

Award Number: W81XWH-12-1-0146

TITLE: Breast Cancer Chemoresistance Mechanisms Through PI 3-Kinase and Akt Signaling

PRINCIPAL INVESTIGATOR: Alex Toker

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Ó[•ç}ÉÁ ÚÆGGÍ Á

REPORT DATE: July 2015

TYPE OF REPORT: 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			<i>Form Approved</i> 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 July 2015		2. REPORT TYPE Final		3. DATES COVERED 1T æ 2012 - 30Apr2015	
4. TITLE AND SUBTITLE Breast Cancer Chemoresistance Mechanisms Through PI 3-Kinase and Akt Signaling			5a. CONTRACT NUMBER W81XWH-12-1-0146		
			5b. GRANT NUMBER Y Ì FÝÝ P È Ç È FÌ Ì		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Alex Toker, Kristin Brown Á Email: atoker@bidmc.harvard.edu			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Beth Israel Deaconess Medical Center Boston, MA 02215			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 We have discovered that the Akt pathway modulates breast cancer cell survival in response to genotoxic agents, and discovered a new substrate of Akt, MERIT40, that is phosphorylated upon exposure of cells to chemotherapeutic drugs. We propose that this represents a major mechanism by which cells exposed to these drugs evade cell death by apoptosis and thus become resistant to the damaging effects of clinically-relevant chemotherapy agents. These findings have important ramifications for the use of chemotherapy drugs in breast cancer patients, and many also suggest that MERIT40 may be used as a clinically relevant biomarker for resistance to doxorubicin.					
15. SUBJECT TERMS Akt, MERIT40, PI 3-K, chemotherapy, signaling, chemoresistance, phosphorylation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 28	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction	4
Key Research Accomplishments.....	13
Reportable Outcomes	14
Conclusion	13
References	15
Appendix.....	16

INTRODUCTION

Genotoxic chemotherapy agents are used to treat breast cancer at all stages of the disease. However, the duration of response is frequently limited by chemotherapy resistance mechanisms. Therefore, resistance has a major impact on breast cancer patient survival. Despite the importance of this issue, the molecular mechanisms underlying resistance are poorly understood and strategies to combat chemotherapy resistance are lacking. The phosphoinositide 3-kinase (PI3K)/Akt pathway has emerged as a major regulator of numerous cellular phenotypes associated with breast cancer. In this project we hypothesized that a major mechanism of resistance to genotoxic chemotherapy agents is activation of the PI3K/Akt signaling cascade. We proposed that *genotoxic drugs induce the activation of Akt to initiate a signaling pathway that renders breast cancer cells resistant to chemotherapy*. This study has two specific aims. In Aim 1 we propose to determine specific contributions of the PI3K/Akt pathway in mediating resistance to chemotherapy drugs. In Aim 2 we propose to identify Akt substrates that mediate the response of breast cancer cells to genotoxic chemotherapy agents. Defining the contribution of the PI3K/Akt pathway to chemotherapy resistance is of great importance as a significant proportion of breast cancer patients harbor mutations in this critical signaling pathway. An understanding of the mechanisms that contribute to chemotherapy resistance will permit the development of novel strategies to treat breast cancer.

KEYWORDS

Chemotherapy

Resistance

Phosphoinositide 3-kinase (PI3K)

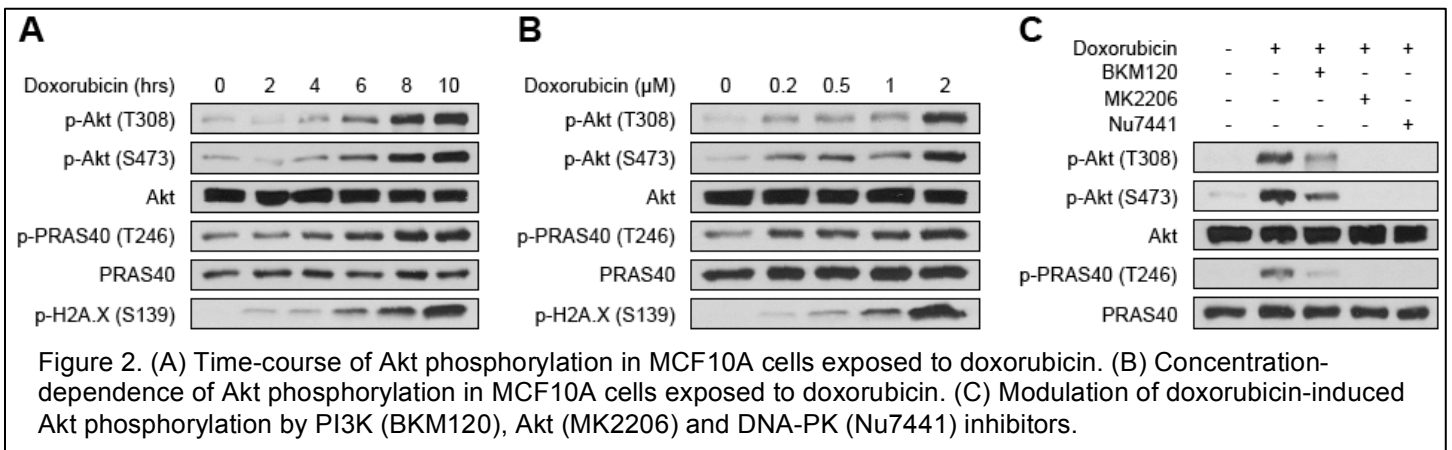
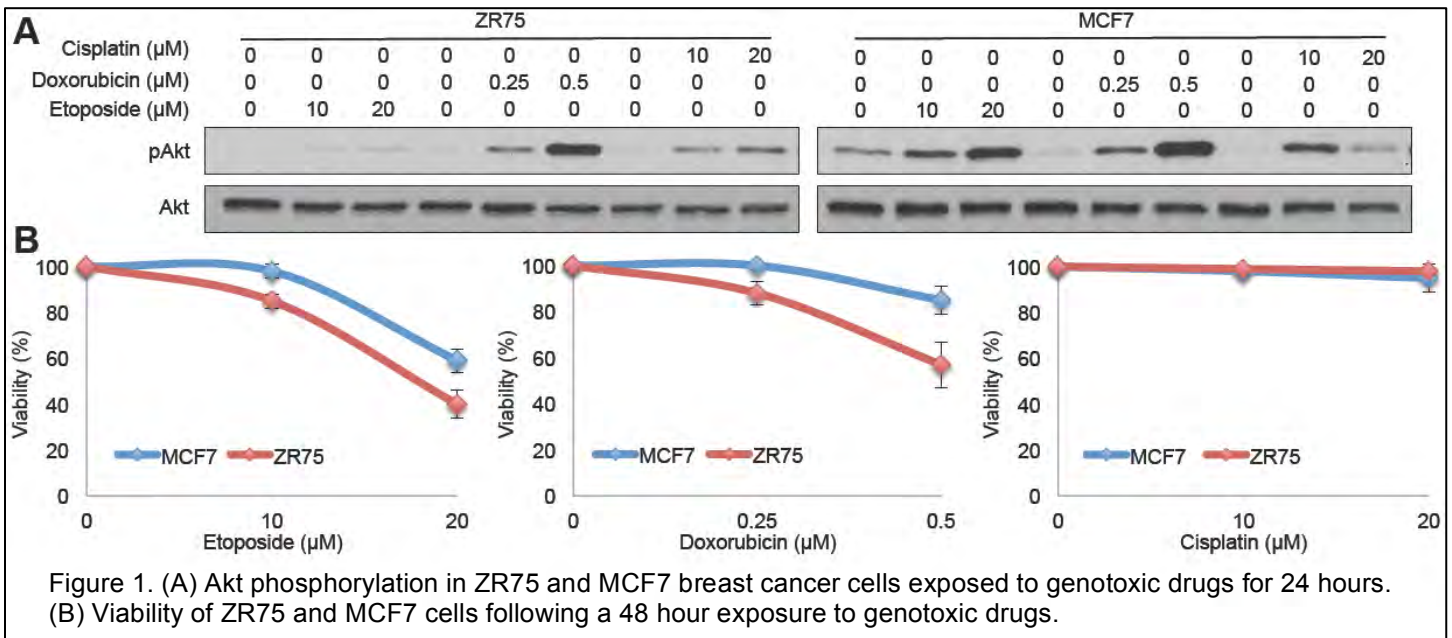
Akt

OVERALL PROJECT SUMMARY

Aim 1: Determine specific contributions of the PI3K/Akt pathway in mediating chemoresistance to genotoxic drugs.

Aim 1, Task 1: Analysis of the ability of genotoxic agents to induce phosphorylation of Akt in breast cell lines. We have demonstrated that genotoxic chemotherapy agents, including etoposide, doxorubicin and cisplatin, induce Akt phosphorylation in both non-tumorigenic breast and cells breast cancer cells (Figure 1A). Akt phosphorylation is triggered by sublethal concentrations of each drug suggesting that activation of the PI3K/Akt pathway could contribute to survival signaling (Figure 1B).

We consistently observed that the DNA topoisomerase II inhibitor doxorubicin is able to induce the most significant increase in Akt phosphorylation in breast cancer cells (Figure 1A). Doxorubicin is frequently used as a single agent therapy for both primary and recurrent breast cancer [1]. However, the duration of response to doxorubicin is frequently limited either by the intrinsic resistance of primary tumors to treatment, or through the emergence of chemotherapy resistance in initially responsive tumors. We therefore decided to examine in greater detail the capacity of doxorubicin to modulate PI3K/Akt pathway activity. Akt phosphorylation occurs within hours of exposure to doxorubicin and is associated with enhanced phosphorylation of the Akt substrate PRAS40 (Figure 2A,B). Akt phosphorylation is also coincident with phosphorylation of histone H2A.X, a specific marker of DNA damage (Figure 2A,B) indicating that Akt phosphorylation occurs as an early response to doxorubicin treatment and within the same time frame as DNA damage. The ability of doxorubicin to induce Akt phosphorylation is prevented by pre-treating cells with a PI3K inhibitor (BKM120) or an inhibitor of DNA-PK (Nu7441) (Figure 2C). Both PI3K and DNA-PK have previously been implicated in mediating Akt phosphorylation in response to DNA damage [2].



