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**14. ABSTRACT** Mutations in one allele TP53 gene in early stages frequently followed by the loss of the remaining wild-type allele (LOH) in later stages of tumor development. Despite the strong notion that p53LOH promotes tumorigenesis, its specific role in acute and long-term response to genotoxic modalities remained unclear. The major innovative findings for the reporting period are the following. We established that wtp53 in mutp53 heterozygous (H/+;ErbB2) tumors might be transcriptionally competent towards a subset of targets (p21, Mdm2) and/or mutp53 may exert dominant-negative effect and suppress subset of wtp53 targets (Gadd45) in response to irradiation. As physiological consequences of p53LOH in vivo and in vitro we found that p53LOH leads to the loss of transcriptional activation of p21 and abrogation of G2/M checkpoint; 2) stabilization of mutp53 protein; 3) aggravation centrosome aberrations leading to increased genomic and chromosomal instability; 4) increased cells proliferation; 5) transcriptional upregulation of genes involved in mitosis, including Nek2 (member of Never in Mitosis (NIMA) Related Kinases family); 6) the increased sensitivity of mutp53 cancer cells to Nek2 inhibition. In the presence of mutp53 allele, the decreased expression of Gadd45 and increased expression of Nek2 may drive cell cycle trough G2/M checkpoint after irradiation leading to increased proliferation, chromosomal aberration, selection the cells with p53LOH, ultimately, leading to tumor progression.

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

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1. INTRODUCTION:

Mutations in the p53 tumor suppressor gene are the most prevalent genetic events in human Her2-positive breast cancer and are associated with poor prognosis and survival.[1] Frequently in the early stages of cancer, a p53 mutation in one allele is followed by loss of heterozygosity (LOH) in the second allele, during tumor progression[1,2]. And despite a strong notion that p53 mutations with subsequent LOH are driving events in breast cancer, the translational significance of p53 mutational and LOH status, and their role in breast cancer development and progression have not been comprehensively evaluated, especially in the context of conventional genotoxic modalities. Previously we found that the heterozygous mutp53 R172H allele increases the frequency of p53 LOH in mammary tumors compared with the p53null allele that correlates with aggravated tumorigenesis in an MMTV/ErbB2 mouse breast cancer model. This phenotype became even more prominent after γ−irradiation of mice with premalignant lesions, which led to a dramatic increase in metastases in the presence of mutp53 allele. These data strongly suggest that DNA damage further augments the oncogenic activity of mutp53.

We found that wtp53 in mutp53 heterozygous (H/+;ErbB2) tumors might be transcriptionally competent towards a subset of targets (p21, Mdm2) and/or mutp53 may exert dominant-negative effect and suppress subset of wtp53 targets (Gadd45) in response to irradiation. As physiological consequences of p53LOH in vivo and in vitro we found that p53LOH leads to the loss of transcriptional activation of p21 and abrogation of G2/M checkpoint; 2) stabilization of mutp53 protein; 3) aggravation centrosome aberrations leading to increased genomic and chromosomal instability; 4) increased cells proliferation; 5) transcriptional upregulation of genes involved in mitosis, including Nek2 (member of Never in Mitosis (NIMA) Related Kinases family); 6) the increased sensitivity of mutp53 cancer cells to Nek2 inhibition.

In the presence of mutp53 allele, the decreased expression of Gadd45 and increased expression of Nek2 may drive cell cycle trough G2/M checkpoint after irradiation leading to increased proliferation, chromosomal aberration, selection the cells with p53LOH, ultimately, leading to 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. We published this results[3.] 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 not expected to be high. Therefore, instead of perusing of this subtask, we dedicated the time and resources to investigate the mechanism, by which mutp53 promotes p53LOH (**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. Since irradiation and the evaluation of p53R248Q/+;Neu mouse model is technically straightforward, we will continue this study after completion of more impactful subtasks.

**Subtask 3. Assess the effect of irradiation of established mutp53;ErbB2 tumors on p53 LOH (neoadjuvant 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, 60% completion).*

In our previous progress report, we demonstrated that a response to irradiation in the adjuvant setting depends on p53 genotype. First, similar to the neoadjuvant setting, adjuvant irradiation (irradiation of 1cm$^3$ tumors) facilitates p53LOH only in the mutp53 heterozygous tumor, but not in p53-/+;ErbB2. To understand the mechanism of the genotype-specific responses to irradiation, we utilized tumor cell lines established from mammary tumors of mice with different genotypes. Importantly, we generated the cell lines from irradiated H/+;ErbB2 tumors , that acquired p53LOH after irradiation and have H/-;ErbB2 genotype (3 biological replicas)

