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| 14. ABSTRACT<br>The Neuroepithelioma transforming gene 1 (Net1) is a RhoA specific guanine nucleotide exchange factor (GEF) that is frequently overexpressed in human cancer, including breast cancer. We have previously reported that DNA damage activates Net1 to control RhoA and p38 MAPK mediated cell survival pathway in response to ionizing radiation (IR). However, others have shown that Net1 activation contributes to RhoB-mediated cell death after IR. Thus, the role of Net1 in controlling IR responses and cell survival is controversial. With the completion of the first year of this fellowship, we have found that the Net1A isoform is specifically required for DNA double-strand break (DSB)-induced signaling and DNA repair. Depletion of Net1A in human breast cancer cells reduced IR-stimulated ATM activation and signaling to its substrates Chk2 and H2AX. In addition, suppression of Net1A expression adversely affected cell survival after IR. Moreover, we observed that overexpression of the Net1A isoform significantly reduced γH2AX foci formation after IR, which required the unique N-terminal region of Net1A. Importantly, this effect did not require Rho GTPase activation by Net1A, and was not recapitulated by overexpression of RhoA or RhoB. Net1A was also found to co-immunoprecipitate with the DNA-PK complex in an IR-regulated manner. Taken together, our current data suggests a model in which Net1A functions as a non-catalytic binding protein to control DNA damage response signaling and DNA repair to affect cell survival after IR. |                  |                                  |                                      |                                                    |                                            |
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## Introduction

In the United States, breast cancer is the second most frequently diagnosed cancer in women (8). Ionizing radiation (IR) is commonly used to treat breast cancer patients after surgery, as well as for care of inoperable metastatic tumors (23). However, despite advances in the use of radiation therapy, a significant percentage of patients experience relapse of their cancers. Exposure to ionizing radiation (IR) causes double strand DNA breaks that are lethal to a cell if not repaired. In response to IR exposure a signal transduction cascade is initiated that activates cell cycle checkpoints to cause cell cycle arrest, thereby allowing the cells time to repair their damaged DNA (12). The ability of a cell to efficiently respond to IR treatment and evade cell death forms the basis for radiation resistance in cancer cells to allow for tumor recurrence. Thus, understanding the mechanisms controlling DNA damage signaling is necessary to devise more efficient treatments for breast cancer.

Rho GTPases control diverse aspects of cell behavior, including organization of the actin cytoskeleton, cell migration, cell cycle progression, and gene expression (11). Rho GTPases acts as molecular switches by cycling between an active GTP-bound and an inactive-GDP bound state (16). The activation of Rho GTPases is regulated by specific guanine nucleotide exchange factors (GEFs) (25). Net1 (Neuroepithelial transforming gene 1) is a RhoA subfamily specific GEF that was originally identified as a transforming gene in NIH3T3 cell focus formation assays (3). Upregulation of Rho protein activity has been shown to contribute to tumor initiation and progression, and aberrant regulation of Rho proteins has been found in tumor cells (2, 26). Recently, we have shown that Net1 is overexpressed in human breast carcinomas and its co-expression with the  $\alpha 6 \beta 4$  integrin is associated with a high risk for distant metastasis in node positive breast cancer patients (9, 22, 28, 31). Previous work in our lab demonstrated that expression of the RhoA activating protein Net1 is required for cell survival following exposure to IR (15). Importantly, depletion of Net1 expression suppressed RhoA activation and induced cell death upon DNA damage (15). These results suggest that Net1 plays a critical role in the cellular response to DNA damage, and indicate that Net1 may be an attractive therapeutic target for sensitization of breast cancer cells to IR. Therefore, we decided to investigate the role of Net1 in DNA damage response signaling in breast cancer.

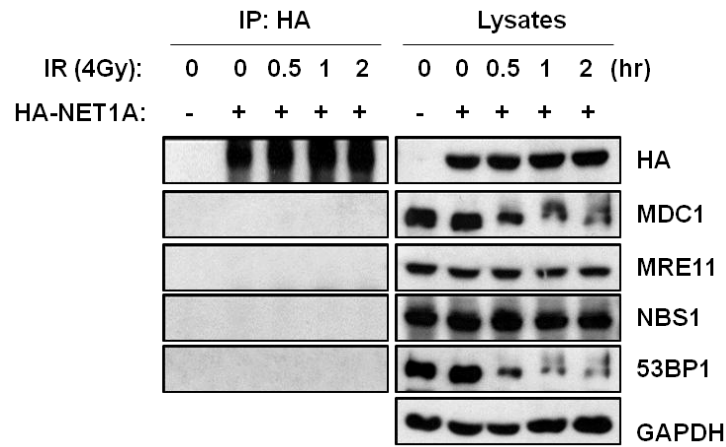
## Body

### **Task 1: To identify the role of Net1 in recruiting early DNA damage response proteins to double strand breaks (Months 13-24).**

