AWARD NUMBER: W81XWH-16-1-0448

TITLE: “Translational Significance of p53 Loss of Heterozygosity in Breast Cancer.”

PRINCIPAL INVESTIGATOR: Natalia Marchenko

CONTRACTING ORGANIZATION:

Stony Brook University
Department of Pathology

REPORT DATE: Sept 2020

TYPE OF REPORT:

Annual progress report

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;

Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

| 1. REPORT DATE Sept 2020 | 2. REPORT TYPE<br>Annual progress report | 3. DATES COVERED<br>09/01/2019 - 08/31/2020 |
|---|---|---|

**4. TITLE AND SUBTITLE**

Translational Significance of p53 Loss of Heterozygosity in Breast Cancer

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-16-1-0448

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Natalia Marchenko Email: Natalia.marchenko@stonybrookmedicine.edu

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) AND ADDRESS(ES)**
Stony Brook University
Department of Pathology
W5510 Melville Library
Stony Brook, NY 11794-3362

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**. In our previously DOD-funded study, we employed a novel MMTV;ErbB2 mouse model carrying the heterozygous R172H p53 mutation (corresponds to R175H p53 mutation in a human, H/+;ErbB2 after that). We have identified novel oncogenic activity of mutant p53 (mutp53) - exacerbated p53LOH, which was profoundly increased and associated with enhanced metastases after irradiation (IR). We established oncogenic outcomes of p53LOH- the loss of p21 expression (the transcriptional target of wtp53) and deficient G2/M checkpoint after genotoxic stress. Hence, p53LOH enables cell cycle re-entry after IR promoting genomic instability. Contrary, in H/+;ErbB2 cells, the wtp53 allele retains the ability to induce p21, G2/M checkpoint, and growth arrest in response to genotoxic stress. Furthermore, we found the strong association of p53LOH with centrosome aberrations in the presence of a mutp53 allele (H/-;ErbB2 cells) indicated by the following phenotypes: (1) centrosome amplification; (2) the increased rate of pseudo-bipolar mitosis via centrosome clustering; (3) aneuploidy and increased chromosomal instability (CIN) as a readout of centrosome malfunction; (4) stabilization of mutp53 protein; (5) upregulation of Nek2 (member of Never in Mitosis (NIMA) Related Kinases family) protein, which localizes to centrosome and regulates its function Markedly, we found that specifically H/-;ErbB2 cells require Nek2 function for their survival. Furthermore, our RNAseq and functional study p53LOH in the presence of mutp53 allele may promote metastases in a gain-of-function manner via complementary mechanisms: 1) modulation ECM-integrin signaling; 2) upregulation Nek2 and its mediated metastatic properties; 3) inducing genomic instability. The main accomplishment of the project up-to-date that based on mechanistic studies, we identified Nek2 as a pharmacological target to prevent p53LOH emergence in mutant p53 heterozygous cells. As a proof-of-principle, we demonstrate that Nek2-specific inhibitor JH295 precludes the loss of wtp53 allele in mutp53 heterozygous cells after irradiation

**15. SUBJECT TERMS**
p53 loss of heterozygosity, a mouse model of Her2 positive breast cancer, radiotherapy, genomic instability,

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON<br>USAMRMC |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | 19b. TELEPHONE NUMBER *(include area code)* |
| Unclassified | Unclassified | Unclassified | Unclassified | 58 | |

**Standard Form 298 (Rev. 8-98)**
**Prescribed by ANSI Std. Z39.18**

**Table of Contents**

**Page**

1. Introduction……………………………………………………….4

2. Keywords………………………………………………………….4

3. Accomplishments………..…………………………………………4-11

4. Impact…………………………...……………………………………11-12

5. Changes/Problems...….……………………………………………… 12-13

6. Products………………………………….……….….……………….13

7. Participants & Other Collaborating Organizations……………14-16

8. Special Reporting Requirements……………………………… 16

9. Appendices…………………………………………………………17-58

1. INTRODUCTION:

P53 is the tumor suppressor that plays a pivotal role in promoting DNA repair, cell death, and growth arrest in response to DNA damage induced by conventional genotoxic modalities. TP53 is the most frequently mutated gene in human breast cancer and, particularly, in Her2(ErbB2)-positive breast cancer (72%), where it is associated with resistance to therapies and poor outcomes for patients. Typically, mutations in the TP53 gene occur through a two-hit mechanism. A missense mutation in one allele is followed by loss of remaining wild type p53 (wtp53) allele (p53LOH, loss of heterozygosity), suggesting the strong selective pressure for p53LOH occurrence during tumor progression. Despite the strong notion that p53LOH is the crucial event in cancer progression, the mechanism of its occurrence, oncogenic outcomes, selective forces are driving p53LOH, and how to prevent its occurrence remain unclear. Our objective for the reporting period was to establish the mechanism p53LOH emergence and the translational approaches of its inhibition.

In our previously DOD-funded study, we employed a novel MMTV;ErbB2 mouse model carrying the heterozygous R172H p53 mutation (corresponds to R175H p53 mutation in a human, H/+;ErbB2 after that). We have identified novel oncogenic activity of mutant p53 (mutp53) - exacerbated p53LOH, which was profoundly increased and associated with enhanced metastases after irradiation (IR). The major innovative findings for the reporting period are the following. We found that the p53LOH in the presence of a mutp53 allele (H/-;ErbB2 cells) enables the following oncogenic phenotypes in the gain-of-function manner: (1) increased proliferation; (2) abrogation of G2/M checkpoint; (3) centrosome amplification; (4) the increased rate of pseudo-bipolar mitosis via centrosome clustering; (5) aneuploidy and increased chromosomal instability (CIN) as a readout of centrosome malfunction; (7) stabilization of mutp53 protein; (5) upregulation of Nek2 (member of Never in Mitosis (NIMA) Related Kinases family) protein, which localizes to centrosome and regulates its function. Markedly, we found that specifically H/-;ErbB2 cells require Nek2 function for their survival. Furthermore, our RNAseq and functional studies demonstrated that p53LOH in the presence of mutp53 allele might promote metastases in a gain-of-function manner via complementary mechanisms: 1) modulation ECM-integrin signaling; 2) upregulation Nek2 and its mediated metastatic properties; 3) inducing genomic instability. The main accomplishment of the project up-to-date is the identification of Nek2 as a pharmacological target to prevent p53LOH onset in mutant p53 heterozygous cells. As a proof-of-principle, we demonstrate that Nek2-specific inhibitor JH295 precludes the loss of wtp53 allele in mutp53 heterozygous cells after irradiation. These findings may have a significant translational impact, as they may provide a foundation for developing a novel therapeutic strategy to curb tumor progression.

**The major accomplishments of the project up-to-date**

2. KEYWORDS: p53, mutant p53, ErbB2, Her2, breast cancer, loss of heterozygosity (LOH), radiation, chemotherapy, Her2 positive breast cancer.