Aim 1, Task 2: Examine the contribution that components of the PI 3-K signaling pathway make to the cellular response to genotoxic stress. We have shown that modulation of PI3K/Akt activity alters cellular sensitivity to genotoxic agents. Hyperactivation of the PI3K pathway, induced by overexpression of the constitutively active catalytic subunit of PI3K (PIK3CA H1047R) (Figure 3A) or PTEN knockdown (Figure 3B), renders MCF10A cells more resistant to doxorubicin. In contrast, silencing of Akt isoforms sensitizes MCF10A

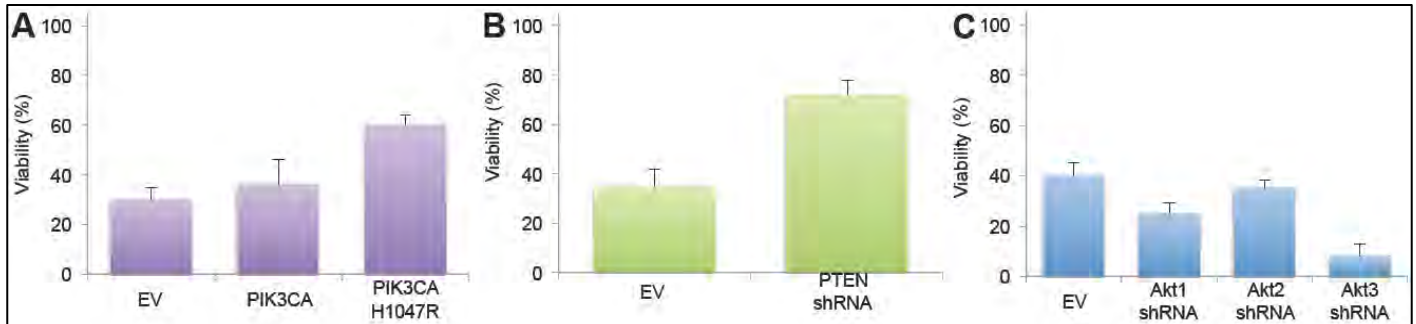


Figure 3. Viability of MCF10A cells exposed to doxorubicin following: (A) overexpression of wild-type PIK3CA or a constitutively active PIK3CA H1047R mutant, (B) shRNA-mediated silencing of PTEN or (C) shRNA-mediated silencing of Akt isoforms.

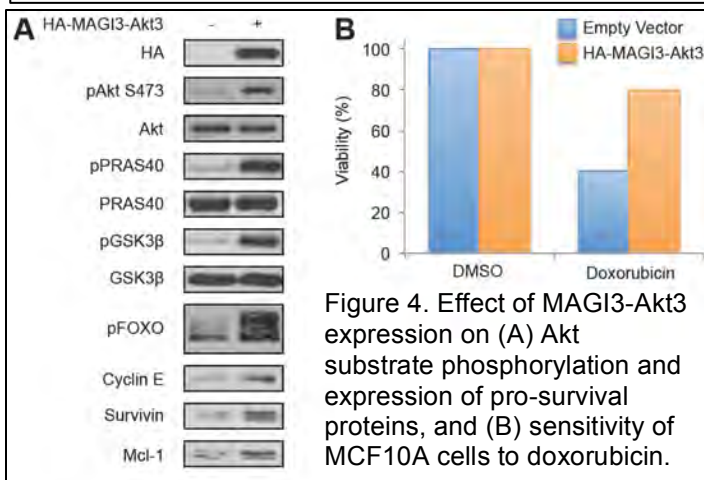


Figure 4. Effect of MAGI3-Akt3 expression on (A) Akt substrate phosphorylation and expression of pro-survival proteins, and (B) sensitivity of MCF10A cells to doxorubicin.

cells to doxorubicin with Akt3 appearing to play a dominant role in regulating cellular viability (Figure 3C).

Having observed that Akt3 makes a significant contribution to doxorubicin sensitivity we decided to investigate the ability of the MAGI3-Akt3 fusion protein to confer resistance to genotoxic agents. A whole-exome and whole-genome sequencing effort to discover mutations and gene rearrangements in human breast cancers identified a translocation event between the *Akt3* gene and the *MAGI3* gene that produces an in-frame fusion protein [3]. This is the first fusion protein to be identified in the PI 3-K/Akt pathway. The translocation event results in significant disruption to the integrity of both Akt3 and

MAGI3. We have demonstrated that MAGI3-Akt3 is constitutively phosphorylated in the Akt3 kinase domain in the absence of growth factors and its expression enhances Akt substrate phosphorylation [3] (Figure 4A). We have also demonstrated that MAGI3-Akt3 elevates the expression of pro-survival proteins including survivin and Mcl-1 (Figure 4A) and renders MCF10A cells more resistant to doxorubicin (Figure 4B). This suggests that Akt3 signaling makes a significant contribution to chemotherapy resistance. Using a combination of knockdown and overexpression studies we are continuing to examine the mechanisms by which Akt3 can influence the response of breast cancer cells to genotoxic drugs.

Aim 1, Task 3: Examine combination therapy with DNA damaging agents and chemical inhibitors of the PI3K/Akt pathway as an approach to kill breast cancer cells. We have observed activation of PI3K/Akt signaling as an early response to genotoxic drug exposure (Figure 2A). The PI3K/Akt pathway is known to contribute to cellular survival by enhancing cell proliferation and blocking apoptosis. We therefore hypothesized that inhibition of PI3K/Akt signaling might sensitize breast cancer cells to genotoxic chemotherapy agents by eliminating a major survival pathway. Indeed, pretreatment with a PI3K inhibitor (BKM120) or an Akt inhibitor (MK2206) rendered MCF10A cells markedly more sensitive to the cytotoxicity of doxorubicin (Figure 6A). Combination treatment with PI3K/Akt pathway inhibitors and doxorubicin was associated with an increase in the abundance of cleaved PARP, a specific marker of apoptosis (Figure 6B). In addition, disruption of PI3K/Akt signaling enhanced doxorubicin-induced DNA damage, as measured by an increase in phosphorylation of H2A.X (Figure 6B). These results indicate that PI3K/Akt signaling contributes to the resolution of DNA damage and maintenance of cell viability following exposure to genotoxic drugs. The ability of PI3K/Akt inhibitors to sensitize to doxorubicin was also observed in T47D, SUM159 and MCF7 breast cancer cells (Figure 7). This data suggests that combining PI3K/Akt inhibitors and genotoxic drugs could be a beneficial strategy for breast cancer therapy.

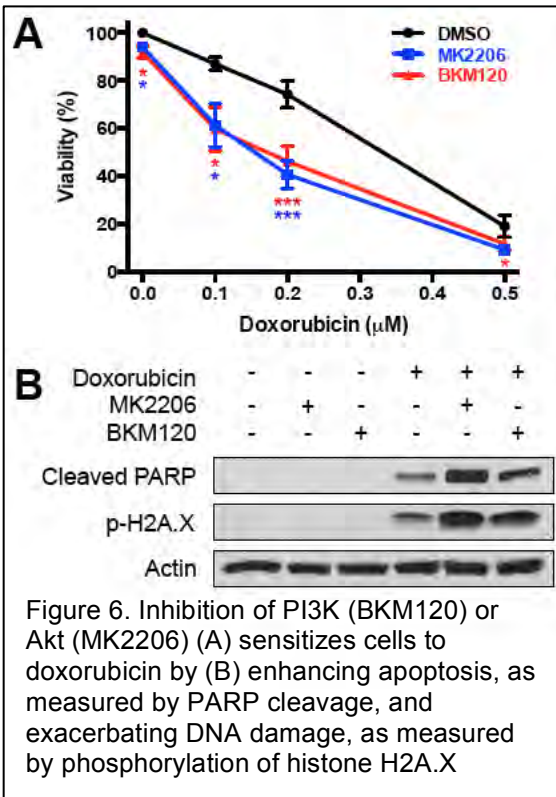


Figure 6. Inhibition of PI3K (BKM120) or Akt (MK2206) (A) sensitizes cells to doxorubicin by (B) enhancing apoptosis, as measured by PARP cleavage, and exacerbating DNA damage, as measured by phosphorylation of histone H2A.X

To examine the ability of PI3K/Akt inhibitors to overcome

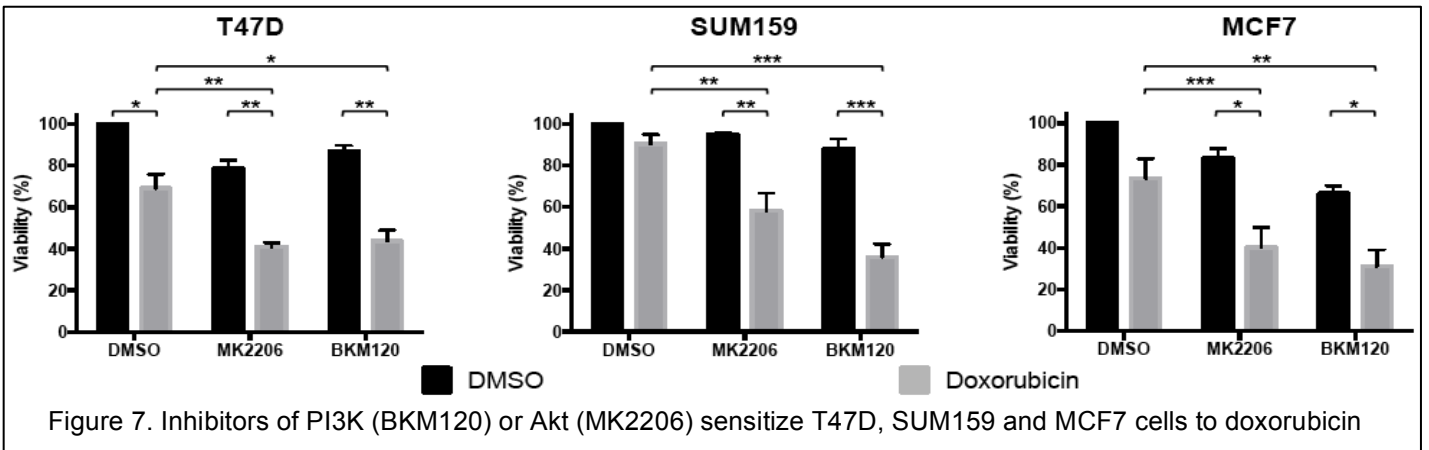


Figure 7. Inhibitors of PI3K (BKM120) or Akt (MK2206) sensitize T47D, SUM159 and MCF7 cells to doxorubicin

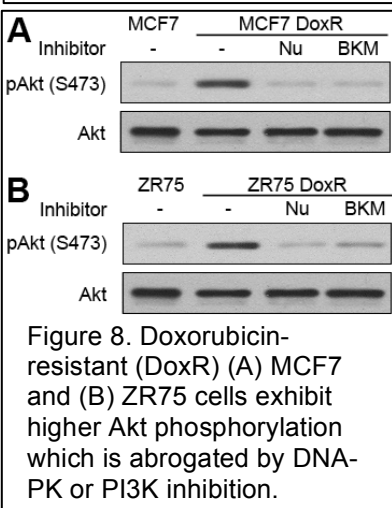
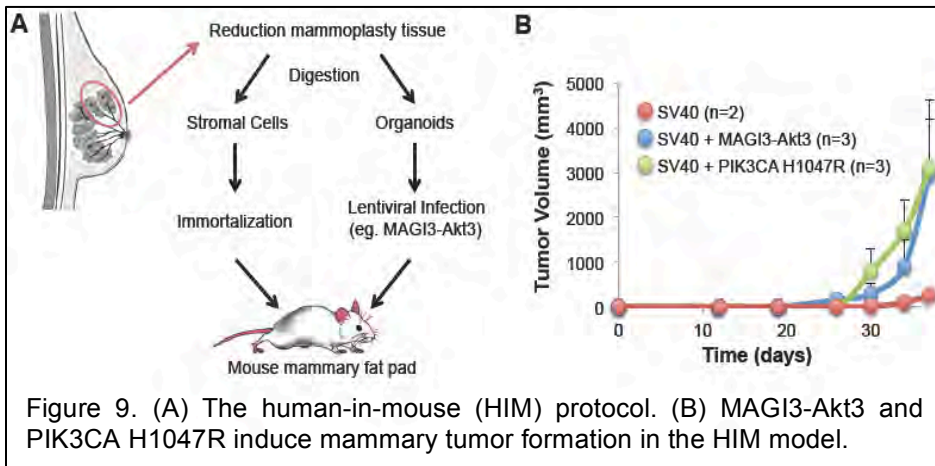


Figure 8. Doxorubicin-resistant (DoxR) (A) MCF7 and (B) ZR75 cells exhibit higher Akt phosphorylation which is abrogated by DNA-PK or PI3K inhibition.

acquired resistance to doxorubicin we have established chemotherapy resistant breast cancer cell lines. The resistant lines were generated by chronic exposure to low dose doxorubicin over a six month period. Basal Akt phosphorylation is significantly higher in the resistant lines (Figure 8). We observed that the enhanced Akt activity in doxorubicin-resistant cells was sensitive to both DNA-PK and PI3K inhibition, suggesting that prolonged exposure to genotoxic agents induces feedback activation of PI3K signaling (Figure 8). We are continuing to investigate the role of feedback activation of the PI 3-K pathway in mediating acquired chemotherapy resistance. We will further explore the idea that downregulation of PI 3-K pathway activity, with shRNA or inhibitors, could resensitize resistant cell lines to chemotherapy agents.

