodies

Immunofluorescence antibodies were: γ-H2AX 1:300 (Upstate, Charlottesville, VA), 53BP1 1:400 (Santa Cruz Biotechnology, Santa Cruz, CA), BRCA1 1:300 (Novus, Littleton, CO), RAD51 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA), Alexa Fluor 488 anti-mouse 1:300, and Alexa Fluor 568 anti-rabbit 1:400 (Invitrogen, Carlsbad, CA).

Kinetics Model and Statistics

Unless otherwise noted, values for all experiments are plotted as the average of 3 independent experiments and error bars are given as +/- 1 standard deviation. The kinetics of γ-H2AX foci formation has been observed to be logarithmic in nature while

the resolution of foci is exponential (195, 196). These characteristics were expressed in a non-linear fit function combining logarithmic growth and exponential decay functions. An Interior-Trust-Region non-linear optimization algorithm was implemented to fit the data with this model (197). A visual reference of this technique is shown in **Figure 6**. P-values are based on the two samples of unequal variance two-tailed student's t-test calculated in Microsoft Excel (Microsoft, Redmond, WA).

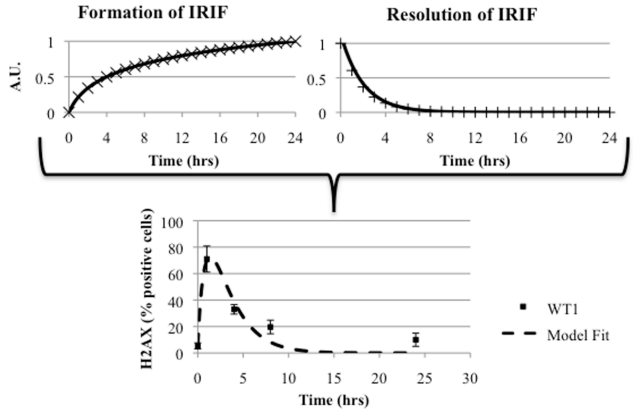

Figure 6. The top panels describe the two components of the model fit to the γ-H2AX kinetic data. The left panel represents the formation of foci component of the model and provides a measure of a cell line's ability to recognize of DSBs, indicated by formation of γ-H2AX foci. The right panel is the component of the model that relates to the resolution of γ-H2AX foci, inferred to be the repair of DSBs. The resolution of foci component provides a measure of the rate and completeness of repair. The bottom panel demonstrates the combination of the two components of the model and the model's fit of a WT repair kinetic.

Results:

NCA performed on whole blood is a promising surrogate assay for the CSA.

Testing radiosensitivity directly from whole blood samples represents an attractive approach for enabling rapid radiosensitivity testing. The NCA protocol outlined by Nahas et al. was adapted for use on whole blood samples and 19 samples submitted for radiosensitivity testing were assayed(184). Consistent with results on LCLs, the NCA correlated with the CSA (**Figure 7**). The linear regression model (CSA = slope(NCA) + intercept) that fit the NCA vs CSA data was used to predict clonogenic survival based on the % DNA repair given by the NCA. The linear model and the NCA to predict radiosensitivity in suspected XCIND patients resulted in a 79% agreement between the NCA prediction and the CSA result and had a positive predictive value (i.e. a finding of radiosensitivity) of 75% and a negative predictive value of 86%. The sensitivity was 90% and the specificity was 67%. These data suggest that the NCA can be performed on whole blood and is a promising rapid surrogate for the CSA in radiosensitive populations.

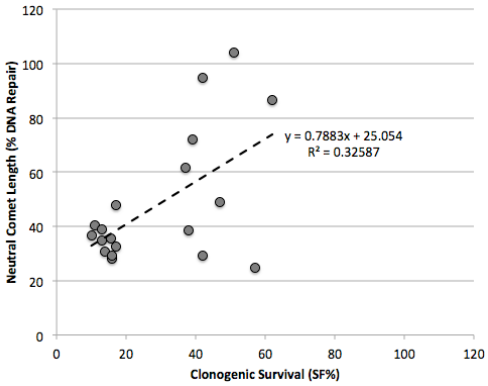

Figure 7. Comparison of the NCA on whole blood and the CSA on LCLs derived from the whole blood samples for 19 suspected XCIND patients submitted for radiosensitivity testing. These data indicate a linear correlation between the CSA and NCA (R = 0.57).

γ-H2AX IRIF kinetics identifies 'fingerprint' curves for known DNA repair disorders.

A secondary assay that could provide information about the specific defect present in a patient sample would be useful for diagnosing individual patients and guiding treatment. Previous studies have indicated that the γ-H2AX biomarker might be useful for identifying cell line or disease specific DNA repair phenotypes, but also have indicated that measuring γ-H2AX foci at single time points post-irradiation has little diagnostic usefulness(55, 184). Confirming my original hypothesis, measurement of γ-H2AX foci at multiple time points, indeed, provided a clearer picture of DSB induction and resolution dynamics in individual cell lines than assessing foci at one or two time points post-IR. The agreement between a finding of radiosensitivity by single time point γ-H2AX foci measurements or foci kinetics and the CSA in these samples was improved from ~50% to 87%, respectively. Further, unique differences were observed between WT-LCLs, RS-LCLs and between known XCIND disorders (**Figure 8**).

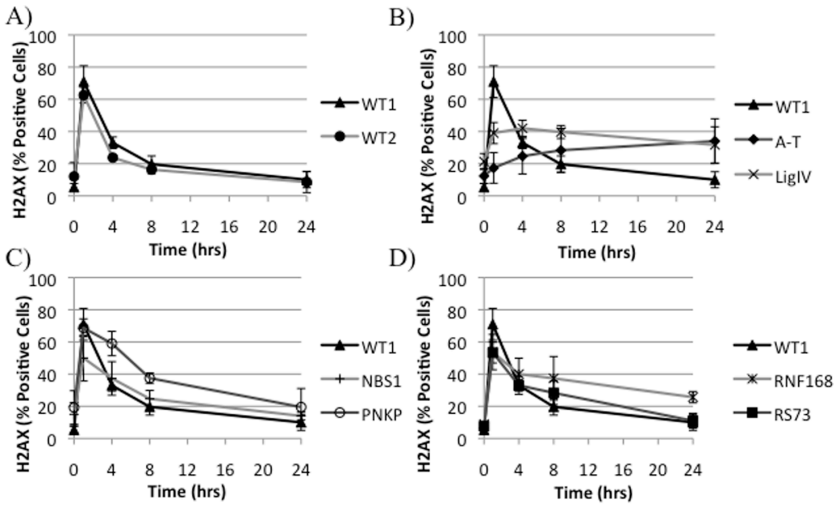

Figure 8. Shown are data of γ-H2AX foci kinetics measured at 0 hr (no-IR) and 1, 4, 8, and 24 hours after 1 Gy. Percent positive cells are presented and are scored as the percentage of nuclei with 4 or more

foci/nucleus. A) WT characteristic γ-H2AX foci curve indicating rapid recognition and repair of DSBs. B) A-T and DNA LigIV characteristic curves indicating persistent damage at 24 hours post-IR. C) γ-H2AX foci kinetic curves for NBS1 and PNKP deficient patients indicating a delay in DNA repair phenotype but a WT shaped curve. D) RNF168 patient and RS73 display a γ-H2AX kinetic curve similar to WT1 until 4 hours post-IR where a slower, linear repair rate is observed at 8 and 24 hours post-IR.

WT-LCLs display a γ-H2AX kinetic curve indicative of rapid recognition of DSBs and recruitment of γ-H2AX to breaks followed by the rapid repair of DNA damage and resolution of foci (**Figure 8A**). A-T LCLs were characterized by a delay and reduction in the formation of γ-H2AX foci and foci persisted at 24 hours (**Figure 8B**). Similarly, DNA LigIV exhibited poor resolution of foci by 24 hours, but formed foci at a higher efficiency than A-T LCLs at 1 hour **(Figure 8B)**. PNKP and NBS, conversely, displayed WT shaped curves characterized, however, by a slower resolution kinetic. Additionally, NBS was characterized by reduced positive cells at 1 hour **(Figure 8C)**. Lastly, the RNF168-LCL displayed WT-like repair rates until four hours post-IR, at which time a slower, linear repair rate was observed **(Figure 8D)**.

'Fingerprint' γ-H2AX kinetic implicates defective repair pathway downstream of 53BP1 in RS73:

To determine if the characteristic curves from known radiosensitivity disorders could be informative for identifying specific pathway defects, I focused on RS73 from my initial screen of RS-LCLs of unknown etiology. RS73 displayed γ-H2AX foci kinetic changes at four hours similar to the RNF168-LCL **(Figure 8D)**. My hypothesis that the

kinetic curves were unique to specific repair pathway defects suggested that RS73 had a defect in the same pathway as the RNF168-LCL. This prompted me to analyze 53BP1 foci kinetics, a biomarker of the entire CULC pathway, which is disrupted in RNF168 deficient patients (189).

In WT-LCLs the γ-H2AX and 53BP1 kinetic curves closely mimicked each other, consistent with the two biomarkers being dependent on similar pathways for formation and resolution (**Figure 9A**)(57, 198). Similarly, in NBS1 and PNKP deficient LCLs, the γ-H2AX and 53BP1 foci kinetics mimicked each other suggesting the DNA repair defect was independent of, or downstream of, both biomarkers (**Figure 9B and C**). A-T and RNF168 LCLs displayed large differences between the γ-H2AX and 53BP1 kinetics suggesting that ATM and RNF168 deficiencies function upstream of the pathways associated with each biomarker (**Figure 9D and E**). The 53BP1 foci kinetics of RS73 mimicked those of the γ-H2AX foci kinetics, suggesting a defect independent of, or downstream of, 53BP1 foci formation (**Figure 9F**).

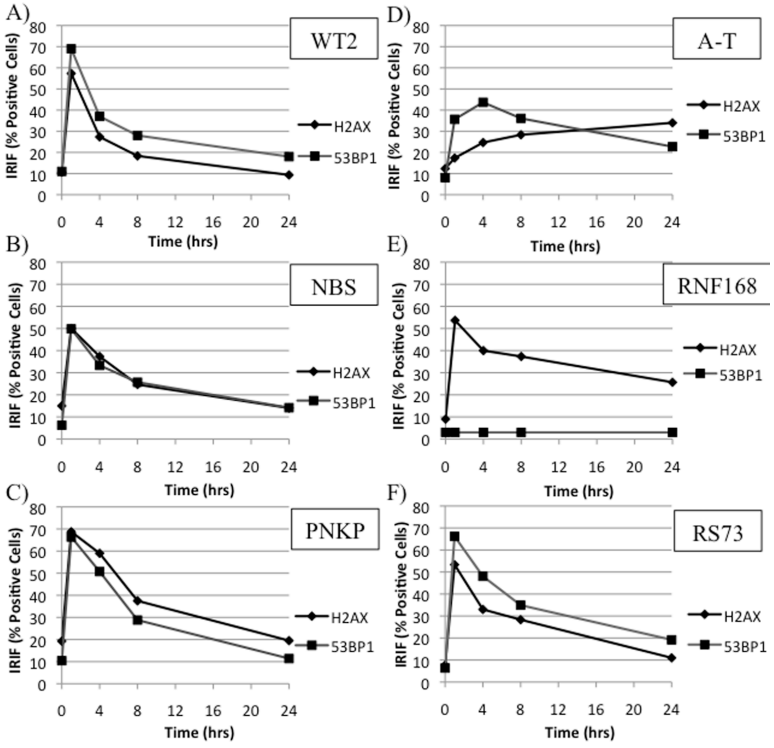

Figure 9. Both the γ-H2AX and 53BP1 foci kinetics are compared for the known XCIND disorders studied. Results are presented as the % cells positive for 4 or more foci/nucleus. A) WT2 is a representative WT LCL and shows that 53BP1 foci kinetics mimicking those of γ-H2AX. B) 53BP1 foci kinetics mimics γ-H2AX foci kinetics, indicating delayed DNA repair in an NBS-LCL. C) The PNKP deficient LCL had delayed resolution of 53BP1 foci which mimicked the response of the γ-H2AX foci kinetics D) Differential 53BP1 and γ-H2AX foci kinetics in a representative A-T LCL illuminating the dependency of both γ-H2AX and 53BP1 foci on ATM signaling. E) Similarly, the RNF168 deficient cell line displayed differential 53BP1 and γ-H2AX foci kinetics due to

dependence of 53BP1 on RNF168 for foci formation, but not γ-H2AX foci formation. F) γ-H2AX and 53BP1 foci kinetics in RS73 indicating that the 53BP1 foci kinetic follows the γ-H2AX foci kinetic.

RS73 confers a reduction in both BRCA1 foci formation and G2/M checkpoint activation after IR:

BRCA1 has been shown to function downstream of 53BP1 foci accumulation through ubiquitin binding and the above results prompted me to test BRCA1 foci kinetics (66)(This model has since changed after the time of study, discussed in Chapter 1). In addition to aiding in the localization of HR proteins, BRCA1 also interacts with 53BP1 at the sites of DSBs to influence the repair pathway utilized for a DSB(46, 64, 68). RS73 displayed a delay in BRCA1 foci formation, but did not display the severe reduction observed in the RNF168-LCL indicating that some BRCA1 recruitment to breaks was retained, though not at WT levels (**Figure 10A**). RAD51 foci formation in RS73 was similar to the WT-LCL, suggesting that the core of the homologous recombination pathway was intact(199) (**Figure 10B**).

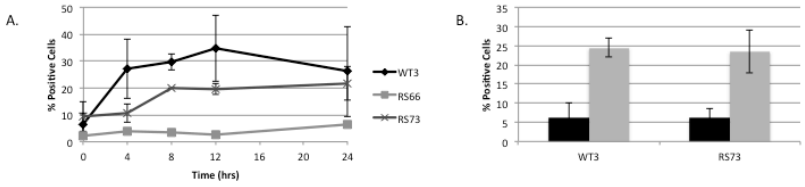

Figure 10. A) BRCA1 foci kinetics evaluated post 12 Gy in WT3, RNF168 and RS73. RNF168 is a negative control for stable BRCA1 foci formation post-IR. RS73 displays a delay and reduction in BRCA1 foci compared to WT3. Values are presented as the percentage of cells positive

for 4 or more foci/nucleus. B) Rad51 foci were evaluated 8 hours post 12 Gy in WT and RS73 cells to assess the fidelity of homologous recombination and are the percentage of cells positive for 4 or more foci/nucleus.

These data suggest that the reduction of BRCA1 foci at DSBs impacted alternative functions of BRCA1 in the DDR beyond its core role in HR. Previous experiments have demonstrated that adequate function of the BRCA1A complex is required for a functioning IR-induced G2/M checkpoint (65, 66). RS73 exhibited reduced activation of the G2/M checkpoint post-IR (**Figure 11**) strongly supporting this operational model of disrupted BRCA1 binding downstream of 53BP1 foci. However, immunoblotting revealed that all members of the BRCA1A complex were expressed in RS73 and full exome sequencing was unable to identify any causative mutations in BRCA1, nor the members of the BRCA1A complex (data not shown).

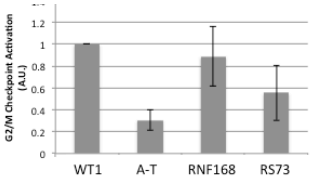

Figure 11. The G2/M checkpoint was assessed by identifying mitotic cells (staining positive for H3pS10) before and after irradiation. The difference between H3pS10 cells before and after irradiation was determined and normalized to WT levels, representing effective activation of the G2/M checkpoint. A-T and RS73 cells display a reduction in G2/M checkpoint activation.

Discussion:

Developing a predictive assay for radiosensitivity is a central challenge in radiation biology(200). Previously, both genetic association and molecular techniques have been applied to this problem (201, 202). The CSA SF% remains the 'gold standard', however, and identifying a more rapid surrogate assay would enable radiosensitivity testing in oncology patients and would also improve diagnostic turnaround times for suspected XCIND patients. The NCA adapted for use on whole blood correlated well with the CSA and represents a potential rapid surrogate for the CSA that could be completed in 2-3 days upon receipt of a blood sample for XCIND patient populations. The prediction of some radionormal cells as radiosensitive, however, suggests that the CSA should still be used as a follow-up assay for patients predicted to be radiosensitive or that follow-up kinetic measures with the γ-H2AX foci assay could be beneficial.

This improvement in turnaround time addresses the initial hurdle for translating radiosensitivity testing beyond diagnosing rare XCIND disorders. The NCA on whole blood could be used to screen oncology patients to determine those at risk for developing severe reactions to radiotherapy within the normal time between initial consult and the first radiotherapy fraction (~ 1 week). However, the general population of oncology patients, even those individuals with severe reactions to radiotherapy, are unlikely to be as hypersensitive to ionizing radiation as XCIND patients. Thus, future studies will be needed on a large cohort (100s to 1000s) of radiotherapy patients to determine if the NCA on whole blood demonstrated in this proof-of-concept study has the sensitivity and specificity to identify the ~5-10% of individuals who develop complications from radiotherapy.

The identification of a specific cause for the radiosensitive phenotype in XCIND patients, especially when it turns out to be a known repair disorder, is of paramount importance for guiding treatment and providing answers for a patient's family. Previous studies have explored, with some success, γ-H2AX foci methods to diagnose radiosensitivity in cell lines. However, in more heterogeneous populations, significant correlations between foci levels and cell survival have been difficult to achieve (195, 196, 202, 203). I found that, while γ-H2AX is not a stronger surrogate than the simpler NCA for clonogenic survival, the repair kinetics in a sampling of known XCIND disorders were unique to each disorder. Defects in repair function, as those found in A-T and LigIV LCLs, were characterized by persistent foci at 24hrs, representing residual, unrepaired DSBs. Reduced positive cells early post-IR in NBS and A-T LCLs illustrated the impact of DSB sensing protein defects on recognition of DSBs and recruitment of repair factors. RNF168 and PNKP deficiencies exhibited delayed resolution kinetics suggesting the presence of a subset of damage that required these proteins for timely repair(57). These characteristics are consistent with the known function of these various proteins in DSB repair pathways. Thus, the utility of the foci kinetic method is its ability to characterize patient specific repair kinetics for comparison against a library of known DNA repair disorders, such as the A-T, DNA LigIV, RNF168, etc. cell lines used in this study.

