AD_____

Award Number: W81XWH-10-1-0985

TITLE: Structural Rearrangements in DNA Repair Genes in Breast Cancer

PRINCIPAL INVESTIGATOR: Adrian V. Lee, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 15213-3320

REPORT DATE: October 2013

TYPE OF REPORT: Addendum to Final

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 *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
October 2013	Addendum to Final	20 September 2012 – 19 September 2013

4. TITLE AND SUBTITLE
Structural Rearrangements in DNA Repair Genes in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0985

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Adrian V. Lee, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Univeristy of Pittsburgh
3520 Fifth Avenue
Pittsburgh
PA 15213-3320

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

The genetic basis of cancer has been firmly established in the last few decades. Genomic instability is a hallmark feature of virtually all breast cancer cells, and is caused either by inherited mutations in genes that control genomic fidelity and stability (particularly in DNA repair pathways), or somatic mutations that are acquired during breast cancer progression. We originally identified translocations in 3 DNA repair genes (RAD51C, EYA2, BRIP1) in MCF7 breast cancer cells, and hypothesized that structural genomic alterations in genes that are themselves actually involved in DNA repair enhance the level of genomic instability and ultimately affect breast cancer progression and prognosis. However, we found that these translocations are specific to MCF7 cells (private mutations) and not present in any other cell lines. Interestingly, we found that BRIP1, a known familial tumor suppressor is amplified in a large portion of sporadic breast cancers. In cells with amplification of BRIP1, knockdown of BRIP1 inhibited cell growth suggesting that BRIP1 might be a driver of breast cancer growth. Mass-spec analysis identified a novel role for BRIP1 as a transcriptional repressor. This study has thus identified a familial tumor suppressor gene which is amplified in sporadic breast cancer and has a novel transcriptional regulatory role.

15. SUBJECT TERMS
Breast Cancer, DNA repair, RAD51C, EYA2, BRIP1, translocation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

BODY

1) Introduction

This addendum to the Final Report contains information on work performed using a no-cost extension (Oct 2012 to Oct 2013). The final year allowed us to develop new insight into a tumor suppressor gene, BRIP1, which has unique functions in breast cancer. For this addendum I will provide a very brief overview of the work from the Final Report (the original work can be found in that report), and then provide the results from the extra year.

2) Initial 2 years of the funding period (Oct 2010 – Oct 2012).

The genetic basis of cancer has been firmly established in the last few decades. Genomic instability is a hallmark feature of virtually all breast cancer cells, and is caused either by inherited mutations in genes that control genomic fidelity and stability (particularly in DNA repair pathways), or somatic mutations that are acquired during breast cancer progression. The importance of DNA repair in breast cancer is highlighted by the fact that inherited breast cancer is associated with germline mutations in ten different genes associated with genome stability and fidelity. Importantly, the central role of DNA double stranded break repair (DSBR) in both hereditary and sporadic breast cancer may provide an Achilles heel that can be targeted therapeutically. Thus, defects in DSBR pathways lead cells to become hypersensitive to DNA damaging agents such as mitomycin C or cisplatin. Using paired end sequencing, we generated a map of breaks in genomic DNA in a breast cancer cell line named MCF-7. This study gave us a unique insight into the genomic instability in MCF-7 cells and showed that a number of genes that had undergone structural change (translocation, deletion, or inversion) were tumor suppressor genes and were mostly repaired by non-homologous end joining an error-prone method of DNA double strand break repair. Intriguingly, we identified translocation of three genes, *RAD51C*, *BRIP1,* and *EYA2*, all of which are all central to DSBR, leading to the novel and exciting IDEA that genes important for genomic integrity and homologous recombination are themselves structurally altered at the genomic level and thus potentially non-functional.

We hypothesized that structural genomic alterations in the genes that are actually themselves involved in DNA repair enhance the level of genomic instability and ultimately affect breast cancer progression and prognosis. We hypothesized that alterations in *BRIP1*, *RAD51C, and EYA2* would render cell hypersensitive to DNA damaging agents and that fidelity of the DSBR pathway, measured at the genomic level, might be a candidate biomarker for personalizing therapy.