Aim 1, Task 4: In vivo assays to determine Akt signaling specificity. For the completion of Aim 1, we had proposed experiments to examine cell lines in which we could silence Akt isoform expression by addition of doxycycline in the context of shRNA. These cell lines were proposed to be used to perform xenograft experiments to examine the contribution of Akt1, Akt2 and Akt3 to the antitumor activity of doxorubicin. Based on our *in vitro* studies (Figure 3C) we proposed to show that Akt isoform depletion enhances the anti-tumor efficacy of doxorubicin. We invested considerable time and effort and time (months 12-18) and beyond into the extension period requested beyond the original funding period, to evaluate the consequence of depletion of AKT1, AKT2 and AKT3 in the anti-tumor activity of doxorubicin. In spite of many repeated attempts *in vitro* aimed at silencing expression of AKT1, AKT2 and AKT3, as proposed, we were unable to obtain quantitative depletion of each individual AKT isoform in the MCF7, T47D and MDA-MB-468 cell lines, although modest depletion was observed. We attempted both constitutive silencing as well as inducible silencing, but this did not result in sufficient quantitative silencing to the extent that would provide a robust effect for *in vivo* xenograft experiments. Regrettably we therefore elected to discontinue this line of investigation and to perform *in vivo* xenograft experiments, the rationale being that any responses observed would not be interpretable. This is also the reason why an ACURO approval was not applied for, since such an approval was negated by the inability to perform the *in vivo* experiments. We instead consider that more quantitative depletion would be achieved using CRISPR/Cas9 mediated knockout of AKT isoforms, followed by xenograft experiments with doxorubicin administration. However this fell below the scope and funding of this particular grant, and will be pursued as a future goal. In spite of the inability to perform this goal of Aim 1, task 4, we were able to publish a new manuscript on the role of MERIT40 in the DNA damage responses to cytotoxic chemotherapy, as described later in this report.



Also as part of this aim, and based on our finding that the MAGI3-Akt3 fusion protein promotes resistance to doxorubicin *in vitro* (Figure 4) we have initiated collaborative studies with Dr. Charlotte Kuperwasser (Tufts University School of Medicine, Boston, MA) to examine the oncogenic potential of MAGI3-Akt3 *in vivo*. Dr. Kuperwasser's lab has pioneered a human-in-mouse (HIM) breast xenograft model (Figure 9A). Our pilot study shows that MAGI3-Akt3 promotes mammary tumor

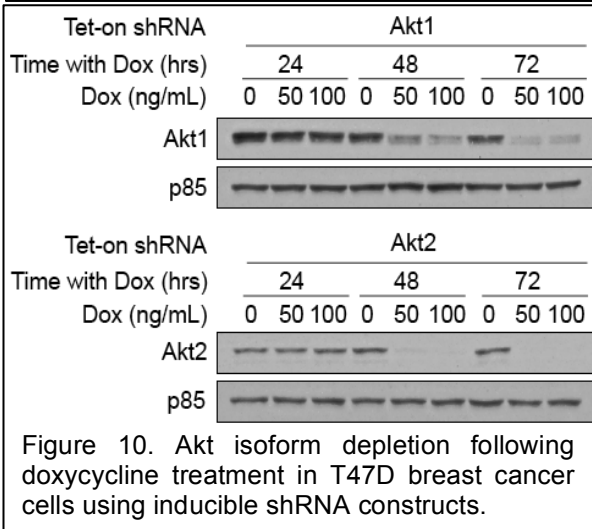
development *in vivo*, in a manner similar to that observed when cells are transformed with the oncogenic PIK3CA H1047R mutant (Figure 9B). We are continuing to collaborate with Dr. Kuperwasser to examine the oncogenic potential of the MAGI3-Akt3 fusion protein *in vivo* and to examine the ability of MAGI3-Akt3 to modulate chemotherapy responses *in vivo*.

Aim 2: Identify isoform-specific substrates that mediate the response of breast cancer cells to DNA damaging chemotherapy.

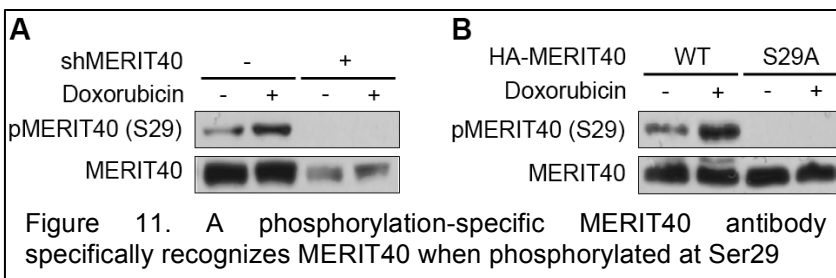
Cell line	PI 3-K pathway mutation	Akt1	Akt2	Akt3
MDA-MB-231	KRas (G13D)	+	+	+
MDA-MB-468	PTEN mutant	+	+	+
ZR-75-1	PTEN missense	+	+	-
T47D	PIK3CA (H1047R)	+	+	-
MCF7	PIK3CA (E545K)	+	+	-
MCF10A	-	+	+	+

Table 1. Breast cell lines utilized for inducible knockdown of Akt isoforms.

Aim 2, Task 1: Silencing Akt isoforms in breast cell lines.
 We have developed highly specific inducible (Tet-on) shRNA constructs to silence Akt1, Akt2 and Akt3. These constructs have been introduced into a variety of breast cancer cell lines and non-tumorigenic MCF10A breast epithelial cells (Table 1). The breast cancer cell lines have been chosen as they are known to contain hyperactivating mutations in the PI 3-K pathway. We have generated stable cell lines and confirmed that doxycycline treatment can specifically silence Akt isoforms (Figure 10). We will utilize the inducible-shRNA system to identify potential Akt isoform-specific substrates.

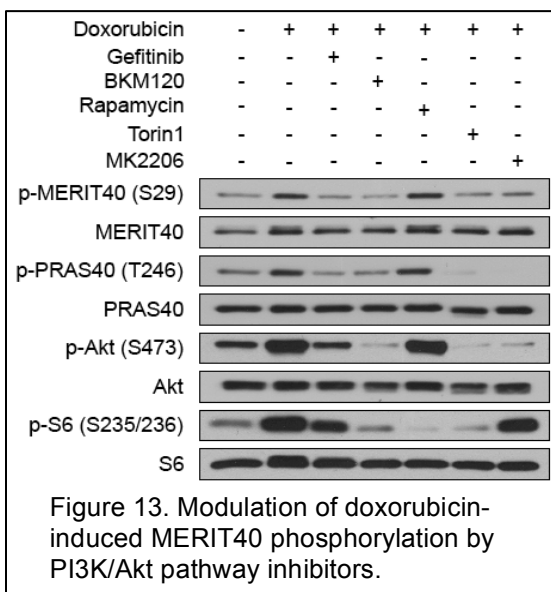
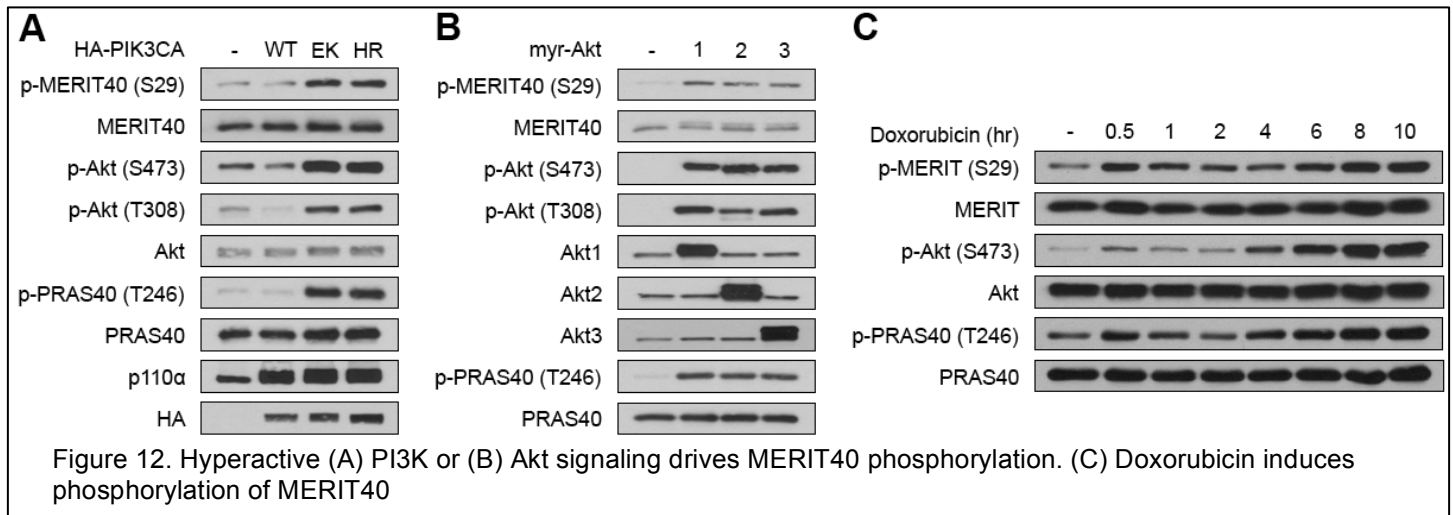


Aim 2, Task 2: Identify Akt substrates downstream of exposure to genotoxic agents. A major goal of this task was to evaluate MERIT40 as a novel Akt substrate contributing to the cellular response to genotoxic chemotherapy treatment. MERIT40 is a component of the nuclear BRCA1 A complex which contains Abraxas, Rap80, BRCC36, BRE and the tumor suppressor protein BRCA1 [4-6]. MERIT40 also participates in a cytoplasmic complex with ABRO1, BRCC36 and BRE [7]. Phospho-proteomic studies have demonstrated phosphorylation of MERIT40 at Ser29 in a consensus sequence that conforms to the Akt phosphorylation motif (RxRxxS/T). Using a combination of biochemical and molecular genetic approaches, we have identified MERIT40 as a novel Akt substrate. We have collaborated with Cell Signaling Technology to develop an antibody that specifically recognizes



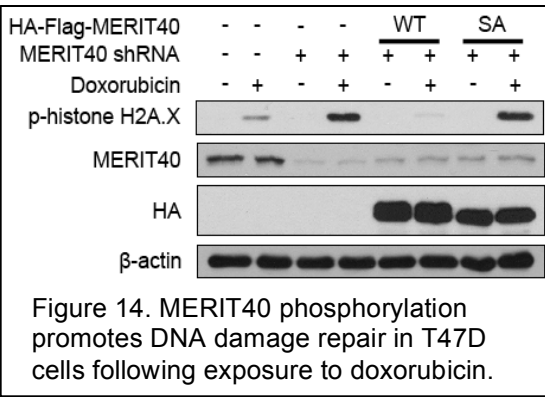
antibody that specifically recognizes MERIT40 when phosphorylated at Ser29. Using a combination of site-directed mutagenesis and shRNA approaches we have confirmed that the phospho-MERIT40 antibody specifically recognizes MERIT40 when phosphorylated at Ser29 (Figure 11). We have also developed an antibody that recognizes total MERIT40 protein.