RS73 illustrates this utility to evaluate unknown repair defects and develop a 'candidate pathway' approach for diagnosing the underlying etiology of a patient's radiosensitivity. Analysis of the γ-H2AX and 53BP1 foci kinetic profiles in the RNF168-LCL and RS73 cells suggested a defect downstream of 53BP1 in RS73. A functioning, or partially functioning, homologous recombination pathway was indicated by the

formation of a) BRCA1 foci (although delayed) and b) RAD51 foci, which may explain the WT-like γ-H2AX foci levels at 24 hours. However, the G2/M checkpoint assay further supports a defect in the G2/M checkpoint pathway in RS73 that is more severe than that of the RNF168-LCL. This G2/M checkpoint defect appears in other deficiencies in the CULC pathway (61, 204) and interactions of the BRCA1A complex at damage sites play a central role in the activation of this checkpoint and guides the interaction between BRCA1 and 53BP1 in repair pathway choices(65, 66, 194). The data for RS73 indicated a defect in the BRCA1A pathway, however, immunoblotting and exome sequencing suggested that the BRCA1A complex was intact in RS73. In Chapter 3, I will revisit RS73, along with additional data, to discuss one of the causes for the DSB repair and clonogenic survival defects in this patient and the change in scientific approach that enabled the discovery.

The data presented in this chapter suggests a two-step diagnostic test involving the NCA and γ-H2AX foci kinetics. Patients would be screened for radiosensitivity by the NCA, initially. The γ-H2AX foci kinetics would be assessed for patients that scored as radiosensitive to determine what type of repair defect may be responsible. This approach could be particularly useful when exome sequencing identified a candidate gene variant predicted to cause radiosensitivity or a specific repair defect, but for which no existing study has confirmed the prediction. Beyond radiosensitivity testing for XCIND disorders, the γ-H2AX foci kinetic method could also be utilized to determine if specific tumors are good candidates for synthetic lethality based treatments, such as treatment with PARP inhibitors(69). This *functional* approach would be especially useful when a patient's tumor lacked BRCA1 or BRCA2 mutations, and would thus be excluded as a

candidate for PARP inhibitors, but exome sequencing identified a genetic defect likely to impact BRCA1 signaling and, thus, likely to respond well to PARP inhibitors. For instance, a BRCA1/2 γ-H2AX foci kinetics 'fingerprint' could be established to screen tumor cells against.

CHAPTER 4

ATM DEPENDENT MICRORNA-335 DISSRUPTS THE DNA DAMAGE RESPONSE BY TARGETING CTIP PROTEIN LEVELS

Previously, studies to identify novel radiosensitivity genes in patients presenting with a XCIND phenotype focused on assaying DNA damage response pathways to identify specific pathway defects. This 'candidate pathway' approach has succeeded in some cases for identifying novel radiosensitivity genes that are involved in DNA repair in the past. However, this approach is tedious, limited by the current level of knowledge for a particular pathway mechanism, and, frequently, falls short of identifying the specific genetic defect because of the lack of detailed mechanisms for most DNA damage response pathways. Thus, our laboratory utilized microarray technologies in an effort to rapidly identify candidate genes first, and then identify the impact of the candidate genes on radiosensitivity and DNA repair. One such study was a microRNA microarray experiment that identified miR-335 as a candidate DNA damage response modulating molecule. In this chapter, I tested whether miR-335 disrupts DNA repair and reduces clonogenic survival after irradiation when overexpressed in cell lines derived from suspected XCIND patients, HeLa cells, and MCF7 cells. Further, I found that miR-335

modulated DNA repair by targeting CtIP protein levels which resulted in reduced BRCA1 foci formation, reduced DNA DSB repair, and radiosensitivity.

Introduction

The DNA damage response (DDR) plays an essential role in deciding cell fate after DNA double strand breaks (DSBs) by arresting the cell cycle to allow evaluation of DNA integrity and signaling for repair or apoptosis(205). In this way, the DDR maintains genomic stability and is an indispensable defense mechanism against cell death or tumor development(206, 207). The molecular mechanisms of DSB-induced DDRs have been extensively characterized and post-translational modifications of proteins, such as by phosphorylation, ubiquitinylation, sumoylation and acetylation, play a crucial role(208). MicroRNAs (miRNAs) have recently emerged as endogenous gene regulators, but their role in the DDR remains largely unexplored. MiRNAs down-regulate protein expression by mRNA cleavage or translation repression(145), suggesting that miRNAs may be a new class of cellular regulators, targeting the protein components of the DDR pathways(132).

ATM (Ataxia-Telangiectasia Mutated) kinase plays a hierarchical role in the DSB-induced DDR(154, 209). ATM coordinates many cellular processes of the ionizing radiation induced DDR starting with the phosphorylation of specific serines or threonines on downstream protein substrates and these interactions are indispensable for recognizing DSBs and initiating repair(209, 210). Three mechanisms have been reported to regulate ATM expression: 1) promoter methylation(211, 212), 2) transcription activators(213) and 3) miRNA interactions with mRNA(131, 144, 214). Such modulation of ATM protein levels can result in the radiosensitization of cells, but, classically, radiosensitization has

been associated with mutations in the *ATM* gene that lead to loss of the protein and failure to activate downstream substrates(212, 215).

Recently, Hu et al. demonstrated that ATM protein levels are reduced by miR-421 overexpression and that this reduction in ATM levels is sufficient to induce a cellular phenotype similar to that found in ATM deficient cells(131). Further, miR-421-mediated ATM down regulation was recently identified as a major factor associated with clinical radiosensitivity in a patient being treated for squamous cell carcinoma(135). Interestingly, the ATM/miRNA interaction is also bidirectional. In addition to phosphorylating downstream protein targets, ATM also regulates the biogenesis of a subset of miRNAs by phosphorylating KH-type splicing regulatory protein (KSRP) which increases mature transcripts of a number of miRNAs(134). Similarly, the breast cancer susceptibility gene, *BRCA1*, has been shown to modulate specific miRNA biogenesis(133). BRCA1 plays an important role in avoiding tumor genesis; likely through the maintenance of genomic stability through cell cycle regulation and localization to DSBs, thereby facilitating DNA repair(72, 194, 216). Thus, ATM and BRCA1 highlight an intriguing connection between miRNA and the DDR.

We have extensively profiled over 100 cell lines derived from patients with A-T-like phenotypes (e.g. reduced clonogenic survival levels characteristic of radiosensitive patients), but of unknown etiology(2, 3). These 'radiosensitive' lymphoblastoid cell lines (RS-LCLs) have been used to interrogate some of the mechanisms of DNA repair in an effort to identify novel molecules involved in the DDR(156, 177, 184, 217). To complement the functional DNA repair assay methodologies used in previous studies, Dr. Hu performed a miRNA expression microarray screen on a panel of RS-LCLs to identify

candidate miRNAs that associate with radiosensitivity and, thus, with DDR defects in an unbiased way. In this chapter, I focus on one of these miRNAs, miR-335, which was down-regulated post-IR in an ATM-dependent manner and which had been associated with increased metastatic and re-initiation potential of breast cancer when down-regulated(139, 140, 148).

MiRNA-335 is located within the mesoderm specific transcript homolog (*MEST)* gene which is a maternally imprinted gene on chromosome 7(218). Dr. Hu confirmed that miR-335 is, indeed, down regulated post-irradiation (IR) in an ATM kinase activity dependent manner and is co-transcribed with *MEST*(148, 219). Further, Dr. Hu's data and Shi et al. indicated that the down regulation of miR-335, by ATM, was mediated by ATM phosphorylation of the transcription factor CREB; an ATM dependent transcription factor predicted to bind to the common promoter of miR-335 and *MEST*(148, 220). CREB, in turn, was shown to transcriptionally promote a significant amount of endogenous miR-335 expression(148).

The regulation of miR-335 by ATM post-IR led us to postulate that miR-335 is involved in the DDR, likely by targeting a protein involved in DSB recognition or repair. *In silico* analysis of the miR-335 sequence predicted that miR-335 might target CtIP protein levels, a protein involved in cell cycle regulation and DNA end resection to promote homologous recombination repair (HRR)(40, 72, 148, 221). It was postulated that miR-335 was linked to the DDR through CtIP. In this chapter, I tested whether or not CREB bound to the *MEST*/miR-335 promoter region in an IR-dependent manner to confirm that this promoter region was a bona fide target of the CREB transcription factor. Further, I tested whether *MEST* promoter methylation contributed to the modulation of

miR-335 levels observed post-IR(148). Finally, I tested whether miR-335 was functionally linked to the DDR and radiosensitivity and whether this link was, indeed, due to miR-335 suppression of CtIP protein levels. The working model for IR induced regulation of miR-335 and the role miR-335 plays in the DDR is summarized in **Figure 12**.

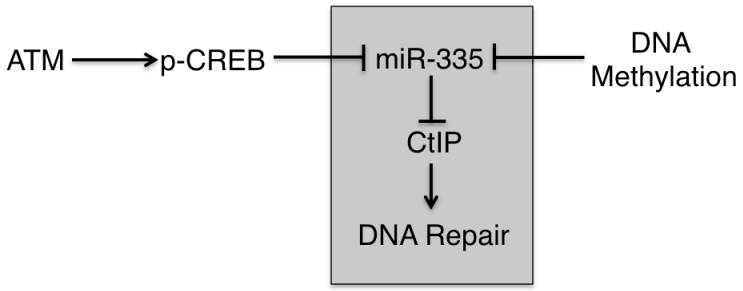

Figure 12. Working model of the ATM--miR-335--CtIP signaling axis. ATM, upon activation after DSBs, phosphorylates CREB, which in turn releases from the promoter region for miR-335, resulting in down regulation. Under normal conditions, the maternal transcript of miR-335 is suppressed by methylation. MiR-335 targets CtIP and results in reduced protein levels that impact upon DNA repair. The grey box highlights the focus of this chapter, the working model of miR-335 modulation of the DDR.

Materials and Methods

Cell culture, miRNA precursors, antisense morpholino oligonucleotides and irradiation.

MCF7 cells were cultured in DMEM media with 10% FBS, 1% streptomycin/penicillin/glutamine (PSG) and 10ng/ml insulin. HeLa cells were cultured in DMEM with 10% FBS and 1% PSG. Lymphoblastoid cell lines were cultured in RPMI1640 media with 10% FBS and 1% PSG. Precursor miR-335 and pre-miR-CTL

were purchased from Applied Biosystems (Foster City, CA). Antisense Morpholino Oligonucleotides (AMO) were custom synthesized based on the pre-miR-335 target sequence and conjugated with non-peptide chemicals that are used to deliver AMO-miR-335 intra-cellularly, i.e. vivo-AMOs (Gene-Tools, Philomath, OR). The sequence of AMO-miR-335 is 5'-ATCAACAGATATAAACAGCAGG. A standard control vivo-AMO (AMO-CTL) was also purchased from Gene-Tools. CtIP without the 3'UTR was amplified by PCR against the cDNA generated from HeLa total RNA and cloned into a pcDNA3 vector. Transfections for miRNA precursors were done with Lipofectamine RNAiMax and plasmids with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. LCLs were pretreated with AMO-miR-335 (1μM) or AMO-CTL (1μM) for 3 days followed by re-treatment and feeding 1 day before collecting cells for experiments. Irradiation was performed using a Mark IV cesium-137 sealed source irradiator at a dose rate of ~4.5 Gy/min.

RNA extraction and Real-time Quantitative PCR (RT-qPCR)

Total RNA from cultured cells was extracted by the mirVana miRNA isolation kit (Applied Biosystems). TaqMan microRNA expression assays (Applied Biosystems) were used to quantitate mature miR-335 expression following the manufacturer's protocol. U6 expression was used as an internal control for miR-335 expression.

Clonogenic survival assay

HeLa cells were transiently transfected with pre-miR-CTL, pre-miR-335, empty vector, and/or CtIP delta-3'UTR vector (50nM) using RNAi Max. After 48 hours, cells were plated at 500 cells/well in 6-well dishes and then incubated for 24 hours. Cells were then treated with a series of IR doses (0, 1, 2 and 5 Gy) and grown for 10-14 days before

staining with 1% crystal violet. Plates were imaged with a VersaDoc Imaging System and clumps of cells containing more than 50 cells were scored as colonies with the Quantity One program (Bio-Rad, Hercules, CA). To generate a radiation survival curve, the surviving fraction at each radiation dose was normalized to that of the non-IR control. The CSA for LCLs was performed as previously described after treatment with AMO-CTRL or AMO-miR-335(3) .

BrdU incorporation assay

To analyze the S-phase checkpoint, HeLa cells were transiently transfected with pre-miR-CTL (50nM) or pre-miR-335 (50nM) using RNAi Max and irradiated with 10 Gy 48 hours after transfection. BrdU was added to cells after a 2h post-IR incubation to allow S-phase arrest and incubated for 2h for BrdU labeling. Cells were collected by trypsinization and centrifugation. Cells were stained with a FITC labeled anti-BrdU antibody following the manufacturer's protocol for BrdU Flow Kits (BD Pharmingen, NJ) and analyzed by FACS.

Neutral comet assay (NCA)

The NCA was performed according to the manufacturer's protocol (Trevigen Inc.) and the comet scoring and calculation was done as previously described (217). Briefly, HeLa cells were transiently transfected with pre-miR-CTL (50nM) or pre-miR-335 (50nM) and irradiated with 15 Gy 48 h after transfection. Cells were embedded in agarose gel, lysed *in situ*, electrophoresed, and stained with SYBR Gold. Slides were imaged using an Olympus fluorescent microscope equipped with AxioVision camera and acquisition software. The images acquired were analyzed with Comet Score software (TriTek Corp.) and scored with the Comet Score software as previously described(217).

A minimum of 50 cells was analyzed per experiment and experiments were performed in triplicate. The Tail Moment (TM) was used as the metric to measure DNA damage and the ratio of non-IR and 5 h post-IR samples were calculated to give the "percent DNA repair". The percent DNA repair was normalized to the WT control. This normalization set the WT control to 100%, or full repair.

BRCA1-irradiation induced foci (IRIF)

Immunofluorescent detection of IRIF was performed as previously described (222). LCLs were irradiated with 12 Gy and BRCA1 antibodies were used at 1:300 (Novus, CO) 8h post-IR. Following primary antibody incubation, the cells were washed 3 times in 0.1% Triton-X-100 in PBS and blocked again in 10% FBS at room temperature for 1 hr. Coverslips were incubated with 1:400 Alexa Fluor Anti- Mouse 488 (Invitrogen, Eugene, OR) for 1 hr. After washing 4 times with PBS, coverslips were mounted onto slides in Vectashield with DAPI. Cells were visualized using an Olympus fluorescent microscope and AxioVision Rel. 4.7 software. A minimum of 100 nuclei was analyzed.

Western blotting

Nuclear lysates from HeLa cells or LCLs were isolated with the NE-PER kit (Pierce, Rockford, IL). Cells were irradiated with 10 Gy, harvested 2 hrs post-IR and then lysed. Equal amounts of total protein for each sample (50μg or 100μg) were loaded onto SDS-PAGE and immunoblot analysis was performed with the following antibodies: rabbit-anti-CtIP (Bethyl Laboratory Inc.TX), mouse-anti-P84 (GeneTex, TX).

Chromatin immunoprecipitation assay

The Chromatin immunoprecipitation (ChIP) assay was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signal Technologies, Boston, MA)

according to manufacturers procedures. Briefly, ~4 x10^7 HeLa cells were irradiated or mock irradiated and incubated for 2 hours. Proteins were cross-linked to chromatin 2 hours post-IR and the cells were collected. DNA was fragmented using enzymatic digestion and collected after cell lysis. The digested chromatin was precipitated using an anti-CREB or anti-IgG antibody (Cell Signal Technologies) and purified. Purified DNA was amplified using primers specific to the MEST promoter region (forward: TGTAAAGGAAACCTGCCCCG, reverse: GTGGGTACTGAACCGTGAGA), which yield a product of 232 base pairs. Samples were run on a 2% agarose gel to visualize PCR fragments.

MEST promoter methylation status

The *MEST* promoter methylation assay was performed using the EpiTech Methyl PCR Assay (Qiagen). 10^7 cells were mock treated or irradiated with 10 Gy and DNA was collected after 2 hours. DNA was processed according the manufactures specifications and amplified using the SybrGreen qPCR assay and MEST primer (Qiagen).

Statistics

All experiments were repeated independently three times (n=3) unless otherwise noted. The Student's t-test was used to evaluate the difference of two groups of data in all the pertinent experiments. P-values of < 0.05 (using the two-tailed, unpaired t-test) were considered significant. Data is presented as the mean +/- one standard deviation.

Results

CREB associates with MEST/miR-335 promoter in an irradiation dependent manner.

The miRNA microarray screen study by Dr. Hu led us to focus on miR-335 because it was differentially expressed post-IR and because this modulation was dependent on ATM, an important molecule in the DDR. The early work by Dr. Hu indicated that the modulation of miR-335 by ATM was not direct, but rather mediated by ATM phosphorylation of the transcription factor CREB(148, 220). siRNA suppression of CREB protein levels resulted in a significant reduction in miR-335 levels and loss of the post-IR down regulation of miR-335(148). It remained unclear, however, if the *MEST*/miR-335 promoter region was a bona fide target of CREB or if the lack of post-IR down regulation of miR-335 levels observed by Dr. Hu was an artifact of CREB siRNA. Thus, I used the chromatin immunoprecipitation (ChIP) assay and primers specific to the promoter region of *MEST* that CREB was predicted to associate with to demonstrate that CREB, indeed, was bound to the *MEST* promoter region pre-IR. Further, I demonstrated that this binding dissociated post-IR, consistent with our model of CREB mediated miR-335 regulation (**Figure 13**).

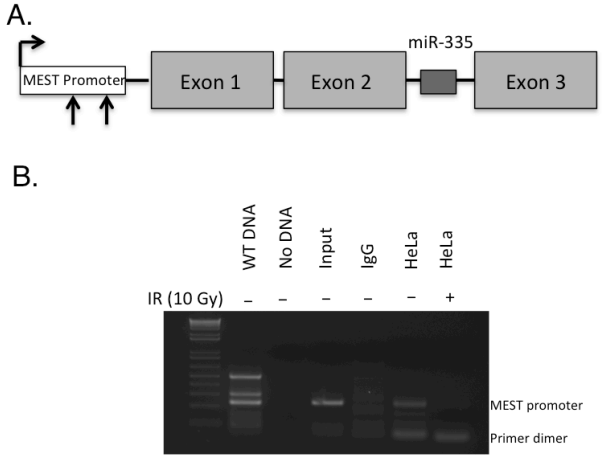

Figure 13. A) Diagram of the first three exons of *MEST* indicating that miR-335 is located in the second intron. The predicted CREB promoter region for miR-335 and *MEST* is approximately located between the black

arrows, which are the locations of the forward and reverse primers. B) DNA fragments bound to CREB pre- and 2 hrs post-IR were immunoprecipitated and a region specific to the *MEST* promoter region was PCR amplified. The resulting captured image indicates binding of CREB to the promoter region before irradiation, but dissociation from the promoter region post-IR.