3. ACCOMPLISHMENTS:

The major goals of the project are:

**Major Task 1. Determine the effect of DNA-damaging therapeutics on p53 LOH and tumorigenesis in ErbB2- driven mutp53 mammary tumors in vivo.**

**Subtask 1. Define the physiological consequences of p53 LOH in ErbB2-driven mammary tumorigenesis.** *Analyze histopathology, the ErbB2/HSF1 signaling by IHC and Western in the established collection of mammary tumors from irradiated and non-irradiated mice with different p53 LOH status. (timeline months: 1-12, 100% completion).*

**Subtask 2. Evaluate the effect of different p53 mutations on p53 LOH in ErbB2-driven mammary tumorigenesis.**

*Test whether similar to R172H, R248Q mutant p53 allele aggravates mammary tumorigenesis compared to p53 null counterparts and promotes p53 LOH after irradiation. (timeline months: 1-24, 50% completion)*

In the previous progress report, we demonstrated that in contrast to R172H p53 mutation, breast cancer latency, and the survival between p53R248Q/+;Neu and p53−/+;Neu siblings were similar. These results implicate p53 mutation-specific effects on mammary cancer development and progression in ErbB2 context. Although further experimental proofs are needed, this data highly suggests that physiological outcomes of irradiation in p53R248Q/+;Neu mice would be similar to -/+;ErbB2 mice, which we extensively evaluated in the current study. If so, the potential impact of these experiments is expected to be low. Therefore, instead of perusing this subtask, we dedicated the time and resources to investigate the mechanism by which mutp53 promotes p53LOH and pharmacological approaches to prevent its occurrence (**Major Task 2, Subtask 3**). Although beyond the originally proposed study, this knowledge may have a significant clinical impact, as it will help to understand how mutp53 heterozygous tumors in early stages respond to DNA damage and to identify the potential targets to prevent the adverse effects of genotoxic therapies in early stages of breast cancer.

**Subtask 3. Assess the effect of irradiation of established mutp53;ErbB2 tumors on p53 LOH (adjuvant setting). Test whether irradiation of established tumors induces LOH and accelerates mammary tumorigenesis in R172H/+;ErbB2 mice.**

*First, we will expand our breeder's colonies to generate 60 females of each genotype: p53-/+;ErbB2, H/+;ErbB2 and +/+;ErbB2. (timeline: months 1-12, 80% completion). 30 mice of each genotype will be irradiated with a single dose of 5Gy irradiation at the time of tumor presentation. The monitoring and analysis as described for the Aim1b. 60 females p53-/+;ErbB2 + 60 females H/+;ErbB2 60 females +/+;ErbB2 = 180 total animals. (timeline: months 12-32, 90% completion).* For the implementation of this task, we irradiated (5Gy) mice with different p53 genotypes in the adjuvant setting and compared to results of neoadjuvant irradiation (Fig. 1). For the adjuvant protocol, mice were irradiated at the time of onset 1cm$^3$ mammary tumors. For the neoadjuvant protocol, mice were irradiated with the indicated doses at 80 days (time of onset of pre-malignant lesions). We found that contrary to neoadjuvant high dose irradiation (5Gy), adjuvant irradiation does not accelerate p53

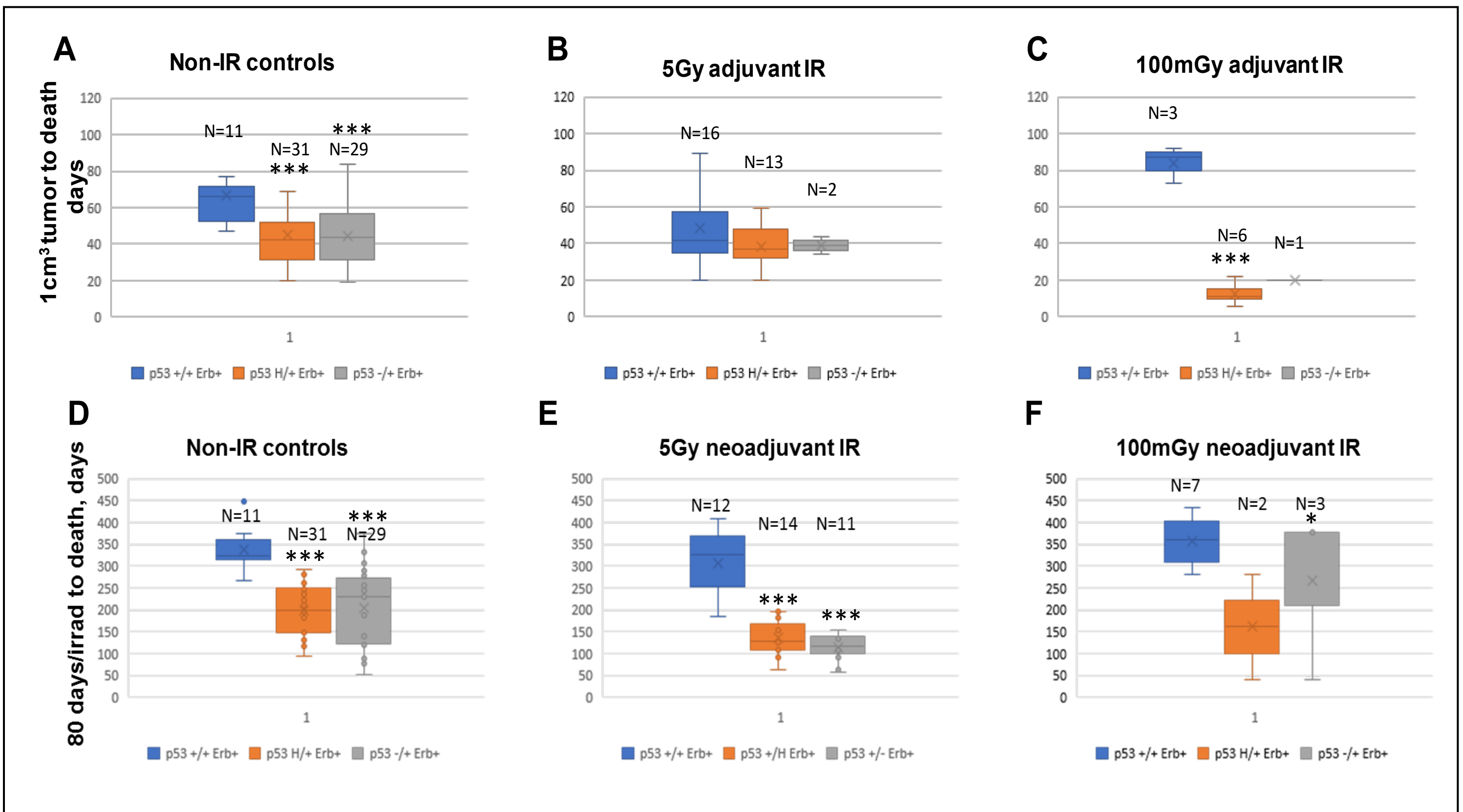


**Fig.1. Irradiation accelerates mammary tumor progression in p53 genotype, stage and dose dependent manner. (A, D)** non-irradiated littermates cohorts. High dose (5Gy) irradiation does not affect tumor progression independent of p53 genotypes **(B)**. Low dose (100mGy) of adjuvant irradiation accelerates mammary tumorigenesis in mutp53;ErbB2 heterozygous mice and does not affect wtp53;ErbB2 mice **(C). (E)** High (5Gy) and **(F)** low (100mGy) doses of neo-adjuvant irradiation accelerate mammary tumorigenesis only in p53 heterozygous mice**,** but don't affect tumor progression in wtp53;ErbB2 mice. p53+/+;ErbB2- blue bars, H/+;ErbB2 mice –orange bars; -/+;ErbB2 –gray bars. For the adjuvant protocol, mice were irradiated when mammary tumors reach 1cm$^3$ volume; for the neoadjuvant protocol, mice were irradiated with the indicated doses at 80 days (the average time of onset of pre-malignant .lesions). * = p<0.05; ** = p<0.01; *** = p<0.001. Comparisons are made to +/+ per treatment.