Aim 2, Task 3: Validate candidates as substrates of the PI 3-K pathway in breast cancer cells. We have utilized the phospho-MERIT40 and total MERIT40 antibodies to validate MERIT40 as a novel Akt substrate. Hyperactivation of the PI3K pathway, resulting from introduction of constitutively active catalytic subunits of PI3K (PIK3CA H1047R or PIK3CA E545K) into MCF10A cells, enhances MERIT40 phosphorylation and phosphorylation of known Akt substrates like PRAS40 (Figure 12A). A similar response is observed when Akt signaling is hyperactivated by introducing constitutively active myristoylated Akt isoforms into MCF10A cells (Figure 12B). We also observe an increase in MERIT40 phosphorylation in cells exposed to doxorubicin (Figure 12C). Phosphorylation of MERIT40, induced upon doxorubicin treatment, is coincident with Akt

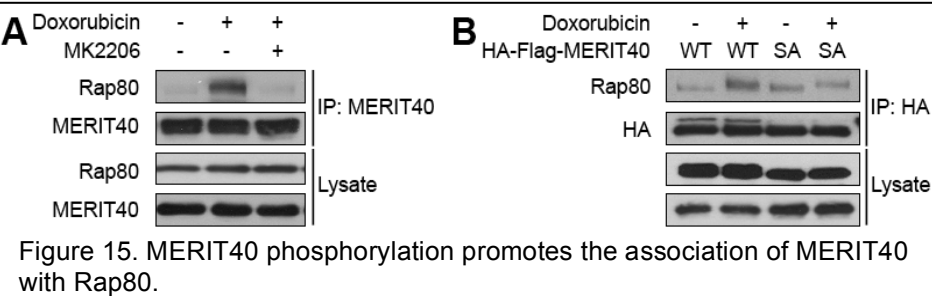


phosphorylation and phosphorylation of the Akt substrate PRAS40. The ability of doxorubicin to induce MERIT40 phosphorylation is disrupted when cells are pretreated with PI3K/Akt pathway inhibitors including the PI3K inhibitor (BKM120) and the Akt inhibitor (MK2206) (Figure 13).

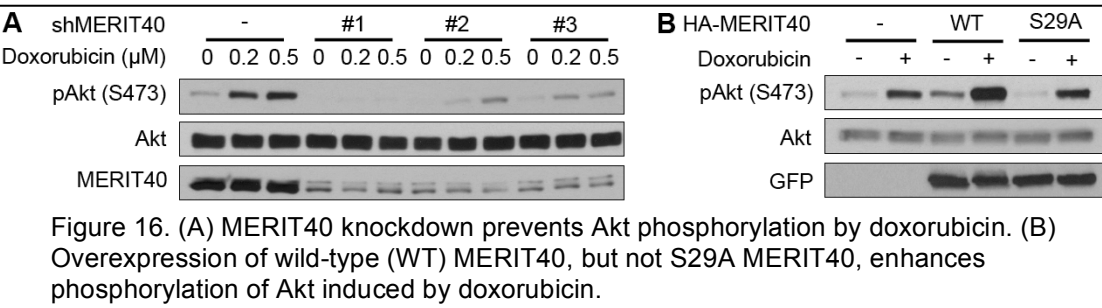
Aim 2, Task 4: Explore the functional consequences of candidate substrate phosphorylation by Akt in the response of breast cancer cells to DNA damaging chemotherapy.



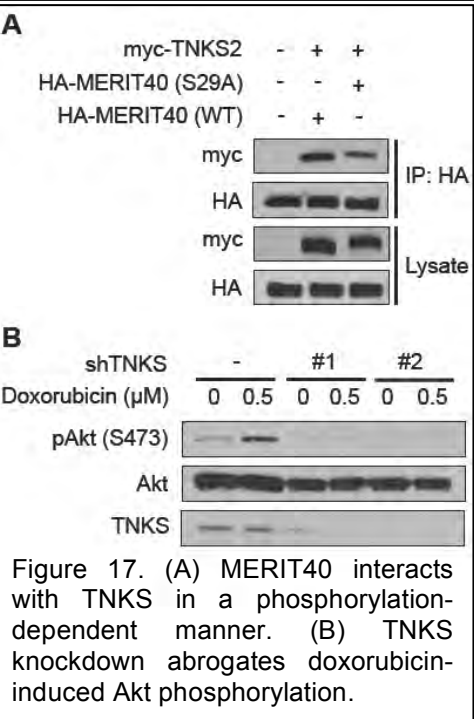
Having characterized MERIT40 as a *bona fide* Akt substrate we sought to determine the contribution of phosphorylation in regulating the cellular functions of MERIT40. As a component of the nuclear BRCA1 A complex MERIT40 contributes to DNA damage repair. We observed that MERIT40 depletion enhances DNA damage in breast cancer cell lines exposed to doxorubicin (Figure 14). Importantly, wild-type MERIT40, but not phospho-deficient MERIT40 Ser29Ala, rescues this response. This data suggests that MERIT40 phosphorylation is required to promote DNA damage repair in cells exposed to genotoxic agents. We have further demonstrated that MERIT40 phosphorylation is required for



stabilization of the BRCA1 A complex (Figure 15). Doxorubicin promotes the association of endogenous MERIT40 with Rap80, another component of the BRCA1 A complex (Figure 15A). This association is sensitive to the Akt inhibitor MK2206. We have also shown that a Ser29Ala MERIT40 mutant is unable to interact



with Rap80 (Figure 15B). Therefore, disruption of MERIT40 phosphorylation likely contributes to the ability of PI3K/Akt inhibitors to sensitize breast cancer cells to doxorubicin by disrupting the BRCA1 A

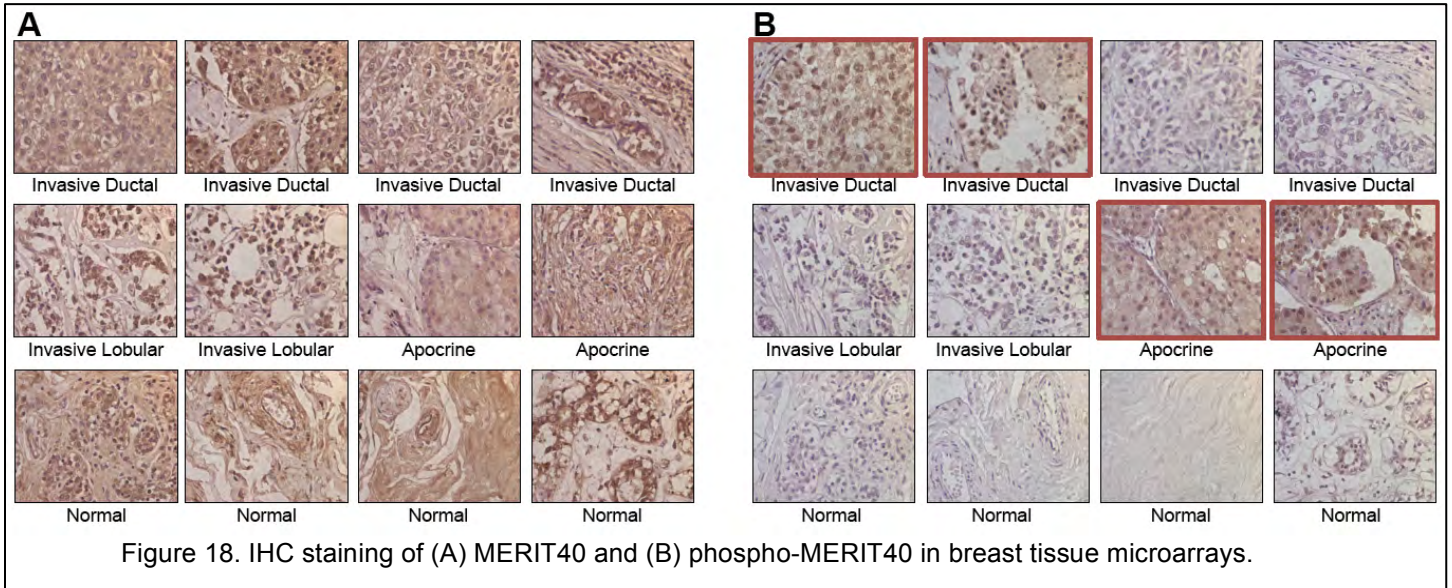


complex which is required for DNA damage repair and cell survival in the face of DNA damage. We have also observed that MERIT40 knockdown significantly inhibits the ability of doxorubicin to induce Akt phosphorylation in breast cancer cells (Figure 16A). In addition, overexpression of wild-type MERIT40, but not a MERIT40 Ser29Ala mutant enhances doxorubicin-induced Akt phosphorylation (Figure 16B). This suggests that phosphorylated MERIT40 participates in the regulation of Akt activity in response to genotoxic drug exposure. We are continuing to investigate this intriguing possibility. MERIT40 has been shown to interact with the poly-(ADP-ribose) polymerase Tankyrase (TNKS) [8]. We have shown that mutation of Ser29 also disrupts the interaction between MERIT40 and TNKS (Figure 17A). Furthermore, knockdown of TNKS prevents doxorubicin-induced Akt phosphorylation in a manner analogous to knockdown of MERIT40 (Figure 16A versus 17B). TNKS has been shown to regulate pro-survival signaling pathways [9, 10] and we are currently exploring the possibility that the phosphorylation-dependent interaction between MERIT40 and TNKS protects cells from the cytotoxicity of genotoxic chemotherapy agents via modulation of Akt activity. Together, our results suggest that the Akt-dependent phosphorylation of MERIT40 induced upon doxorubicin exposure significantly contributes to the cellular response to

DNA damage via multiple mechanisms. In addition to investigating MERIT40 as a novel Akt substrate we are

currently in the process of initiating phosphoproteomic studies to identify additional novel substrates involved in the response to DNA damage.

Aim 2, Task 5: Examine candidate substrate phosphorylation in human breast tissue (non-tumor and tumor) microarrays. Having identified and characterized MERIT40 as a novel Akt substrate that contributes to the cellular response to genotoxic drugs, we sought to examine the expression of MERIT40 and phospho-MERIT40 in human breast cancer specimens using human breast tissue microarrays and immunohistochemistry. All tumor and normal breast tissue samples showed staining for total MERIT40 (Figure 18A). In contrast, phospho-MERIT40 was only detected in breast tumor samples, specifically in two cases of Invasive Ductal Cancer and two cancers of Apocrine Cancer. We also expanded our tissue array studies to further explore the possibility that phospho-MERIT40 could be utilized as a biomarker to identify patients that might benefit from combination therapy with PI 3-K/Akt inhibitors and genotoxic chemotherapy.