#### **Task 1a: Identify the interaction of Net1 with MRN complex after IR (Months 13-15).**

In response to DNA damage, DNA double strand breaks lead to activation of ataxia-telangiectasia mutated (ATM), which phosphorylates downstream targets to initiate cell cycle arrest, DNA repair, and apoptosis. The MRE11/RAD50/NBS1 (MRN) complex acts not only as a DSB sensor for ATM but also as a substrate of ATM (30). However, the mechanism of ATM activation is not entirely clear. In Year 1 of the fellowship we showed that knockdown of the Net1A isoform attenuated ATM phosphorylation after IR treatment in breast cancer cells. To determine whether Net1 controls MRN function, we first examined whether Net1 is able to interact with MRN complex proteins in IR treated cells using coIP/Western blot assays.

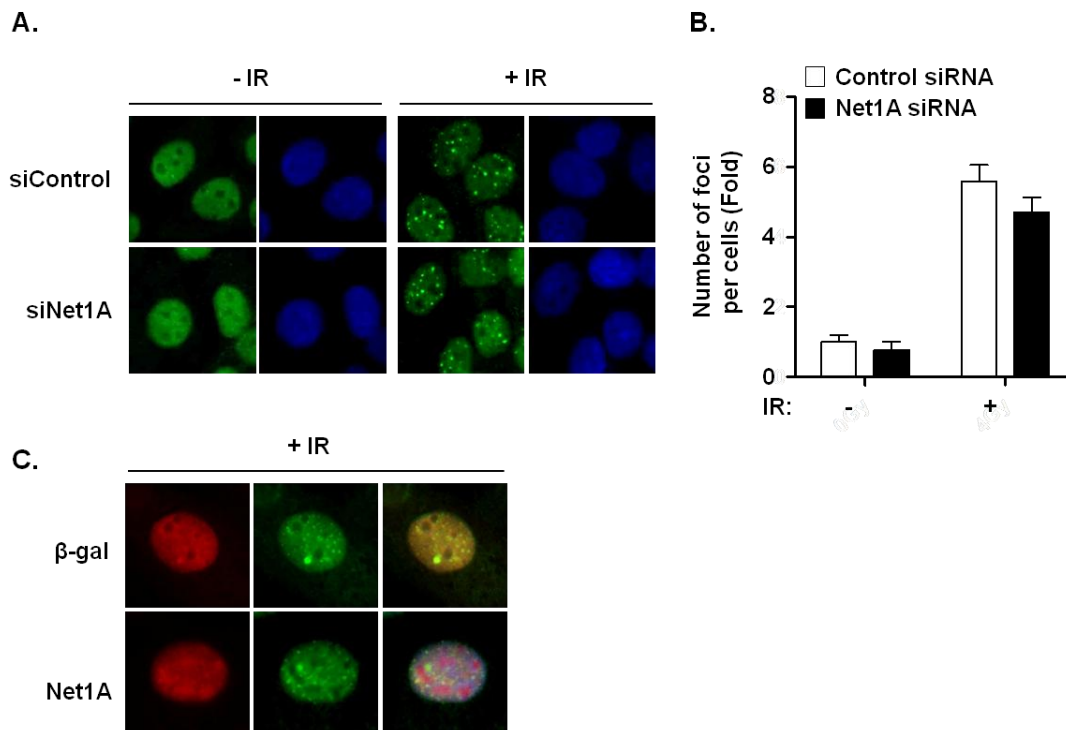
We transfected MCF7 breast cancer cells with HA-epitope tagged Net1A and immunoprecipitated it after treatment with IR. We found that Net1A did not interact with MRN components in MCF7 cells in the presence or absence of IR (Figure 1). This finding suggests that Net1A directly affects ATM activation without affecting association with MRN complex in breast cancer cells.



**Figure 1. Test for interaction of Net1A with MRN complex proteins in response to IR.** MCF7 cells were transfected with HA-epitope tagged Net1A and cells were harvested at the indicated time points after IR treatment. The cells were then subjected to IP with anti-HA rabbit antibody and blotted with indicated antibodies.

#### Task 1b: Identification of the role of Net1A in MRN complex recruitment to DSBs (Months 16-18).

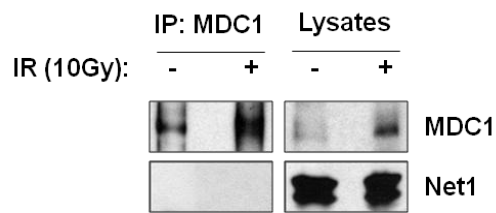
To further characterize the regulation of DNA damage signaling by Net1A, we examined whether Net1A regulates MRN complex recruitment to IRIF by measuring the levels of NBS1 within IRIF in Net1A knockdown or Net1A overexpressing cells using an immunofluorescence assay. MCF7 cells were transfected with control or Net1A isoform specific siRNAs, exposed to IR, and cells were stained for NBS1. We observed NBS1 foci formation after 6h post IR treatment. We counted foci numbers per cell and compared NBS1 IRIF between control and Net1A depleted cells. We did not see significant effect of loss of Net1A on NBS1 IRIF formation (Figure 2A and B). We also tested effects on NBS IRIF by Net1A overexpression. Overexpression of  $\beta$ -Gal control and Net1A showed similar NBS1 foci formation after IR treatment (Figure 2C). Taken together these experiments suggested that the regulation of ATM activation by Net1A is not mediated by effects on the MRN complex association with DSBs.