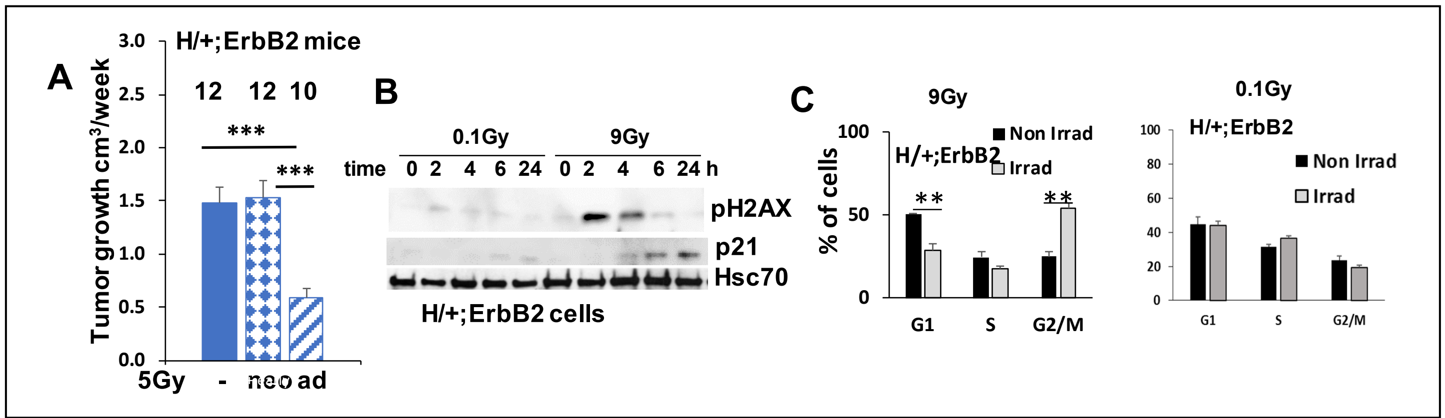


**Fig.2. A.** H/+;ErbB2 tumor growth rate after irradiation in adjuvant setting (middle bar) or at the neoadjuvant setting (80 days old mice, right bar). 1cm$^3$tumors were irradiated (adjuvant) or/not. Growth kinetic was measured for 2 weeks in all experimental cohorts after tumors reach 1cm$^3$ volume. **B.** High dose irradiation of H/+;ErbB2 cells (9 Gy) induces robust DNA damage (pH2Ax), upregulates p21 and partially induces G2/M arrest. (**C**). Low dose (0.1Gy) irradiation fails to induce p21-mediated growth arrest in the presence of low levels of DNA damage (**B**) and does not affect cell cycle (C, left panel). * = p<0.05; ** = p<0.01; *** = p<0.001.

heterozygous mammary tumor progression (Fig. 1). Although we observed the decrease in the kinetic of tumor growth within the first two weeks after adjuvant irradiation (Fig. 2A), the overall time of tumor growth (from time of irradiation until reach 3.5 cm$^3$ (Fig. 1)) did not change. Predicted on our data outlined in the previous progress report, we hypothesized that wtp53 allele in H/+;ErbB2 tumors is transcriptionally functional to induce reversible growth arrest in response to high dose irradiation with the subsequent relapse of tumor growth. To evaluate the threshold of irradiation dose, causing the growth-inhibiting effect in the adjuvant setting, we tested low doses of irradiation. Although out of the scope of the original application, we decided to test the impact of low dose irradiation on mammary tumorigenesis with respect to p53 genotype, as a clinically relevant experimental setting, as all breast cancer patients undergo screening procedures associated with recurring exposure to low dose of irradiation.

Therefore, we irradiated mice with 100 mGy at both neo-adjuvant protocol (80 days old mice) and adjuvant protocol (established tumors, 1cm$^3$). Results from limited amount of mice from ongoing experiments demonstrate that similar to high dose, 100mGy irradiation accelerates mammary tumorigenesis in p53 heterozygous mice with similar to 5Gy irradiation kinetic (Fig. 1D-F). Although it needs to be confirmed on larger cohorts, we observed that low dose accelerates tumorigenesis more profoundly in H/+; ErbB2 than -/+;ErbB2 mice in neo-adjuvant setting (Fig. 1F). In contrast, neither high nor low dose of irradiation doesn't affect tumorigenesis in wtp53;ErbB2 mice (Fig. 1D). Strikingly, we found that compared to 5Gy adjuvant protocol, low dose irradiation exacerbates tumorigenesis more robustly in p53 heterozygous mice (compare Fig. 1B and C). To understand the mechanism underlying this observation, we explored the kinetics of response to irradiation *in vitro* in the experimental setting of adjuvant treatment. Consistent with *in vivo* data, we observed growth arrest after high dose irradiation of H/+;ErbB2 cells *in vitro* (9 Gy) associated with the robust DNA damage (pH2Ax) and p21 upregulation, indicating that cells undergo growth arrest (Fig. 2B,C). That data implies that despite the strong notion of the dominant-negative effect of mutp53 over the wtp53 allele in heterozygosity, the wtp53 allele

is transcriptionally active, at least partially, to enable growth arrest to allow DNA repair, thus, enabling the maintenance of the genomic integrity. Contrary, a low dose of irradiation fails to reach the threshold of DNA damage (pH2Ax, Fig. 2B), necessary for G2/M checkpoint (Fig. 2C, left panel), allowing cell cycle entry in the presence of unrepaired DNA, thus leading to genomic and chromosomal instability. Subsequently, genomic aberrations provide a rich tapestry for a further selection of more aggressive clones, therefore, accelerating tumor progression (Fig. 2C). We expect that enhanced chromosomal and genomic instability correlates with an increased rate of p53LOH and tumor progression. Although needed further validation in human specimens, our *in vivo* data suggests a cautious use of low dose irradiation attributed by screening modalities in a particular subset of early stages breast cancer patients carrying mutp53 heterozygous tumors.

As we presented the results from ongoing experiments, we are continuing to collect mouse data and tumor samples to finalize in a statistically appropriate manner the association of irradiation-induced tumor progression in the adjuvant setting with p53LOH. We will also complete testing the rate of p53LOH occurrence under different protocols, i.e., low *vs.* high dose and adjuvant *vs.* neo-adjuvant, and will correlate the outcomes with the severity of the disease.

**Subtask 4. Determine whether generic genotoxic drug doxorubicin promotes p53 LOH in established R172H/+;ErbB2 tumors in the adjuvant setting.**

*To test whether commonly used for Her2 positive breast cancer treatment genotoxic drug doxorubicin similar to irradiation induces LOH in mutp53 dependent manner, 30 H/+;ErbB2 females, 30 p53-/+;ErbB2 and 30+/+;ErbB2 females will be treated with 4mg/kg doxorubicin (dox) in PBS intraperitoneally at the time of tumor onset (0.5 $cm^3$, volume) once daily for 5 consecutive days. Monitoring and analysis will be performed as described in Aim 1b. 60 females p53-/+;ErbB2 + 60 females H/+;ErbB2 + 60 females +/+;ErbB2 = 180 total animals (timeline: months 12-32, 80% completion).*

We performed the outline in subtask 4 experiment- doxorubicin treatment in the adjuvant setting. Similar to adjuvant high dose irradiation (Fig. 1 A, B), we did not observe the difference in the survival of p53 heterozygous mice. The median survival after drug administration was ~40 days for both H/+;ErbB2 and -/+;ErbB2 mice. We established the collection of mammary tumors for the future p53LOH analysis, which was delayed due to the shutdown of Stony Brook University during the March-July 2020 COVID pandemic. Currently, we are performing p53LOH analysis for the implementation of the proposed subtask.