KEY RESEARCH ACCOMPLISHMENTS

We have demonstrated that:

1. genotoxic chemotherapy agents induce Akt phosphorylation.
2. hyperactivation of the PI 3-K pathway renders cells more resistant to genotoxic chemotherapy drugs.
3. silencing of Akt isoforms sensitizes cells to genotoxic chemotherapy drugs. In particular, Akt3 may play a dominant role in regulating cellular sensitivity to these drugs.
4. the MAGI3-Akt3 fusion protein renders cells more resistant to genotoxic drugs.
5. inhibition of PI3K/Akt signaling enhances DNA damage and induction of apoptosis following exposure to genotoxic drugs suggesting a novel combination therapy strategy for breast cancer.
6. chemotherapy-resistant breast cancer cells exhibit enhanced basal Akt phosphorylation suggesting that prolonged exposure to genotoxic drugs induces feedback activation of PI 3-K/Akt signaling.
7. we have developed highly specific inducible shRNA constructs to silence Akt1, Akt2 and Akt3.
8. we have developed an antibody that specifically recognizes MERIT40 when phosphorylated at Ser29 by Akt.
9. MERIT40 phosphorylation is induced when cells are exposed to genotoxic drugs.
10. MERIT40 phosphorylation regulates the interaction of MERIT40 with additional components of the BRCA1 A complex, including Rap80.
11. MERIT40 phosphorylation contributes to the resolution of DNA damage following genotoxic drug exposure.
12. knockdown of MERIT40 abrogates Akt phosphorylation induced by genotoxic drugs, suggesting that MERIT40 contributes to the regulation of Akt.
13. MERIT40 interacts with tankyrase in a phosphorylation-dependent manner.
14. MERIT40 phosphorylation can be detected in human breast tumors but not in normal breast tissue.

CONCLUSION

We have discovered that the Akt pathway modulates breast cancer cell survival in response to genotoxic agents, and discovered a new substrate of Akt, MERIT40, that is phosphorylated upon exposure of cells to chemotherapeutic drugs. We propose that this represents a major mechanism by which cells exposed to these drugs evade cell death and thus become resistant to the damaging effects of clinically-relevant chemotherapy agents. These findings have important ramifications for the use of chemotherapy drugs in breast cancer patients, and also suggest that MERIT40 may be used as a clinically relevant biomarker of resistance to doxorubicin.

PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

a. Manuscripts

1. Brown KK, Montaser-Kouhsari L, Beck AH and Toker A. MERIT40 is an Akt substrate that promotes resolution of DNA damage induced by chemotherapy. *Cell Reports* 11:1358, 2015.

b. Presentations

The research findings in this report and supported by this grant have been presented at the following symposia during the last year

1. Alex Toker and Kristin Brown, Keystone Symposia on PI 3-Kinase Signaling Pathways in Disease, Vancouver, Canada, January 13-18, 2015.
Brown KK, Montaser-Kouhsari L, Beck AH and Toker A. MERIT40 is an Akt substrate that promotes resolution of DNA damage induced by chemotherapy.
2. Alex Toker and Kristin Brown, 2015 Annual AACR (American Association for Cancer Research) Meeting in Philadelphia, PA, April 18-22, 2015.
Brown KK, Montaser-Kouhsari L, Beck AH and Toker A. MERIT40 is an Akt substrate that promotes resolution of DNA damage induced by chemotherapy.

INVENTIONS, PATENTS AND LICENSES

Nothing to report.

REPORTABLE OUTCOMES

Nothing to report

OTHER ACHIEVEMENTS

Nothing to report

PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Kristin Brown, Ph.D.
Alex Toker, Ph.D.

REFERENCES

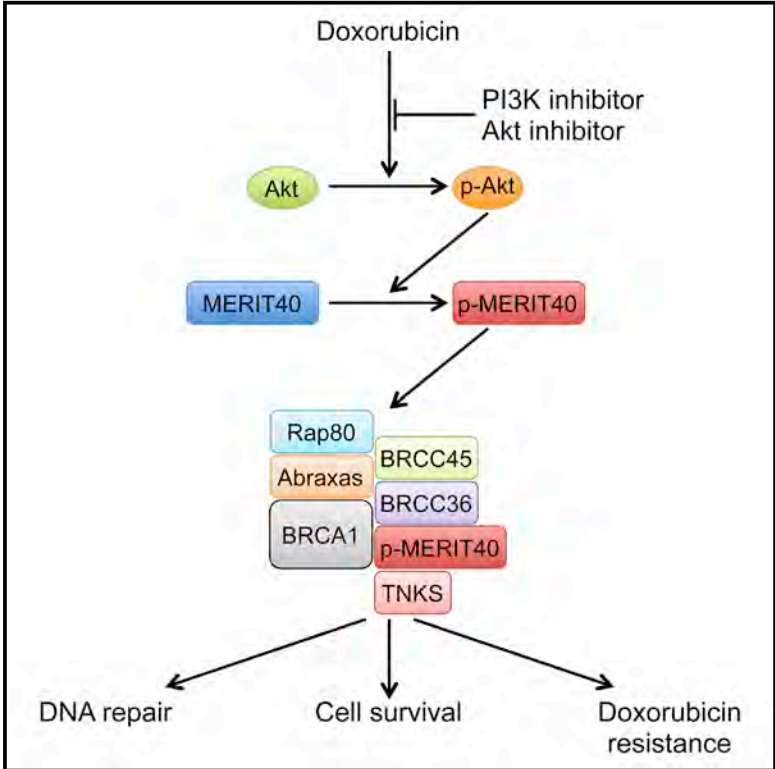
1. Paridaens, R., Biganzoli, L., Bruning, P., Klijn, J.G., Gamucci, T., Houston, S., Coleman, R., Schachter, J., Van Vreckem, A., Sylvester, R., Awada, A., Wildiers, J., and Piccart, M., *Paclitaxel versus doxorubicin as first-line single-agent chemotherapy for metastatic breast cancer: a European Organization for Research and Treatment of Cancer Randomized Study with cross-over*. Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2000. **18**:724-33.
2. Bozulic, L., Surucu, B., Hynx, D., and Hemmings, B.A., *PKBalpha/Akt1 acts downstream of DNA-PK in the DNA double-strand break response and promotes survival*. Molecular cell, 2008. **30**:203-13.
3. Banerji, S., Cibulskis, K., Rangel-Escareno, C., Brown, K.K., Carter, S.L., Frederick, A.M., Lawrence, M.S., Sivachenko, A.Y., Sougnez, C., Zou, L., Cortes, M.L., Fernandez-Lopez, J.C., Peng, S., Ardlie, K.G., Auclair, D., Bautista-Pina, V., Duke, F., Francis, J., Jung, J., Maffuz-Aziz, A., Onofrio, R.C., Parkin, M., Pho, N.H., Quintanar-Jurado, V., Ramos, A.H., Rebollar-Vega, R., Rodriguez-Cuevas, S., Romero-Cordoba, S.L., Schumacher, S.E., Stransky, N., Thompson, K.M., Uribe-Figueroa, L., Baselga, J., Beroukhim, R., Polyak, K., Sgroi, D.C., Richardson, A.L., Jimenez-Sanchez, G., Lander, E.S., Gabriel, S.B., Garraway, L.A., Golub, T.R., Melendez-Zajgla, J., Toker, A., Getz, G., Hidalgo-Miranda, A., and Meyerson, M., *Sequence analysis of mutations and translocations across breast cancer subtypes*. Nature, 2012. **486**:405-9.
4. Feng, L., Huang, J., and Chen, J., *MERIT40 facilitates BRCA1 localization and DNA damage repair*. Genes Dev, 2009. **23**:719-28.
5. Wang, B., Hurov, K., Hofmann, K., and Elledge, S.J., *NBA1, a new player in the Brca1 A complex, is required for DNA damage resistance and checkpoint control*. Genes Dev, 2009. **23**:729-39.
6. Shao, G., Patterson-Fortin, J., Messick, T.E., Feng, D., Shanbhag, N., Wang, Y., and Greenberg, R.A., *MERIT40 controls BRCA1-Rap80 complex integrity and recruitment to DNA double-strand breaks*. Genes Dev, 2009. **23**:740-54.
7. Hu, X., Kim, J.A., Castillo, A., Huang, M., Liu, J., and Wang, B., *NBA1/MERIT40 and BRE interaction is required for the integrity of two distinct deubiquitinating enzyme BRCC36-containing complexes*. The Journal of biological chemistry, 2011. **286**:11734-45.
8. Guettler, S., LaRose, J., Petsalaki, E., Gish, G., Scotter, A., Pawson, T., Rottapel, R., and Sicheri, F., *Structural basis and sequence rules for substrate recognition by Tankyrase explain the basis for cherubism disease*. Cell, 2011. **147**:1340-54.
9. Casas-Selves, M., Kim, J., Zhang, Z., Helfrich, B.A., Gao, D., Porter, C.C., Scarborough, H.A., Bunn, P.A., Jr., Chan, D.C., Tan, A.C., and DeGregori, J., *Tankyrase and the canonical Wnt pathway protect lung cancer cells from EGFR inhibition*. Cancer research, 2012. **72**:4154-64.
10. Huang, S.M., Mishina, Y.M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G.A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., Hild, M., Shi, X., Wilson, C.J., Mickanin, C., Myer, V., Fazal, A., Tomlinson, R., Serluca, F., Shao, W., Cheng, H., Shultz, M., Rau, C., Schirle, M., Schlegl, J., Ghidelli, S., Fawell, S., Lu, C., Curtis, D., Kirschner, M.W., Lengauer, C., Finan, P.M., Tallarico, J.A., Bouwmeester, T., Porter, J.A., Bauer, A., and Cong, F., *Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling*. Nature, 2009. **461**:614-20.
11. Quinn, J.E., Kennedy, R.D., Mullan, P.B., Gilmore, P.M., Carty, M., Johnston, P.G., and Harkin, D.P., *BRCA1 functions as a differential modulator of chemotherapy-induced apoptosis*. Cancer Res, 2003. **63**:6221-8.

APPENDIX

Cell Reports

MERIT40 Is an Akt Substrate that Promotes Resolution of DNA Damage Induced by Chemotherapy

Graphical Abstract



Authors

Kristin K. Brown, Laleh Montaser-Kouhsari, Andrew H. Beck, Alex Toker

Correspondence

atoker@bidmc.harvard.edu

In Brief

Brown et al. show that doxorubicin triggers activation of Akt signaling and phosphorylation of MERIT40, a component of the BRCA1-A DNA repair complex. MERIT40 phosphorylation contributes to DNA repair and cell survival following doxorubicin exposure. Inhibition of Akt signaling and MERIT40 phosphorylation sensitize breast cancer cells to doxorubicin.