**Figure 2. Identification of the role of Net1A in MRN complex recruitment to DSBs.** (A) MCF7 cells were transfected with control or Net1A isoform specific siRNAs. Three days later, the cells were then treated with 4Gy IR and fixed after 6h. NBS foci formation was detected with anti-NBS and Alexa 488 mouse antibodies. (B) Quantification of NBS1 foci in Net1A knockdown cells. Errors are SEM. (C) MCF7 cells were transfected with the control Myc-NLS-β-Gal or HA-epitope tagged wild type Net1A. The cells were untreated or treated with 10Gy IR, fixed and immunostained for anti-NBS1 (green), HA and Myc antibodies (red). Shown are IR-treated cells.

### Task 1c: Identification of the role of Net1 in MDC1 recruitment to DSBs (Months 19-21).

MDC1 is an ATM substrate and mediates the accumulation of many DDR factors at the sites of DSBs, including the ATM, the MRN complex, BRCA1, and 53BP1. Specifically, accumulation of the MRN complex at sites of DSBs is dependent on MDC1 (5). Regarding this, Net1 may regulate MDC1 recruitment to IRIF. We first examined whether Net1A associates with MDC1 in response to IR using a co-immunoprecipitation (coIP) /western blot assay. We tested whether endogenous Net1 interacts with MDC1 in MCF7 cells after treatment with IR. However, we were not able to detect their association under these conditions (Figure 3). Therefore, we further tested this by Net1A overexpression. We transfected MCF7 breast cancer cells with HA tagged Net1A and immunoprecipitated the Net1A after IR treatment. We found that Net1A did not interact with MDC1 in MCF7 cells in the presence or absence of IR (Figure 1). These results suggest that MDC1 is not involved in Net1A-mediated ATM signaling following IR in breast cancer cells.



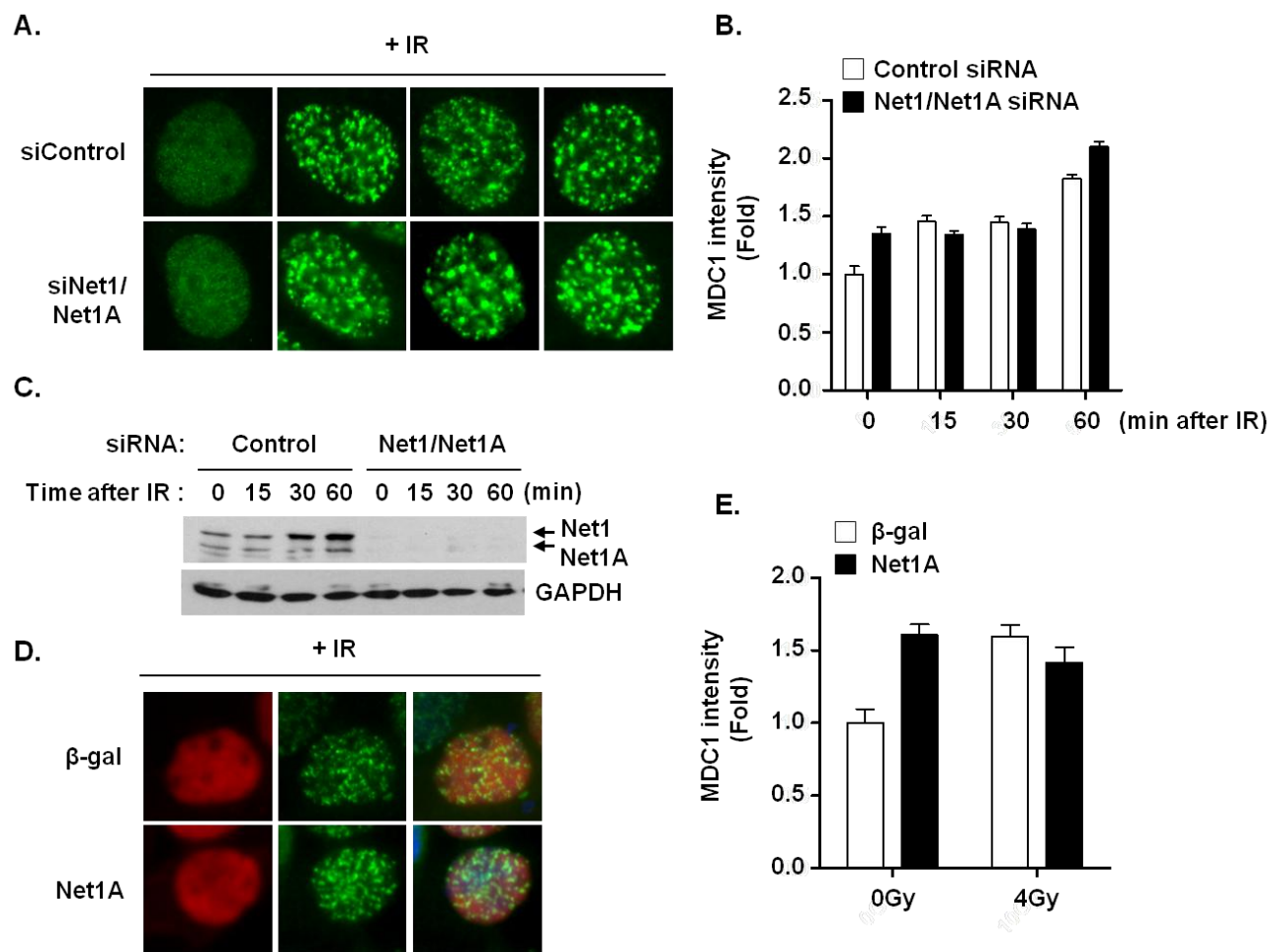
**Figure 3. Identify the interaction of Net1A with MDC1 in response to IR.** MCF7 cells were irradiated or not with 10Gy IR. The cells were then subjected to IP with anti-MDC1 mouse antibody and blotted with indicated antibodies.