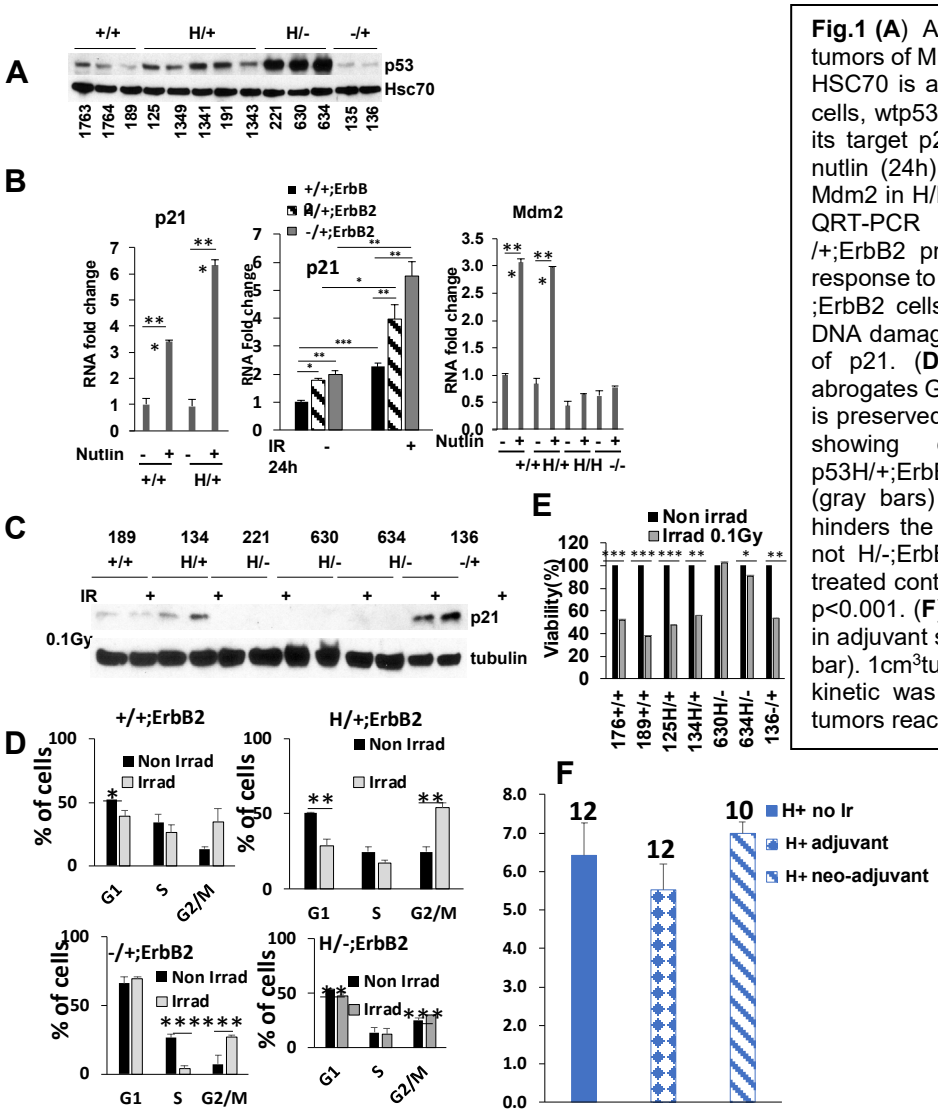


**Fig.1 (A)** A panel of cell lines established from mammary tumors of MMTV-ErbB2 mice with different p53 genotypes. HSC70 is a loading control. **(B)** In mutp53 heterozygous cells, wtp53 retains its transcriptional activity and induces its target p21 and Mdm2 in response to Mdm2 inhibitor nutlin (24h) and 9Gy irradiation. Nutlin does not induce Mdm2 in H/H;ErbB2 and -/-;ErbB2 MECs (*right, bars 5-8*). QRT-PCR 24h post-irradiation. (**C**) H/+;ErbB2 and -/+;ErbB2 preserve the ability to induce p21 protein in response to LDIR (0.1Gy), which is lost after p53LOH in H/-;ErbB2 cells. Consistent with the efficient recovery from DNA damage +/+;ErbB2 cells show marginal upregulation of p21. (**D**) p53LOH in H/-;ErbB2 cells completely abrogates G2/M checkpoint (FACS cell cycle analysis) that is preserved in the presence of wtp53 allele. Bar graphs showing cell cycle analysis of p53+/+;ErbB2 p53H/+;ErbB2, and p53 -/+;ErbB2 cell lines 9 Gy irradiated (gray bars) or not (black bars). (**E**) Irradiation (0.1Gy) hinders the proliferation of H/+;ErbB2 and -/+;ErbB2, but not H/-;ErbB2 cells. % viable cells compared to mock-treated control, CTB assay. * = p<0.05; ** = p<0.01; *** = p<0.001. (**F**) H/+;ErbB2 tumor growth rate after irradiation in adjuvant setting (middle bar) or at the tumor onset( right bar). 1cm$^3$tumors were irradiated (adjuvant) or/not. Growth kinetic was measured in all experimental cohorts after tumors reach 1cm$^3$ volume.

(Fig.1A). These cell lines are highly instrumental in deciphering complex biological effects of mutp53 allele after irradiation *in vivo*.