MEST promoter methylation is not modulated by irradiation.

MEST is a maternal imprinted gene and the maternal transcript is silenced by DNA methylation. Promoter methylation has previously been shown to regulate *MEST* expression(218). While the data suggested that CREB was responsible for both endogenous miR-335 expression and the post-IR down regulation of miR-335 levels, it was possible that changes in promoter methylation might also contribute to the post-IR modulation of miR-335. I confirmed that promoter methylation did not change post-IR, indicating that change in methylation status was not responsible for the post-IR down regulation of miR-335 levels (**Figure 14A and B**).

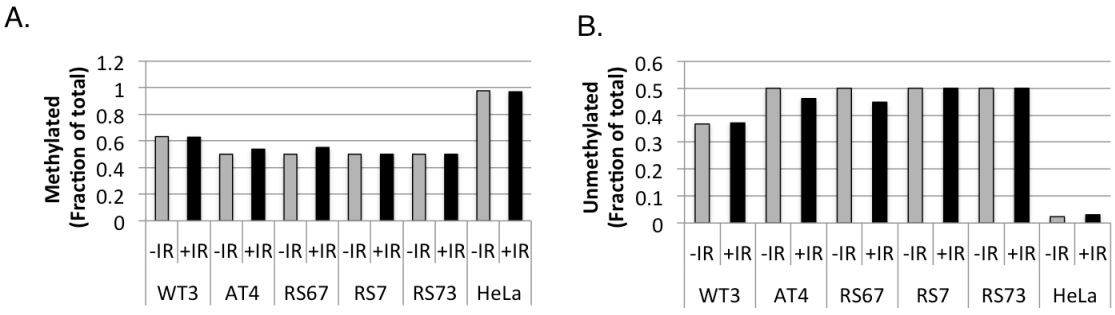

Figure 14. Representative data from the *MEST* promoter methylation status assay. A) The proportion of total transcripts that are methylated is displayed. Approximately 50% of transcripts are methylated, consistent

with a maternally imprinted gene. Further, the proportion of methylated transcripts does not change 2 hrs post-10 Gy. B) Approximately 50% of transcripts are unmethylated and that the proportion of unmethylated transcripts are not modulated after 10 Gy.

DNA repair and cell cycle checkpoints are modulated by miR-335 overexpression.

MiR-335 was predicted to target CtIP by the MicroCosm software. CtIP regulates DNA end resection during HRR, influences the choice of DNA repair pathway after DSBs, and is involved in cell cycle checkpoint activation (40, 221, 223). Thus, we hypothesized that the DDR may be modulated by miR-335 through resulting changes in CtIP protein levels. Confirming the prediction that miR-335 targets CtIP protein levels, Dr. Hu demonstrated that miR-335 overexpression targeted the 3'UTR of CtIP and reduced CtIP protein levels when miR-335 was overexpressed in HeLa cells(148). However, it remained unclear if miR-335 targeting of CtIP protein levels had a functional impact on the DDR and cell survival. Radiation resistant DNA synthesis (RDS) is a characteristic of A-T LCLs, which have a defective intra-S phase cell cycle arrest when responding to IR; a checkpoint for which CtIP also plays a role (223-225). Consistent with a functional consequence of reduced CtIP levels due to miR-335 overexpression, an RDS phenotype was induced in HeLa cells by overexpressing miR-335 (**Figure 15A**). Further, overexpression of miR-335 in HeLa cells induced a delay in DNA repair visualized by residual comet tails 5 hours post-IR in the neutral comet assay (**Figure 15B**).

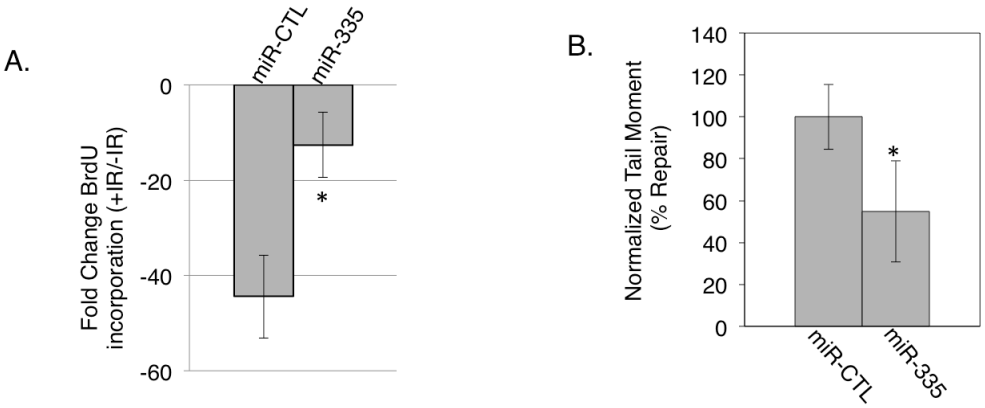

Figure 15. A) BrdU was used to stain cells undergoing synthesis 2 hrs post-10 Gy after overexpression of miR-335 or miR-control (CTL) in HeLa cells and indicates activation of the intra-S phase cell cycle checkpoint (i.e. reduction in BrdU incorporation) in miR-CTL transfected cells, but not in miR-335 overexpressing cells. Values are given as the fold change in BrdU incorporation post-IR (+IR) compared to pre-IR levels (-IR). An asterisk indicates significance at $p<0.05$. B) The neutral comet assay was used to assess DNA repair 5 hrs post-15 Gy. MiR-335 overexpressing HeLa showed delayed repair kinetics compared to miR-CTL transfected cells. Values are given as % Repair which signifies the percent return to baseline comet tail lengths 5 hrs post-IR. An asterisk indicates significance at $p<0.05$.

MiR-335 reduction of CtIP protein levels induced DNA repair defect and radiosensitive phenotypes.

BRCA1 interacts with CtIP to facilitate DNA repair (221, 226). Assessment of BRCA1 foci formation at DNA breaks post-IR in HeLa cells demonstrated that miR-335 overexpression reduced BRCA1 focus formation or retention at DSBs. This defect was

rescued by transfecting CtIP without the 3' UTR in to the cells, supporting the working model that miR-335 modulates the DDR through CtIP (**Figure 16A**). The DDR defects caused by miR-335 overexpression in HeLa cells suggested that miR-335 may sensitize cells to ionizing radiation. I observed reduced clonogenic survival when miR-335 was overexpressed in HeLa cells, a trend that was most notable at 5 Gy, indicating the DDR defects induced by overexpression of miR-335 culminate in radiosensitization of cells. Similar to the BRCA1-IRIF results, re-expression of CtIP without the 3' UTR was able to abrogate the radiosensitization of HeLa cells by miR-335-overexpressing (**Figure 16B**).

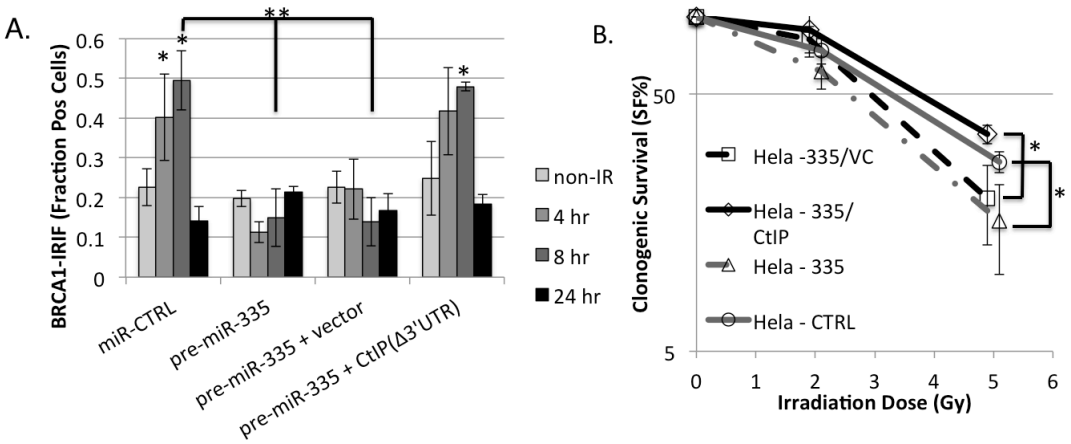

Figure 16. A) BRCA1-irradiation induced foci (IRIF) kinetics were assessed post-12 Gy in HeLa cells. Significant BRCA1 foci were induced in miR-CTL transfected cells while miR-335 overexpression, miR-335 and miR-335-vector, abolished BRCA1 foci recruitment or retention at DSBs. Addition of CtIP without its 3'UTR (delta3'UTR) abrogated the BRCA1 foci formation or retention defect. Values are presented as the percentage of cells positive for 4 or more foci/nucleus. A single asterisk indicates significance at $p<0.05$ level comparing post-IR foci values to

non-IR values within the same cell condition. Two asterisks indicate significance at $p<0.05$ level comparing the 8 hr BRCA1 foci levels across cell conditions. B) Clonogenic survival was assessed in HeLa cells post-2 and -5 Gy. The x-axis values for each data point are offset to improve viewing of the data. MiR-335 overexpressing cells (Hela – 335 and Hela-335/vector control) were sensitized to radiation while addition of CtIP without the 3'UTR (Hela-335/CtIP) abrogated the radiosensitization of miR-335 overexpression. An asterisk indicates significance at the $p<0.05$ level.

miR335-CtIP axis was disrupted in two RS-LCLs.

In addition to observing different post-IR changes in miR-335 expression between WT and A-T LCLs, the miRNA microarray study identified two patient-derived RS-LCLs with elevated endogenous levels of miR-335. To further support the working model of the proposed ATM-miR335-CtIP pathway and the impacts of miR-335 expression on the DDR, we chose to focus on the two RS-LCLs previously described by our lab with constitutive miR-335 overexpression indicated by the microarray data, RS7(184) and RS73(156). MiR-335 overexpression was validated by RT-qPCR and miR-335 levels were elevated >10 fold when compared to WT and A-T LCL controls (**Figure 17**). Significant reduction of CtIP protein expression was observed in RS7, similar to HeLa cells overexpressing miR-335(148).

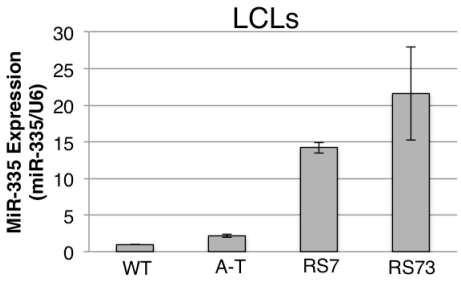

Figure 17. RT-qPCR results showing overexpression of miR-335 levels in RS7 and RS73 relative to WT and A-T levels. The WT level is the average of three different WT-LCLs. A-T, RS7, and RS73 values were normalized to the WT average and represent the fold increase in miR-335 expression compared to the WT pool. MiR-335 expression was normalized to U6 as an internal control.

To further demonstrate the inverse relationship between miR-335 and CtIP protein levels in the naturally miR-335 overexpressing RS-LCLs, an antisense morpholino oligonucleotide (AMO-miR-335) was used to suppress miR-335 expression. AMO-miR-335 was designed to be complementary to miR-335, thereby inhibiting the processing of pre-miR-335 into mature transcripts and also blocking binding of mature transcripts to mRNA targets. Treatment of RS7 and RS73 LCLs with AMO-miR-335 increased CtIP protein levels, confirming that overexpression of miR-335 was responsible for reductions in CtIP in both RS7 and RS73 LCLs (**Figure 18**).

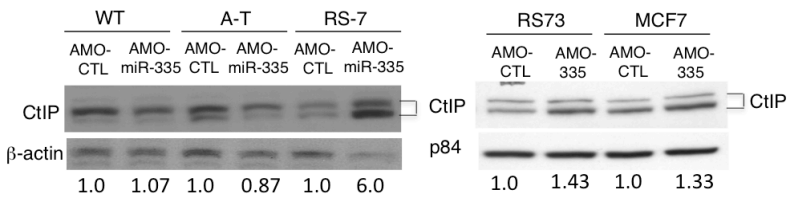

Figure 18. Western blotting results for nuclear CtIP protein levels in LCLs treated with an AMO designed to inhibit miR-335 (AMO-miR-335) or a control AMO (AMO-CTL). RS7, RS73, and MCF7 (also miR-335 overexpressing) cells show relative increases in nuclear CtIP protein levels after treatment with AMO-miR-335. The relative change in CtIP protein levels after AMO-miR-335 treatment was determined by densitometry and is indicated under the respective lanes and is normalized to the cell line AMO-CTL value.

MiR-335 disruption of CtIP results in BRCA1 foci defects and 'radiosensitivity' in RS7 and RS73 LCLs.

In a previous study, RS7 cells demonstrated a RDS phenotype and a delay in DNA repair, similar to HeLa cells overexpressing miR-335(184). RS73 also displayed delays in DNA repair demonstrated by γ-H2AX foci kinetics and a G2/M checkpoint defect-a checkpoint that is also dependent on CtIP(156, 223). In addition, RS73 LCLs were found to have a defect in BRCA1 foci formation(156), similar to miR-335 overexpressing HeLa cells, and subsequent study of RS7 LCLs also showed reduced BRCA1 foci formation (**Figure 19**). Taken together, these are consistent with our operational model in which miR-335 impacts the DDR through CtIP and BRCA1.

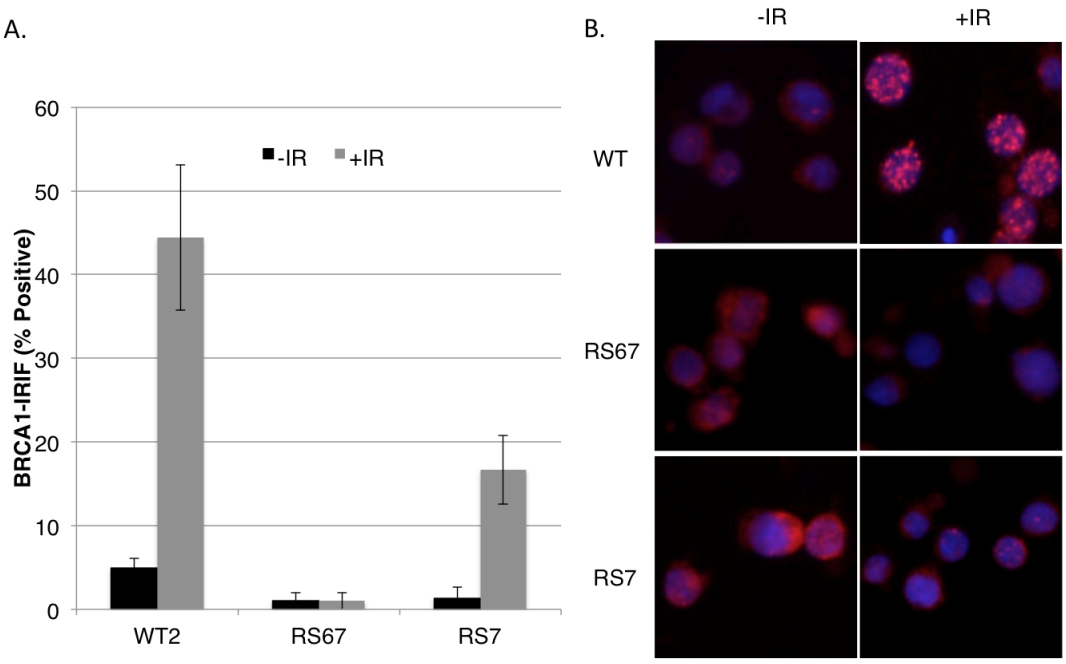

Figure 19. BRCA1 irradiation induced foci (IRIF) in RS7-LCLs. A) BRCA1 foci were quantified 8 hrs post 12 Gy and indicate reduced post-IR induced BRCA1 foci in RS7. RS67 represents a negative control cell line for BRCA1 foci. Results are presented as the percent of cells positive for 4 or more foci/nucleus. B) Representative images of BRCA1 foci (red) within nuclei (blue, dapi staining) pre- and 8 hrs post-12 Gy.

To test this hypothesis, the constitutively miR-335 overexpressing RS7 and RS73 LCLs were treated with AMO-miR-335, which resulted in the partial restoration of BRCA1 foci (**Figure 20A**). The DDR defects observed in RS7 and RS73 suggest that miR-335 modulates the DDR in patient derived cells, and when strongly overexpressed, reduces DNA repair and provides one possible explanation for the reduced colony survival (i.e. radiosensitivity) observed in these RS-LCLs(46, 194). To directly demonstrate the connection between miR-335 overexpression and radiosensitivity, I treated RS7 and RS73 cells with AMO-miR-335 and found that clonogenic survival post-

1 Gy significantly improved with AMO-miR-335 treatment but was not affected by AMO-Control (AMO-CTL) treatments (**Figure 20B**).

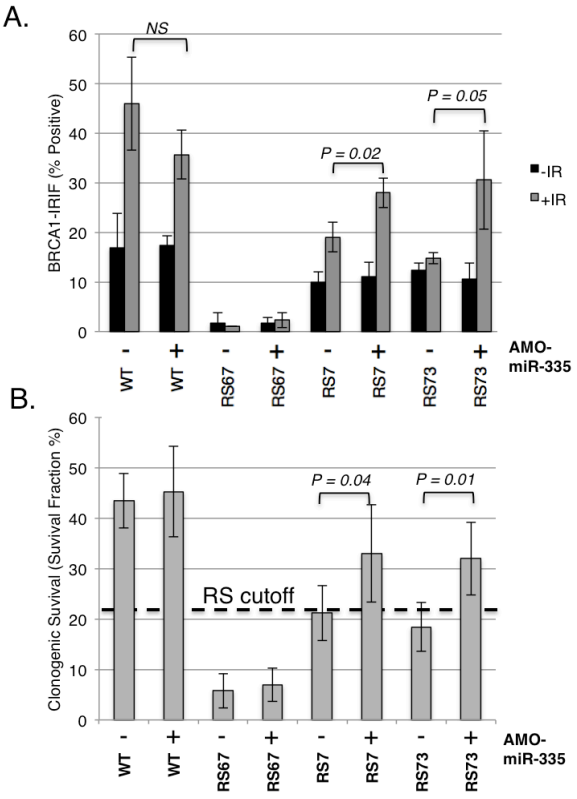

Figure 20. AMO-miR-335 abrogates BRCA1 foci and radiosensitive defects. A) BRCA1 irradiation induced foci (IRIF) were assessed 8 hrs post-12 Gy in LCLs treated with AMO-miR-335 (+) or AMO-CTL (-). AMO-miR-335 abrogated the BRCA1 foci defect in miR-335 overexpressing cells, RS7 and RS73. Results are given as the percent of cells positive for 4 or more foci/nucleus. B) Clonogenic survival was assess post-1 Gy with AMO-miR-335 (+) or AMO-CTL (-) treatment. AMO-miR-335 treatment partially rescued the radiosensitive (i.e. reduced clonogenic survival) phenotype in RS7 and RS73.