**Major Task 2. Mechanistically assess the physiological consequences of p53 LOH in heterozygous mutp53 mammary cells in vitro.**

**Subtask 1. Examine the frequency and time of p53 LOH onset in the existing collection of cell culture of primary mammary epithelial cells and mammary tumors culture derived from mice with different p53 genotypes.** *Serial passaging of R172H/+ErbB2 vs p53-/+;ErbB2 vs p53+/+;ErbB2 MECs and mammary tumors cultured cells. (timeline: months 12-24, 100% completion).*

We previously reported that we successfully established R172H/+ErbB2 vs. p53-/+;ErbB2 vs. p53+/+;ErbB2 cell lines (3 biological replicas per genotype) and validated our *in vivo* data that high dose of irradiation promotes p53LOH in mutp53 heterozygous cells but not p53-/+;ErbB2 cells. Therefore, this subtask is completed.

**Subtask 2. Test the effect of irradiation on the frequency and time of p53 LOH onset in primary mammary epithelial cells (MECs) and mammary tumors culture derived from mice with different p53 genotypes.** *Serial passaging of R172H/+;ErbB2 vs p53-/+;ErbB2 vs p53+/+;ErbB2 MECs and mammary tumors cultured cells after single dose of irradiation in vitro at passage 1.* (timeline: months 24-32, 100% completion). Previously we reported the completion of this subtask.

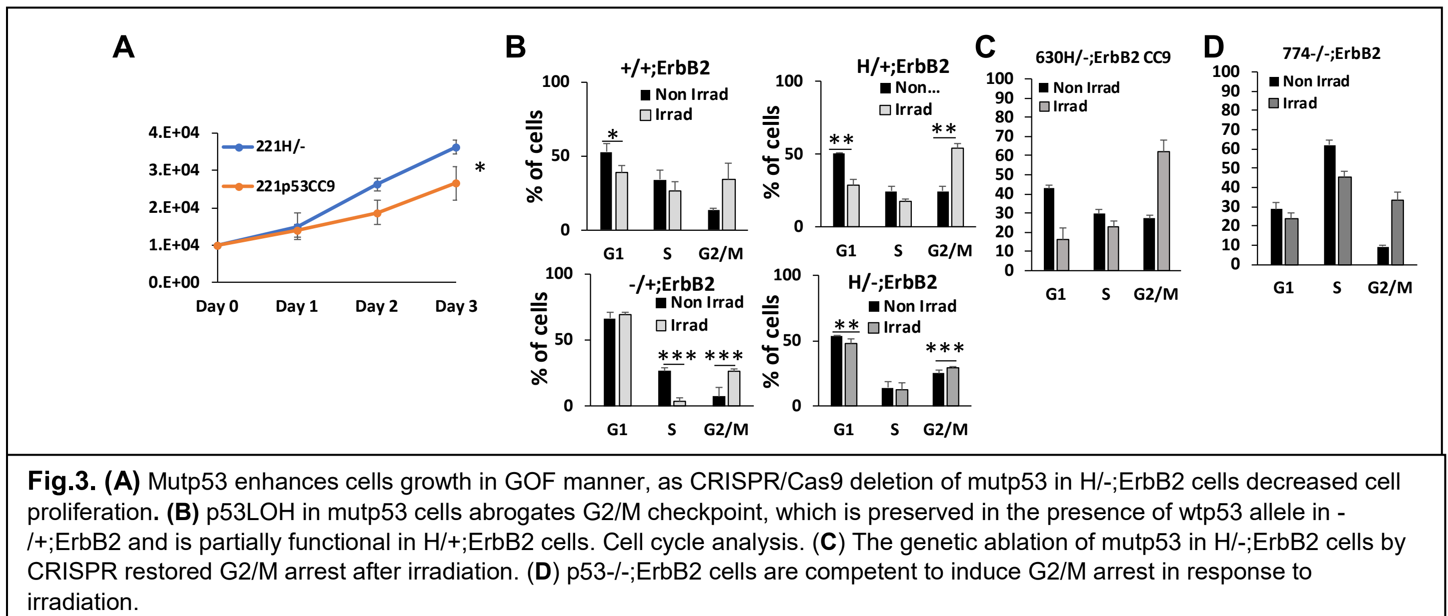


**Fig.3. (A)** Mutp53 enhances cells growth in GOF manner, as CRISPR/Cas9 deletion of mutp53 in H/-;ErbB2 cells decreased cell proliferation**. (B)** p53LOH in mutp53 cells abrogates G2/M checkpoint, which is preserved in the presence of wtp53 allele in -/+;ErbB2 and is partially functional in H/+;ErbB2 cells. Cell cycle analysis. (**C**) The genetic ablation of mutp53 in H/-;ErbB2 cells by CRISPR restored G2/M arrest after irradiation. (**D**) p53-/-;ErbB2 cells are competent to induce G2/M arrest in response to irradiation.

**Subtask 3. Correlate the p53 LOH status of R172H/+;ErbB2 vs. p53-/+;ErbB2 vs. p53+/+;ErbB2 MECs and mammary tumors cultured cells with cellular properties (proliferation, chemoresistance, allografts) and with biochemical characteristics.** *(timeline: months 24-32, 100% completion).*

To gain insight into the mechanism of p53LOH and to develop pharmacological approaches of its targeting, we extended the research outlined in this subtask. As was shown in the previous report, we generated cell lines from murine mammary tumors with various p53 genotypes. The functional and RNAseq studies demonstrated that p53LOH in H/+;ErbB2 cells (H/-;ErbB2) causes the loss of p21 expression (the transcriptional target of wtp53) and deficient G2/M checkpoint after irradiation (IR) (Fig.3B). Hence, p53LOH enables cell cycle re-entry after IR, leading to genomic instability. Contrary, in H/+ cells, the wtp53 allele retains the ability to induce p21, G2/M checkpoint, and partially arrest in response to genotoxic stress. In addition to the loss of the G2/M checkpoint, we found the strong association of p53LOH with increased proliferation, stabilization of mutp53, centrosome amplification in the presence of a mutp53 allele (H/-;ErbB2 cells), increased chromosomal instability (CIN); upregulation of Nek2 (member of Never in Mitosis (NIMA) Related Kinases family) protein, which localizes to the centrosome and regulates its function.

In the course of the reported period, we focused on the mechanistic aspect of mutp53 regulation of p53LOH. We found that contrary to H/-;ErbB2 cells, p53-/-;ErbB2 cells retain functional G2/M checkpoint in response to irradiation (Fig.3D). Consistently, genetic ablation of mutp53 CRISPR/Cas9 in H/-;ErbB2 cells decreased cell proliferation and restored G2/M checkpoint after irradiation (Fig.3C), indicating that mutp53 obliterates G2/M enabling cell proliferation (Fig.3A) in the gain-of-function (GOF), but not loss-of-function manner.