Previous p53 ectopic overexpression studies proposed that in heterozygosity mutp53 may exert its oncogenic activities via dominant-negative (DN) mechanism, by inhibiting the tumor-suppressive function of wtp53 allele[4]. However, the understanding of the DN effect of endogenous mutp53 in heterozygosity has been hindered by the lack of suitable *in vitro* models. Therefore, we took advantage of our panel of cell lines that continuously maintain heterozygosity during passaging in culture under normal condition. Despite a strong notion of DN effect of mutp53, we found that wtp53 allele is transcriptionally competent in H/+;ErbB2 cells, at least, for a subset of wtp53 target genes including p21 and Mdm2. Mdm2 and p21 are upregulated in H/+;ErbB2, but not in H/H;ErbB2 and -/-;ErbB2 cells, in response to Mdm2 inhibitor nutlin (induces wtp53 without genotoxic stress) and irradiation (9Gy) (Fig.1B). Consistent with qRT-PCR data, p53 heterozygous cells (H/+;ErbB2 and -/+;ErbB2) preserve the ability to induce p21 in response to low doses of irradiation (0.1Gy), which gets lost after p53LOH (Fig.1C). Consistent with the efficient recovery from DNA damage (data were shown in the previous report), +/+;ErbB2 cells show only marginal upregulation of p21 (Fig.1C). Since the high doses irradiation may have p53-independent effects on p21, in this experiment, we utilized low doses of irradiation to decipher p53 (mut and wt)

specific activities. Importantly, we found that p53LOH (H/-;ErbB2 cells) correlates with abrogated G2/M checkpoint that is preserved in cells that carry wtp53 allele (Fig.1D). Consistent with transcriptional activity of wtp53 in heterozygosity and the partially preserved G2/M checkpoint, even low dose of irradiation (0.1 Gy) suppresses the proliferation of H/+;ErbB2 and -/+;ErbB2, but not H/-;ErbB2 cells (Fig.1E).

These results are consistent with our *in vivo* data. We found that adjuvant irradiation of H/+;ErbB2 tumors inhibit tumor growth in comparison to the growth of tumors, which were irradiated in the neoadjuvant setting (80 days old mice) and had H/-;ErbB2 genotype (Fig.1F).

**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, 30% completion).*

For the implementation of this subtask, we expanded the colonies of mice with different p53 genotypes. At the present moment, we are expecting the emergence of tumors to start the treatment protocol and the evaluation of tumor growth kinetics in the context of p53LOH.

**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.

**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 (ErbB2 and HSF1 signaling).** *(timeline: months 24-32, 100% completion).*

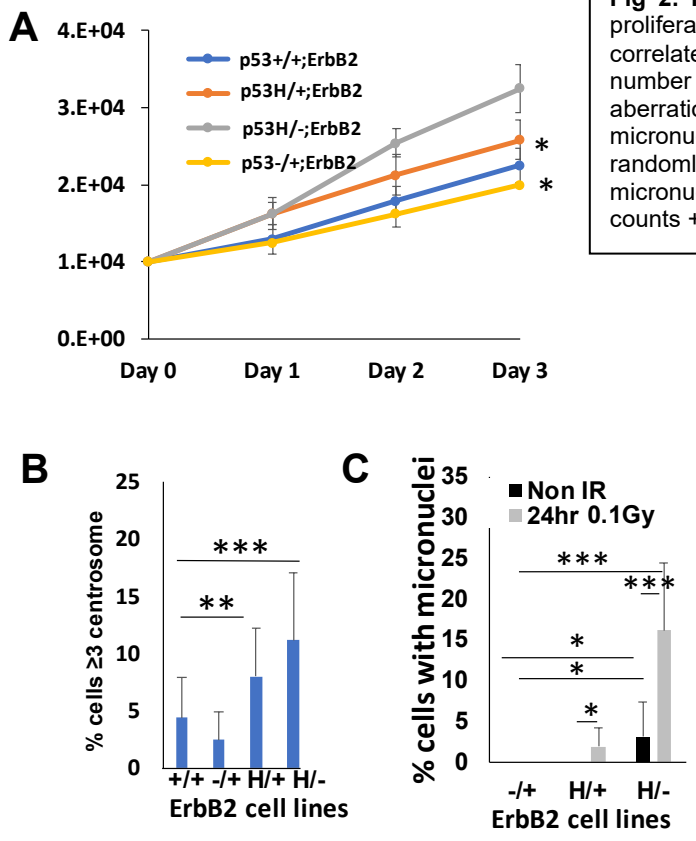


**Fig 2. Physiological outcomes of p53LOH.** (**A**) p53LOH enhances cell proliferation. Average of 3 biological replicas per genotype. (**B**) Mutp53 allele correlates with an increased percentage of cells with abnormal centrosome number (≥3) in mouse cancer cell lines. (**C**) p53LOH leads to chromosomal aberrations. Nuclei were stained with DAPI and visualized to score for micronuclei presence. Per treatment (+/- 0.1Gy), cells were counted from 5 randomly selected fields, for a total of at least 100 cells. Percent of cells with micronuclei was then calculated. Bars represent average of 3 independent counts +/-SD.* = p<0.05; ** = p<0.01; *** = p<0.001.