We measured mRNA expression of RAD51C, BRIP1 and EYA2 by Q-RT-PCR on a panel of 32 breast cancer cell lines and used MCF10A (immortalized but non-transformed) and normal female breast RNA (purchased from Life Technologies) as a control (Figure 1). Our hypothesis was that RAD51C expression would be elevated as it is found as an expressed fusion mRNA (RAD51C:ATXN7) and indeed we found this to be the case. For BRIP1 and EYA2 the translocation we originally reported (1) resulted in truncation of the gene with the final exons being replaced by non-genic DNA. As this eliminates the mRNA polyA tail, we believed this would result in an unstable mRNA that is rapidly degraded, thus resulting in reduced mRNA levels. Cells with this translocation would thus have one mutant allele and thus would be expected to have either half the level of mRNA compared to normal cells, or if the other allele is also genomically rearranged or mutated they might have no transcript at all. We thus hypothesized that MCF7 cells would have low levels of expression compared to other cell lines, and that breast cancer cells would be lower than MCF10A and normal RNA. However, for both BRIP1 and EYA2 we found that MCF7 had the highest level of expression and that most cell lines had expression that was higher than MCF10A and normal RNA. This result didn't fit with our hypothesis of these genes being mutated with reduced expression (e.g. tumor suppressor genes) in breast cancer.

The translocations in BRIP1 and EYA2 which we originally reported resulted in truncation of the gene with the final exons being replaced by non-genic DNA. As this eliminates the mRNA polyA tail we believed this would result in an unstable mRNA that is rapidly degraded. Cells with this translocation would thus have one mutant allele and thus would be expected to have either half the level of mRNA compared to normal cells, or if the other allele is also genomically rearranged or mutated they may have no transcript at all. This would be consistent with the classic two-hit hypothesis for tumor suppressor genes. However, to examine this, we needed to directly examine the mRNA produced from specific alleles. To do this, we performed restriction fragment

length polymorphism (RFLP) analysis on mRNA isolated from a panel of breast cancer cell lines. For this assay we identified unique single nucleotide polymorphisms (SNPs) in restriction sites (either introducing or deleting a restriction site) that will thus affect restriction enzyme digestion of DNA. We thus amplified BRIP1 and digested the PCR product from a panel of breast cancer cell lines with the specific restriction enzyme to identify cell lines with heterozygous alleles (to allow us to investigate allele specific expression). Figure 4 shows the panel of cell lines and indicates if they have are homozygous for A allele, homozygous for G allele, or are heterozygous with both an A and G. Note that some cells including MCF7 have a ? indicating an imbalance in their alleles as each allele had a band with a different intensity.

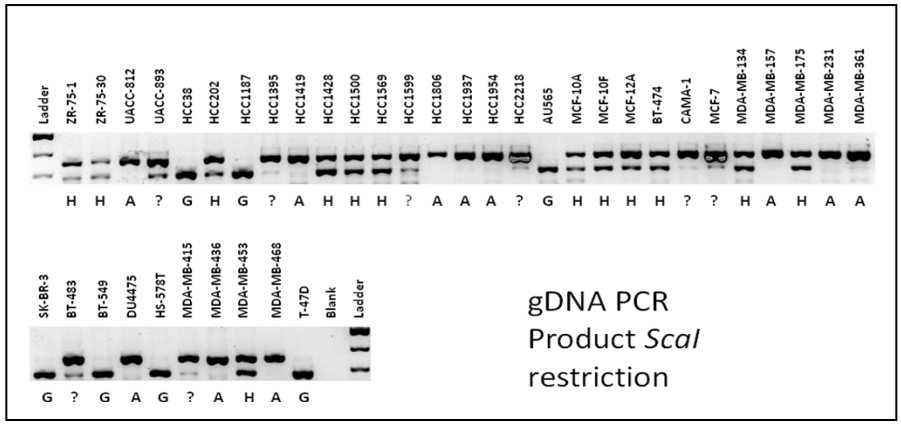

Figure 1: RFLP to examine heterozygosity of BRIP1 in a panel of breast cancer cell lines. PCR for BRIP1 was performed on a panel of breast cancer cell lines. The PCR product was digested with *Sca1* enzyme and visualized by gel electrophoresis. Note that some cells are homozygous (A) for A allele (e.g. UACC-812), homozygous (G) for G allele (e.g. HCC1995), or heterozygous (H) with both A and G (e.g. HCC 1428).