Following our previous observation of the strong association of p53LOH and CIN, we found that IR increases CIN in the presence of a mutp53 allele that is further augmented by p53LOH. As CIN may arise from abnormal chromosome segregation in mitosis, we investigated centrosome aberration with respect to p53 status. We found that the presence of the mutp53 allele in heterozygous cells significantly increases centrosome amplification compared to -/+;ErbB2 cells (Fig.4A) in an apparent DN fashion. As centrosome amplification was implicated in chromosomal rearrangements (amplifications and deletions), the elevated centrosome number in H/+;ErbB2 cells may cause the increased incidence of spontaneous p53LOH compared to -/+;ErbB2 cells. On the other hand, the excessive centrosome amplification within tumor cells can be deleterious. It may lead to multipolar mitosis, generating sufficiently high levels of aneuploidy to pose a risk for cell viability. As a pro-survival mechanism, cancer cells adapt to avoid multipolar mitosis and mitotic catastrophe by clustering their extra centrosomes at the two poles of the spindle during mitosis, thus ensuring bipolar chromosome segregation.

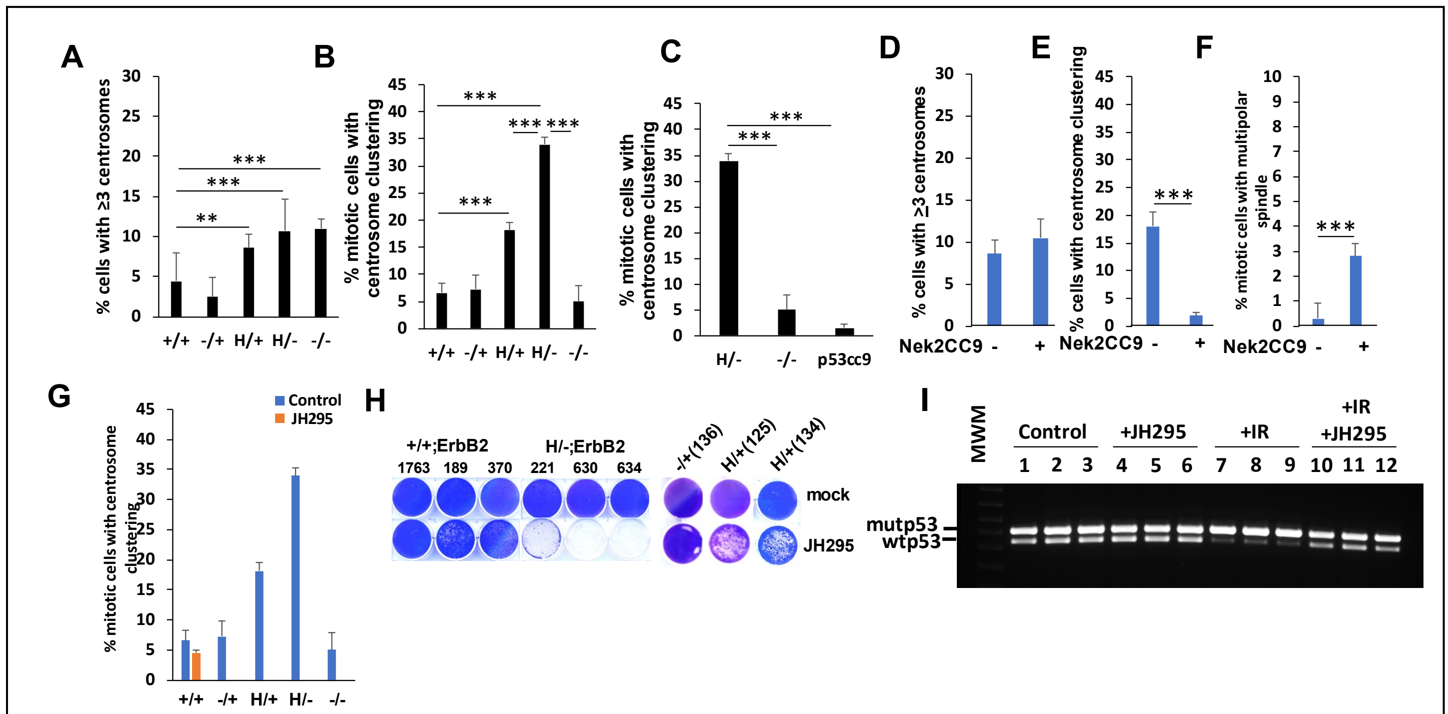


**Fig.4. (A)** the presence of mutp53 allele in heterozygous cells significantly increases centrosome amplification compared to -/+;ErbB2 cells in the dominant negative manner. **(B)** p53LOH drives centrosome clustering (compare H/-;ErbB2 vs. H/+;ErbB2 cells). (**C**) genetic (-/-;ErbB2) and CRISPR/Cas9- mediated deletion of mutp53 by significantly reduces centrosome clustering. CRISPR/Cas9- mediated deletion of Nek2 (**D**) does not affect centrosome amplification (**D**), but significantly reduced centrosome clustering (**E**) with concomitant increase in multipolar mitosis (**F**). (**G**) Nek2 inhibitor JH295 abrogates centrosome clustering in H/+;ErbB2 and H/-;ErbB2 cells. (**H**) JH295 shows the highest sensitivity in mutp53 cells with p53LOH (H/-;ErbB2) as compared to cells with wtp53 allele (+/+;ErbB2, -/+;ErbB2, H/+ErbB2)( the colony formation assay). **(I)** JH295 significantly protects H/+;ErbB2 cells from irradiation-induced p53LOH (each lane represents a biological replica.

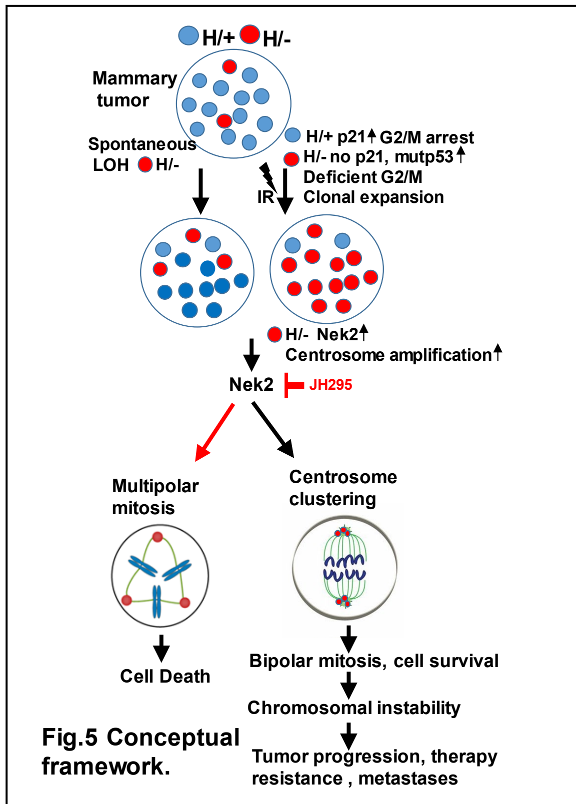

**Fig.5 Conceptual framework.**

However, pseudo-bipolar spindle formation through centrosome clustering causes slower mitosis, increased frequency of lagging chromosomes during anaphase, and thus to CIN. Strikingly, we observed the highest incidence of centrosome clustering in H/-;ErbB2 cells (Fig.4B; compare H/- vs. H/+cells) in mutp53 GOF manner. The genetic (-/- cells) or CRISPR/Cas9 deletion of mutp53 by significantly reduced centrosome clustering (Fig. 4C) and inhibits cell growth (Fig. 3A), but does not affect centrosome amplification or multipolar spindle formation (data not shown).