To complete this subtask, we generated H/-;ErbB2 cell lines from mammary tumors which acquired p53LOH after irradiation *in vivo* (3 biological replicas). This panel of cell lines (Fig.1A) allowed us to evaluate the physiological outcomes of p53LOH *in vitro*. Our results demonstrated that p53 LOH augments cells proliferation, providing a growth advantage to H/-;ErbB2 over H/+;ErbB2 and -/+;ErbB2 cells (average of 3 biological replicas per genotype) (Fig.2A). Consistent with the proliferation, H/-;ErbB2 cells show the highest percentage of cells in mitosis compared to other p53 genotypes (Fig.4F). As we have shown in the previous report, the main physiological outcome of p53LOH that we observed *in vivo* is an elevated level of chromosomal instability measured by high percent of cells with anaphase bridges. As chromosomal instability was implicated in tumor progression and metastasis[2,5,6], we followed this observation and interrogated *in vitro* panel cell lines with different p53 genotypes. We found that the presence of mutp53 allele correlates with an elevated level of centrosome amplification, which may underlie the mechanism of high chromosomal instability in mutp53 cells (Fig.2B). Moreover, the centrosomal aberrations were even further increased after p53LOH (Fig.2B). Importantly, we found that in response to irradiation, mutp53 allele drives chromosomal instability in heterozygous cells that is further augmented by p53LOH as indicated by micronuclei formation (Fig.2C). Hence, we concluded that *p53LOH leads to chromosomal instability due to centrosomal aberration that can be further aggravated by irradiation.*

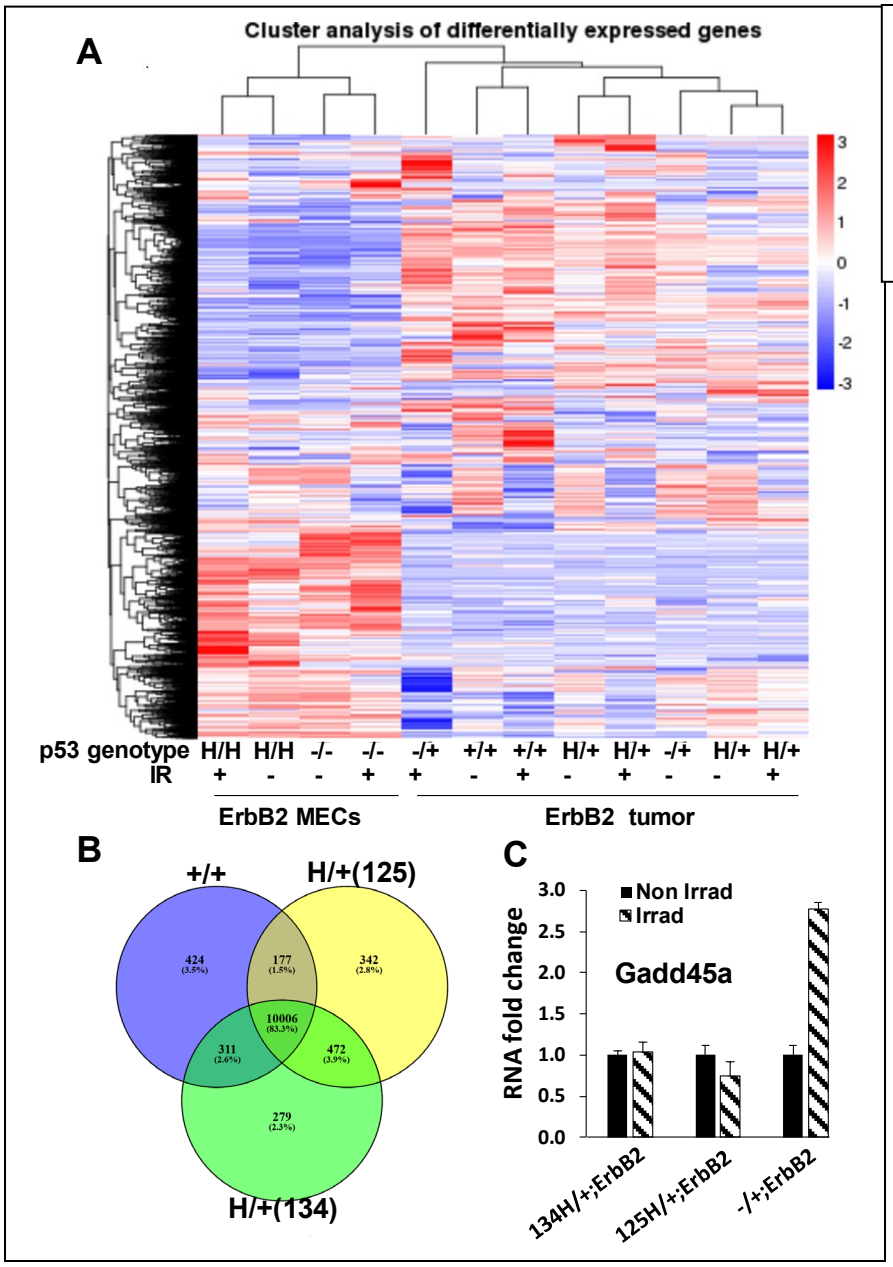


**Fig 3. Transcriptome analysis of murine cells with various p53 genotype 24h post irradiation**. (**A**). Heat map showing the expression of genes altered after irradiation depending on p53 genotype. (**B**). Venn diagrams showing the number of genes differentially expressed in -/+;ErbB2 vs. H/+;ErbB2 (125 and 134) after irradiation. (**C**). Gadd45, p53 target gene expression shows DN effect of mutp53 allele in H/+;ErbB2 cells 24 after irradiation.