Cells should either have one band (homozygous) or two bands (heterozygous) of equal intensity. Some cell lines showed a complex pattern with an imbalance in the alleles (e.g. HCC38, HCC1599). These cell lines were labeled with a ?. Note that MCF-7 cells had an extremely high level of the A allele (so high the black band turned white in the center) and a similar pattern was seen in HCC2218. This suggested that this allele in MCF7 cells maybe amplified, an odd observation for a tumor suppressor gene which should in theory be lost in breast cancer.

We then performed RT-PCR on cells with were heterozygous for BRIP1 and digested the cDNA product to reveal the relative abundance of mRNA coming from each allele (Figure 2). Consistent with our original observation that one allele of MCF-7 cells has a translocation, and the hypothesis that this results in a null allele, we found allele specific mRNA expression in MCF7 cells. However, note that the expression from this allele is much higher than all other cell lines and significantly higher than MCF10A cells. This would be consistent with the amplification of this allele mentioned previously, and a result highly unexpected for a tumor suppressor gene. Note also that all cell lines have mRNA expression higher than MCF10A cells, again a result that is contradictory to BRIP1's role as a tumor suppressor gene.

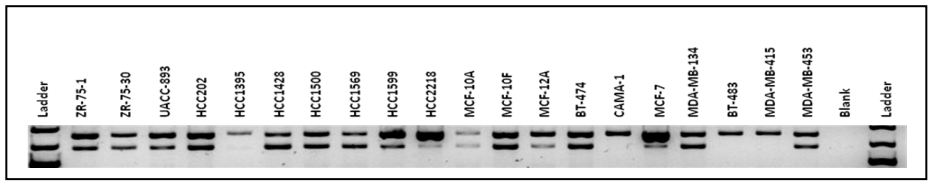

Figure 2: RFLP analysis of mRNA in a panel of breast cancer cell lines. Following RT-PCR, cDNA was digested with Sal1 enzyme and visualized by gel electrophoresis. Note that MCF7 cells have an abundance of mRNA from a single allele. However, also note that all cell lines have increased mRNA compared to MCF10A immortalized cells, a finding partly inconsistent with BRIP1s proposed role as a tumor suppressor gene.

3) Results from additional year no cost extension

3.1) BRIP1 amplification and overexpression in a large panel of breast cancer cell lines

To further examine the amplification and overexpression of BRIP1 in breast cancer, we carefully measured DNA and mRNA levels in an extended panel of breast cancer cell lines. As shown in Figure 3, BRIP 1 was amplified in several HER2+ and luminal cell lines. Interestingly, some cell lines showed heterozygous loss (i.e. 1 copy) of BRIP1 (e.g. AU565) and this correlated with the RFLP analysis which showed single alleles in these cell lines - Figure 1 and 2).