The data on Nek2 as the regulator centrosome structure and function set us to investigate whether p53LOH-associated Nek2 upregulation (as we presented in the previous report) drives centrosome clustering (Fig. 4B) by utilizing CRISPR/Cas9 technology. Notably, we were able to generate H/+/Nek2-/-, but not H/-/Nek2-/- cell lines by CRISPR/Cas9, suggesting the obligatory requirement of Nek2 for the survival of mutp53/LOH cancer cells. The genetic ablation of Nek2 did not alter the proportion of H/+ cells with centrosome amplification (Fig. 4D); however, centrosome clustering was significantly reduced (Fig. 4E) with a concomitant increase in cells carrying multipolar mitotic spindle (Fig. 4F). Likewise, the selective Nek2 inhibitor JH295, diminishes centrosome clustering in H/+ and H/- cells (Fig. 4G), while the proportion of mitotic cells with supernumerary centrosomes did not change (data not shown).

We investigated the effects of Nek2 inhibition with respect to p53 genotypes. In our study, we utilized JH295 (oxindole propynamide, IC50=770nM), which is a highly specific and irreversible Nek2 inhibitor that blocks Nek2 activity via alkylation of residue Cys22, and does not affect the activities of other mitotic kinases (CDK1, PLK1, Aurora B, or Mps1). Moreover, JH295 does not perturb bipolar spindle assembly or the spindle assembly checkpoint. Given this selective profile, we thought that JH295 is as useful for identifying the biological roles of Nek2 as RNA interference approach. The data that JH295 does not affect the proliferation of wtp53 cells (Fig.4H) and the lack of organ defects in Nek2 knockout mice (https://www.mousephenotype.org/) suggests potential low toxicity of Nek2-selective inhibitors with no off-target effects for breast cancer therapy. Strikingly, we observed the p53 genotype-specific inhibitory effect of JH295, with the highest sensitivity in mutp53 cells with p53LOH (H/-;ErbB2) as compared to cells with wtp53 allele (+/+, -/+, H/+) by the colony formation assay (Fig. 4H). JH295 shows an intermediate inhibitory effect on H/+ cells (Fig.4H). The increased sensitivity of H/-;ErbB2 cells to Nek2 inhibition set us to test whether JH295 prevents the expansion of mutp53 cells with p53LOH after irradiation. Hence, we irradiated (9 Gy) or not H/+ cells, and then treated them with JH295/vehicle for 10 days. Remarkably, JH295 significantly abolishes irradiation-induced p53LOH in H/+ cells (Fig. 4I). In sum, our findings support the following mechanism of how mutp53 augments the onset of p53LOH. In heterogeneous tumor populations,

spontaneous p53LOH generates the clonal pool of genetically unstable cells prone to expand after genotoxic stress due to loss G2/M checkpoint and p21 expression, leading to the selection of therapy refractory aggressive clones enabling tumor progression (Fig. 5). The loss of mitotic checkpoint leads to accumulation of cells harboring centrosome abnormalities. Following p53LOH, mutp53-mediated upregulation of Nek2 provides the competitive survival advantage to mutp53/LOH (H/-;ErbB2) over mutp53 heterozygous cells (H/+;ErbB2). As a pro-survival mechanism of escape from mitotic catastrophe in the presence of centrosome amplification, mutp53/LOH cells adapt Nek2-mediated pseudo-bipolar mitosis and evasion of G2/M checkpoint by centrosome clustering. We propose that p53LOH generates a more aggressive population by two complementary mechanisms: (i) abrogation of G2/M checkpoint followed by unrestricted mitotic entry and the expansion clones with p53LOH and (ii) enhanced genomic instability that provides the genomic plasticity for the emergence of more aggressive metastatic clones.

**Major Task 3. Determine whether p53 LOH promotes metastatic behavior in ErbB2 cancer cells.**

**Subtask 1. Establish whether p53 LOH enhances the motility and invasion of cancer cells in vitro.** *Test the motility and invasive properties of primary mammary epithelial cells and tumor cultures derived from H/+;ErbB2 and p53-/+;ErbB2 mice before and after LOH in vitro. Boyden chamber assay, wound healing assay. (timeline: months 24-32, 60% completion)*

As we previously reported, the main phenotype associated with p53LOH in the presence of mutp53 is enhanced metastases *in vivo*. Our RNAseq data identified Nek2 as a novel putative target of mutp53, that promotes genomic and chromosomal instability. Genomic instability has long been proposed to be a mechanism by which a cell may acquire the necessary properties for invasion and metastasis.

The clonal diversity, produced by genomic instability, leads to intratumor clonal competition, clonal evolution, and acquisition of the necessary properties required for metastasis (Fig.5). Therefore, we hypothesized that mutp53-Nek2 axis mediates p53LOH that further produce genomic instability, generating a variety of genetically distinct clones with de novo genetic evolutionary changes that allow clones to metastasize. On the other hand, recent advances in understanding Nek2 biology suggest that Nek2 itself is an important regulator of cancer cell migration and metastasis beyond promoting genomic instability through the activation of a variety of oncogenic pathways. Consistently, our retrospective analysis of clinical data revealed that the high level of Nek2 expression is prognostic of poor survival of breast cancer patients (http://kmplot.com/) (Fig.6A). As metastatic disease remains the leading cause of cancer-related mortality in humans, this clinical observation confirms the association of the high Nek2 level and metastasis in humans.

To evaluate the impact of mutp53, p53LOH, and Nek2 on phenotypic characteristics related to metastatic dissemination, we established the panel cell lines from mammary tumors of mice with different p53 and LOH genotypes and performed RNAseq analysis. The transcriptome analysis of enriched gene sets differentially