The understanding of how p53LOH enables the proliferation of mutp53 cells and disrupts the mitotic checkpoint (Fig.2A) in the presence of chromosomal aberrations (Fig.2B,C) would provide an important insight into how to prevent the clonal expansion of p53LOH-acquired cells. Therefore, we performed RNAseq of mammary tumor cell lines with various p53 genotypes (Fig.1A) irradiated/or not to evaluate the contributions of dominant-negative, gain-of-function and loss-of-function effects of mutp53 in H/+;ErbB2 (n=2), -/+;ErbB2 (n=1), +/+;ErbB2 (n=1) cells. Cells were harvested 24h post-irradiation (9Gy), and qRT-PCR was performed prior to RNAseq to ensure 1:1 heterozygosity state. To compensate for intra-genotype variations, we considered only genes similarly deregulated in both H/+;ErbB2 cell lines (Fig.3B).

We identified differentially expressed genes between irradiated/non-irradiated H/+;ErbB2 vs. -/+;ErbB2 and +/+;ErbB2 cells (Fig.3A). Strikingly, we found that while mutp53 exerts DN effect on a subset of wtp53 targets, e.g., Gadd45), yet mutp53 DN effect is not global. A number transcriptional targets of wtp53 (Mdm2, Sestrins1, 2, p21, CCNG1) were efficiently upregulated in response to irradiation in H/+;ErbB2 cells were suggesting that wtp53 allele retains the transcriptional activity towards selective genes. However, we identified putative GOF subset of genes that are not wtp53 targets, and are differentially expressed only in H/+;ErbB2 vs. -/+;ErbB2 and +/+;ErbB2 irradiated cells. They are involved in the regulation of cell cycle: Nek2, Rb, Wee1; DNA repair: Lig4, Ercc6l2; and transcription factors: Klf7, Tfa2b, and were not previously connected to mutp53 functions. RNAseq

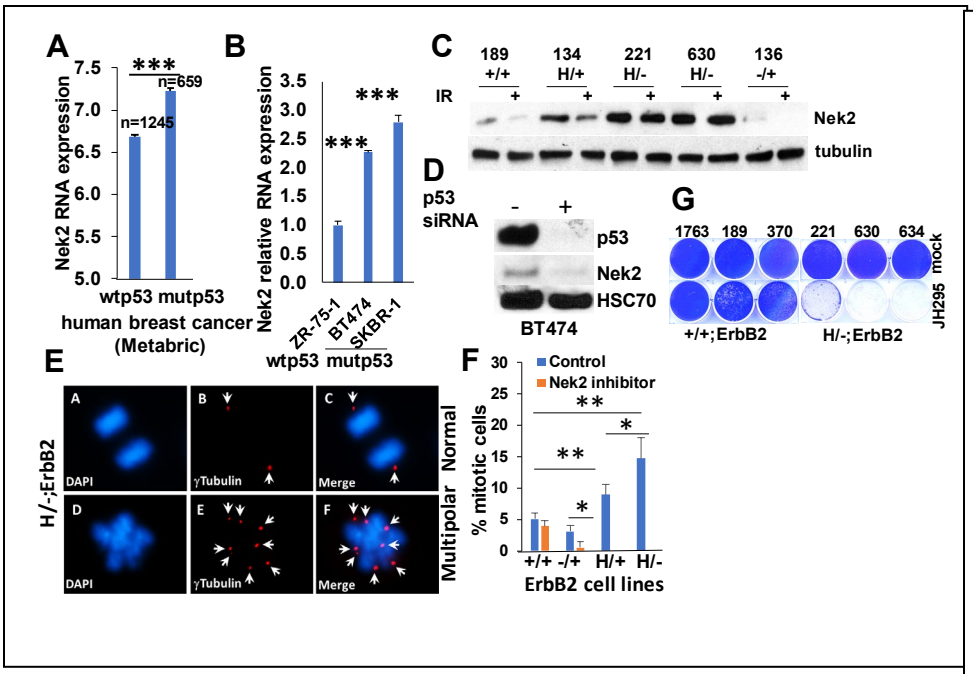


**Fig 4. Mutp53 is associated with the elevated Nek2 function.** Nek2 mRNA expression level in (**A**) breast cancer patients with wtp53 or mutp53, Metabric cohort, and (**B**) human breast cancer cell lines. (**C**) p53LOH elevates Nek2 protein even after 9Gy irradiation. (D) mutp53 ablation by siRNA in human BT474 cells downregulates Nek2 protein. (**E**) Mutp53 allele correlates with an increased percentage of cells with abnormal centrosome number (≥3) in mouse cancer cell lines. Centrosome staining (red, γ-tubulin) in mitotic p53H/-;ErbB2 cells. Upper panel, normal bipolar mitosis with one centrosome at each side. Lower panel, abnormal multipolar mitosis with supernumerary centrosomes. The Nek2 inhibitor (JH295, 1μM) blocks mitosis (24h) (**F**) and inhibits cell growth (14 days, crystal violet staining) (**H**) only in H/+;ErbB2 and H/-;ErbB2 cells, but does not affect +/+;ErbB2 cells. Nuclei were stained with DAPI, and mitotic cells were scored in 5 random fields per treatment (total of at least 100 cells). Percent of mitotic cells. Bars represent an average of 3 independent counts. ±SD **=p<0.01, ***=p<0.001.

data were validated by qRT-PCR analysis. Some of wtp53 targets were validated in response to nutlin treatment to avoid potential unspecific effects of irradiation.