Interestingly, MCF7 breast cancer cells also have high levels of miR-335 expression and treatment with AMO-miR-335 increased CtIP protein levels (**Figure 18**). Pre-IR treatment of MCF7 cells with AMO-miR-335 increased BRCA1 foci at 8 hours post-IR and also enhanced the clonogenic survival of cells at different doses of irradiation, further supporting the working model of DDR disruption by miR-335 overexpression (**Figure 21**).

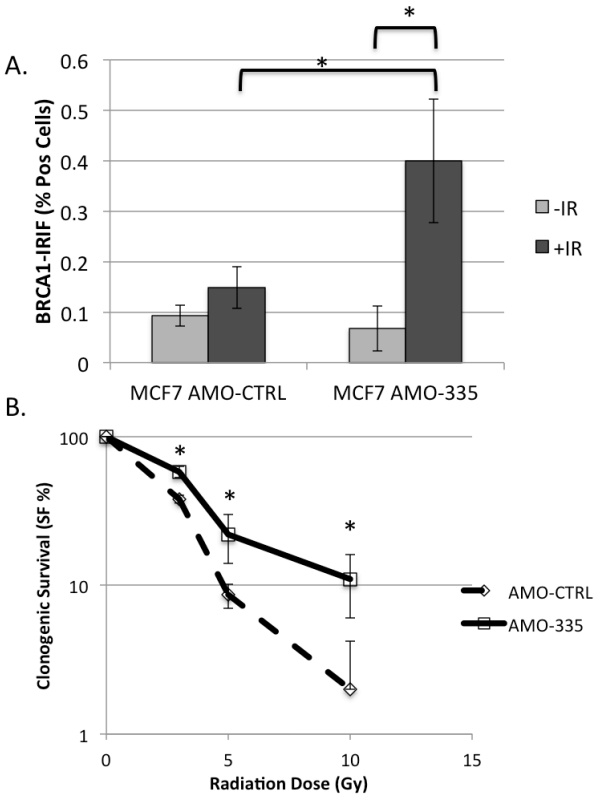

Figure 21. Suppression of elevated miR-335 levels in MCF7 cells with AMO-miR-335 elevated BRCA1 foci formation levels and rendered cells more resistant to irradiation. A) BRCA1 irradiation induced foci (IRIF) levels were assessed 8 hrs post-12 Gy in MCF7 cells treated with AMO-miR-335 (AMO-335) or AMO-CTL. MCF7 cells treated with AMO-miR-335 formed BRCA1 foci with greater efficiency than AMO-CTL treated

cells. Results are reported as the percentage of cells positive for 4 or more foci/cell. An asterisk indicates significance at $p<0.05$ level. B) Clonogenic survival was assessed in MCF7 cells treated with AMO-miR-335 (AMO-335) or AMO-CTL. AMO-miR-335 rendered MCF7 cells more resistant to radiation treatment. An asterisk indicates significance at $p<0.05$.

Discussion

Dr. Hu and I have identified a novel ATM-mediated DDR pathway involving a CREB--miR-335--CtIP axis. The proposed pathway impacts upon BRCA1 focus formation and BRCA1 retention at DSBs, cell cycle checkpoint regulation, DNA repair, and cell survival. This regulatory pathway is responsive to DSBs and our working model places ATM kinase, and the inferred subsequent phosphorylation of CREB, as a novel down-regulating mechanism of *MEST/*miR-335, which supplements the suppression of maternally-derived transcripts by promoter hypermethylation(139, 140, 220). The methylation status of the *MEST/miR-335* promoter region remained largely unchanged post-IR, indicating that methylation does not play a significant role in the IR-induced modulation of miR-335 and likely only functions to suppress the maternal *MEST* transcript. The ChIP assay confirmed that CREB binds directly to the promoter region of *MEST/miR-335.* This data, taken with the observation that CREB siRNA significantly reduced basal miR-335 expression, suggests that CREB is responsible for a substantial portion of the basal miR-335 expression. Previous studies indicated that ATM phosphorylates CREB post-IR and that this phosphorylation reduces the transcription activity of CREB(220). Our data demonstrating the removal of CREB from the MEST/miR-335 promoter region post-IR suggested that CREB is likely responsible for

the IR-induced and ATM-dependent down-regulation of miR-335. However, the extreme reduction of miR-335 levels after CREB siRNA made it technically difficult to fully assess the IR-induced response in cells where CREB was already knocked down.

Tavazoie et al. discovered that miR-335 expression levels were significantly lower in some metastatic breast cancers and that overexpression of miR-335 suppressed tumor metastasis(140). Subsequent studies have confirmed that miR-335 plays an important role in tumor initiation, propagation, and responses to genotoxic therapies(136, 139, 146, 147). The MCF7 breast cancer cell line used in our study strongly expressed miR-335 and had reduced BRCA1 foci levels. BRCA1 mutations are associated with a large increase in the risk of breast cancer and the data suggest that miR-335 overexpression may be another mechanism by which the functions of this cancer-associated gene can be modulated. My results also indicated that miR-335 overexpressing cells are more sensitive to IR-induced damage and that the significantly lower miR-335 expression found by Tavazoie et al. may render breast cancer cells more resistant to ionizing radiation. The importance of miR-335 expression levels, such as these two examples, might further explain why some metastatic breast cancers are more resistant to radiotherapy or chemotherapy while others are more sensitive. These studies would also suggest that miR-335, due to its dual ability to target DDR and metastasis genes, is a promising candidate as a radiosensitizer for radiotherapy or chemotherapy. For example, overexpression of miR-335 should cause tumor cells to become more sensitive to ionizing radiation or DSB-inducing agents, as observed in my study, and continued overexpression should suppress tumor metastasis and tumor re-initiation leading to improved short and long term tumor control.

Previous *in vitro* studies have described CtIP functions in the DDR by promoting DSB end-resection via the MRN complex(40), and that this role promotes selection of the HRR pathway via end-resection and BRCA1 recruitment to DSBs(221). Thus, reductions in CtIP protein expression by miR-335 overexpression had far-reaching consequences in the DDR, such as the effects on RDS, BRCA1 focus formation, and other DNA repair mechanism defects observed when miR-335 was overexpressed in HeLa cells or in the endogenously overexpressing RS7 and RS73 LCLs(156, 184). Transfection of CtIP to miR-335 overexpressing HeLa cells rescued the BRCA1 foci formation and clonogenic survival in these cells, further solidifying the notion that miR-335 modulates DDRs through CtIP. MiR-335 overexpression also changed cell cycle distribution, consistent with previous studies showing that CtIP functions in cell cycle regulation(194, 226, 227). It will be interesting to learn whether the miR-335-CtIP-regulated cell cycle changes affect the choice of DNA repair between non-homologous end joining and HRR when methodologies exist to interrogate these mechanisms together in asynchronous cells.

Our working model suggested that the down regulation of miR-335 by ATM--CREB in WT-LCLs could increase in CtIP protein expression post-IR and subsequently enhance the DDR. However, significant changes in CtIP protein levels post-IR were not observed in WT-LCLs, using the methods in this study. This suggested instead, in WT-LCLs, the ATM--CREB--miR-335--CtIP axis plays a subtle role in the DDR and most likely functions to 'fine tune' DNA repair. In contrast, RS7, RS73 and MCF7 cells illustrated the substantial influence that miR-335 can have on the DDR when grossly dysregulated, such as by strong miR-335 overexpression. RS7 and RS73 had defects in

post-IR DNA repair and cell cycle regulation, and both, similarly, did not efficiently form or retain BRCA1 foci at the sites of DNA damage(156, 184). Interestingly, both reduced BRCA1 foci and colony survival were responsive to suppression of miR-335 levels by AMO-miR-335. The partial abrogation of the defective DDR cellular phenotypes in these cells, and in MCF7 cells (e.g. BRCA1 foci), suggests that miR-335 overexpression is responsible for many of the DDR defects observed in these cells. Human CtIP mutations have been implicated in Seckel and Jawad syndromes and cells from these patients present with hypersensitivity to DNA damage(228). The present study suggests that miR-335 may be an additional mechanism by which CtIP can be disrupted in disease phenotypes and could present with similar clinical features (i.e., phenocopies) while lacking *CtIP* mutations.

CHAPTER 5

MTPAP MUTATION RESULTED IN INCREASED INDUCTION OF DNA DOUBLE STRAND BREAKS, REDUCED DNA REPAIR, AND RADIOSENSITIVITY

Similarly to microarray technologies in the past, full exome sequencing is emerging as a powerful tool for rapidly identifying disease associated genes, especially in cases where the etiology of a clinical phenotype remains elusive. To complement previous microarray studies, such as the study described in Chapter 3, our laboratory performed exome sequencing on 60+ radiosensitive cell lines of unknown etiology in an effort to identify novel genes associated with radiosensitivity. In this chapter, I describe two Amish siblings, RS63-3 and -7, for whom exome sequencing suggested that mutation of the mitochondrial poly-A-polymerase (*MTPAP*) was the cause of the radiosensitivity

observed in lymphoblastoid cell lines derived from the patients. I tested the hypothesis that the *MTPAP* mutation was, indeed, responsible for the radiosensitivity observed in the patients' cells by transfecting WT MTPAP back into the patients' cell lines. Additionally, I profiled DNA double strand break recognition and repair, apoptosis, reactive oxygen species, and mitochondrial function in the patients' cells to determine if patients with mutated *MTPAP* fit a classical model of XCIND (i.e. radiosensitivity arising from a DNA double strand break recognition or repair defect).

Introduction:

XCIND syndrome (x-ray sensitivity, cancer predisposition, immunodeficiency, neurologic involvement, and DNA repair deficiency) is a pleiotropic syndrome that includes a spectrum of radiosensitivity disorders resulting from mutation of DNA damage recognition and repair genes(1, 149). Ataxia-telangiectasia (A-T) is the archetypal XCIND syndrome disorder and results from mutation of the ataxia-telangiectasia mutated (*ATM*) protein kinase gene(229). A-T patients manifest both *cellular* and *clinical* hypersensitivity to ionizing radiation, which results from disruption of the nuclear function of ATM in the recognition and response to DNA double strand breaks (DSBs)(2, 3). Additional radiosensitivity disorders have been identified since A-T was first characterized, and, to date, have involved mutations in DNA DSB recognition or repair genes (5, 149).

Recognition of DSBs and their rapid repair is a complex and dynamic process involving at least two main repair pathways, non-homologous end joining (NHEJ) and homologous recombination (HR) repair. The MRN complex (Mre11, Rad50, and NBS1) and ATM are initial sensors of these lesions and are recruited to nuclear foci surrounding

DSBs(53). Loss of a single member of the MRN complex results in cellular radiosensitivity and clinical features that overlap with A-T(166, 230-232). Recognition of DNA double-strand breaks by the MRN complex and ATM initiate a signaling cascade leading to the activation of cell cycle checkpoints and the recruitment of other repair proteins, such as γ-H2AX and 53BP1, to the sites of DSBs(50, 51, 60, 233).

γ-H2AX forms nuclear foci that persist throughout the repair process and disengage from the repair site once the repair process has been completed(56). The initial recruitment of MRN, ATM, and γ-H2AX is followed by recruitment and retention of the 53BP1 repair protein via a chromatin ubiquitin ligase cascade (177, 188, 189). 53BP1 is important for modulating the choice of repair pathway between NHEJ, the dominant DSB repair mechanism, and HR, an error free repair mechanism that is only active in late S and G2 phase cells because it requires homologous chromatids (68, 234). When 53BP1 is displaced from the sites of DSBs by the breast cancer susceptibility protein, BRCA1, end resection via CtIP and the MRN complex occurs to initiate homologous recombination repair(64, 69, 234). Recently, reduction of CtIP protein levels was shown to disrupt BRCA1 focus accumulation leading to DSB repair defects and cellular radiosensitivity in two patient-derived cell lines(148).

While much of what is known about the cellular response to ionizing radiation induced damage is focused on molecules that accumulate at the sites of damage, the cellular environment would also be expected to play an important supporting role in facilitating these responses and maintaining genomic stability. Ionizing radiation generates DSBs via direct interaction with DNA. Many more breaks are generated from reactive oxygen species (ROS) that also cause oxidative and mitochondrial stress(104,

126). Cellular ROS homeostasis and ATP levels are required for supporting DNA repair and protecting against the generation of DSBs, and mitochondria are the main mediators of those levels(11, 99, 235, 236). Additionally, mitochondria act as central modulators of apoptotic cell death, which plays an important role in removing cells with persistent or oncogenic DNA lesions(126, 237). Thus, mitochondria would be expected to play a central role in the DDR. Several studies focusing on patients with mutations in mitochondrial genes have indicated dysfunction of mitochondrial ATP production, dysregulation of DSB repair gene expression, and genomic instability(115, 236, 238, 239). However, the exact mechanism by which mitochondrial dysfunction, such as that induced by mutation of genes important for mitochondrial function, can reduce survival after radiation exposure remains unclear(115, 238, 239).

In the present study, exome sequencing identified a homozygous 1432A>G DNA variant encoding the missense change N478D in the mitochondrial poly-A-polymerase (*MTPAP*) gene in two siblings of an Amish family affected with spastic ataxia. Lymphoblastoid cell lines (LCLs) derived from these siblings were radiosensitive, based on colony survival fraction post-irradiation (IR). Although the homozygous 1432A>G *MTPAP* variant was previously associated with spastic ataxia in an Amish pedigree, the listed clinical features of the affected individuals did not include radiosensitivity. Therefore, the association of homozygous 1432A>G with sensitivity to ionizing radiation was tested by transfecting the patient derived LCLs with wild type (WT) *MTPAP* and assaying clonogenic survival after irradiation. The *MTPAP* variant LCLs were also assessed for levels of DNA repair foci, DNA damage and repair, ROS, mitochondrial

respiration, and apoptosis in order to elucidate pathways by which mtPAP influences cellular responses to ionizing radiation.

Methods:

Cell culture, irradiation, and α-LA and NAC treatment

LCLs were derived from lymphocytes isolated from whole blood submitted for radiosensitivity testing as previously described(3). LCLs were cultured in RPMI1640 media with 10% FBS, 1% streptomycin/penicillin/glutamine (PSG) (Invitrogen, Carlsbad CA) and maintained at 37 ^{0}C in 5% CO_2. Cells were harvested for experiments when growing under log growth phase conditions and irradiation was carried out using a Mark IV Cs-137 sealed source irradiator at a dose rate of ~4.5 Gy/min. Where indicated, LCLs were treated overnight with α-LA in DMSO or n-acetyl cysteine (NAC) in H2O(Sigma Aldrich, St. Louis MO) before irradiation.

WT mtPAP plasmid, and transfection

The open reading frame of human mtPAP (1749bp) was PCR amplified from WT LCLs using the following primers: Xba-mtPAP forward (**gacTCTAGA**ATGGCGGTTCCCGGCGTGGGGCTCT) and Mlu-mtPAP reverse (**gacACGCGT** TCATGTCTGAGTACTAATTGTTCTC) and subcloned into a TA-vector using the manufacture's protocol (Invitrogen, Carlsbad CA) to generate the WT mtPAP plasmid. The resulting product was sequenced to confirm WT mtPAP cDNA sequence. The TA-cloned mtPAP cDNA was then digested with XbaI and MluI restriction enzymes and the purified fragment was ligated into XbaI-MluI digested (New England Biolabs, Ipswich, MA), SAP treated (Clontech, Mountain View, CA), lenti 308 plasmid vector containing GFP (UCLA Vector Core, Los Angeles, CA). Once the

plasmid was generated, ~15x10^6 LCLs were transiently transfected with 15ug of Lenti-mtPAP/GFP or Lenti-GFP control expression constructs using electroporation (250V, 1180 uF) in a Cell-Porator (Invitrogen, Carlsbad CA). Cells were harvested for experiments two days after transfection. Transfection efficiencies were estimated at ~80% by fluorescent microscopy imaging of GFP positive cells.

Clonogenic survival assay

The clonogenic survival assay (CSA) for cellular radiosensitivity testing was performed on LCL samples as previously described(3). Briefly, LCLs were seeded in a 96-well plate. The plates were treated with 1 Gy or sham-IR and returned to the incubator for 10-14 days. Wells positive for at least a single colony of >32 cells (i.e. 5 generations) were scored as positive and results were compared to sham treated plates to determine the survival fraction percentage (SF%)(3).

Neutral comet assay

The NCA for assessing DNA repair capacity of LCLs has been previously optimized by Nahas et al.(184). The Comet Assay kit (Trevigen Inc, Gaithersburg MD) was used under neutral conditions according to the manufacturer's specifications. LCLs were treated with 15 Gy or sham treated and collected at 0-hr (no-IR), 30 minutes, and 5-hours post-IR. LCLs were re-suspended in 1% low melting point agarose (Sigma Aldrich, St. Louis MO) and plated on 20-well comet assay slides. Once the agarose solidified, the slides were added to a bath of cell lysis solution (Trevigen Inc, Gaithersburg MD) overnight at 4 ^{0}C. The following day, the samples (i.e., the free DNA fragments) on the slides were electrophoresed and stained using SYBR Gold (Invitrogen, Carlsbad CA). Comets were visualized using an Olympus fluorescent microscope equipped with an

AxioVision camera and software. The tail moments (TM) of comets were scored using CometScore software (TriTek, Sumerduck VA). Percent repair (% repair) was determined by monitoring the return to baseline TM levels ($TM_{5\ hrs}/TM_{0\ hrs}$). Where no specific WT-LCL line is denoted, % repair values are normalized to multiple WT samples used across experimental replicates to avoid any unexpected idiosyncrasies specific to a particular WT line. Initial induction of DNA breaks (i.e. without time for DSB repair) was also measured by the NCA. LCLs were treated with 2 and 5 Gy and immediately placed on ice after IR to assess the level of induced damage. Samples were then re-suspended in 1% low melting point agarose and assayed as described above to determine the initial induced levels of DSBs, and the results are reported as the TM without IR (0 Gy) and post-2 and -5 Gy.