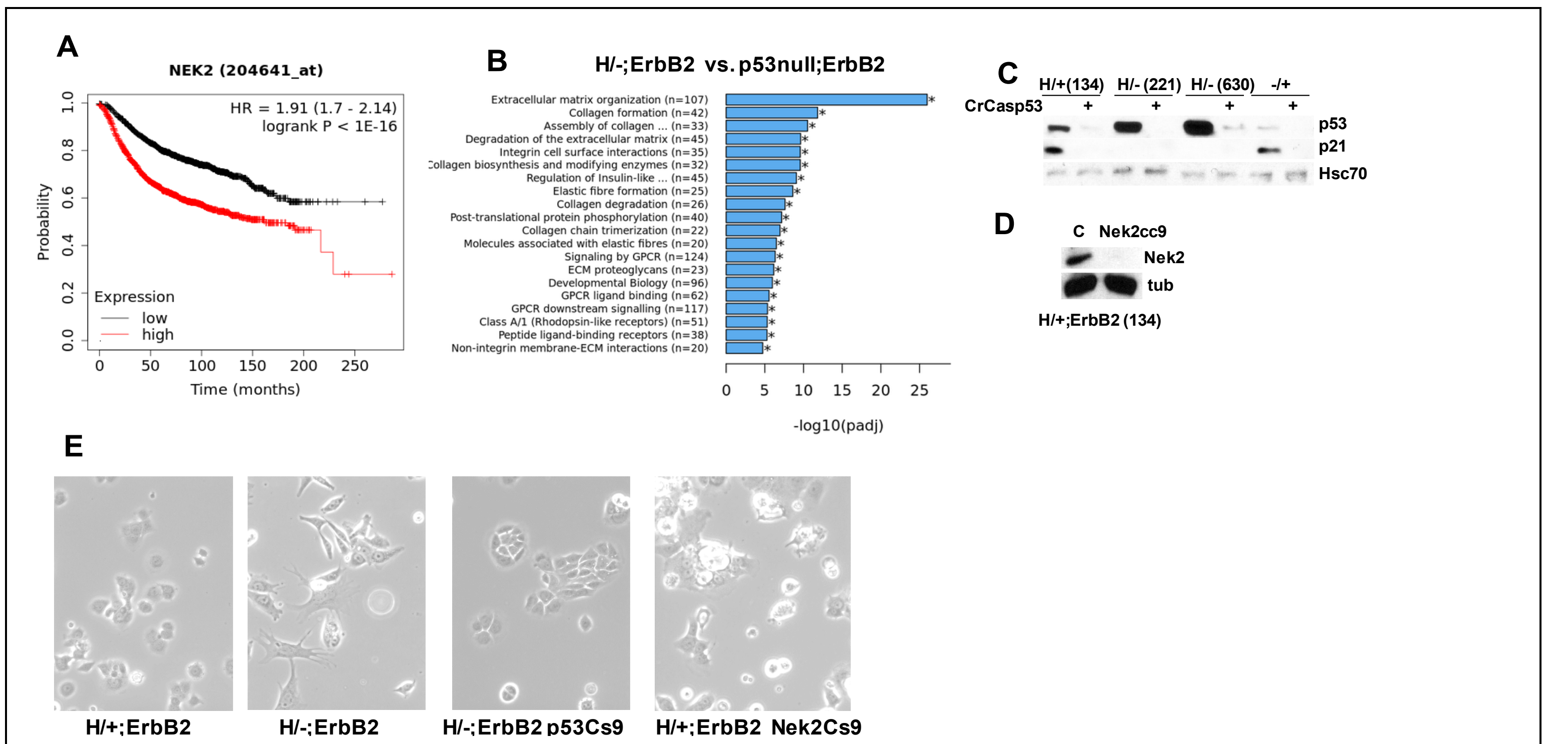


**Fig.6. A.** high level of Nek2 expression is prognostic of poor survival of breast cancer patients (http://kmplot.com/). **B.** ECM-remodeling and integrin signaling are the most upregulated pathways in the presence of mutp53 allele. The transcriptome analysis of enriched gene sets differentially expressed in H/-;ErbB2 vs. p53null;ErbB2 cells. (**C**) Panel of isogenic CRISPR derivatives of murine ErbB2 tumor cell lines with depleted p53 (**C**) and Nek2 (**D**). (**E**) The loss of wtp53 allele shifts cells phenotype from epithelial (H/+;ErbB2) to mesenchymal (H/-;ErbB2). Panel 3: CRISPR-cas9 mediated depletion of mutp53 from H/-;ErbB2 cells revert mesenchymal (H/-;ErbB2) phenotype to epithelial. Panel 4: Nek2 CRISPR-cas9 depletion mitigate cells adhesion.

expressed in H/-;ErbB2 vs. p53null;ErbB2 cells highlighted ECM (extracellular matrix)-remodeling and integrin signaling as the most upregulated pathways in the presence of mutp53 allele (Fig.6B). ECM proteins produced by both tumor and stromal cells, were shown to contribute significantly to alteration in integrin signaling to promote invasive growth and metastasis.

To build upon these observations, we generated isogenic CRISPR derivatives of murine tumor cell lines: with depleted p53 (Fig.6C) and Nek2 (Fig.6D). Consistent with our in vivo data, loss of wtp53 allele in cells shifts cells phenotype from epithelial (H/+;ErbB2) to mesenchymal (H/-;ErbB2) (Fig.6E). Furthermore, CRISPR-cas9 mediated depletion of mutp53 from H/-;ErbB2 cells reverts mesenchymal (H/-;ErbB2) to epithelial phenotype (Fig.6E). Significantly, Nek2 CRISPR-cas9 depletion dramatically affects cell adhesion (Fig.6E, panel 4), indicating the interception of ECM-integrin signaling. Currently, we are utilizing the isogenic panel of ErbB2 mammary cells to analyze EMT (epithelial-mesenchymal transition) markers, adhesion, motility, and invasion to identify how phenotypical alterations affect physiological outcomes related to metastatic behavior. In sum, our experiments imply that p53LOH in the presence of a mutp53 allele may promote metastases in GOF manner via complementary mechanisms: 1) modulation ECM-integrin signaling; 2) upregulation Nek2 and its mediated metastatic properties; 3) inducing genomic instability. We hypothesize that after p53LOH mutp53-mediated upregulation of Nek may alter ECM-integrin signaling and, thus, metastases. As a future development of this

project, we will determine whether genetic and pharmacological ablation of Nek2 prevents p53LOH associated metastases, which we observed *in vivo.*

**Subtask 2. Determine whether p53 LOH enhances the ability of tumor cells to metastasize in vivo. Isolate metastatic cells from lungs of irradiated and non-irradiated R172H/+;ErbB2 vs. p53-/+;ErbB2 vs. p53+/+;ErbB2 mice. Assess p53 LOH status in metastases in comparison with primary tumors. (timeline: months 24-32, 10% completion).**

We have made numerous attempts to isolate metastatic cells from the lungs of mice with different genotypes, as we described in the original grant application (ErbB2 FACS sorting). We also attempted to dissect metastatic lesions from the paraffin-embedded section of lungs using a laser-capture microscope. However, the low yield of metastatic cells and contamination with normal tissues precluded the implementation of the proposed task in a reproducible and the statistically appropriate manner. To evaluate how p53LOH affects the metastatic behavior of cells in vivo, we will use the alternative approach by employing an established panel of isogenic cell lines with different p53 genotype, which recapitulate p53LOH in p53 heterozygous cells (Fig.6C,D).

To complete the proposed subtask, we will test whether p53LOH promotes metastasis in proof-of-concept competition experiments. We will use H/+;ErbB2 and H/-;ErbB2 cells with lentiviral overexpression of derivatives of pLOCTurboRFP (Dharmacon), which contains two tracers: red (RFP) and green (GFP). We modified the original vector and generated two constructs: 1) red -pLOCTurboRFP by deleting GFP and 2) green-pLOCTurboGFP by deleting RFP sequence. This model has the same vector backbone and ensures well-controlled and quantitative tracing of labeled cells. We generated and validated stable cell lines by transducing H/+;ErbB2 with pLOCTurboGFP to obtain the GFP-labelled population and by transducing H/-;ErbB2 with pLOCTurboRFP (red population) following the Dharmacon protocol. To minimize genetic/epigenetic heterogeneity, we will use the pools of transduced clones (FACS sorted) rather than individual clones. This model will enable us to compare clonal expansion under two circumstances: i) normal condition (Protocol 1); ii) after IR (Protocol 2). We will test whether H/-;ErbB2 cells outcompete H/+;ErbB2 cells in mammary tumors and exhibit metastatic behavior. We will mix H/+;ErbB2-GFP and H/-;ErbB2-RFP cells in the ratio 1:1. $10^6$ of total mixed cells will be transplanted into a mammary fat pad. The same amount of H/-;ErbB2-RFP cells and of H/+;ErbB2-GFP cells will be implanted independently (no mix) as control arms. 10 mice per arm will be utilized. We will focus on the kinetics of tumor growth, metastases, and the change in the ratio of H/+;ErbB2-GFP vs. H/-;ErbB2-RFP in primary tumors and metastases. Tumors and lungs will be collected at the endpoint and divided into two parts. One part will be dissociated and analyzed by FACS analysis for the quantification of the GFP/RFP ratio. p53LOH status will be validated by qPCR. The second part of the tumor and lungs will be fixed and evaluated by fluorescent microscopy for the enrichment analysis of RFP vs. GFP expressing cells. Hence, we will test whether metastases are generated by specific sub-population of cells (RFP or GFP). We expect by this proof-of-principle experiment to definitely establish whether p53LOH enables metastatic behavior, whether irradiation augments it, and whether mutp53 genetic ablation prevents the onset of metastasis *in vivo*.