Hence, our RNAseq data identified several putative pathways involved in the abrogation of G2/M arrest in response to irradiation in the presence of mutp53 allele.

First, we found that in heterozygosity mutp53 allele may inhibit the transcriptional activity of wtp53 allele via DN effect. Indeed, we found that irradiation induces transcriptional target of wtp53 Gadd45 only in -/+;ErbB2, but not in H/+;ErbB2 cells. We validated RNA seq data by qRT-PCR (Fig.3C). Gadd45 was shown to inhibit the kinase activity of the CDK1/CyclinB1 complex and promotes G2/M arrest. Therefore, abrogation of Gadd45 expression in H/+;ErbB2 cells may negatively affect G2/M checkpoint leading to the accumulation of chromosomal aberrations and p53LOH. We will test this hypothesis during the next funding period.

Also, RNAseq analysis of genes involved in the regulation of mitosis identified Nek2 among the top 10 differentially up-regulated genes in the presence of mutp53. Never in Mitosis (NIMA) Related Kinases (Neks) are a family of serine/threonine kinases that are highly expressed in various cancers, including Her2 positive breast cancer, where it predicts poor overall survival.

We focused on studying of Nek2 for the following reasons. Nek2 plays an indispensable role for the entry into mitosis and G2/M progression. Nek2 is required for centrosome assembly/maintenance, spindle formation, and chromosome segregation. Previous reports suggest that overexpression of Nek2 promotes centrosome amplification and aneuploidy by disrupting the mitotic checkpoint, leading to malignant transformation[7,8]. Silencing Nek2 with siRNA inhibited proliferation, induced cell death (due to mitotic errors) and dramatically increased the susceptibility of breast cancer cells to DNA-damaging modalities[7,8]. Collectively, these results

identify Nek2 as a potential therapeutic target in breast cancer. However, the link between Nek2 and mutp53 has not been investigated.

Our data suggest the following associations between mutp53 and Nek2 activity. **1)** According to our RNASeq data, Nek2 RNA is overexpressed in the presence of mutp53 allele (data not shown); **2)** A retrospective analysis of the publically available Metabric cohort of breast cancer patients demonstrated significantly higher median of Nek2 mRNA expression in mutp53 (all mutations) patients as compared to patients with wtp53 (Fig.4A). **3**) Human mutp53 Her2-positive human breast cancer lines (BT474 (E285K), SKBR3 (R175H)) showed significantly higher expression of Nek2 mRNA as compared to ZR-75-1(wtp53) (Fig.4B). **4**) Consistent with human cells, H/+;ErbB2 cells show much higher Nek2 protein level compared to +/+;ErbB2 and -/+;ErbB2 mouse tumor cells, while p53LOH in H/-;ErbB2 further stabilizes Nek2 (Fig.4C). Importantly, in the presence of wtp53 allele irradiation downregulates Nek2 protein levels, whereas, after p53LOH, Nek2 does not respond to irradiation (Fig.4C). These results are in line with previous observations implicating wtp53 as a repressor of Nek2 expression20. **5**) We found that mutp53 ablation in human BT474 cells downregulates Nek2 protein, suggesting that mutp53 may upregulate Nek2 as GOF mechanism (Fig.4D). **6**) The overexpression of Nek2 was shown to induce centrosome amplification in breast cancer cells19,20. As a functional readout of Nek2 activity, we observed the highest level of centrosome amplification (Fig.2B) and anaphase bridges after p53LOH (data was shown in the previous report). **7**) The Nek2 inhibitor (JH295) profoundly blocks mitosis only in H/+;ErbB2 and H/-;ErbB2 cells, but does not affect +/+;ErbB2 cells (Fig.4F). Strikingly, we observed strong inhibitory effect of JH295 only in H/-;ErbB2 cells and negligible toxicity in +/+;ErbB2 cells (3 biological replicas per genotype, Fig.4G). Based on these results, we hypothesized, that after p53LOH mutp53 may upregulate Nek2 in GOF manner, disabling G2/M checkpoint, inducing proliferation, centrosome amplification, ultimately promoting chromosomal instability and cancer progression. After p53LOH, cells may obligatory depend on Nek2 expression for unrestricted mitosis. Hence, Nek2 inhibition may prevent the outgrowth of H/-;ErbB2 cells by inducing mitotic failure, and whereby curb the expansion cells with p53LOH. We will test this hypothesis in the next funding period by pharmacological and genetic Nek2 ablation in the context of different p53 statuses.