### Task 1d: Identification of the role of Net1A in MDC1 complex recruitment to DSBs (Months 22-24).

We then determined whether Net1A affects MDC1 recruitment to IRIF using immunofluorescence analysis. In year 1, overexpression of Net1A significantly suppressed phosphorylated ATM (pATM) in response to IR in MCF7 cells. Thus, we wanted to further characterize the regulation of DNA damage signaling by Net1A in breast cancer. To investigate whether Net1A controls the recruitment of MDC1 to IRIF, we examined whether siRNA mediated knockdown of Net1A or overexpression of Net1A affects MDC1 localization to IRIFs by immunofluorescence analysis.

We first determined whether Net1A might be required for IR-induced foci formation. We examined the ability of MDC1 to form foci in Net1A siRNA transfected cells after IR. We observed MDC1 foci formation after 30 min post IR treatment. We measured MDC1 intensity and compared MDC1 IRIF between control and Net1A depleted cells. However, we did not see a significant effect of Net1A knockdown on MDC1 IRIF in MCF7 cells (Figure 4A-C).

To further test whether overexpression of Net1A affects the recruitment of MDC1 to IRIFs, we transfected Net1A and measured MDC1 intensity after IR. IR treatment induced a rapid increase of MDC1 foci formation but we did not observe a significant effect of Net1A overexpression on MDC1 recruitment to IR-induced foci. This data suggests that Net1A does not affect this downstream pathway of pATM (Figure 4D and E).



**Figure 4. Identification of the role of Net1 in MDC1 recruitment to DSBs.** (A) MCF7 cells were transfected with control or Net1/Net1A dual isoform specific siRNAs. Three days later, the cells were then treated with 10 Gy IR and fixed at the indicated time points. MDC1 foci formation was detected with anti-MDC1 and Alexa 488 mouse antibodies. (B) Quantification of MDC1 intensity in both Net1/Net1A knockdown cells. Mean fluorescence image intensities were quantified using Image J software. Errors are SEM. (C) Representative Western blot of siRNA transfected cells. (D) MCF7 cells were transfected with Myc-NLS-β-Gal, HA-epitope tagged wild type Net1A. The cells were untreated or treated with 10Gy IR, fixed and immunostained for anti-NBS1 (green), HA and Myc (red) antibodies. Shown are IR-treated cells. (E) Quantification of MDC1 intensity. Errors are SEM.