Irradiation-induced foci assay

The γ-H2AX, 53BP1, and BRCA1 (Millipore, Billerica MA, Santa Cruz Biotechnology, Santa Cruz CA, and Novus Biologics, Littleton CO, respectively) IR-induced foci (IRIF) assay was performed as previously described(156). For γ-H2AX and 53BP1 foci, cells were treated with 2 Gy and collected at 1, 4, 8, and 24 hours. Cells were collected at 4, 8, and 24 hours post-12 Gy for assessment of BRCA1 foci. Cells were plated on coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X 100 (Sigma Aldrich, St. Louis MO), blocked in 10% FBS, and incubated with primary antibodies (1:300) for 1 hour at room temperature. The coverslips were washed, blocked with 10% FBS and incubated with a AlexaFluor-488 secondary antibody (1:400, Invitrogen, Carlsbad CA) for 45 minutes at room temperature. Coverslips were washed a final time and mounted on slides using ProlongGold anti-fade reagent containing DAPI (Applied Biosystems,

Grand Island NY). Foci were imaged using an Olympus fluorescent microscope equipped with an AxioVision camera and software. Cells were scored as positive if they contained 4 or more foci/nuclei and results are presented as percent positive cells (% Pos).

Rezasurin assay

Rezasurin is reduced to a fluorescent dye as mitochondria respire and has been used to monitor mitochondrial respiration in radiosensitive cells(160, 184). Cells were grown at low density, collected, and plated in a 96-well plate. Prior to plating, cells were resuspended in fresh media containing 3 mM of the rezasurin dye (Sigma Aldrich, St. Louis MO). The plates were measured at 0, 1, 2, and 3 hours post-plating to monitor the relative increase in fluorescence. Fluorescence levels were measured on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale CA).

Reactive oxygen species assessment and Annexin V assay.

DCHF-DA was used to assess the presence of a broad array of cellular ROS levels, as previously described(240). DHE preferentially detects superoxide moieties and was used to assess superoxide levels(241). Cells were collected and re-suspended in 1X PBS at 37 ^{0}C containing 50 μM DCHF-DA dye (Sigma) for 20 minutes or for 30 min in RPMI media containing 20 μM DHE dye (Life Technologies). For the DCHF-DA dye, samples were treated with 10 Gy or sham treated and immediately placed on ice and analyzed within 8 minutes by FACS (FL-1). Samples incubated with DHE were also sham treated or irradiated with 10 Gy and analyzed by FACS (FL-2) within 8 minutes. Annexin V has been previously described to label apoptotic cells for rapid analysis by FACS(242). The flow cytometry-based Annexin V Apoptosis kit was used per manufacturer's protocol

(BD Biosciences). Cells were collected 48 hours post-10 Gy and re-suspended in 1X binding buffer containing Annexin V and propidium iodide. Cells positive for Annexin V staining were scored as positive for apoptosis.

Mitochondrial bioenergetics assay.

The mitochondrial bioenergetics assays was performed on the XF24 Extracellular Flux Analyzer (Seahorse Biosciences) as previously described(243, 244). Briefly, $3X10^5$ cells were re-suspended in RPMI media and added to each poly-D-lysine coated well in an XF24 Analyzer 24-well plate. 4 wells (i.e. 4 replicates) were used for each cell line and condition measured. The plate was then centrifuged for 1 minute and incubated at 37 ^oC and 5% CO_2 for 30 minutes to allow suspension cells to attach to the plate prior to analysis. Measurements were carried out after addition of oligomycin, FCCP, and rotenone/myxothiazol at concentrations of 0.75 μM for each compound to act as an ATP synthase inhibitor, respiratory chain uncoupler, and mitochondrial respiration inhibitors, respectively. A 2-minute mix and 2-minute wait period preceded the measurement of the oxygen consumption rate (OCR) during a 4-minute measurement period for each cycle of the XF24 analyzer.

Exome sequencing and variant calling (performed by Dr. Concannon)

At the time of initial sequencing, only an LCL was available from RS63-3. Exonic sequences from RS63-3 were captured using Agilent SureSelect v1 and submitted for 50 base pair, paired end sequencing on an Illumina HiSeq instrument by Drs. Concannon and Quinlan. A total of 9GB of sequence was obtained and aligned to the human reference genome (GRCh37). The median read depth was 78x; 91.68% of the exome was sequenced to a depth of 20x or greater. After alignment to the human reference genome,

duplicate molecules arising during PCR were removed with the MarkDuplicates utility in Picard (version 1.60). In the interest of improved insertion-deletion (INDEL) discovery, alignments supporting candidate INDELs were realigned with the GATK (version 1.4.9) IndelRealigner utility(245). Both single-nucleotide and INDEL polymorphisms were identified with the GATK UnifiedGenotyper (version 1.4.9) using default settings and all variants were annotated and prioritized with GEMINI(246).

Statistics

Unless otherwise noted, data is presented as the mean of three independent measurements (n = 3) and error bars represent +/- 1 standard deviation. Statistical analysis was determined by the Student's t-test for two independent samples of equal variance and p-values of $p<0.05$ were considered significant.

Results:

Radiosensitivity segregates with bi-allelic mtPAP mutations in RS63 family.

Two affected siblings from a consanguineous Amish family were tested for reduced colony survival levels post-IR, a radiosensitive phenotype, because they displayed a clinical phenotype similar to A-T. Both affected siblings, RS63-3 and RS63-7, were characterized by ataxia with initiation tremors. Growth failure, developmental delay, and spasticity were also noted, while no immune deficiencies nor tumors were observed to date in RS63-3 and -7. There was reduced colony survival in RS63-3 and -7, similar to radiosensitive LCLs from A-T patients(3). Unaffected siblings (RS63-4, -5, -6) had normal colony survival post-IR (**Figure 22A**). The NCA is a useful method for assessing DNA DSB repair fidelity in LCLs and correlates moderately well with the CSA(184). RS63-3 and -7 demonstrated an AT-like DSB repair defect, consistent with

the CSA result, and the unaffected siblings of the RS63 family were found to have normal levels of DNA repair (**Figure 22B**)(184).

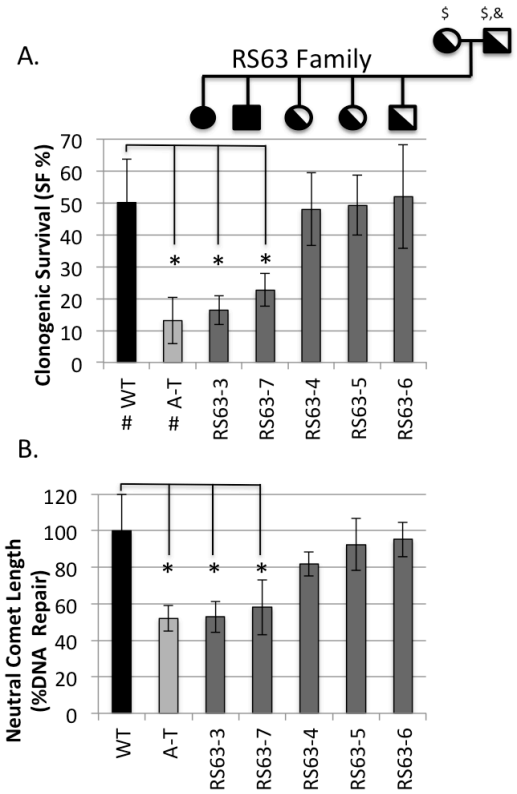

Figure 22. Cellular radiosensitivity and DNA repair defect segregates with affected members of RS63 family with a homozygous mutation of mtPAP. A. The survival fraction (SF%), measured by the CSA (at 1 Gy) is reduced in the two members of the RS63 family, RS63-3 and -7, who are homozygous for the *MTPAP* mutation. The '#' indicates SF% representative of WT and highly radiosensitive (A-T) cells used for comparison in clinical radiosensitivity testing based on Sun et al (3). The '$' denotes patients for whom we were not able to generate an LCL. The '&' denotes that we were unable to obtain DNAs for *MTPAP* sequencing analysis; however, the father is an obligate heterozygote. B**.** The neutral

comet assay, measuring predominately DNA DSBs, reveals an AT-like DNA repair defect in RS63-3 and -7. Results are displayed as percent DNA repair (% repair) post-15 Gy and represent the return of comet tails to baseline levels at 5 hours post-IR. An asterisk denotes statistical significance at $p<0.05$.

The AT-like cellular and clinical phenotype strongly suggested a diagnosis of A-T. However, ATM protein expression in cells from family members was normal. Exome sequencing was employed by Dr. Concannon as an untargeted approach to identify candidate causative mutations in other genes. At the time of initial sequencing, only an LCL derived from RS63-3 was available. Comparison of the RS63-3 exome sequence to the human reference sequence confirmed the absence of ATM mutations, but identified 36,563 coding variants relative to the reference, 690 of which were unique to RS63-3. Based upon the consanguinity in the RS63 family, the variants were filtered by Dr. Quilan's group for homozygosity, yielding 26 coding variants. Those variants predicted to be deleterious because they would result in gain or loss of a stop codon, a frameshift or a non-synonymous substitution at a conserved amino acid position and were not observed in either the NHLBI Exome Sequencing Project nor the 1000 Genomes Project were identified, yielding *HRNR* and *MTPAP* as the resulting candidate variants. These two genes affected by these putative deleterious variants were manually examined to determine if the genes might play a role in DNA damage responses or in cerebellar development or function. The homozygous missense mutation (c.1432A>G, N478D) in the *MTPAP* gene was singled out because it had been previously associated with spastic ataxia and optic atrophy in an Old Order Amish population and was the only of the two

possible variants predicted to be deleterious according to Polyphen (247, 248). The 1432A>G *MTPAP* mutation, and lack of ATM mutations, was later confirmed in RS63-7 once the LCL was available. The unaffected siblings (RS63-4, -5, -6) and mother were heterozygous for the *MTPAP* mutation, suggesting an autosomal recessive inheritance pattern.

Mutation of mtPAP is causally linked to the radiosensitive and persistent DNA damage phenotype by mtPAP transfection.

mtPAP, although encoded in the nucleus, poly-adenylates mitochondrial transcripts and, unlike nuclear poly-adenylation polymerases, has a variable affect on transcript stability(247, 249, 250). Thus, it was unclear how mutation of *MTPAP* might result in radiosensitivity. The AT-like radiosensitivity (**Figure 23A**) and DNA repair defect (**Figure 23B**) observed in RS63-3 and -7 were rescued when WT mtPAP cDNA was transfected into the patient's LCLs, confirming that mutation of *MTPAP* was responsible for the cellular radiosensitivity and defective DNA repair.

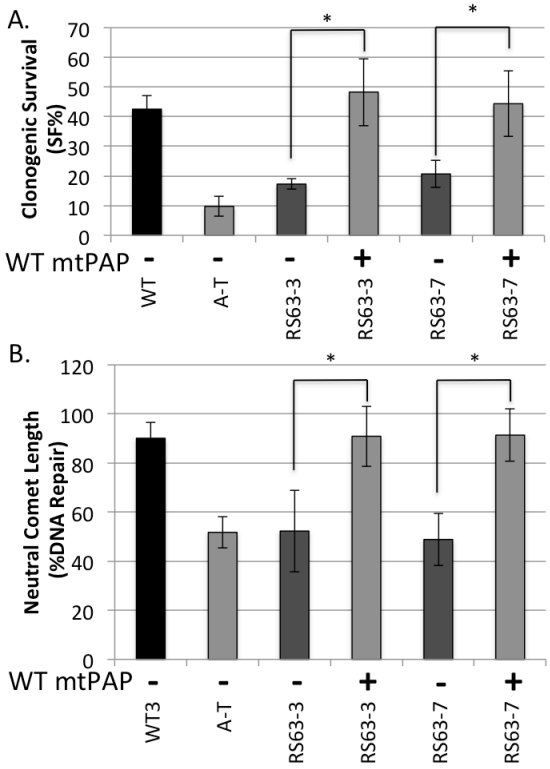

Figure 23. Transfection of WT mtPAP into RS63-3 and -7 rescued the radiosensitivity and DNA repair defect. A. Transfection of WT mtPAP cDNA (+) into RS63-3 and -7 rescued the radiosensitive (i.e. reduced SF%) phenotype measured by the CSA (at 1 Gy). A (+) denotes transfection with WT mtPAP cDNA and (-) denotes transfection with an empty vector. B. WT mtPAP (+) or control plasmid (-) was transfected into RS63-3 and -7; the WT mtPAP plasmid rescued the DNA DSB repair defect as measured by the NCA (at 15 Gy). '% DNA repair' represents the return to baseline tail moment (TM) measure of DNA damage 5 hours post-IR and is the $TM_{5hours}/TM_{no\ IR}$. An asterisk denotes statistical significance at $p<0.05$.

Increased post-IR DNA double strand break induction in RS63-3 and -7.

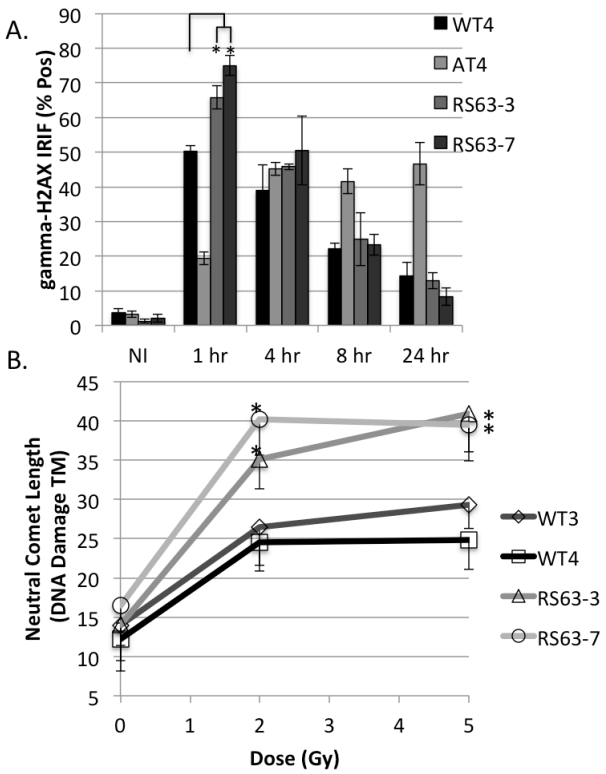

Figure 24. Increased induction of DNA damage post-IR in RS63-3 and -7. A. γ-H2AX irradiation induced foci (IRIF) were increased at 1 hour (post-2 Gy) in RS63-3 and -7 compared to WT levels. The results are expressed as the percentage of cells positive for 4 or more foci/nuclei (% Pos). WT4 is a representative WT response. Asterisks denote statistical significance at $p<0.05$. B. DNA DSB levels were assessed immediately post-2 and -5 Gy. Increased levels of DSBs were observed in RS63-3 and -7. Asterisks indicate statistical significance at the $p<0.05$ level comparing RS63-3 and -7 (n=4, each) to both WT3 and WT4 (n=4, each).

The similarities in clinical phenotype between RS63-3 and RS63-7 and A-T patients led us to consider that mutation of *MTPAP* might impact the ATM signaling cascade. I screened a panel of ATM kinase activity biomarkers including ATM

autophosphorylation and downstream phosphorylation of SMC1, KAP1, and NBS1, which are necessary for the rapid detection of DSBs and transmission of the DDR signal(51, 53, 233, 251). A WT-like response to IR was observed in the phosphorylation of ATM, SMC1, KAP1, and NBS1 in cell lysates from RS63-3 and -7, indicating that mutation of mtPAP does not likely impact early ATM kinase signaling in the DDR (data not shown).

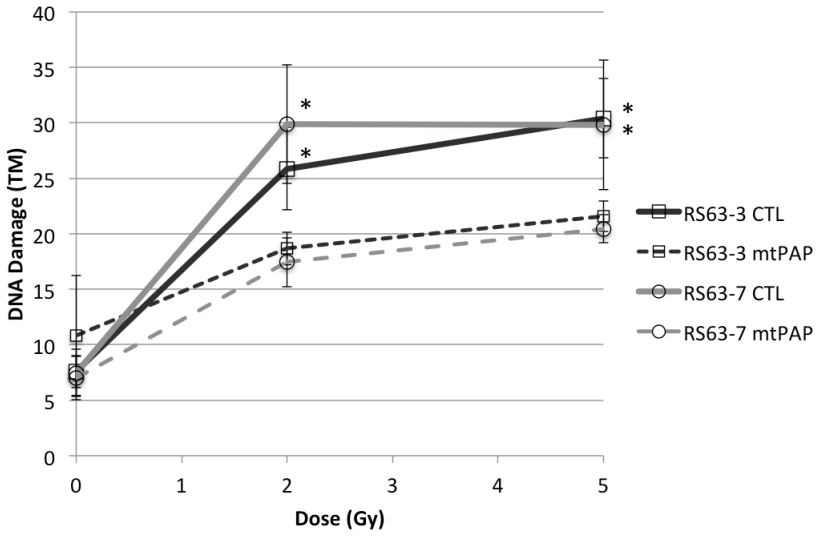

Figure 25. Transfection with WT *MTPAP* rescues increased DNA damage phenotype post-IR in RS63-3 and -7. Comet tail lengths were assessed immediately post-2 and -5 Gy in RS63-3 and -7 LCLs 48 hours after transfection by electroporation with WT *MTPAP* (mtPAP) or empty vector (CTL) plasmids. The results are presented as the average tail moment (TM) and represent initial, induced DNA damage levels. An asterisk indicates significance at the $p<0.05$ level, comparing *MTPAP* transfected sample to control transfected sample for each condition.

In chapter 2, I have shown that monitoring γ-H2AX, 53BP1, and BRCA1 foci kinetics post-IR can be used to characterize DDR defects in radiosensitive patients of unknown etiology by comparing kinetic curves to those of known radiosensitivity disorders(156). RS63-3 and -7 displayed normal basal levels of γ-H2AX foci and normal repair kinetics at 4, 8 and 24 hrs; however, the 1 hr post-IR response was increased in both LCLs (**Figure 24A**). Similarly, increased DSB levels in RS63-3 and -7 were noted by the NCA, confirming increased DSB induction immediately post-IR (**Figure 24B**). Transfection of WT *MTPAP* rescued the increased level of DSBs illustrated by the NCA (**Figure 25**). Increased levels of 53BP1 foci were observed at 1 hr post-IR and persisted at 4 hrs (**Figure 26A**). Finally, BRCA1 foci were assessed as a marker of HR. A trend towards reduced BRCA1 foci positive cells, compared to WT, at 4 hours was observed followed by a significant reduction in foci numbers at 8 hours post-IR suggesting either a defect in recruitment of BRCA1 to DSBs or a shift away from initiating HR (**Figure 26B**).