Highlights

- Doxorubicin triggers activation of Akt signaling in breast cancer cells
- MERIT40 is phosphorylated by Akt in response to doxorubicin exposure
- MERIT40 phosphorylation contributes to DNA repair and cell survival
- PI3K and Akt inhibitors sensitize breast cancer cells to doxorubicin

MERIT40 Is an Akt Substrate that Promotes Resolution of DNA Damage Induced by Chemotherapy

Kristin K. Brown,¹ Laleh Montaser-Kouhsari,¹ Andrew H. Beck,¹ and Alex Toker^{1,*}

¹Departments of Pathology and Medicine and Cancer Center, Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School, Boston, MA 02215, USA

*Correspondence: atoker@bidmc.harvard.edu

<http://dx.doi.org/10.1016/j.celrep.2015.05.004>

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

SUMMARY

Resistance to cytotoxic chemotherapy drugs, including doxorubicin, is a significant obstacle to the effective treatment of breast cancer. Here, we have identified a mechanism by which the PI3K/Akt pathway mediates resistance to doxorubicin. In addition to inducing DNA damage, doxorubicin triggers sustained activation of Akt signaling in breast cancer cells. We show that Akt contributes to chemotherapy resistance such that PI3K or Akt inhibitors sensitize cells to doxorubicin. We identify MERIT40, a component of the BRCA1-A DNA damage repair complex, as an Akt substrate that is phosphorylated following doxorubicin treatment. MERIT40 phosphorylation facilitates assembly of the BRCA1-A complex in response to DNA damage and contributes to DNA repair and cell survival following doxorubicin treatment. Finally, MERIT40 phosphorylation in human breast cancers is associated with estrogen receptor positivity. Our findings suggest that combination therapy with PI3K or Akt inhibitors and doxorubicin may constitute a successful strategy for overcoming chemotherapy resistance.

INTRODUCTION

As a component of both monotherapy and combination therapy regimens, the anthracycline antibiotic doxorubicin is the primary route of treatment in a wide range of cancers, including breast cancer. The cytotoxicity of doxorubicin is primarily mediated by the inhibition of DNA topoisomerase II and subsequent generation of DNA double-strand breaks (Tewey et al., 1984). The extent of DNA damage induced by doxorubicin exceeds the DNA repair capacity of tumor cells, leading to cell cycle arrest and cell death. Although breast tumors are often initially responsive, the clinical efficacy of doxorubicin is severely limited by intrinsic and acquired resistance. Characterization of mechanisms that contribute to drug resistance and the identification of novel strategies to circumvent doxorubicin resistance would provide new and more effective therapies in the management of breast cancer.

The phosphoinositide 3-kinase (PI3K) pathway plays a critical role in virtually all aspects of tumor biology by regulating fundamental cellular functions, including cell proliferation and survival. The PI3K pathway is frequently hyperactivated in breast cancer, and numerous small molecule inhibitors have been developed to specifically inactivate this pathway for cancer therapy (Baselga, 2011). Elevated PI3K pathway activity has been associated with diminished sensitivity to conventional chemotherapy agents, and the class I PI3K inhibitor GDC-0941 enhances the anti-tumor activity of doxorubicin in breast and ovarian cancer cells that depend on PI3K for survival (Isakoff et al., 2005; Wallin et al., 2010). A major effector of the PI3K pathway is the serine-threonine kinase Akt. Phosphorylation at two sites, Thr308 and Ser473, increases the enzymatic activity of Akt and leads to the phosphorylation of numerous substrates containing the consensus RxxRxxS/T motif. Akt activation, in response to oncogenic PI3K pathway mutations and in response to growth factor signaling, has been well documented. However, Akt also is regulated by the PI3K-related kinase family member DNA-dependent protein kinase (DNA-PK), which phosphorylates Akt at Ser473 in response to DNA damage (Bozulic et al., 2008). The mechanisms by which Akt influences cell survival following DNA damage are poorly understood.

Here we demonstrate that doxorubicin induces Akt activation in breast cancer cell lines, and we show that PI3K and Akt inhibitors dramatically enhance the cytotoxicity of doxorubicin. Mechanistically, we identify MERIT40 as an Akt substrate, and we demonstrate that MERIT40 phosphorylation contributes to the resolution of DNA damage following doxorubicin exposure. We propose that the inhibition of MERIT40 phosphorylation and disruption of DNA damage repair contribute to the efficacy of combination therapy with PI3K/Akt inhibitors and doxorubicin.

RESULTS

PI3K and Akt Inhibitors Sensitize Cells to Doxorubicin-Induced Death

We first examined the ability of doxorubicin to induce Akt activation. Exposure of MCF10A breast epithelial cells to doxorubicin results in elevated Akt phosphorylation at both Ser473 and Thr308 in a time- and concentration-dependent manner (Figures 1A and 1B). Akt phosphorylation is accompanied by an increase in phosphorylation of the Akt substrate PRAS40 and is also coincident with phosphorylation of histone H2A.X, a marker of DNA

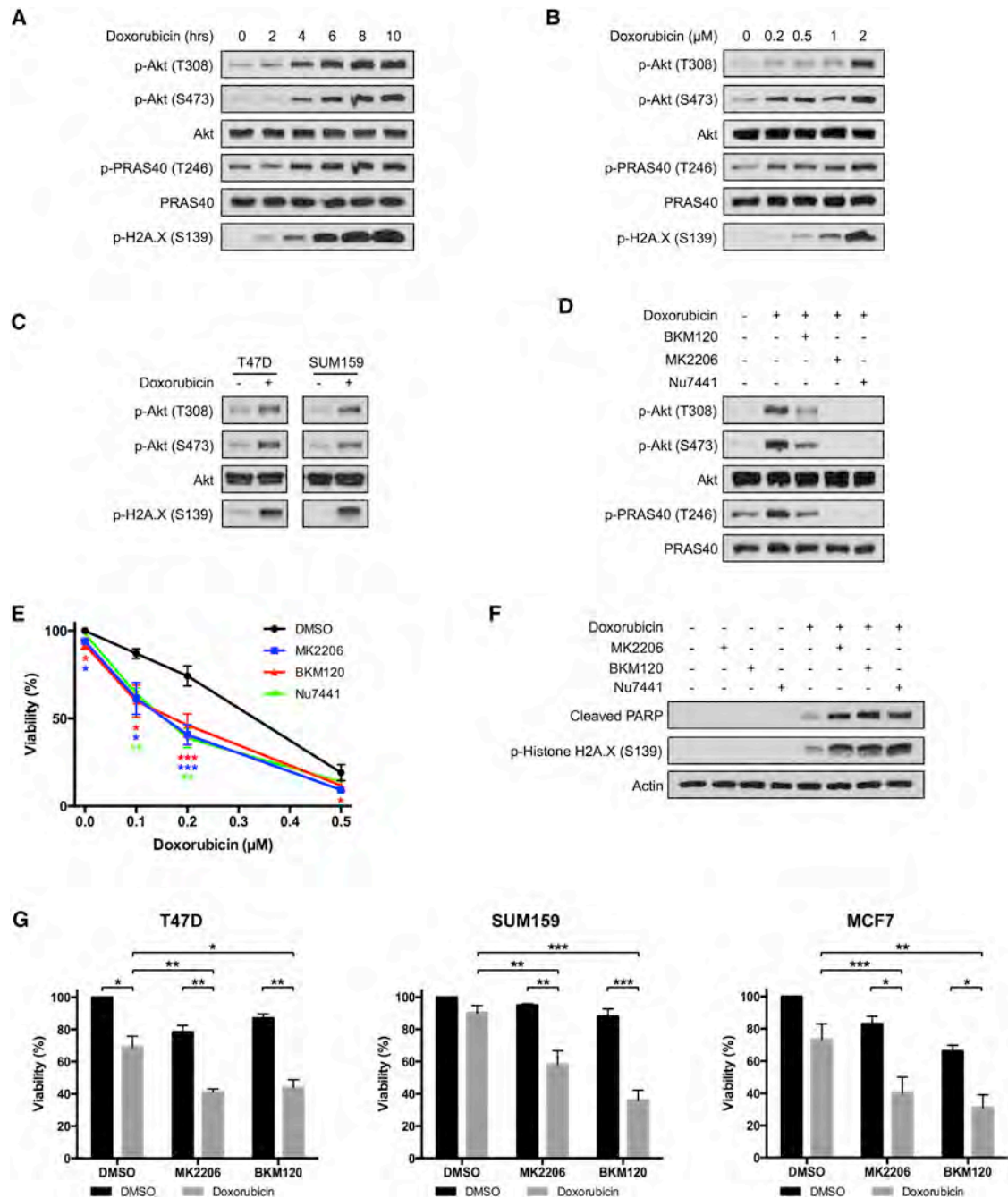


Figure 1. Influence of Doxorubicin on Akt Activity and Contribution of PI3K/Akt Signaling toward Cell Survival following Doxorubicin Exposure

(A and B) MCF10A cells were serum-starved and treated with (A) 2 μ M doxorubicin over a 10-hr time course or (B) increasing concentrations of doxorubicin for 10 hr.

(C) T47D and SUM159 cells were serum-starved and treated with 0.5 μ M doxorubicin for 24 hr.

(D) MCF10A cells were serum-starved and pre-treated with 2 μ M BKM120, 2 μ M MK2206, or 2 μ M Nu7441 for 30 min before exposure to 2 μ M doxorubicin for 10 hr.

(E) MCF10A cells were pre-treated with 1 μ M MK2206, 1 μ M BKM120, or 1 μ M Nu7441 for 24 hr before exposure to increasing concentrations of doxorubicin for an additional 48 hr. Cell viability is expressed as a percentage of viability observed in untreated cells (t test; *p < 0.05, **p < 0.01, ***p < 0.001).

(F) MCF10A cells were pre-treated with 1 μ M MK2206, 1 μ M BKM120, or 1 μ M Nu7441 for 24 hr before the addition of 0.5 μ M doxorubicin for an additional 24 hr.

(G) T47D, SUM159, and MCF7 cells were pre-treated with 1 μ M MK2206 or 1 μ M BKM120 for 24 hr before exposure to 0.5 μ M doxorubicin for an additional 48 hr. Cell viability is expressed as a percentage of viability observed in untreated cells (t test; *p < 0.05, **p < 0.01, ***p < 0.001).

damage and genomic instability. Doxorubicin also enhances Akt phosphorylation in T47D and SUM-159 breast cancer cells (Figure 1C), despite the fact that both cell lines harbor activating mutations in the *PIK3CA* gene that promote constitutive PI3K pathway activity. The ability of doxorubicin to induce Akt activation is blocked by the DNA-PK inhibitor Nu7441, consistent with the notion that DNA-PK is directly involved in activating Akt downstream of DNA damage (Figure 1D). Interestingly, Akt phosphorylation is also largely disrupted by the class I PI3K inhibitor BKM120, consistent with a recent study demonstrating that doxorubicin induces activation of receptor tyrosine kinase (RTK)/PI3K/Akt signaling (Bezler et al., 2012).