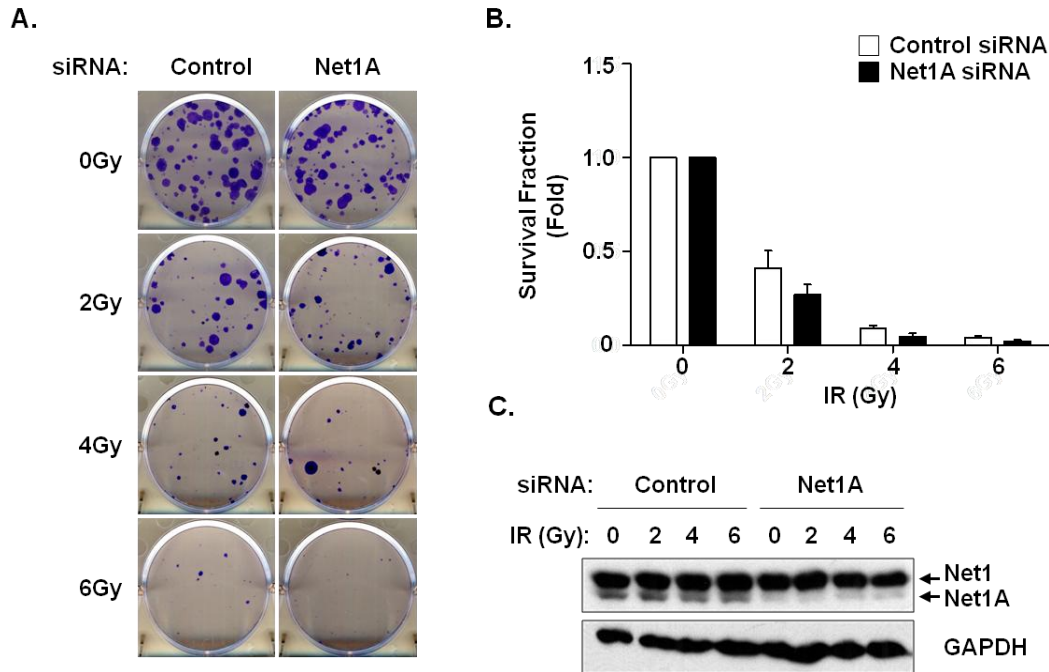
## **Task2: To determine the physiological role of Net1 in cellular responses to IR. (Months 13-24).**

### **Task 2a: Determine the role of Net1 in breast cancer cell survival in response to IR (Months 13-16).**

Our previous studies have shown that inhibition of Net1 expression sensitizes cells to the apoptotic effect of double strand DNA damage (15). Thus, this study indicates that Net1 regulates cell survival in response to double strand DNA damage. To determine whether this affects the long term viability of cells, we conducted clonogenic survival assays following Net1 knockdown in MCF7 breast cancer cells. Clonogenic survival assays are the accepted standard for discerning effects on radiation sensitivity (20). For these assays we used MCF7 cells, which are reflective of the luminal class of breast tumors that are often effectively treated by IR (21).

Cells were transfected with control or Net1A specific siRNAs and then treated with different doses of IR. The cells were then plated and allowed to form colonies for two weeks. We observed that knockdown of

Net1A reduced the number of colonies after IR (Figure 5). Therefore, this finding suggests that Net1A is required for MCF7 cell survival after exposure to IR.

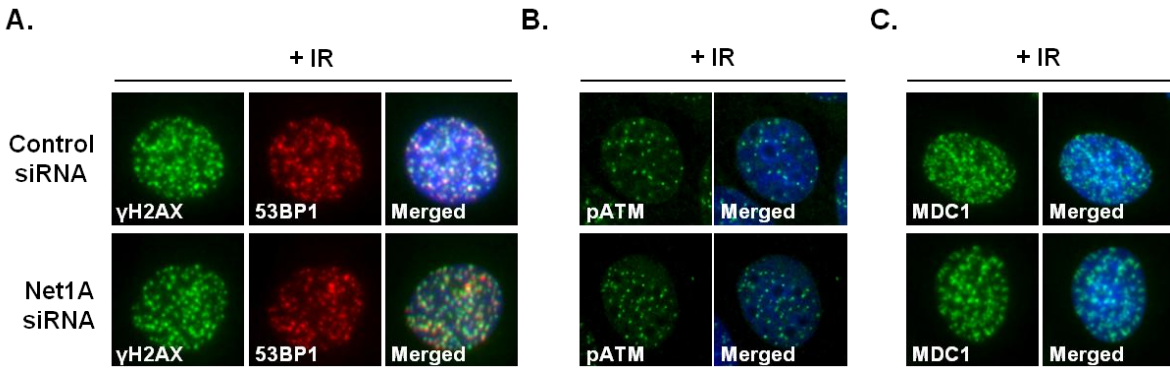


**Figure 5. Net1A expression is required for cell survival after IR.** (A) MCF7 cells were transfected with control or Net1A siRNAs. The cells were irradiated, replated and allowed to form colonies. (B) Quantification of survival fraction after IR. Shown is the mean of 3 independent experiments. Errors are SEM. \* = p<0.05. (C) Representative Western blot of siRNA transfected cells.