**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, metastases in allografts. (timeline: months 24-32, 40% completion)*

As we previously reported, the main phenotype associated with p53LOH in mutp53 heterozygous cells is enhanced metastases *in vivo*. Also, we demonstrated that irradiation induces migration in all genotypes, but

**Table1. EMT genes that are overexpressed in mutp53 heterozygous cells**

| | H/+;ErbB2(125) | H/+;ErbB2(134) | -/+;ErbB2(136) |
|---|---|---|---|
| **Cdh2** | 9.4945 | 9.2689 | -4.0326 |
| **Vegfc** | 6.6434 | 9.5535 | -3.1181 |
| **Mmp7** | 6.4454 | 6.0173 | 0 |

more profoundly in the presence of mutp53 allele (H/+;ErbB2 cells) *in vitro*. To identify the mechanism of mutp53-induced metastases, we performed the RNAseq analysis of cells with different genotypes (Fig. 3A).

RNAseq analysis identified three top candidates which were highly overexpressed in H/+;ErbB2 (n=2) vs. -/+;ErbB2 cells: Cdh2 (N-cadherin), VEGFC and MMP7 (Table 1). We validated the RNAseq data by qRT-PCR. N-cadherin (a member of the family of Ca2+ dependent cell-cell adhesion molecules) is involved in multiple processes including inducing invasion, migration, promoting survival of cancer cells, regulating adhesion and, ultimately, metastasis. N-cadherin and Her2 were found to be co-expressed in human invasive breast carcinomas, where they associated with lymph node-positive disease, distant metastases, and high risk of metastatic brain relapse. Importantly, the Her2 amplified subtype, the frequency of brain metastasis has been reported to be as high as 35% that represents a significant clinical problem[9]. Hence, in the next funding period, we will focus on mutp53-N-cadherin link in the context of Her2 positive breast cancer and radiation-induced p53LOH.

Vascular endothelial growth factor (VEGF-C ) (Table 1) overexpression in breast cancer cells shown to be associated with increased intratumoral lymphangiogenesis, resulting in significantly enhanced metastasis to regional lymph nodes and to lungs *in vivo*. The expression of VEGF-C mRNA has recently been shown to correlate with the rate of metastasis to lymph nodes in human breast cancer[10].

Matrix metalloproteinase-7 (MMP-7) (Table 1) is a small secreted proteolytic enzyme with broad substrate specificity. Its expression has been shown to be associated with tumor invasion, metastasis, and survival for a variety of cancers including metastatic breast cancer, where it has been shown to be correlated with disease progression, and decreased survival[11].

In the next funding period, we will continue this study, focusing mainly on the potential link between mutp53 and N-cadherin. We will assess the impact of p53LOH on N-cadherin expression and metastasis in the functional in vitro assays ( Boyden chambers, wound healing assay) in isogenic *in vitro* model (Fig.1A) with upregulated and downregulated N-cadherin in the context of p53 genotype. Also, we will focus on VEGF-C and MMP7 as an alternative approach.

**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 of R172H/+;ErbB2 vs. p53-/+;ErbB2 vs. p53+/+;ErbB2 mice. Assess p53 LOH status in metastases in comparison with primary tumors. (timeline: months 24-32, 0% 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). However, the extremely low yield of ErbB2 positive cells and contamination with normal lung cells complicated the definitive conclusion. Currently, we are working on the completion of this subtask using the alternative approach. We are utilizing laser capture microscope, which is located at the Pathology Department Stony Brook University. We plan to accomplish this study in the next funding period. We plan to test the E-cadherin/N-cadherin ratio in mammary tumors of mice with different p53 genotypes by IHC. Also, we will test, whether depletion of N-cadherin affects pulmonary metastases in the context of p53 status by mammary fat pad transplantation assay.

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

Malik Padellan, undergraduate student, Stony Brook University (September 2018- August 2019) has received professional on-hand training while working on this project.

- How were the results disseminated to communities of interest?
  Oral presentations: 8th International mutant p53 Workshop, Lyon, France, 15-18 May, 2019
  Poster presentation: "Molecular mechanisms of p53 loss of heterozygosity in breast cancer in response to irradiation", Amr Ghaleb, Alisha Yallowitz, and Natalia Marchenko. 8th International mutant p53 Workshop, Lyon, France, 15-18 May, 2019.

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

For the next reporting period, we will continue experiments described in Major Task 1 Subtask 3,4 to determine the effect of doxorubicin and irradiation on p53LOH in the neoadjuvant setting. We will also focus on the implementation of Major Task 3 to determine 1)the potential link between mutp53 and N-cadherin. We will assess the impact of p53LOH on N-cadherin expression and metastasis in the functional in vitro assays ( Boyden chambers, wound healing assay) in isogenic in vitro model with upregulated and downregulated N-cadherin in the context of p53 genotype. Also, we will focus on VEGF-C and MMP7 as an alternative approach. 2) We will utilize laser capture microscope to identify p53LOH in metastases vs. primary tumors. We also plan to test the E-cadherin/N-cadherin ratio in mammary tumors of mice with different p53 genotypes by IHC. Further, we will test whether depletion of N-cadherin affects pulmonary metastases in the context of p53 status by mammary fat pad transplantation assay.