We next determined the contribution of Akt activity to cell survival following doxorubicin exposure. Single-agent doxorubicin limits the viability of MCF10A cells in a concentration-dependent manner (Figure 1E). However, the cytotoxicity of doxorubicin is significantly enhanced when MCF10A cells are pre-treated with Nu7441, BKM120, or MK2206, an allosteric pan-Akt inhibitor, suggesting that DNA-PK/PI3K/Akt signaling contributes to cell survival following DNA damage. Strikingly, Akt inhibition with MK2206 is as effective at sensitizing cells to doxorubicin as DNA-PK or PI3K inhibition, indicating that Akt plays a critical role in regulating cell survival after DNA damage (Figure 1E). It should be noted that the effect of single-agent Nu7441, BKM120, or MK2206 on the viability of MCF10A cells is minimal. Doxorubicin-induced cell death by apoptosis is exacerbated in cells pre-treated with Nu7441, BKM120, or MK2206, as demonstrated by enhanced cleavage of poly (ADP-ribose) polymerase (PARP), a signature marker of apoptosis (Figure 1F). Inhibition of DNA-PK, PI3K, or Akt also increases phosphorylation of histone H2A.X following doxorubicin exposure, suggesting that Akt directly contributes to DNA damage repair. Moreover, inhibition of PI3K or Akt sensitizes PI3K mutant breast cancer cell lines T47D, SUM159, and MCF7 to doxorubicin, and in all cases the combination of Akt inhibitor and doxorubicin is as effective as the combination of PI3K inhibitor plus doxorubicin (Figure 1G). Taken together, these data demonstrate that Akt drives a survival pathway that promotes DNA repair and thereby desensitizes cells to the toxicity of doxorubicin.

Akt Phosphorylates MERIT40 in Response to Doxorubicin Exposure

As part of three distinct protein complexes, the tumor suppressor breast cancer susceptibility gene 1 (BRCA1) plays a critical role in regulating the cellular response to DNA damage. The BRCA1-A complex, containing BRCA1, Abraxas, Rap80, BRE, BRCC36, and MERIT40, forms at sites of DNA double-strand breaks and contributes to the resolution of DNA damage (Feng et al., 2009; Shao et al., 2009; Wang et al., 2009). A global phosphoproteomic screen identified that MERIT40 is phosphorylated in a sequence that conforms to the optimal Akt consensus motif, RxRxxS/T (Figure 2A; Moritz et al., 2010).

Using a phospho-MERIT40 Ser29-specific antibody, we found that purified recombinant Akt1, Akt2, or Akt3 can directly phosphorylate MERIT40 at Ser29 (Figure 2B). Moreover, IGF-1 stimulation promoted phosphorylation of MERIT40 and this was blocked in cells pretreated with BKM120, MK2206, and the mTORC1/2 inhibitor Torin1, but not the mTORC1 inhibitor rapa-

mycin, thereby implicating Akt as the kinase responsible for MERIT40 phosphorylation at Ser29 (Figure 2C). Specific knock-down of Akt isoforms also prevented MERIT40 phosphorylation in response to IGF-1 stimulation (Figure 2D). Expression of constitutively active *PIK3CA* alleles into MCF10A cells promoted hyperactivation of Akt and enhanced MERIT40 phosphorylation in the absence of growth factors (Figure 2E). Constitutively active, myristoylated Akt1, Akt2, and Akt3 constructs also were able to induce phosphorylation of MERIT40 in the absence of growth factor (Figure 2F). Strikingly, doxorubicin also induced rapid and sustained phosphorylation of MERIT40 at Ser29 (Figures 2G and S1), and this was blocked by Nu7441, Torin1, and MK2206, but not rapamycin (Figure 2H). Consistent with the notion that doxorubicin induces activation of RTK/PI3K/Akt signaling, the EGFR inhibitor gefitinib and BKM120 blocked doxorubicin-induced MERIT40 phosphorylation. In addition, specific knockdown of Akt isoforms completely inhibited MERIT40 phosphorylation and exacerbated histone H2A.X phosphorylation in response to doxorubicin exposure (Figure 2I). These data show that MERIT40 is an Akt substrate phosphorylated in response to growth factor-induced Akt activation, hyperactivation of Akt via PI3K oncogenic mutations, and downstream of DNA damage-induced activation of Akt.

MERIT40 Phosphorylation Promotes Assembly of the BRCA1-A Complex

MERIT40 is an integral component of the nuclear BRCA1-A complex and is required for BRCA1-A complex stability and DNA damage resistance (Feng et al., 2009; Shao et al., 2009; Wang et al., 2009). MERIT40 harbors two binding motifs for the PARP family member tankyrase (TNKS) (Figure 2A; Guettler et al., 2011). Interestingly, one TNKS-binding motif includes the Ser29 residue. We therefore determined if TNKS is a component of the BRCA1-A complex, and we examined the consequence of phosphorylation on the ability of MERIT40 to interact with TNKS. Previous studies have shown that MERIT40 is required to maintain the stability of components of the BRCA1-A complex (Hu et al., 2011). In addition to destabilization of Rap80, Abraxas, BRE, and BRCC36, MERIT40 depletion caused a dramatic reduction in TNKS expression (Figure 3A). TNKS expression, as well as the expression of additional components of the BRCA1-A complex, was fully rescued by re-expression of either wild-type MERIT40 or a phosphorylation-deficient MERIT40 Ser29Ala mutant (Figure 3A). This implies that MERIT40 phosphorylation did not impact the ability of MERIT40 to influence the stability of BRCA1-A complex components or TNKS. These data, however, do suggest that TNKS could represent a previously uncharacterized component of the BRCA1-A complex.

Consistent with this model, co-immunoprecipitation experiments revealed that endogenous TNKS interacts with endogenous BRCA1, and this interaction was enhanced following doxorubicin exposure (Figure 3B). Co-immunoprecipitation experiments also showed that MERIT40 interacts with TNKS (Figure 3C). However, whereas wild-type MERIT40 binds TNKS with high affinity, MERIT40 Ser29Ala showed dramatically reduced binding to TNKS (Figure 3C). In cells exposed to doxorubicin, an increase in the interaction of wild-type MERIT40 with endogenous Rap80 and TNKS was observed (Figure 3D). By contrast,

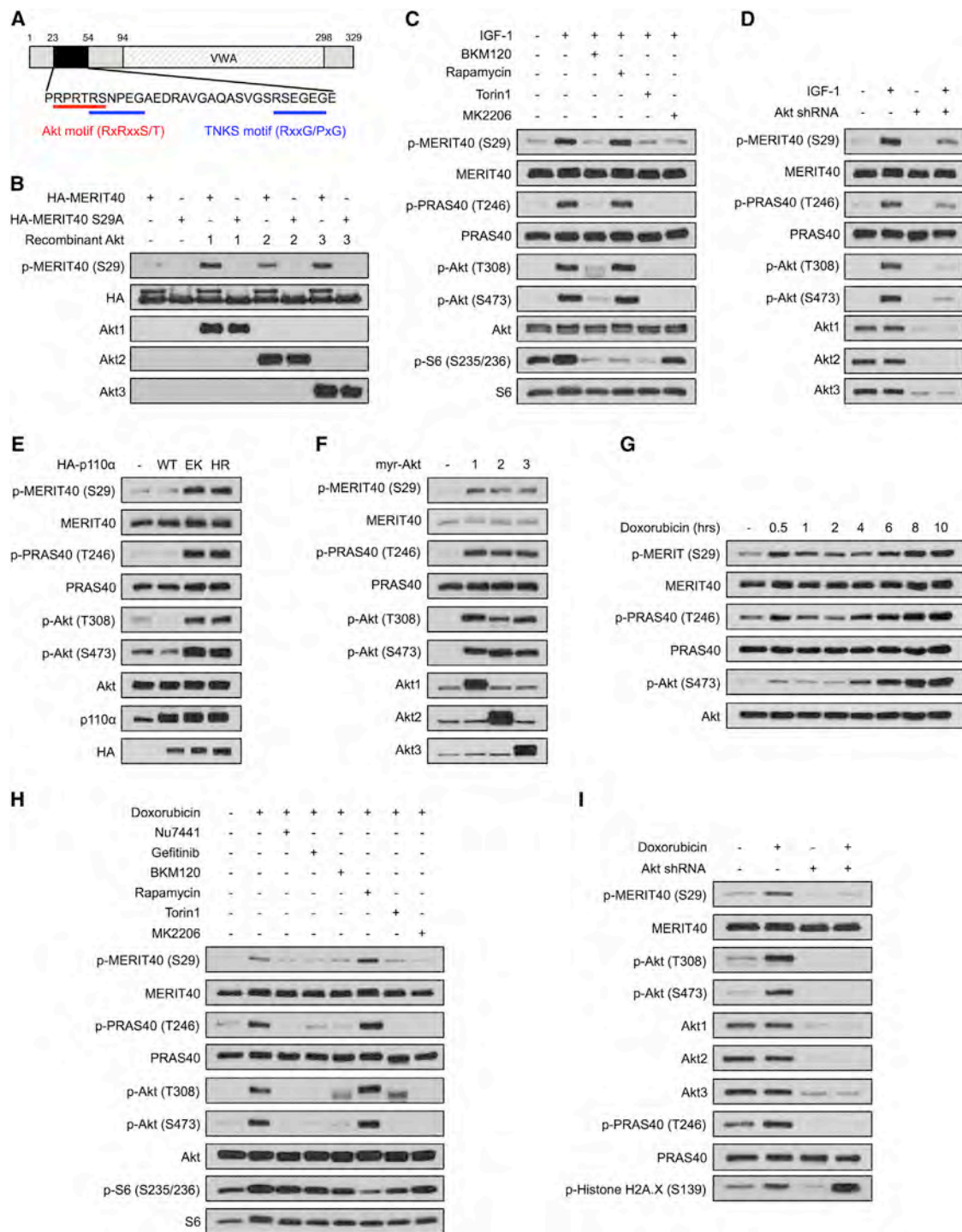


Figure 2. MERIT40 Is an Akt Substrate

(A) Domain structure of MERIT40 highlights the Akt consensus phosphorylation motif (₂₄RPRTRS₂₉), two TNKS-binding motifs (₂₈RSNPEGAE₃₅ and ₄₈RSEGE₅₄), and a von Willebrand A domain (VWA).

(B) HA-Flag-MERIT40 and MERIT40 Ser29Ala were used as substrates in an in vitro kinase assay with recombinant active Akt1, Akt2, or Akt3.

(C) MCF10A cells were serum-starved and pre-treated with the indicated inhibitors for 30 min before stimulation with IGF-1 for 30 min.

(D) MCF10A cells were infected with empty vector or Akt small hairpin RNA (shRNA) constructs. Cells were serum-starved before stimulation with IGF-1 for 30 min.

(E) MCF10A cells were infected with vector control, PIK3CA wild-type (WT), PIK3CA E545K (EK), or PIK3CA H1047R (HR) constructs and serum-starved.

(legend continued on next page)

doxorubicin did not stimulate the association of either Rap80 or TNKS with MERIT40 Ser29Ala, indicating that phosphorylation of MERIT40 promotes the association of BRCA1-A complex components in response to DNA damage. This conclusion is also supported by the observation that doxorubicin triggered an increase in the association of endogenous TNKS and Rap80 with endogenous MERIT40 (Figure 3E). Strikingly, the interaction of MERIT40 with TNKS and Rap80 in response to DNA damage was disrupted by the Akt inhibitor MK2206, indicating that MERIT40 phosphorylation was required to enhance the association of BRCA1-A complex components following DNA damage (Figure 3E). Importantly, a phosphorylation-dependent increase in the association of BRCA1-A complex components was not observed in cells stimulated with IGF-1, indicating that BRCA1-A complex formation specifically requires a DNA damage signal in addition to Akt-dependent phosphorylation of MERIT40 (Figure 3F). Consistent with previous studies, MERIT40 depletion compromised Rap80 focus formation upon DNA damage (Figure 3E; Feng et al., 2009). Rap80 focus formation was completely rescued by re-expression of wild-type MERIT40, but not by phosphorylation-deficient MERIT40 Ser29Ala. These data demonstrate that MERIT40 phosphorylation plays an integral role in assembly of the BRCA1-A complex following doxorubicin exposure.