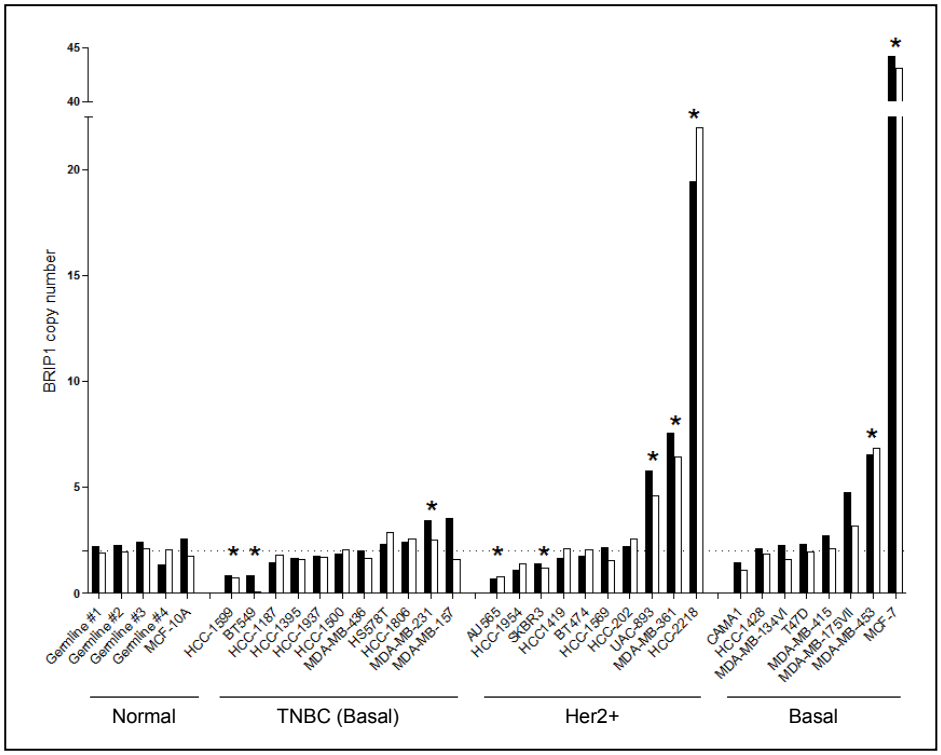

Figure 3: BRIP1 copy number in a large panel of breast cancer cell lines. BRIP1 copy number was measured with the CNV assay from Q biomarkers using a stable region on Chr17 as a control. A line highlights the normal 2 copies. Black and white bars represent two independent measurements. * significantly different from 2 copies

We also measured total BRIP1 mRNA in the same panel of cell lines.

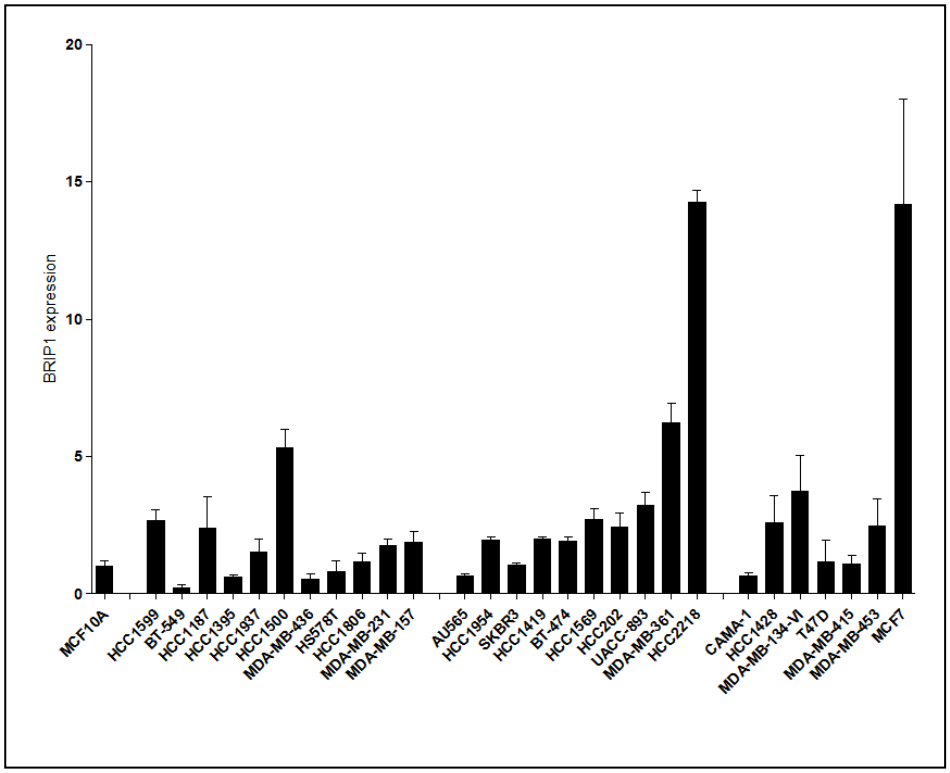

Figure 4: BRIP1 mRNA levels in a large panel of breast cancer cell lines. BRIP1 mRNA was measured by Q-RT-PCR and normalized to B-actin. Note that cell lines are represented in the same order as above.

3.2) BRIP1 is amplified and overexpressed in human tumors

BRIP1 (chr17:57114766-57295537) is present on a breast cancer amplicon at 17q23 (1). Increased copy number of 17q23 is associated with tumor progression (2) and poor prognosis (3, 4). The 17q23 amplicon is large and comlex, covering aapproximately 4-5 Mb and consisting of approximately 50 genes which are both amplified and rearranged (5). By correlating amplification of genes in this region with increased mRNA expression, several putative oncogenes have been proposed (6). These genes include *RPS6KB1*, *APPBP2*, *RAD51C*, *TBX2*, *TRIM37*, *THRAP1*, *PPM1D*, and *BRIP1*. *Many of these genes do indeed show properties of oncogenes when tested in culture and animal model systems, although no studies have been performed on BRIP1, likely because it is a known tumor suppressor gene and thought unlikely to serve as an*

oncogene. A search of Tumorscape at the Broad Institute showed high level BRIP1 amplification in breast cancer (Q-value 5.6E10-19), however, the presence of the wide range of amplification at this region likely points to collaborating oncogenes. Interestingly, however, there has been relatively little study of this region in the past 5 years (when the ability to resolve copy number change has increased dramatically), and there have been no studies of BRIP1 as an oncogene, likely because it is a known tumor suppressor gene in hereditary breast cancer.