## Task 2b: Determine the role of Net1 in ATM-mediated DNA repair (Months 13-24).

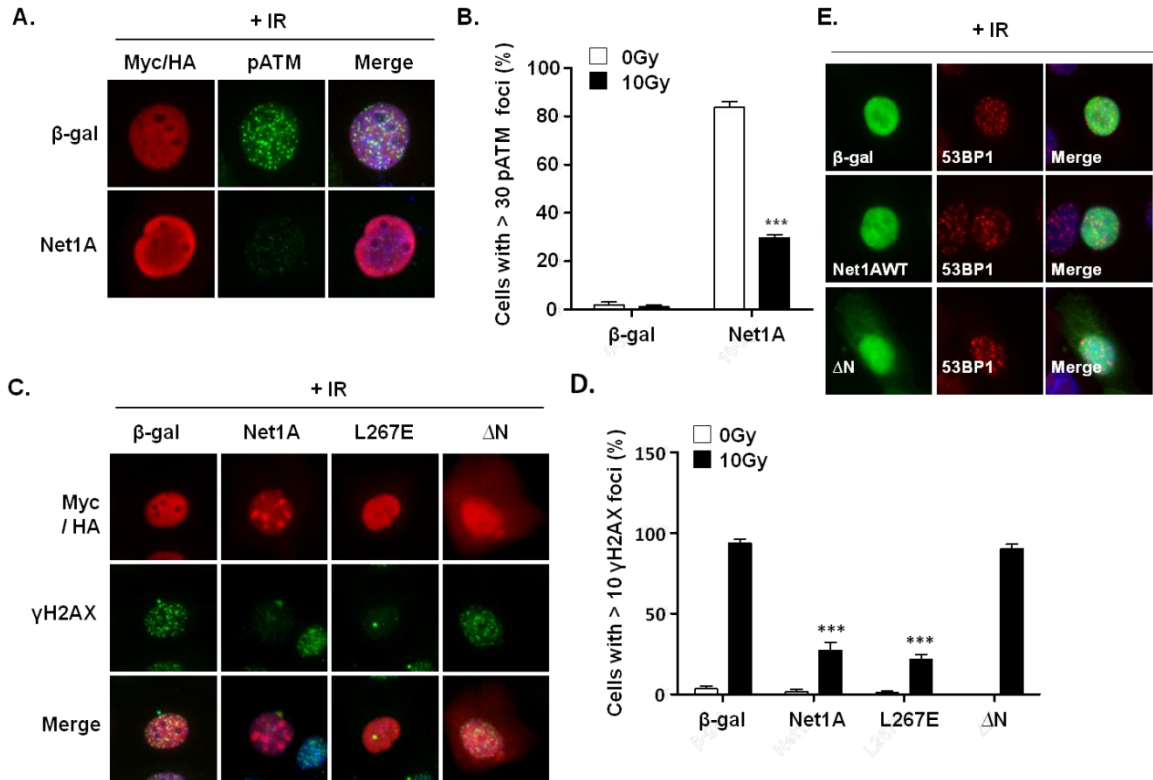
The DNA damage response (DDR) is a signal transduction pathway leading to transient cell cycle arrest, DNA repair, and elimination of damaged cells by apoptosis (12). DDR is primarily mediated by the phosphatidylinositol 3-kinase-like protein kinases (PIKKs), which include ATM, ATR, and DNA-PK (8). ATM phosphorylates many downstream targets to control DNA damage signaling, including Chk2 and p53 (32). This activation leads to transient cell cycle arrest. At the same time, activated ATM phosphorylates a histone protein variant called H2AX (6). H2AX phosphorylation recruits the MDC1 protein, which is a mediator of DNA damage checkpoint activation. Phosphorylated MDC1 and H2AX allow the recruitment of many additional factors, including p53, BRCA1, and 53BP1, to cause cell cycle arrest and begin the process of DNA repair. Unrepaired DNA damage can lead to permanent cell cycle arrest and apoptosis (12).

In Year 1, we found that knockdown of Net1A, but not Net1, significantly reduced activation of ATM activation in MCF7 cells following IR. Thus, we investigated whether Net1A is required to the recruitment of ATM downstream targets to IRIF. MCF7 cells were transfected with control or Net1A isoform specific siRNAs, exposed to IR, and detected of phosphorylated ATM (pATM), H2AX ( $\gamma$ H2AX), 53BP1, and MDC1. However, we observed that knockdown of the Net1A isoform did not affect to IRIF formation of ATM and its downstream targets after IR (Figure 6). These results indicate that Net1 isoform may compensate for the loss of Net1A on IR-induced foci formation of ATM and its targets in breast cancer cells.