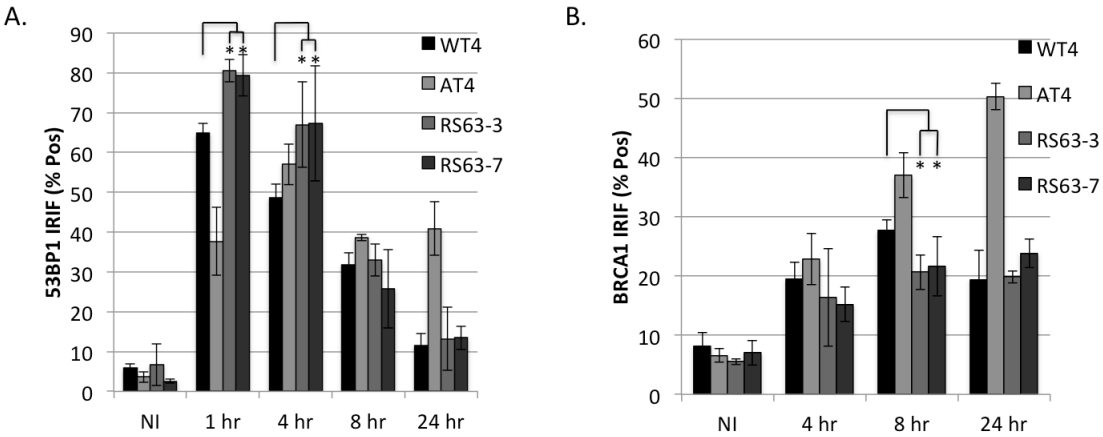

Figure 26. Increased 53BP1 foci and decreased BRCA1 foci post-IR in RS63-3 and -7. A. 53BP1 irradiation induced foci (IRIF) kinetics

indicating increased foci at 1-hour post-2 Gy and persistent foci at 4-hours in RS63-3 and -7. B. Foci kinetics for BRCA1 displayed reduced BRCA1 foci accumulation in RS63-3 and -7 8-hours post-12 Gy.

HR is only active in the late S and G2 phases of the cell cycle and, thus, I hypothesized that cell cycle perturbations might impact the reduction observed in BRCA1 foci numbers at 8 hrs. Cell cycle distribution was assessed in RS63-3 and -7 and was found to be WT-like before and after IR (data not shown). Similarly, the fidelity of the S-phase and G2/M phase checkpoints were WT-like (data not shown). This suggested that the reduction in BRCA1 foci was not due to large differences in cell cycle distribution nor checkpoint activation and that cell cycle control was not significantly impacted by mutation of *MTPAP*.

Normal mitochondrial respiration, increased ROS levels and increased apoptosis in RS63-3 and -7 post-IR.

Mitochondria are closely involved in the DDR by at least three mechanisms: 1) maintaining ROS homeostasis, 2) mediating apoptotic signals, and 3) producing energy needed for DSB repair(121, 126, 236). Due to the known function of mtPAP in the mitochondria, I postulated that mutation of *MTPAP* might result in dysfunctional mitochondria and subsequent disruption of the DDR. Mitochondrial respiration levels were assessed with the rezasurin dye. Contrary to my working model, mitochondrial respiration was normal in RS63-3 and -7 (**Figure 27A**). Mitochondrial bioenergetics were further probed by measuring the oxygen consumption rate (OCR) after addition of oligomycin (Oligo, ATP synthase inhibitor), FCCP (respiratory chain uncoupler), and rotenone/myxothiazol (R/M, mitochondrial respiration inhibitors). Basal respiration

(time 1-3), ATP linked respiration (time 4-6), and total mitochondrial respiration levels (time 10-14) were largely similar to WT levels in RS63-3 and -7, consistent with the findings of the rezasurin assay. However, maximal mitochondrial respiration levels (time 7-9), induced by addition of FCCP, were greatly increased in RS63-3 and -7, compared to WT3 and AT7 (**Figure 27B**). Transfection of WT mtPAP cDNA into RS63-3 and -7 was sufficient to partially abrogate the increased mitochondrial respiration phenotype after addition of the respiratory chain decoupler, FCCP (**Figure 28**).

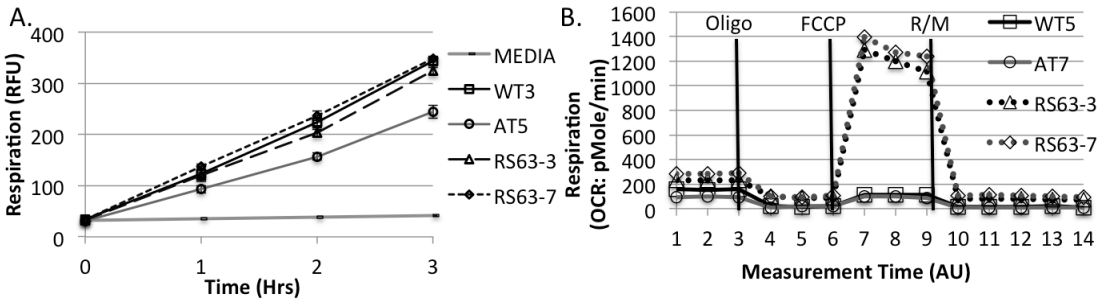

Figure 27. RS63-3 and -7 LCLs had normal basal, but increased maximal, mitochondrial respiration. A. Mitochondrial respiration was measured by monitoring metabolism of the rezasurin dye and indicated WT-like, or normal, respiration in RS63-3 and -7. The results are displayed as relative fluorescent units (RFU) of the metabolized rezasurin dye. WT3 is a representative WT-LCL response. AT5 is a representative A-T cell with a characteristic mitochondrial defect (37). B. Mitochondrial bioenergetics were profiled and RS63-3 and -7 had increased maximal respiration levels after addition of FCCP compared to WT-LCLs. WT5 is a representative WT-LCL response. The oxygen consumption ratio (OCR) was measured as an indicator of mitochondrial respiration.

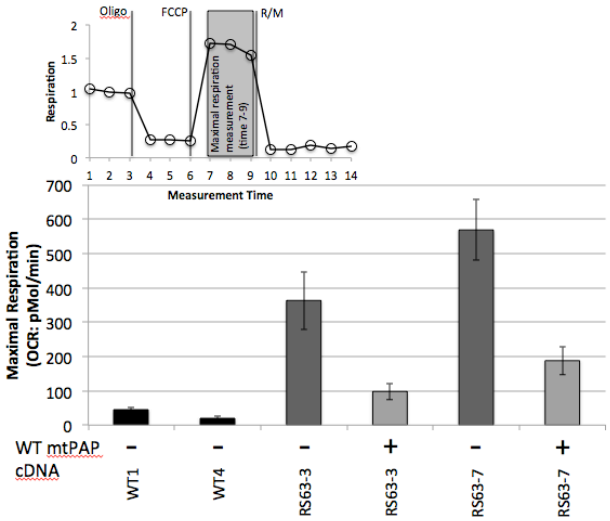

Figure 28. Mitochondrial bioenergetics were assessed using the XF24 Extracellular Flux Analyzer and representative data of the oxygen consumption ratio (OCR) after addition of the respiratory chain decoupler, 0.75 μM FCCP, are displayed. Control (-) transfected RS63-3 and -7 cells displayed increased maximal mitochondrial respiration, while WT mtPAP cDNA (+) transfection partially rescued maximal respiration levels to near WT-like levels and demonstrated a link between *MTPAP* mutation and the dysfunctional mitochondrial respiration phenotype observed. Inset in the upper left corner of the figure is a depiction of the curve assessed by the mitochondrial bioenergetics assay. For clarity of presentation, the timeframe outlined by the grey box, which signifies maximal mitochondrial respiration levels after addition of the respiratory chain decoupler FCCP, is being displayed as a bar graph in this figure. Values presented are the average respiration levels of the three measurements taken at measurement time 7-9. Error bars are +/- 1 standard deviation.

Abnormal ROS levels have been linked to reduced colony survival and DSB repair and mitochondria are known to be the main producers, and reducers, of many ROS moieties (126, 165). ROS levels were assessed and increased levels were observed in RS63-3 and -7 post-IR, similar to those of A-T LCLs (**Figure 29A**)(157). Consistent with cells undergoing oxidative stress, increased apoptosis levels were found in RS63-3 and -7 post-IR (**Figure 29B**)(126, 130). The mitochondrial bioenergetics assay suggested that there was likely a defect with the mitochondria in RS63-3 and -7 and that the defect may lie with the respiratory chain. DCHF-DA reacts to a number of ROS species and the predominate ROS specie that would be released from dysfunctional mitochondria is superoxide(96). Thus, I used the ROS dye, DHE, that is preferentially modified by superoxide as a marker of superoxide levels in RS63-3 and -7(241). RS63-3 and -7 showed increased levels of superoxide before irradiation and these levels increased post-IR while WT cells did not demonstrate a substantial increase in superoxide levels (**Figure 30A**). Transfection of RS63-3 and -7 with WT mtPAP cDNA abrogated both the increased basal levels of superoxide and the IR-induced increase in superoxide levels demonstrating a link between *MTPAP* mutation and increased ROS levels in these cells(**Figure 30B**).

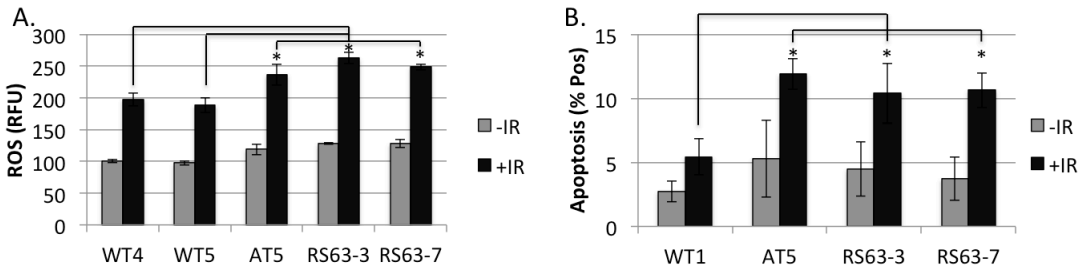

Figure 29. RS63-3 and -7 had increased ROS and apoptosis levels post-IR. A. Reactive oxygen species (ROS) levels were assessed using DCHF-DA dye (RFU). Increased IR-induced levels of ROS were noted in RS63-3 and -7. Asterisks denote statistical significance at the $p<0.05$ level comparing the +IR condition of the indicated sample to both of the WT4 and WT5 +IR conditions. B. Apoptosis was determined by FACS analysis of cells positive for Annexin V staining 48 hours post-10 Gy, and indicates increased, post-IR apoptosis in RS63-3 and -7. Asterisks indicate statistical significance at the $p<0.05$ level.

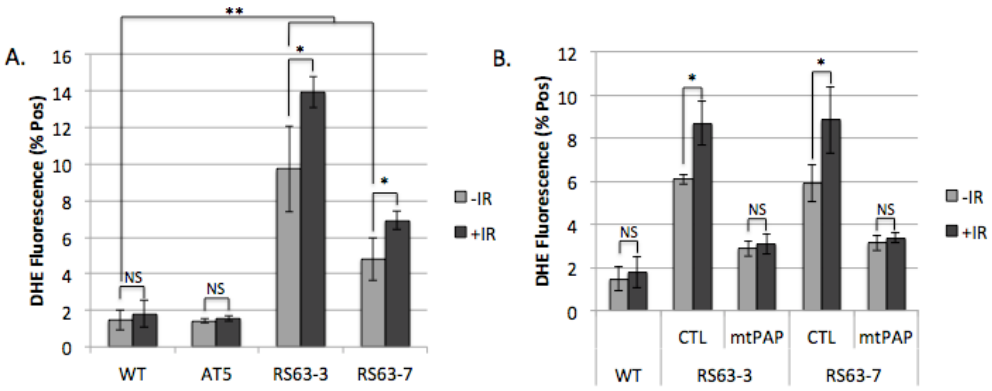

Figure 30. A. DHE is a cell permeable dye that preferentially detects superoxide levels in living cells. Increased DHE staining was observed in RS63-3 and -7 without irradiation (-IR) and these cells demonstrated increased superoxide levels post-10 Gy (+IR), while there was not a significant IR-induced response in WT or A-T cells. The 'WT' sample represents the averaged value of three different WT-LCLs. DHE fluorescent levels were analyzed by FACS (FL-2) and the results are presented as the percentage of cells staining positive (% Pos) for larger than WT -IR levels of superoxide. An asterisk denotes statistical

significance at the $p<0.05$ level comparing –IR to +IR conditions. Two asterisks denotes statistical significance at the $p<0.05$ level comparing the –IR WT condition to the –IR RS63-3 and -7 conditions. B. RS63-3 and -7 were transfected with control (CTL) or WT mtPAP cDNA (mtPAP) and superoxide levels were assessed with the DHE dye 2 days later. Transfection of WT mtPAP cDNA reduced superoxide levels to near WT-like levels while the control plasmid gave no improvement for superoxide levels. Further, the IR-induced increase in superoxide levels observed in RS63-3 and -7 were abrogated by WT mtPAP cDNA. DHE fluorescent levels were analyzed by FACS (FL-2) and the results are presented as the percentage of cells staining positive (% Pos) for larger than WT-levels of superoxide. An asterisk denotes statistical significance at the $p<0.05$ level.

Antioxidants, alpha-lipoic acid and n-acetyl cysteine, rescued mtPAP deficient cellular radiosensitivity and DNA repair phenotype.

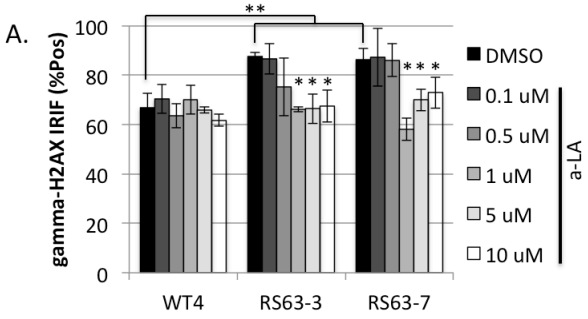

Figure 31. alpha-LA rescues elevated levels of γ-H2AX foci in RS63-3 and -7. γ-H2AX foci were assessed 1-hour post 2 Gy after overnight treatment with α-LA or vehicle control (DMSO). Pre-IR treatment with α-LA >1 μM abrogated the increased γ-H2AX foci phenotype in RS63-3

and -7, restoring WT-like levels of γ-H2AX foci positive cells. One asterisk indicates statistical significance at the $p<0.05$ level comparing α-LA treated samples to vehicle control samples for each cell line. Two asterisks indicate significance at the $p<0.05$ level comparing vehicle control samples across cell lines

The increased levels of ROS were intriguing, but it was unclear whether this contributed to the cellular phenotype of RS63-3 and -7. To test this, α-LA, a potent antioxidant, was used to lower ROS levels(252). First, γ-H2AX foci levels were assessed at 1-hour post-IR to determine if the increased levels of foci in RS63-3 and -7 could be returned to WT levels by α-LA pre-treatment. Indeed, pre-treatment with α-LA at concentrations >1 μM reduced γ-H2AX foci levels to those of the WT (**Figure 31**). Interestingly, α-LA treatment had no effect on γ-H2AX foci levels in WT LCLs at the concentrations tested. When assessed by the NCA, the increased levels of DSBs initially induced post-IR in RS63-3 and -7 were also abrogated by pre-IR treatment with α-LA, confirming protection against elevated IR-induced damage (**Figure 32**).

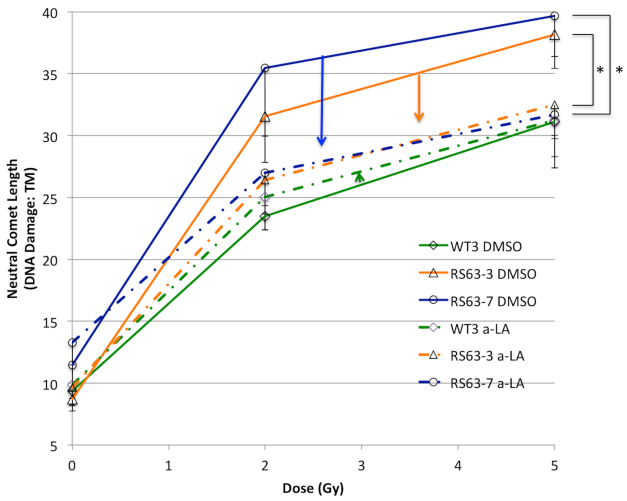

Figure 32. Increased levels of DNA damage in RS63-3 and -7 are reduced by pre-treatment with α-LA. The initial induction of DNA breaks was assessed by the NCA after pre-IR treatment with α-LA or DMSO control post-2 and -5 Gy. The initial levels of DNA breaks were reduced to WT levels in RS63-3 (blue arrow) and -7 (orange arrow) by pre-IR treatment with α-LA. Values are presented as the tail moments (TM) post-2 and -5 Gy. Asterisks indicate significance at the $p<0.05$ level.

The DNA repair defect measured by the NCA was also rescued in RS63-3 and -7 by pre-IR treatment with 1 μM α-LA. Further, pre-IR α-LA treatment also rescued the radiosensitive phenotype, but it is interesting that this had no affect on WT or A-T LCLs (**Figure 33A and B**). The lack of SF% improvement with α-LA treatment in A-T LCLs is consistent with previous findings and further elucidates the differential dependency of radiosensitivity and ROS levels in WT, A-T and mtPAP deficient LCLs(253). To rule out the possibility that the improvement in the cellular phenotype for RS63-3 and -7 was due to a phenomena specific to α-LA, especially a phenomena not associated with it's antioxidant function, I treated cells with another potent antioxidant, n-acetyl cysteine (NCA). Pre-treatment with NAC abrogated the increased level of DNA damage, DNA repair defect, and reduced clonogenic survival in RS63-3 and -7 confirming that antioxidants are sufficient to reduce oxidative stress in the cells and improve the cellular phenotype (**Figure 34A-C**).