4.IMPACT

- Major innovative findings and achievements for this reporting period:

  The major innovative findings for the reporting period are the following. We established that wtp53 in mutp53 heterozygous (H/+;ErbB2) tumors might be transcriptionally competent towards a subset of targets (p21, Mdm2) and/or mutp53 may exert dominant-negative effect and suppress subset of wtp53 targets (Gadd45) in response to irradiation. As physiological consequences of p53LOH in vivo and in vitro we found that p53LOH leads to the loss of transcriptional activation of p21 and abrogation of G2/M checkpoint; 2) stabilization of mutp53 protein; 3) aggravation centrosome aberrations leading to increased genomic and chromosomal instability; 4) increased cells proliferation; 5) transcriptional upregulation of genes involved in mitosis, including Nek2 (member of Never in Mitosis (NIMA) Related Kinases family); 6) the increased sensitivity of mutp53 cancer cells to Nek2 inhibition.

  In the presence of mutp53 allele, the decreased expression of Gadd45 and increased expression of Nek2 may drive cell cycle trough G2/M checkpoint after irradiation leading to increased proliferation, chromosomal aberration, selection the cells with p53LOH, ultimately, leading to tumor progression.

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

We summarized our previous and current research related to Major task1 Subtask1 in book chapter : Ghaleb A. Marchenko N. “Mutant p53-Hsp90 axis in human cancer”, book chapter, “Heat Shock Protein 90 in Human Diseases and Disorders”, 2019, Springer Nature Publishers, Vol. 19, Alexzander A. A. Asea and Punit Kaur (Editors), book chapter.

The manuscript that summarized the experimental results described above is accepted for publication in Communications Biology.

- 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 a problem with the isolation of metastatic cells from mouse 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). However, the extremely low yield of ErbB2 positive cells and contamination with normal lung cells complicated the definitive conclusion. Currently, we are working on the completion of this subtask using the alternative approach. We are utilizing laser capture microscope, which is located at the Pathology Department Stony Brook University. 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:

1. Ghaleb A. Marchenko N. “Mutant p53-Hsp90 axis in human cancer”, book chapter, “Heat Shock Protein 90 in Human Diseases and Disorders”, 2019, Springer Nature Publishers, Vol. 19, Alexzander A. A. Asea and Punit Kaur (Editors), book chapter.

2. Ghaleb A., Yallowitz A, Marchenko N. Molecular mechanisms of p53 loss of heterozygosity in breast cancer in response to irradiation. 2019. Communications Biology, accepted for publication.

Both publications contain the 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. |
| Funding Support: | |
| Name: | Malik Padellan |
| Project Role: | Undergraduate Student, Stony Brook University |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 1 months |
| Contribution to Project: | Malik performed Western blot analysis of cell lines, mice genotyping and assessment of p53 LOH status in cell lines. |
| Funding Support: | none |
| Name: | |
| Project Role: | |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 2 months |
| Contribution to Project: | |
| Funding Support: | |
| 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

**References**


1 Borresen-Dale, A. L. TP53 and breast cancer. *Hum Mutat* **21**, 292-300, doi:10.1002/humu.10174 (2003).

2 Cancer Genome Atlas, N. Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61-70, doi:10.1038/nature11412 (2012).

3 Alexandrova, E. M. *et al.* p53 loss-of-heterozygosity is a necessary prerequisite for mutant p53 stabilization and gain-of-function in vivo. *Cell Death Dis* **8**, e2661, doi:10.1038/cddis.2017.80 (2017).

4 Brosh, R. & Rotter, V. When mutants gain new powers: news from the mutant p53 field. *Nat Rev Cancer* **9**, 701-713, doi:10.1038/nrc2693 (2009).

5 Ghadimi, B. M. *et al.* Centrosome amplification and instability occurs exclusively in aneuploid, but not in diploid colorectal cancer cell lines, and correlates with numerical chromosomal aberrations. *Genes Chromosomes Cancer* **27**, 183-190 (2000).

6 Isola, J. *et al.* Genetic alterations in ERBB2-amplified breast carcinomas. *Clin Cancer Res* **5**, 4140-4145 (1999).

7 Hayward, D. G. & Fry, A. M. Nek2 kinase in chromosome instability and cancer. *Cancer Lett* **237**, 155-166, doi:10.1016/j.canlet.2005.06.017 (2006).

8 Fang, Y. & Zhang, X. Targeting NEK2 as a promising therapeutic approach for cancer treatment. *Cell Cycle* **15**, 895-907, doi:10.1080/15384101.2016.1152430 (2016).

9 Qian, X. *et al.* N-cadherin/FGFR promotes metastasis through epithelial-to-mesenchymal transition and stem/progenitor cell-like properties. *Oncogene* **33**, 3411-3421, doi:10.1038/onc.2013.310 (2014).

10 Skobe, M. *et al.* Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* **7**, 192-198, doi:10.1038/84643 (2001).

11 Beeghly-Fadiel, A. *et al.* Genetic polymorphisms in the MMP-7 gene and breast cancer survival. *Int J Cancer* **124**, 208-214, doi:10.1002/ijc.23859 (2009).

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Dear Dr Marchenko,

Your manuscript entitled "Molecular mechanisms of p53 loss of heterozygosity in breast cancer in response to irradiation." has now been evaluated by the editors and I am delighted to say that we are happy to publish a suitably revised version in Communications Biology under the open access CC BY license (Creative Commons Attribution v4.0 International License).

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