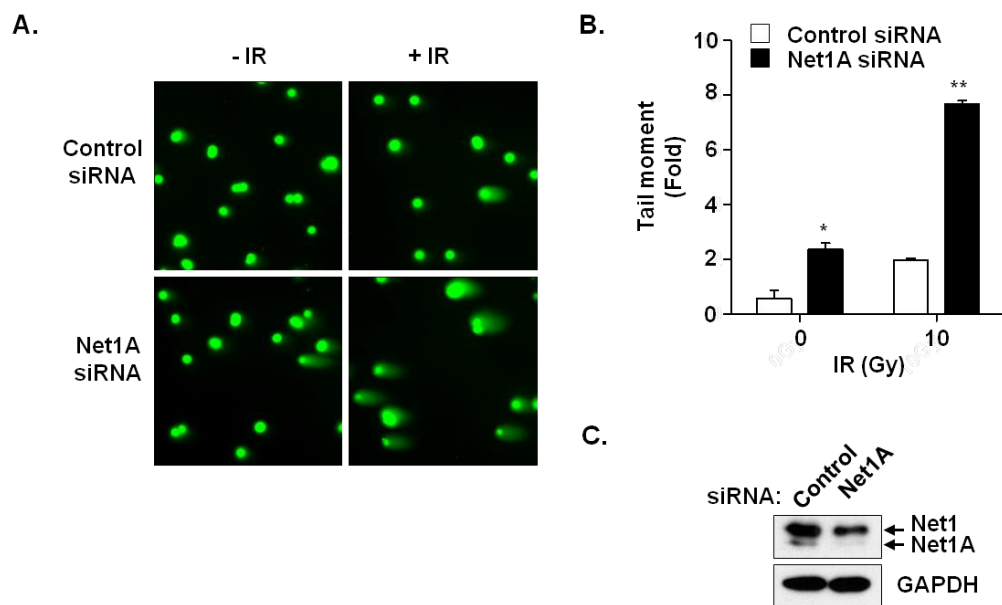
**Figure 6. Effect of Net1A in the recruitment of ATM downstream substrates to DSBs.** (A) MCF7 cells were transfected with control or Net1A isoform specific siRNAs. Three days later, the cells were fixed after 10 Gy IR treatment and then immunostained with anti- $\gamma$ H2AX and 53BP1 antibodies. (B) MCF7 cells were transfected with control or Net1A isoform specific siRNAs. Three days later, the cells were fixed after 2 Gy IR treatment and then immunostained with anti-ATMpS1981 antibody. (C) MCF7 cells were transfected with control or Net1A isoform specific siRNAs. The cells were untreated or treated with 10 Gy IR, fixed and immunostained for anti-MDC1 antibody. Shown are IR-treated cells.

We further investigated whether Net1A overexpression affected ATM signaling. For these experiments we measured the localization of pATM or  $\gamma$ H2AX to IR induced nuclear foci (IRIF) using wild type or N-terminal deletion mutant of Net1A. Surprisingly, we observed that overexpression of Net1A, but not nuclear targeted  $\beta$ -galactosidase (NLS- $\beta$ -Gal), dramatically inhibited the formation of pATM and  $\gamma$ H2AX, but not 53BP1 IRIF (Figure 7). Taken together, these data indicate that overexpressed Net1A suppresses ATM activation and phosphorylation of its downstream substrate H2AX in breast cancer in response to IR.



**Figure 7. Effect of Net1A in the recruitment ATM downstream substrates to DSBs.** (A) MCF7 cells were transfected with control or Net1/Net1A dual isoform specific siRNAs. Three days later, the cells were then treated with 10 Gy IR and fixed at the indicated time points. pATM foci formation was detected with anti-pATM and Alexa 488 mouse antibodies. (B) Quantification of pATM intensity in Net1/Net1A knockdown cells. Mean fluorescence image intensities were quantified using Image J software. Errors are SEM. (C) MCF7 cells were transfected with Myc-NLS- $\beta$ -gal, HA-epitope tagged wild type Net1A. The cells were untreated or treated with 10Gy IR, fixed and immunostained for anti- $\gamma$ H2AX (green), HA and Myc antibodies (red). Shown are IR-treated cells. (D) Quantification of  $\gamma$ H2AX intensity. Errors are SEM.

In response to DNA damage, mammalian cells have to arrest the cell cycle to allow time for DNA repair. Double strand breaks are repaired by two main pathways, including non-homologous end joining (NHEJ) and homologous recombination (HR) (18). ATM plays important role in HR. For HR, double strand breaks are detected and processed by the MRN complex. This leads to recruitment and activation of ATM. ATM then phosphorylates many substrates required for arrest and repair. Because of its pivotal role in IR responses, ATM represents a critical target of breast cancer therapy and downregulation of ATM has been implicated as a therapeutic strategy for radiosensitization of breast cancer (7, 14, 28, 29,). In addition, a low level of ATM expression is associated with advanced breast cancer (14). Thus, we further tested for the role of Net1 in DNA repair pathway.