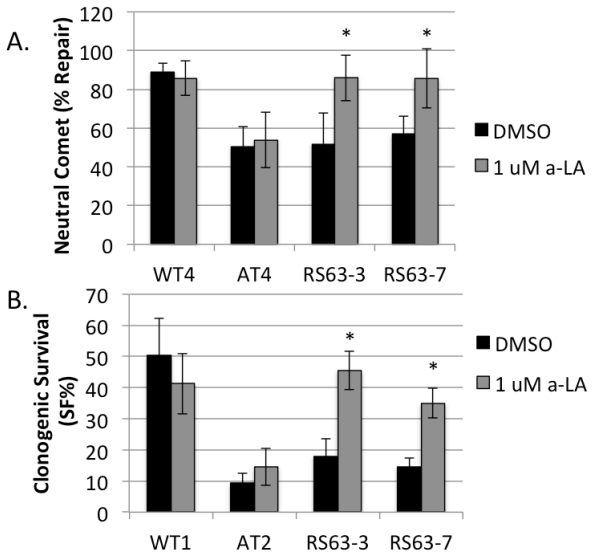

Figure 33. Pre-treatment with α-LA rescues DNA repair defect and radiosensitive phenotype in RS63-3 and -7. A. Pre-IR treatment of RS63-3 and -7 with 1 μM α-LA abrogated the post-IR DNA repair defect measured by the NCA. Comets resulting from unrepaired DSBs were assessed post-15 Gy and results are presented as '% Repair', which is the return to baseline levels of damage 5-hours post-IR ($TM_{5hours}/TM_{no\ IR}$). Asterisks indicate statistical significance at the $p<0.05$ level when comparing α-LA treated samples to vehicle control samples. B. Cellular radiosensitivity, indicated by reduced SF% post-1 Gy, was rescued in RS63-3 and -7 by pre-treatment with 1 μM α-LA. Asterisks denote statistical significance at the $p<0.05$ level when comparing α-LA treated samples to vehicle control samples.

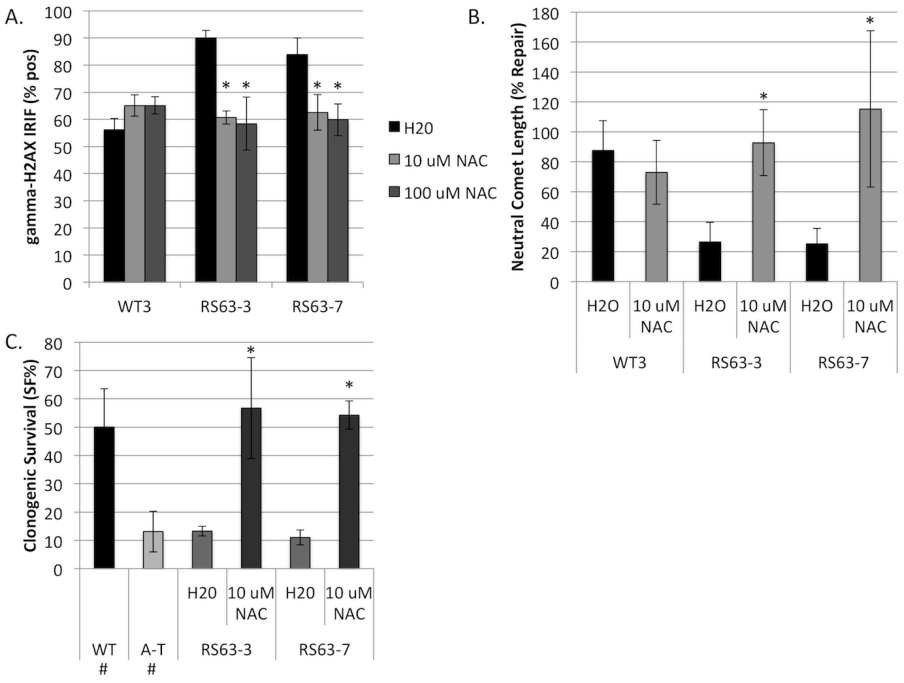

Figure 34. A. WT, RS63-3 and -7 LCLs were pre-treated overnight with 10 and 100 μM NAC and DNA damage levels induced by 2 Gy were assessed by measuring γ-H2AX foci levels at 1 hour post-IR. NAC pre-treatment rescued increased γ-H2AX foci levels in RS63-3 and -7 while NAC had no impact on foci levels in the WT-LCL. Results are presented as the percentage of cells positive (% Pos) for 4 or more foci/nucleus. B. DNA repair efficacy was assessed 5 hrs post-15 Gy by the neutral comet assay after pre-treating cells with 10 μM NAC. Reduced DNA DSB repair was rescued in RS63-3 and -7 by pre-treatment with NAC while NAC had no impact on WT3 repair levels. Results are presented as percent repair (% Repair), which are the tail moments without irradiation (TM_{0hrs}) divided by the residual tail moments (i.e. unrepaired DNA) at 5

hrs post-IR (TM_{5hrs}). C. Clonogenic survival was assessed using the CSA post-1 Gy, and pre-treatment with 10μM NAC rescued the reduced levels of clonogenic survival in RS63-3 and -7 to WT-like levels. Results are presented as the survival fraction percentage (% SF) and the WT and AT values are the ranges established by Sun et al. (3). For **A-C**, an asterisk represents statistical significance at the $p<0.05$ level when using the Student's t-test to compare NAC treated conditions to the control (H2O).

Discussion:

I describe two Amish patients (RS63-3 and -7) with a homozygous *MTPAP* mutation that had been previously associated with spastic ataxia in an Old Order Amish population and can now be causally linked to radiosensitivity and modulation of the DDR (247, 254). Identification of *MTPAP* as a candidate gene responsible for the defects observed in these patients was enabled by exome sequencing of DNA from these patients. However, as is common with exome sequencing, many variants were identified that were potentially disease causing. With the expectation of homozygosity in the disease-causing variant, the variant list was narrowed by Drs. Concannon and Quinlan and led to the N478D missense mutation in mtPAP, which was previously shown to segregate with spastic ataxia in the Old World Amish population(247). This would be first radiosensitivity associated gene identified by exome sequencing approaches.

Colony survival testing indicated that LCLs derived from RS63-3 and -7 were radiosensitive, a new clinical phenotype for spastic ataxia associated with *MTPAP* mutation. The NCA further indicated a DNA repair defect in RS63-3 and -7. Crucially, transfection of WT *MTPAP* into RS63-3 and -7 cells abrogated both the DSB repair

defect and the reduction in clonogenic survival, confirming that mutation of *MTPAP* is responsible for the cellular radiosensitivity phenotype.

mtPAP is a nuclear encoded mitochondrial polyadenylation polymerase that was not an obvious candidate for causing radiosensitivity(247, 250). Examination of ATM expression and kinase activity indicated that it was unlikely that mutation of mtPAP disrupted ATM mediated signaling and the early recognition of DSBs. Increased γ-H2AX and 53BP1 foci at 1 hr, and persistent 53BP1 foci at 4 hrs were observed, suggesting disruption of the DDR early post-IR, but downstream of the initial sensing of DSB damage by ATM. Increased induction of DNA damage post-IR relative to WT levels was suspected in RS63-3 and -7, and was confirmed by the observation of increased comet tails measured by the NCA. Further, transfection of WT *MTPAP* into RS63-3 and -7 LCLs rescued the increased level of DSBs post-2 and -5 Gy. These results indicated that mutation of mtPAP likely affects the induction of DSBs rather than their repair, a potential deviation from a classical presentation of radiosensitivity/XCIND.

Interestingly, ROS levels were increased in RS63-3 and -7. It has been known that mitochondrial function is important for maintaining ROS homeostasis and that ROS levels can impact upon the number and complexity of DSBs through the induction of single or many clustered single strand breaks(99). The increased level of 53BP1 foci and decrease in BRCA1 foci post-IR may indicate a shift away from HR repair, which may fail with complex breaks, and a preference towards NHEJ repair.

Based on the known function of mtPAP, I postulated that the increased ROS levels were due to dysregulation of mitochondria function. Contrary to my prediction, basal respiration levels were WT-like in RS63-3 and -7. However, using the cellular

bioenergetics assay, I found that there was a significant difference in maximal respiration levels in RS63-3 and -7 compared to WT levels when the respiratory chain decoupling agent, FCCP, was added to cells. The mitochondrial respiratory chain produces ROS moieties and, when deregulated, can leak ROS into the cellular compartment producing a state of oxidative stress. This may be a source of the increased ROS levels observed in RS63-3 and -7. The predominate ROS moiety that can be leaked into the cytosol from dysfunctional mitochondria are superoxide ions. Indeed, increased levels of superoxide ions were observed in RS63-3 and -7 and increases in these levels were induced by irradiation. WT cells demonstrated no significant increase in superoxide levels after irradiation consistent with previous results and our working model that the increase in ROS species observed in WT cells with the DCHF-DA dye likely derive from radiolysis of water and the generation of hydroxyl radicals(107). RS63-3 and -7 would appear to leak superoxide radicals from dysfunctional mitochondria and this effect is exacerbated by an external stress such as irradiation. Transfection with WT mtPAP cDNA abrogated both the increased superoxide levels and maximal mitochondrial respiration phenotypes and provided evidence that RS63-3 and -7 do likely have dysfunctional mitochondria that contribute to a cellular state of oxidative stress.

I explored the importance of ROS for modulating the RS63-3 and -7 cellular phenotype by pre-treating LCLs with the antioxidants, α-LA and NAC. Pre-treatment with α-LA and NAC abrogated the increased DNA damage levels, reduction in repair, and radiosensitivity in RS63-3 and -7. The possibility of clinical treatment of patients with *MTPAP* mutations with α-LA and NAC is clinically intriguing as well. α-LA and NAC are FDA approved antioxidants that come in many formulations and represent an

attractive potential therapeutic approach for patients harboring mutations in the *MTPAP* gene to protect against DSBs and chromosomal instability induced by oxidative stress(99, 165). Pre-treatment of WT and A-T LCLs with α-LA had no affect on DNA repair nor clonogenic survival, while previous studies have indicated that antioxidants can improve other aspects of the A-T cellular phenotype(160, 253). The contrasting response to α-LA between A-T and mtPAP deficient patient cells highlights the differential causes of the radiosensitive phenotype in these patients (160, 253).

The A-T LCLs illustrate a classic model of radiosensitivity and XCIND whereby increased levels of ROS are secondary to a central DSB recognition and repair defect arising from mutation of a core DDR gene. Thus, modulating ROS levels has little impact on DSB repair or clonogenic survival(160). Conversely, the data for RS63-3 and -7 indicate that ROS levels post-IR increased DSB induction, resulted in reduced or delayed repair, and lead to reduced clonogenic survival in these cells. The data suggest a potentially atypical mechanism of radiosensitivity and XCIND, where there is not mutation of a core DNA damage response gene but, rather, a mutation in a gene leading to amplified induction of DNA damage by ionizing radiation. This might also account for the absence of immunodeficiency and cancer in mtPAP deficient patients that would be expected if these patients followed a more classical presentation of the XCIND syndrome.

CHAPTER 6

SUMMARY AND CONCLUDING THOUGHTS

Translating radiosensitivity testing for non-XCIND oncology patients.

There is great interest in measuring a patient's sensitivity to ionizing radiation so that tailored radiotherapy plans can be designed based on patient specific biology, rather than on population averages. Radiotherapy plans based on patient specific biology parameters could reduce adverse reactions to radiotherapy, thus improving patient care and reducing health care costs incurred during treatment of adverse reactions. Understanding an individual patient's biologic responses to irradiation could also improved tumor control for some individuals by identifying radioresistant patients who may benefit from dose escalation.

To date, a central roadblock for exploring radiosensitivity screening for personalized radiotherapy plans has been the long turnaround times needed for the current, 'gold standard', testing methodology, the clonogenic survival assay. To fit within the radiotherapy workflow timeline, a diagnostic test for radiosensitivity needs to be performed in less than the approximately one week between consultation with a patient and initiation of a radiotherapy plan (personal communication with Percy Lee, MD). A second roadblock has been understanding the connection between radiation hypersensitivity in a patient's *cells*, or *cell line*, and how the *patient* will react to irradiation. Thirdly, and often the most overlooked obstacle, determining *how* radiosensitive an individual may be remains a significant challenge for determining how much to change current radiotherapy doses based on radiosensitivity testing. Oncologists will need a diagnostic test that indicates *how* much to reduce or increase radiation dose for a test to be useful in the clinic (personal communications with Drs. Percy Lee and Steve Lee).

In Chapter 3, I demonstrated that the neutral comet assay could be adapted to assess DNA repair in primary lymphocytes from whole blood samples submitted for radiation hypersensitivity testing. The results from the neutral comet assay testing were promising correlated reasonably well with the current gold standard, the clonogenic survival assay, for predicting hypersensitive to ionizing radiation and represents a laboratory test that could be performed in 2-3 days, well within the time frame needed for a clinical assay. γ-H2AX foci assays and gene expression based assays have also now been adapted for use on whole blood and could be performed within the time frame needed for clinical implementation of a radiosensitivity diagnostic(255). Based on our previous experience, however, the neutral comet assay is likely to be the best rapid assay to predict radiation hypersensitivity(184, 256). Thus, these studies suggest that turnaround time for biologic assays is no longer a significant hurdle for implementing radiation sensitivity testing for radiotherapy populations.

The second hurdle for implementing radiosensitivity diagnostics, the connection between *cellular* and *clinical* radiosensitivity, has been addressed for XCIND patients by Pollard et al.(2). Pollard et al. performed a retrospective study of several types of known XCIND disorder patients who had been irradiated to treat tumors that had developed. The authors found that the patients with *cellular* radiosensitivity responded very poorly to radiotherapy, i.e. also *clinical* radiosensitivity(2). Thus, there is evidence that XCIND-like patients indicated to be *cellular* radiosensitive by the clonogenic survival assay, or a surrogate assay such as the neutral comet assay, are likely *clinically* radiosensitive. This is not particularly surprising in XCIND, or suspected XCIND, patients because the hypothesis is that there is an inherited, biallelic mutation of a DNA repair gene.

Therefore, every cell in the patient carries the DNA repair defect and, according to our working model, will be more sensitive to ionizing radiation that a radionormal individual, irrespective of tissue type and location.

The connection between cellular and clinical radiosensitivity becomes harder to interpret for routine oncology patients, however. Routine oncology patients are unlikely to carry bialleic mutations of genes required for DNA repair and the moderate sensitivity to ionizing radiation, compared to XCIND patients, in these patients may be due to many factors that are difficult to identify. This notion seems to be supported by the many inconsistencies reported in the literature by previous attempts at radiosensitivity testing. Previous studies attempting to correlate cellular endpoints, such as the neutral comet or γ-H2AX foci assays, and reactions to radiotherapy in non-XCIND patients have described correlations between *cellular* and *clinical* outcomes(183, 215, 257, 258). However, variability between individual patients was significant in these studies and resulted in assays that were not *predictive* or, in other larger scale studies, resulted in negligible correlations between *cellular* and *clinical* endpoints(2, 183, 259). Thus, the question of whether *cellular* results are indicative of *clinical* outcomes remains open.

The correlations found in some studies, and the lack of correlations in other studies, may suggest that there are two or more subsets of patients who develop normal tissue complications after radiotherapy. Patients who have inherited DDR gene mutations, such as XCIND patients, which impact normal tissue response to ionizing radiation constitute the first subset of patients. These patients fit the current model of radiosensitivity and are effectively identified by current testing methodologies; methodologies *designed*, with the exception of the clonogenic survival assay, to test only

the DNA DSB response. The inability of any of the current assays, when applied beyond XCIND patients to routine oncology patients, to robustly predict normal tissue complications suggests that there may be a second subset of patients who have unforeseen environmental modifiers of their radiation response, or harbor mutations in genes that affect aspects of the radiation response not related to DNA DSBs or repair. It may also be that the penetrance of heterozygous mutations in DNA repair genes is low or variable and result in some patients who respond adversely to conventional doses of radiotherapy, while others tolerate conventional doses well.

Advancements in genome sequencing technologies and concurrent reduction in sequencing costs now enables exome sequencing to be combined with rapid *functional* assays, such as the adapted neutral comet assay described in Chapter 3, to study the correlation between cellular and clinical outcomes in large numbers radiotherapy patients. The combination of *genomic* and *functional* approaches can begin to study the subtleties of the radiation response and begin to identify under which conditions cellular DNA repair defects identified by the assays described in Chapter 3 predict clinical radiosensitivity, and under which conditions the two are not associated. These studies may begin to identify alternative signaling pathways that have significant impact upon clinical radiation responses in the complex genetic backgrounds of routine oncology patients and could begin to guide development of more comprehensive radiosensitivity diagnostic packages.

While fully characterizing the connection between *cellular* testing and *clinical* outcome in non-XCIND patients remains to be done, present evidence provides some confidence that cellular testing could identify a portion of individuals who are more

likely to react poorly to standard doses of radiotherapy(2, 183, 257). Due to the hypersensitivity of XCIND patients to ionizing radiation and the strong recommendation to avoid irradiating XCIND patients, stratifying patients into one of two bins, radiosensitivity or radionormal, is sufficient for diagnostic purposes. However, routine radiotherapy patients likely lie along a spectrum of sensitivities and doses for radiotherapy plans will be needed to be adjusted along a similar dose spectrum based on individual patient responses, rather than eliminating radiotherapy like with XCIND patients. Thus, determining *how* sensitive a patient is remains a key, unaddressed roadblock for implementing radiosensitivity testing in oncology clinics. Conveniently, this challenge is likely to be addressed, in some part, while further studying the connection between cellular assays and clinical outcomes discussed above.

Similar to the challenge of connecting cellular assays with clinical outcome, a key hurdle to predicting *how* sensitive an individual is to ionizing radiation is developing a metric that accounts for the majority of individual variability and correlates with clinical outcome, with high confidence. The literature to date and data presented in this thesis suggests that this metric is likely to be an aggregate of measures from several assays, rather than a single assay. The current conceptual model of radiosensitivity suggests that *how* radiosensitive a patient is should be directly related to a measure of DNA repair efficiency or efficacy. The data presented in Chapter 3 indicated that, for XCIND patients, monitoring DNA repair by the neutral comet assay could be used to determine, broadly, *how* sensitive a patient is to ionizing radiation. The predicted survival fraction based on the model of the relationship between neutral comet assay % DNA repair and the clonogenic survival assay SF% could be used to establish a relative biologic effect

(RBE) value for an individual patient and used to guide adjustment of radiotherapy dose, in most cases suggesting elimination of radiotherapy for XCIND levels of sensitivity.