We thus examined BRIP1 copy number changes and gene expression in several recently reported large publicly available breast cancer datasets. Two large datasets (TCGA and METABRIC) both showed significant BRIP1 amplification (Figure 3 and data not shown). Importantly, we found that BRIP1 amplification was subtype specific, being higher in HER2+ and luminal B tumors. This indicates that the amplification is unlikely a passenger event and that there is likely some interaction with breast cancer subtype. A recent intriguing study showed that ras downregulates BRIP1 levels to inhibit BRCA1 activity and promote senescence, and that ectopic BRIP1 can inhibit ras-induced senescence (7). We hypothesize that BRIP1 amplification and overerexpression in sporadic breast cancer functions to block HER2-induced senescence, and this may explain the enrichment for amplification of BRIP1 in HER2+ and luminal B breast tumors.

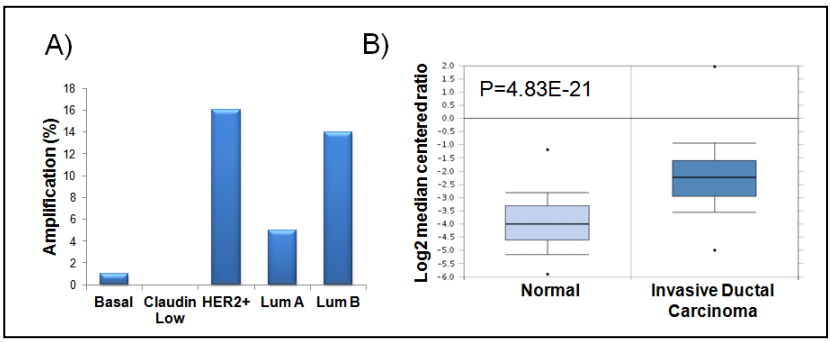

Figure 5: BRIP1 is amplified in HER2+ and luminal B breast cancers and mRNA is highly overexpressed compared to normal. Analysis of TCGA data for A) BRIP1 DNA levels and B) BRIP1 mRNA. Data was generated using cBio and Oncomine.

Confirming that the amplification of BRIP1 actually leads to increased gene expression we examined TCGA and found that BRIP1 is one of the top 8% overexpressed genes in this dataset with 3.2 fold overexpression comparing breast cancer to normal breast (p=4.8E-21). A recent study of BRIP1 protein levels by IHC in 101 invasive breast cancers showed that expression was higher in Grade 3 cancers compared to Grades 1 and 2 (8). The BRIP1 promoter has binding sites for FOXM1 and E2F and these transcription factors can drive expression (8, 9). We examined expression of BRIP1, FOXM1 and E2F1 in TCGA. BRIP1 mRNA is overexpressed 3.2 fold in breast cancers compared to normal (p=4.8E-21), FOXM is overexpressed 5.2-fold (p1.7E-49), and E2F1 is overexpressed 2.7-fold (p=1.7E-22). However, most importantly, the genes show highly significant co-occurrence with a Fisher's exact test showing BRIP1 and E2F (p<0.0001), BRIP1 and FOXM1 (p<0.0001) and E2F and FOXM1 (p<0.0001). It is thus possible that BRIP1 acts as an oncogene both following amplification and also overexpression in a network with FOXM1 and E2F.

3.3) A reduction of BRIP1 levels in cells with amplification of BRIP1 slows growth

Breast cancers often undergo multiple copy number changes, and identifying the critical driver events versus simple passengers remains a challenge. In experiments to determine if the amplification and overexpression of BRIP1 has biological significance we knocked down BRIP1 using siRNA (Figure 6). The knockdown was successful as shown by the complete loss of BRIP1 protein.