- What opportunities for training and professional development has the project provided?

Paige Brook, High School student, (June 2020- August 2020) has received professional on-hand training in literacy while working on this project.

- How were the results disseminated to communities of interest?

In May 2020 we planned to present our findings at the p53 International Workshop in Israel. The workshop was cancelled due to pandemic.

Oral presentations: Amr Ghaleb "Centrosome clustering as a survival mechanism in breast cancer cells carrying mutant p53". Stony Brook Cancer center, grant development seminar.

- What do you plan to do during the next reporting period to accomplish the goals?

For the next reporting period we will finish experiments described in Major Task 1 Subtask 3,4 to determine the effect of doxorubicin and irradiation on p53LOH in the adjuvant setting. We collected all tumor samples from treated and control cohorts. We plan to finalize the outlined experiments within 2 months period.

We will also focus on the implementation of Major Task 3. As the approaches outlined in the original application did not yield definitive results, we will use the alternative approach by employing an established panel of isogenic cell lines with different p53 genotype, which recapitulate p53LOH in p53 heterozygous cells. We will use this isogenic system in vivo competition assay. This proof-of-principle experiment will definitively establish whether p53LOH enables metastatic behavior, whether irradiation augments it, and whether mutp53 genetic ablation prevents the onset of metastasis in vivo. Also, we plan to determine whether genetic and pharmacological ablation of Nek2 prevents the onset p53LOH in vivo and inhibits metastases.

We plan to prepare 2 manuscripts for publication; on effects low and high doses irradiation in the adjuvant setting, and summarizing our data on how p53LOH affects metastatic properties of cancer cells in context mutp53 gain-of-function and loss-of-function.

## 4.IMPACT

- Major innovative findings and achievements for this reporting period:

The major innovative findings for the reporting period are the following. We found that the p53LOH in the presence of a mutp53 allele (H/-;ErbB2 cells) enables the following oncogenic phenotypes in the gain-of-function manner: (1) increased proliferation; (2) abrogation of G2/M checkpoint; (3) centrosome amplification; (4) the increased rate of pseudo-bipolar mitosis via centrosome clustering; (5) aneuploidy and increased chromosomal instability (CIN) as a readout of centrosome malfunction; (7) stabilization of mutp53 protein; (5) upregulation of Nek2 (member of Never in Mitosis (NIMA) Related Kinases family) protein, which localizes to centrosome and regulates its function. Markedly, we found that specifically H/-;ErbB2 cells require Nek2 function for their survival. Furthermore, our RNAseq and functional studies demonstrated that p53LOH in the presence of mutp53 allele might promote metastases in a gain-of-function manner via complementary mechanisms: 1) modulation ECM-integrin signaling; 2) upregulation Nek2 and its mediated metastatic properties; 3) inducing genomic instability. The main accomplishment of the project up-to-date is the identification of Nek2 as a pharmacological target to prevent p53LOH onset in mutant p53 heterozygous cells. As a proof-of-principle, we demonstrate that Nek2-specific inhibitor JH295 precludes the loss of wtp53 allele in mutp53 heterozygous cells after irradiation. These findings may have a significant translational impact, as they may provide a foundation for developing a novel therapeutic strategy to curb tumor progression.

- What was the impact on the development of the principal discipline(s) of the project?

We summarized our previous and current research in the following publications:

Ghaleb A., Yallowitz A, Marchenko N. 2019. Irradiation induces p53 loss of heterozygosity in breast cancer expressing mutant p53. Commun Biol. 2(1):436.

Ghaleb A, Padellan M., Marchenko N. 2020. Mutant p53 drives the loss of heterozygosity by the

upregulation of Nek2 in breast cancer cells. Breast Cancer Research, under final revision. Manuscript Number: BRCR-D-20-00462

- What was the impact on other disciplines?

Nothing to Report.

- What was the impact on technology transfer?

Nothing to Report.

- What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

As we described above, we encountered problem with the isolation of metastatic cells from lungs *in vivo*. We have made numerous attempts to isolate metastatic cells from the lungs of mice with different genotypes, as we described in the original grant application (ErbB2 FACS sorting). Unfortunately, the alternative approach of using laser capture microscope did not yield satisfactory results. Now we plan to perform in vivo competition assay as a proof-of-principle experiment establishing p53LOH as critical event in the switch to metastatic phenotype. We plan to accomplish this study in the next funding period

- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

- Significant changes in use or care of human subjects

Nothing to Report.

- Significant changes in use or care of vertebrate animals.

Nothing to Report.

- Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

Publications:

Ghaleb A., Yallowitz A, **Marchenko N.** 2019. Irradiation induces p53 loss of heterozygosity in breast cancer expressing mutant p53. **Commun Biol.** 2(1):436.

Ghaleb A, Padellan M., **Marchenko N**. 2020. Mutant p53 drives the loss of heterozygosity by the upregulation of Nek2 in breast cancer cells. **Breast Cancer Research**, under final revision. Manuscript Number: BRCR-D-20-00462

Both publications contain acknowledgement of DOD support.

Other Products

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

| Name: | Natalia Marchenko |
|---|---|
| Project Role: | PI |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 12 months |
| Contribution to Project: | Dr. Marchenko was responsible for the overall administration, data analysis, coordination and direction of the project and lab work. Dr. Marchenko performed breeding and mouse colony maintenance, tumor specimens analysis, mammary epithelial cells isolation, manuscript preparation. |
| Funding Support: | DOD # BC151569 |
| Name: | Euvgenia Alexandrova |
| Project Role: | Collaborator |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 3 months |
| Contribution to Project: | As a collaborator Dr. Alexandrova was involved in generation, specimen tissue preparation and data analysis of R248Q;ErbB2 mice. |
| Funding Support: | NCI gran t# K22CA190653-01A1 |

| | |
|---|---|
| Name: | Amr Ghaleb |
| Project Role: | investigator |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 12 months |
| Contribution to Project: | Amr was responsible for breeding and mouse colony maintenance, mouse genotyping, performed tissue embedding, cutting and IHC staining, QRT-PCR, in vitro experiments. |
| Name: | Ute Moll |
| Project Role: | Collaborator |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 1 month |
| Contribution to Project: | As a collaborator Dr. Moll participated in planning of experiments, discussions of data interpretations, manuscript preparation. |
| Funding Support: | NCI grant R01CA176647 |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |

| | |
|---|---|
| | |
| | |
| | |
| | |

- What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report