**Figure 8. Effect of Net1A in the recruitment ATM downstream substrates to DSBs.** (A) Net1A knockdown delays DNA repair after IR. MCF7 cells were transfected with control or Net1A siRNAs. Three days later the cells were treated with 10 Gy IR. Four h later the cells were tested for DNA damage by comet assay. Shown is a representative experiment from three independent experiments. (B) Quantification of comet assays. Results are the average of three independent experiments. At least 100 cells were analyzed per sample. Errors are SEM; \* =  $p < 0.05$ , \*\* =  $p < 0.005$ . (C) Representative Western blot of siRNA-transfected cells.

We originally planned to measure DNA repair using DR-GFP DNA repair reporters in MCF7 cells, however we had trouble to detect a DNA repair after establishment of the stable cell lines. Alternatively, we examined the rate of DNA damage repair using neutral comet assay. The neutral comet assay (single-cell gel electrophoresis) is a sensitive and rapid technique for quantifying and analyzing DNA damage in individual cells. We observed that loss of Net1A expression resulted in an increase in the amount of damaged DNA in both non-irradiated cells, as well as in cells four hours after irradiation (Figure 8A-C). These data suggest that Net1A expression may be required for efficient DSB repair.

As a second assay to check for effects on DNA repair, we will use a well established DSB repair assay (19). We received a fibroblast cell line stably expressing DR-GFP composed of two mutated GFP cassettes, SceGFP and iGFP. Induction of DSBs by expression of endonuclease I-SceI results in the activation of ATM signaling pathway. We will test which DNA repair pathway is regulated by Net1A following IR in breast cancer cells using the cell lines. Then we will determine the percentage of GFP-positive cells between control and Net1A depleted cells. This experiment will address whether Net1A regulates HR or NHEJ DNA repair following IR.

## Key Research Accomplishments

- I determined that Net1A did not associate with the MRN complex and that Net1A did not affect to IR-induced DNA damage foci formation of the MRN complex protein NBS1 after IR treatment.
- I determined that Net1A did not associate with MDC1 and that Net1A did not affect to IR-induced DNA damage foci formation of MDC1 after IR treatment.
- I determined that Net1A overexpression inhibits IR-induced pATM and  $\gamma$ H2AX foci formation independent of its catalytic activity.
- I determined that Net1A expression is important for cell survival in response to IR.
- I determined that Net1A expression is required for DNA repair after DNA damage.

## Reportable Outcomes

Oh, W, Frost, JA (2014) Rho GTPase independent regulation of ATM activation and cell survival by the RhoGEF Net1A. *EMBO Rep.* (submitted, see Appendix 1).

## Conclusions

In our published studies Net1 expression is required for cell survival in response to DNA damage. However, there has been no molecular mechanism describing how Net1 regulates DNA damage responses. In the second year of this fellowship we showed that the Net1A isoform did not associate with the MRN complex or MDC1, which are downstream substrates of ATM following IR exposure. Furthermore, we did not detect the effect of Net1A knockdown or overexpression on the recruitment of MRN complex or MDC1 to IRIF following IR treatment. Interestingly, Net1A overexpression inhibits IR-induced DNA damage foci formation independent of its ability to stimulate the activity of its substrates RhoA or RhoB. Thus, we found that Net1A affect ATM

activation but not its further downstream signaling in response to IR. A second key finding of this study is that Net1A reduced cell survival in breast cancer cells after IR exposure. This suggests that Net1A expression is important for cell survival in response to IR. A third key finding of this study is that depletion of Net1A inhibits DNA repair activity after IR, suggesting that Net1A expression is critical for DNA repair after IR exposure. Together, these findings support a role for Net1A in DNA damage signaling and repair. Overall, the preliminary findings obtained during the second year of the current funding period will provide important knowledge on breast cancer research by identifying Net1A as a novel regulator of the double strand DNA damage response and repair. The research conducted in this year will lead to the publication of a manuscript in the future. It may also serve as the basis for the discovery of new therapeutic strategies for breast cancer.

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## Appendix

### Appendix 1:

#### **Rho GTPase independent regulation of ATM activation and cell survival by the RhoGEF Net1A**

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ATM activation following DNA damage is a critical event which is required for efficient DNA repair and cell survival, yet signaling mechanisms controlling its activation are incompletely understood. The RhoGEF Net1 has previously been reported to be activated by double strand DNA damage to control Rho GTPase activation and downstream cell survival outcomes. As most cells express two isoforms of Net1 that regulate distinct physiological events, we assessed their roles in ATM activation, DNA repair and cell survival. We observed that the Net1A isoform expression was specifically required efficient activation of ATM, but not the related kinase DNA-PK, after ionizing radiation. Surprisingly, Net1A overexpression also potently suppressed ATM activation and phosphorylation of its substrate H2AX. This effect did not require catalytic activity towards RhoA or RhoB, and neither Rho GTPase affected ATM activation on its own. Importantly, Net1A expression was required for DNA repair and impaired cell survival. Taken together, these data indicate that Net1A plays a previously unrecognized, Rho GTPase-independent role in controlling ATM activity and downstream signaling after DNA damage to impact cell survival.