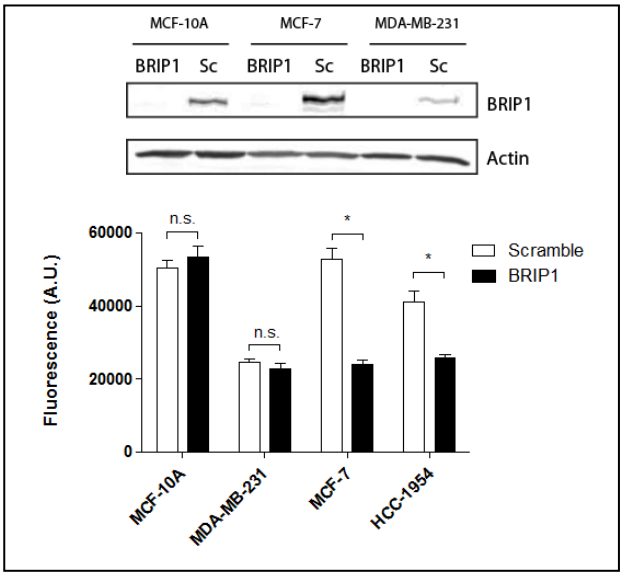

Figure 6: siRNA knockdown of BRIP1 slows growth of MCF7 and HCC-1954 breast cancer cells. Cells were transiently transfected with siRNA (BRIP1 or scrambled control - sc) and examined for protein expression by immunoblot and cell proliferation measured with fluorescence (arbitrary units – a.u.)

Measuring cell growth following BRIP1 knockdown we found a decrease in proliferation in MCF7 and HCC-1954, cell that harbor amplification of BRIP1. BRIP1 knockdown had no effect in MCF10A or MDA-MB-231 cells, both of which don't have amplification of BRIP1. This suggests that

BRIP1 is important for the proliferation of cells which show amplification (perhaps oncogene addiction).

3.4) BRIP1 is present in a transcriptional co-regulator complex

We hypothesized that BRIP1 amplification may result in gain-of-functions that are outside of its well-known action in DNA repair. To investigate this, we identified proteins interacting with BRIP1 by immunoprecipitating endogenous BRIP1 from MCF-7 cells and performing mass spectrometry (IgG served as a negative control). We identified 179 proteins which included BRIP1 itself and the three major BRIP1 interacting proteins BRCA1, MLH1, and PMS2, thus validating the assay. Of the other interacting proteins, gene ontology (GO) analysis revealed the strongest enrichment for the Mediator complex (p=4.8E-36) with identification of 22 members and CDK8. In fact approximately 80% of the gene list consisted of transcription factors, co-regulators, and chromatin modifiers. To confirm that BRIP1 has a transcriptional function, we cloned BRIP to a GAL4 DNA binding domain and performed a GAL4 transcription assay. In this assay BRIP1 showed very strong transcriptional repressor function (Figure 7). Using a mutant BRIP1 that either 1) lacks helicase activity (K52R) or fails to bind BRCA1 (S900A) showed the same repression indicating that this function is outside of the two main known actions of BRIP1.

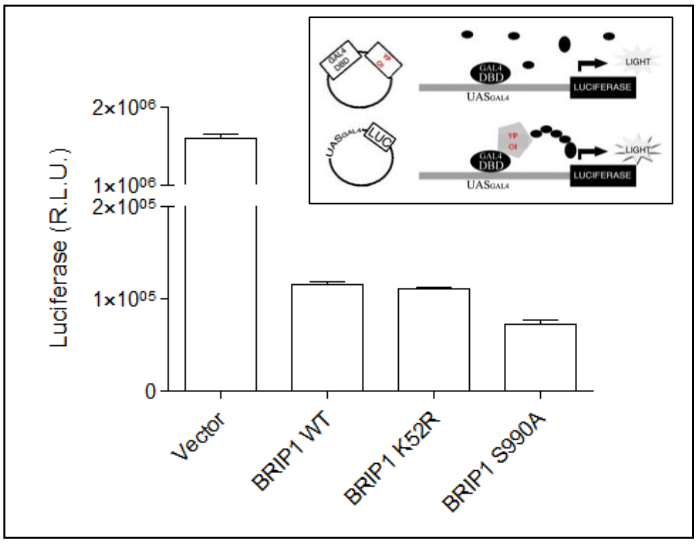

Figure 7: BRIP1 is a transcriptional repressor. Wild type BRIP1 and two mutants (K52R and S900A) were cloned upstream of a GAL4 DNA binding domain (top left plasmid). This was then transfected into MCF7 cells with a reporter (UAS-Luc) and luciferase measured and corrected for renilla (relative light units – RLU). Note that expression of BRIP1 causes a reduction in luciferase, and the same is seen with both BRIP1 mutant proteins.