However, genome wide association studies aimed at identifying radiation over reactor, i.e. non-XCIND clinical radiosensitivity, associated genes and my data presented in Chapter 5 suggest that additional mechanisms, especially mitochondrial function and oxidative stress responses, likely play a significant role in routine oncology patients' risk for adverse reactions(260, 261). The studies aimed at associating DNA repair assays with clinical radiosensitivity in similar patient populations discussed above also suggest that DNA repair assays, alone, are unlikely to account for the variability in radiation responses observed in oncology patients(192, 193). Thus, to address the challenges for determining *how* sensitive an individual patient may be, additional assays that test mitochondrial function, oxidative stress responses, and/or the level of induced DNA damage will be needed to develop a robust combined metric for predicting adverse reactors to radiotherapy in non-XCIND populations.

Finally, the qualitative criteria for scoring radiotherapy toxicity are also a likely contributor to inter-individual variability. In early studies, a consistent measure of radiotherapy toxicity was not available and was a confounding factor for functional biologic assays to predict toxicity(262). Now, standardized criteria have been developed which score patient's reactions to radiotherapy on a graded scale. The criteria for determining a patient's score remain qualitative, subjective and bias' between individual clinicians and treatment sites are likely a significant source of the inter-individual variability observed in previous studies(183). Thus, exploration of unbiased methods to describe and quantitate normal tissue response to doses of ionizing radiation may prove

to be as important as identifying an ideal molecular biomarker package for confidently predicting patient outcome before radiotherapy is administered.

Candidate gene approaches for identifying novel DNA damage response modulators.

The candidate gene vs. the candidate pathway approach.

During clinical radiosensitivity testing for suspected XCIND patients, approximately 20 percent of blood samples submitted are identified as radiosensitive, but of unknown etiology. Our laboratory has utilized functional assays that test specific DDR pathways to profile these radiosensitive patient cell lines in past efforts to identify novel genes involved in the DDR and radiosensitivity. This 'candidate pathway' approach involved screening a panel of radiosensitive cell lines with a functional assay for a specific DDR pathway (i.e. the S-phase checkpoint). Cell lines with a defect in the pathway would be studied further in an effort to determine which gene in the respective signaling pathway was mutated.

In our lab, this approach successfully identified the second patient deficient for RNF168 by profiling 53BP1 foci in a panel of radiosensitive cell lines(177). However, in another similar study, this approach fell short of identifying a specific gene defect, likely due to incomplete knowledge of the signaling pathway being tested at the time of the study(156, 184). This second example outlines a central difficulty of the candidate pathway approach; identifying specific gene defects is heavily dependent on the availability of accurate knowledge for specific signaling pathway mechanisms. In my experience, candidate pathway approaches often hit dead ends due to incomplete knowledge of a signaling mechanism or conflicting information in the literature.

Additionally, this approach rarely gives substantial insights into novel DDR mechanisms because the discovery phase is dependent on what is already known about a particular signaling pathway.

Recent advancements in technology have brought 'big data' techniques, the ability to simultaneously measure many dimensions of cellular function or integrity, to biology through microarray and exome sequencing approaches. It was our laboratory's hypothesis that identifying abnormal gene expression or unique gene mutations in a panel of radiosensitive cell lines with these technologies would enable a 'candidate gene' approach for identifying novel molecules that modulate the cellular response to ionizing radiation. Further, we postulated that this unbiased candidate gene approach would enable more efficient identification of novel molecules in the DDR. Chapters 4 and 5 summarize two studies that tested this approach.

The microarray approach utilized in Chapter 4 successfully identified a novel molecule in the DDR, miR-335, though this approach was not more efficient than the candidate pathway approach. The efficiency of arriving at a candidate gene was hampered by high false positive rates and difficulties interpreting biologic impact based on changes in microRNA expression because little is known, currently, about most microRNA. Further, each microRNA can target many genes giving rise to difficulties predicting which microRNA-RNA target pairs will result in the cellular phenotype being studied. However, despite these challenges, this study illustrated that microarray technologies could be useful for identifying novel molecules in the DDR, especially molecules not readily predicted to play a role in the DDR. Additionally, microarrays are sensitive to changes in expression of transcripts and can uniquely identify molecules that

differentially impact the DDR based on expression level, rather than gene mutation, which has been the central focus of XCIND study to date.

Chapter 4 illustrates the power and efficiency of exome sequencing for identifying novel molecules in the DDR. Sequencing the exomes of RS63-3, and later RS63-7, and filtering the results based on the assumption of an autosomal recessive inherited trait and homozygocity suggested that *MTPAP* was associated with radiosensitivity. *MTPAP* was not an obvious radiosensitivity gene, and would not have been associated with a radiosensitive phenotype without exome sequencing. I was able to quickly establish a causal link between *MTPAP* mutation and radiosensitivity by transfecting wild type *MTPAP* back into the patients' cells, which rescued the radiosensitivity. It will be interesting to sequence more of the radiosensitive cell lines of unknown etiology to determine if there are more surprises and insights into novel modulators of the DDR waiting in these Experiments of Nature(254).

As 'big data' technologies become more affordable and bioinformatics analyses improve, it is likely that both exome sequencing and gene expression-sequencing hybrid assays (i.e. RNA sequencing arrays) will play a larger role in clinical diagnostics. It will be interesting to see if the current working model of XCIND and radiosensitivity holds true as more radiosensitivity candidate genes are identified by technologies which are not, by design, limited to identifying DSB repair defects. We may find that the XCIND model needs to be modified, or a new model conceptualized, to incorporate mechanisms tangentially related to DNA DSB signaling that we know mechanistically contribute to cellular responses to ionizing radiation, but have not been observed to be defective in patients. Chapters 4 and 5 would suggest that, indeed, we can expect to uncover more

unexpected molecules involved in the DDR, thus gaining a broader understanding of mechanisms that play a significant role in human radiosensitivity.

Tools for confirming candidate gene approaches.

Exome sequencing and microarray approaches identify many potential abnormalities in a single patient. Often, bioinformatics approaches are not sufficient to identify a disease causing gene or expression change with high confidence. The impact of this challenge on patient care is being discussed frequently to reach consensus on what is ethically responsible and useful for reporting results to patients(263, 264). Thus, *functional* confirmation techniques that demonstrate a causal link between a candidate molecule and a disease state are often needed to return results that are meaningful and actionable for patient care.

An example of this common predicament with exome sequencing is outlined in Chapter 5 where many potential disease-causing variants were identified in RS63-3. Previous knowledge of consanguinity in the family and the hypothesis that the disease-causing variant would contain a homozygous mutation narrowed the potential variants to two, which were further narrowed by comparing the clinical phenotype to a previous study(247). However, the final predicted disease-causing variant was not an obvious candidate for modulating radiosensitivity and it was not possible, based on the current literature, to confirm that mutation of *MTPAP* was responsible for the radiosensitivity observed in RS63-3 and -7 cells. Transfection of WT *MTPAP* into LCLs from RS63-3 and -7 confirmed the causal link between *MTPAP* mutation and radiosensitization of cells. Subsequent profiling of the DDR in RS63-3 and -7 with additional *functional* assays began to illuminate the impact of *MTPAP* mutation on the DDR and also

identified a potential therapeutic approach for the patients; using FDA approved antioxidants to offset the chronic oxidative stress measured in the patient's cells.

The study summarized in Chapter 4 also illustrates that 'big data' techniques, such as miRNA microarrays, can be powerful tools for identifying unexpected radiosensitivity associated molecules. The initial microarray experiment identified many changes in miRNA expression signatures in A-T, RS7, and RS73 LCLs and bioinformatics approaches, alone, did not sufficiently demonstrate a causal link to the DDR. Transfection of miR-335 into a cell model, HeLa cells, suggested that miR-335 overexpression could, indeed, sensitize cells to ionizing irradiation and demonstrating a causal link between miR-335 and radiosensitivity in *patients* was all that remained to be demonstrated. Antisense morpholino oligonucleotides (AMOs) were utilized to suppress miR-335 expression, abrogating the cellular phenotype in RS7 and RS73 and demonstrating the causal link between miR-335 and radiosensitivity in patients. Thus, AMOs are an attractive tool for biologic research because they are highly customizable and can be delivered into hard to transfect cells, such as LCLs, through addition of chemical moieties that improve uptake by cells. In addition to using AMOs for expression suppression, AMOs have also proven useful for remedying aberrant splicing in the *ATM* gene, demonstrating the powerful customizability of AMOs for use in radiosensitivity research(131, 135, 148).

The neutral comet assay to support RNAi driven candidate gene approaches.

Some of the early discoveries that identified potential DNA repair genes, and often their function, were due to gene mutation or knockdown in yeast(265). Once candidate genes that modulated DNA repair were identified, the human homologue

would be identified and the function of the candidate gene would be verified in human cells. Yeast models were, and still are, attractive model organisms for high-throughput screens due to the ease of genetic manipulation and robust and easily measured endpoints(265-267). In recent years, however, human RNAi libraries and accompanying high-throughput platforms have been developed leading to candidate gene identification directly in human cells(268-270). For the DNA repair field, and specifically radiosensitivity, high-throughput screens have been difficult because endpoints, such as clonogenic survival, have been challenging to robustly measure in human cells(268).

The neutral comet assay described in this thesis and by Nahas et al. is an attractive surrogate for the clonogenic survival assay and could be adapted for high-throughput experimental and analysis technologies which could enable high-throughput radiosensitization, or radioresistance, RNAi or chemical screens directly in human cells (271). Further, the neutral comet assay could be adapted as it was in Chapter 5 to measure the amount of induced DNA damage to identify genes or compounds that increase or protect against induction of DNA DSBs by ionizing radiation or other environmental inducers of DSBs. This approach could be used to generate a more comprehensive list of radiosensitizing genes and would constitute a panel of genes to screen XCIND and routine oncology patients for by exome sequencing approaches.

Evolving concepts of XCIND

Classical presentation of XCIND.

The central hypothesis underlying XCIND syndrome is that the radiosensitivity, immunodeficiency and neurologic involvement observed in these patients stem from a DNA double strand break recognition or repair defect(1, 149). Consistent with this

hypothesis, patients identified as XCIND of known etiology, to date, harbor mutations in genes that code for the core members of DNA DSB responses(149, 272). The genes fall into two broad categories based on their function in the DDR. ATM, NBN, MRE11, RAD50, and DNA-PKcs belong to the category of genes responsible for recognizing the presence of DSBs and activating downstream repair factors(50, 51, 53, 233, 273). DNA LigIV, RNF168, Artemis, Cerunnos, and the Fanconi Anemia complementation group genes are involved in downstream events crucial for the direct repair of breaks in specific pathways of DNA repair(175, 189, 274, 275).

The role of many of these genes in V(D)J and class switch recombination likely explain the immunodeficiency observed in many of the XCIND disorders(276, 277). Both V(D)J and class switch recombination occur through specific induction of DSBs in genes encoding for the heavy or light chains, removal of various sized fragments, and the ligation of the remaining fragments to generate antibodies and immune receptors(278-280). ATM plays a crucial role in stabilizing DSB complexes in V(D)J recombination to avoid abnormalities during repair and is necessary for error free class switch recombination(278, 279). DNA-PKcs, DNA LigIV, and Artemis are required for V(D)J recombination and deficiencies in these proteins in patients result in a SCID phenotype(280). These patients pose serious challenges for bone marrow transplantation to abrogate the SCID phenotype and have responded adversely to the genotoxic therapies used to remove the patient's existing bone marrow niches(1, 4, 5, 225).

It has also been postulated that aberrant, or deficient, DNA repair mechanisms are also the root cause of the neurologic involvement observed in many XCIND patients(129, 158). In A-T patients, loss of Purkinje cells in the cerebellum are suggested to be

responsible for the ataxia observed and it is postulated that disrupted DNA DSB signaling is responsible for this cell loss(157, 158, 281). However, it remains to be shown by which mechanism Purkinje cells die in A-T patients and whether the cell loss is due to defects in Purkinje cells, or functional defects in other cell types necessary to support Purkinje cell development or function(282). While the role of ATM in DSB recognition suggests the neurodegeneration in A-T is also linked to this function, it is unclear how necessary DNA DSB repair is for cells that are not actively progressing through the cell cycle, such as the various, terminally differentiated neuronal cells found in the brain(283).

Post-mitotic neural cells, due to lack of G2 or M phase cells, do not rely on homologous recombination repair, but recent evidence suggests that post-mitotic neural cells initiate a pseudo cell cycle reentry to provide an environment where NHEJ repair factors can become active. This pseudo cell cycle reentry is also used to activate, or results in, apoptotic responses, however(284-286). We have shown recently that ATM becomes activated after irradiation in neural progenitor cells derived from A-T patient fibroblasts in a manner similar to fibroblasts and LCLs(162). ATM inhibition of nuclear HDAC4 accumulation has also been shown to modulate cell cycle re-entry in post-mitotic neurons(286). It remains to be shown, however, that this role for ATM in neural progenitor cells and post-mitotic neurons is necessary for neuronal function, survival, and/or differentiation.

Current evidence would suggest that single strand break repair is likely a more important and active mechanisms in neural cells than DSB repair, despite observations that NHEJ, or a NHEJ-like mechanism, and ATM are activated in post-mitotic neural

cells in the presence of DSBs(162, 283). Mutations in ATM and other DDR genes under the XCIND umbrella also result in a cellular environment characterized by oxidative stress and increased cell death, in addition to genomic instability(114, 157, 160, 165, 287). This cellular environment, under chronic oxidative stress, may turn out to be the core driver of neurologic degeneration in many XCIND patients and mouse models would support this hypothesis(157). Analogous ataxias and neurodegeneration in adults, such as Parkinson's and Alzheimer's diseases, further suggest that chronic oxidative stress may play a significant role in the development of neural deficits and abnormalities, though at an accelerated rate in XCIND patients(127, 288, 289).

MTPAP deficient patients and an evolving concept of XCIND.

The data presented in Chapter 5 on two Amish siblings with *MTPAP* mutations suggested that these patients are an atypical presentation of XCIND. Both patients presented with radiation sensitivity, neurologic involvement, and the neutral comet assay indicated that the patients' cells had a DNA repair defect characteristic of XCIND patients. However, neither patient has presented with immunodeficiency or any evidence suggesting cancer predisposition, to date. This suggested that *MTPAP* was not involved in V(D)J or class switch recombination and was not particularly surprising because only a subgroup of suspected XCIND patients present with immunodeficiency. However, the lack of cancer in the patients was intriguing because gene defects that lead to XCIND syndrome generally result in genomic instability, and we often observe malignancies in the classical XCIND cohorts we have studied.

Further probing into the cellular phenotype of RS63-3 and -7 indicated that, indeed, these patients were likely an atypical presentation of XCIND. My results

suggested that the residual, unrepaired DSBs observed initially were actually DSBs with slower kinetics of repair, which was confirmed by profiling γ-H2AX and 53BP1 foci. Analysis of induced DNA damage levels post-IR indicated that both RS63-3 and -7 had elevated levels of DNA damage induction post-irradiation that were due to mutation of *MTPAP* and a cellular state of increased oxidative stress. The data indicate that increased levels of DNA damage induction and reduced oxidative stress homeostasis were responsible for the radiosensitivity observed, rather than persistent, unrepaired DSBs observed in most classical XCIND patients. The lack of immunodeficiency and cancer predisposition observed in these patients is consistent with the working model that they represent an atypical presentation of XCIND, and further indicates that it is unlikely that mutation of *MTPAP* directly impacts the recognition or repair of DSBs.

The neurologic features of XCIND are of central importance for the quality of life of patients and their families, and remain the least understood aspect of the XCIND phenotype. The overlapping ataxic neurologic phenotype of *MTPAP* deficient patients and A-T patients is intriguing. In Chapter 5, I demonstrated that the DNA repair defect and radiosensitivity in *MTPAP* deficient patients and A-T patients stem from different mechanisms. However, similar cellular states of oxidative stress in both sets of patients raise the question: is oxidative stress the common cellular phenotype associated with ataxia in both of these types of patients(160, 165, 290)? A number of other XCIND disorders with neurologic involvement, and non-XCIND disorders characterized by neural degeneration, also have cellular phenotypes characterized by oxidative stress(104, 126, 130, 165, 286). As better neurologic model systems become available for XCIND work, such as the patient derived human induced pluripotent stem cell model(162), it will

be interesting to probe the mechanistic and phenotypic overlap in neuronal cell types from similar neural pathologies in XCIND and XCIND-related disorders. A-T and *MTPAP* deficient patients may be an interesting set of patients to study the importance of oxidative stress in neuronal cell function because of the very different roles ATM and mtPAP play in the DDR, especially contrasted against other XCIND genes which function in ATM related pathways.

MiRNA: regulators of the radiation response and potential inducers of an XCIND phenotype.

miRNA are emerging as powerful regulators of cellular processes and increasing numbers of miRNA are being discovered that modulate tumor genesis, resistance or sensitivity to cancer therapeutics and modulation of DNA damage responses(131, 135, 148, 291). Expression levels of miRNA can modulate the DDR by targeting DNA repair proteins and can induce a cellular phenotype that is remindful of a specific genetic defect, such as A-T, without any gene mutations. An example of this paradox is summarized in a study of miR-421 overexpression in a patient being treated for squamous cell carcinoma who presented with a cellular phenotype similar to A-T, but lacked *ATM* mutations(135). miR-421 targeted ATM protein levels and reduced ATM protein levels to those of A-T cells, rendering the cells radiosensitive(131, 135).

The study summarized in Chapter 4 identifies another miRNA, miR-335, that can modulate the DDR by targeting a DSB repair protein. miR-335 reduced CtIP protein levels and miR-335 overexpression in HeLa cells and LCLs from two radiosensitive patients induced a cellular phenotype similar to that of CtIP deficient cells. These studies illustrate the powerful role miRNA can have in DNA damage signaling mechanisms. It

will be interesting to determine if miRNA, or miRNA mimics can be harnessed for therapeutic purposes as discussed in Chapter 4. In addition to demonstrating the role miRNA can play in modulating the DDR, the miR-335 and miR-421 studies illustrate an aspect of cellular regulation in disease that will not be identifiable by exome sequencing techniques. In the future, it will be interesting determine if there is overexpression of miRNA in patients with clinical phenotypes suggestive of a specific gene defect, but lack mutations in the suspected gene.

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