4) Key Research Accomplishments

- BRIP1 is amplified in luminal B and HER2+ cell lines and human tumors
- Knockdown of BRIP1 in cell lines with amplified BRIP1 slows growth
- BRIP1 co-precipitates with a large transcriptional complex
- BRIP1 has transcriptional repressor activity which is independent of its ability to bind BRCA1 or its helicase activity

5) Reportable Outcomes

None

6) Conclusion

BRIP1 is a regulator of BRCA1 action, and like BRCA1 is a tumor suppressor gene which is mutated in hereditary breast cancer. However, while BRCA1 is reduced in sporadic breast cancer, we found that BRIP1 is amplified. Amplification is enriched in HER2+ and luminal B cell lines and human breast cancers. A reduction of BRIP1 levels in those cells with amplification caused a reduction in cell growth, suggesting that in this instance BRIP1 maybe acting as an oncogene. In a search for a novel function of BRIP1 in cells with amplification, we found that BRIP1 is present in a large complex of transcriptional regulators, and validated that BRIP1 can cause transcriptional repression in a GAL4 reporter assay. This is a novel function for BRIP1 and in part may explain why it is amplified in sporadic breast cancer

7) References

1. Kallioniemi A, Kallioniemi OP, Piper J, Tanner M, Stokke T, Chen L, Smith HS, Pinkel D, Gray JW, Waldman FM. Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. Proceedings of the National Academy of Sciences of the United States of America. 1994;91:2156-60. PMCID:43329
2. Andersen CL, Monni O, Wagner U, Kononen J, Barlund M, Bucher C, Haas P, Nocito A, Bissig H, Sauter G, Kallioniemi A. High-throughput copy number analysis of 17q23 in 3520 tissue specimens by fluorescence in situ hybridization to tissue microarrays. The American journal of pathology. 2002;161:73-9. PMCID:1850703
3. Isola JJ, Kallioniemi OP, Chu LW, Fuqua SA, Hilsenbeck SG, Osborne CK, Waldman FM. Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer. The American journal of pathology. 1995;147:905-11. PMCID:1871005
4. Barlund M, Forozan F, Kononen J, Bubendorf L, Chen Y, Bittner ML, Torhorst J, Haas P, Bucher C, Sauter G, Kallioniemi OP, Kallioniemi A. Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. Journal of the National Cancer Institute. 2000;92:1252-9.
5. Sinclair CS, Rowley M, Naderi A, Couch FJ. The 17q23 amplicon and breast cancer. Breast Cancer Res Treat. 2003;78:313-22.
6. Parssinen J, Kuukasjarvi T, Karhu R, Kallioniemi A. High-level amplification at 17q23 leads to coordinated overexpression of multiple adjacent genes in breast cancer. British journal of cancer. 2007;96:1258-64. PMCID:2360139
7. Tu Z, Aird KM, Bitler BG, Nicodemus JP, Beeharry N, Xia B, Yen TJ, Zhang R. Oncogenic RAS regulates BRIP1 expression to induce dissociation of BRCA1 from chromatin, inhibit DNA repair, and promote senescence. Developmental cell. 2011;21:1077-91. PMCID:3241855
8. Eelen G, Vanden Bempt I, Verlinden L, Drijkoningen M, Smeets A, Neven P, Christiaens MR, Marchal K, Bouillon R, Verstuyf A. Expression of the BRCA1-interacting protein Brip1/BACH1/FANCJ is driven by E2F and correlates with human breast cancer malignancy. Oncogene. 2008;27:4233-41.
9. Monteiro LJ, Khongkow P, Kongsema M, Morris JR, Man C, Weekes D, Koo CY, Gomes AR, Pinto PH, Varghese V, Kenny LM, Charles Coombes R, Freire R, Medema RH, Lam EW. The Forkhead Box M1 protein regulates BRIP1 expression and DNA damage repair in epirubicin treatment. Oncogene. 2012.

8) Appendix

None