MERIT40 Phosphorylation Contributes to the Resolution of DNA Damage

To further evaluate the functional significance of MERIT40 phosphorylation by Akt, we investigated the ability of MERIT40 to influence DNA damage repair. Depletion of MERIT40 from T47D cells dramatically enhanced doxorubicin-induced DNA damage, as revealed by an increase in histone H2A.X phosphorylation (Figure 4A) and phospho-H2A.X focus formation (Figures 4B and 4C). This response was rescued by re-expression of wild-type MERIT40, but not MERIT40 Ser29Ala. These data demonstrate that MERIT40 phosphorylation at Ser29 is required for the resolution of doxorubicin-induced DNA damage. In addition, MERIT40 depletion dramatically enhanced spontaneous DNA damage in MCF10A cells, and this could be rescued by re-expression of wild-type MERIT40, but again not by MERIT40 Ser29Ala (Figure S2). Importantly, MERIT40 depletion sensitized cells to doxorubicin, and this response was rescued by re-expression of wild-type MERIT40, but not MERIT40 Ser29Ala (Figures 4D and 4E). Taken together, these data demonstrate that Akt-dependent phosphorylation of MERIT40 promotes DNA repair and that MERIT40 phosphorylation contributes to cell survival following doxorubicin exposure.

Elevated MERIT40 expression has been identified in epithelial ovarian cancer, and SNPs within the MERIT40 gene are associated with breast cancer risk (Antoniu et al., 2010; Bolton et al., 2010). However, the expression of MERIT40 and phospho-MERIT40 in human breast tumors has not been examined. Tissue microarrays containing cores of invasive breast cancer tissue obtained from archival pathology specimens were used to examine

expression of MERIT40 and phospho-MERIT40 by immunohistochemistry (IHC). This analysis revealed, in a subset of cases, strong phospho-MERIT40 staining that was present in both the nuclei and cytoplasm (Figure 4F, positive/strong). A subset of cases showed no phospho-MERIT40 staining (Figure 4F, negative/weak). Overall, the total MERIT40 staining showed more intermediate staining with fewer strongly positive cases (Figure 4F, intermediate). We also used computational analysis to assess the association of phospho-MERIT40 nuclear staining with breast cancer subtypes. This analysis revealed a strong, statistically significant association of nuclear phospho-MERIT40 with estrogen receptor (ER) status ($p = 3.3 \times 10^{-5}$) (Figure 4G).

DISCUSSION

Resistance to cytotoxic chemotherapy agents is a common phenomenon in breast cancer that has a drastic impact on patient survival. This underscores the need to identify strategies to overcome drug resistance. One approach that has been favored in recent years is the development of drug regimens combining cytotoxic chemotherapy with molecularly targeted drugs that inhibit signaling cascades critical to breast cancer survival and progression. The PI3K/Akt pathway is hyperactive in more than 70% of breast tumors and is critical for tumor progression and resistance to anti-cancer drugs (Courtney et al., 2010). In addition, as highlighted in this study, an undesirable response to chemotherapy exposure is activation of PI3K/Akt signaling. Rationally designed small molecule inhibitors that target either PI3K or Akt recently have been developed and are currently in phase I or II clinical trials (Dienstmann et al., 2014). Unfortunately, modest anti-tumor responses have been reported following PI3K and Akt inhibitor monotherapy and substantial tumor regression is rarely observed (Rodon et al., 2013). In an attempt to improve response rates to PI3K and Akt inhibitors, clinical trials that incorporate these inhibitors and traditional chemotherapeutic drugs are now in progress (Paplomata and O'Regan, 2014). Our study provides evidence that PI3K and Akt inhibitors dramatically sensitize breast cancer cells to the DNA-damaging chemotherapy agent doxorubicin. The frequency of PI3K pathway alterations in breast cancer combined with the ability of doxorubicin to induce PI3K/Akt activity provides rationale to assess combination therapy with doxorubicin and PI3K inhibitors in the clinic.

In chemotherapy-sensitive cancer cells, DNA damage resulting from doxorubicin exposure leads to cell death via the induction of apoptosis. The ability of the PI3K/Akt pathway to promote cell survival has been widely reported and a number of substrates that contribute to the inhibition of apoptosis have been identified (Manning and Cantley, 2007). Although it is well established that Akt activity promotes resistance to DNA damage, the substrates that directly impact DNA damage repair are poorly characterized (Xu et al., 2012). Our study identifies MERIT40, a component of the BRCA1-A DNA damage repair complex, as an Akt substrate that directly influences the cellular response

(F) MCF10A cells were transfected with vector control or myristoylated Akt alleles (myrAkt1/2/3) and serum-starved.

(G) MCF10A cells were serum-starved and exposed to 2 μ M doxorubicin over a 10-hr time course. See also Figure S1.

(H) MCF10A cells were serum-starved and pre-treated with the indicated inhibitors for 30 min before exposure to 2 μ M doxorubicin for 10 hr.

(I) MCF10A cells were infected with empty vector or Akt shRNA constructs. Cells were serum-starved before exposure to 2 μ M doxorubicin for 10 hr.

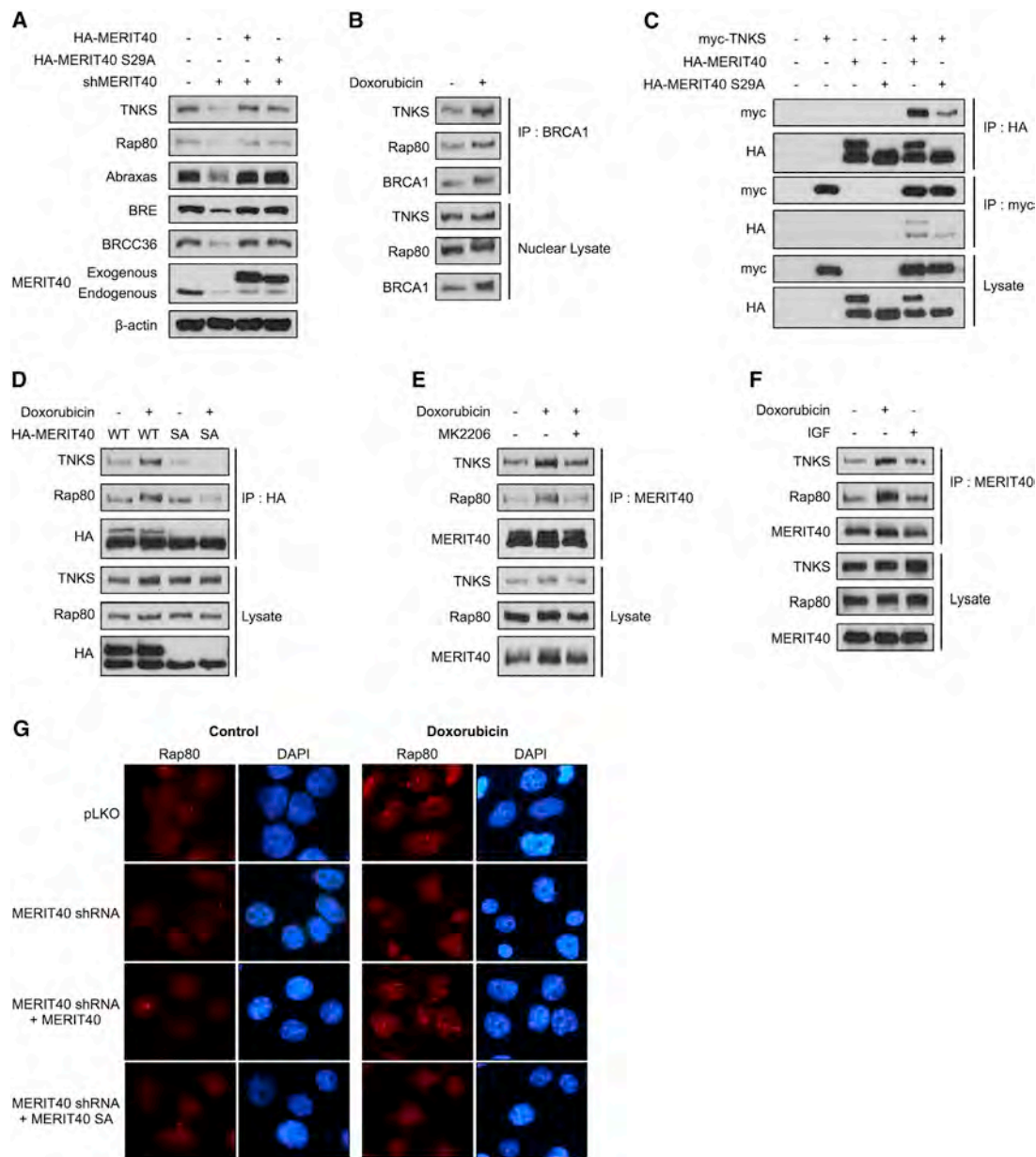


Figure 3. Effect of MERIT40 Phosphorylation on Stability and Formation of the BRCA1-A Complex

(A) MCF10A cells were infected with empty vector or MERIT40 shRNA constructs and empty vector, WT MERIT40, or MERIT40 S29A mutant expression constructs.

(B) MCF10A cells were serum-starved before exposure to 2 μ M doxorubicin for 1 hr. Endogenous BRCA1 was immunoprecipitated from nuclear extracts and co-immunoprecipitation with endogenous TNKS and Rap80 was monitored.

(C) HEK293T cells were transfected with WT or mutant MERIT40 constructs and a WT TNKS expression plasmid. HA-tagged MERIT40 was immunoprecipitated from cells and co-immunoprecipitation of myc-tagged TNKS was monitored. Alternatively, myc-tagged TNKS was immunoprecipitated from cells and co-immunoprecipitation of HA-tagged TNKS was monitored.

(D) MCF10A cells were infected with WT or mutant MERIT40 constructs. Cells were serum-starved and exposed to 2 μ M doxorubicin for 1 hr. HA-tagged MERIT40 was immunoprecipitated from cells and co-immunoprecipitation with endogenous TNKS and Rap80 was monitored.

(E) MCF10A cells were serum-starved and pre-treated with 2 μ M MK2206 for 30 min before exposure to 2 μ M doxorubicin for 1 hr. Endogenous MERIT40 was immunoprecipitated from cells and co-immunoprecipitation with endogenous TNKS and Rap80 was monitored.

(F) MCF10A cells were serum-starved and treated with 2 μ M doxorubicin for 1 hr or IGF-1 for 30 min. Endogenous MERIT40 was immunoprecipitated from cells and co-immunoprecipitation with endogenous TNKS and Rap80 was monitored.

(G) T47D cells were infected with empty vector or a MERIT40 shRNA construct and empty vector, WT MERIT40, or MERIT40 S29A mutant expression constructs. Cells were exposed to 0.5 μ M doxorubicin for 1 hr, washed with PBS, and incubated in fresh media for an additional hour. Rap80 focus formation was monitored by immunofluorescence.

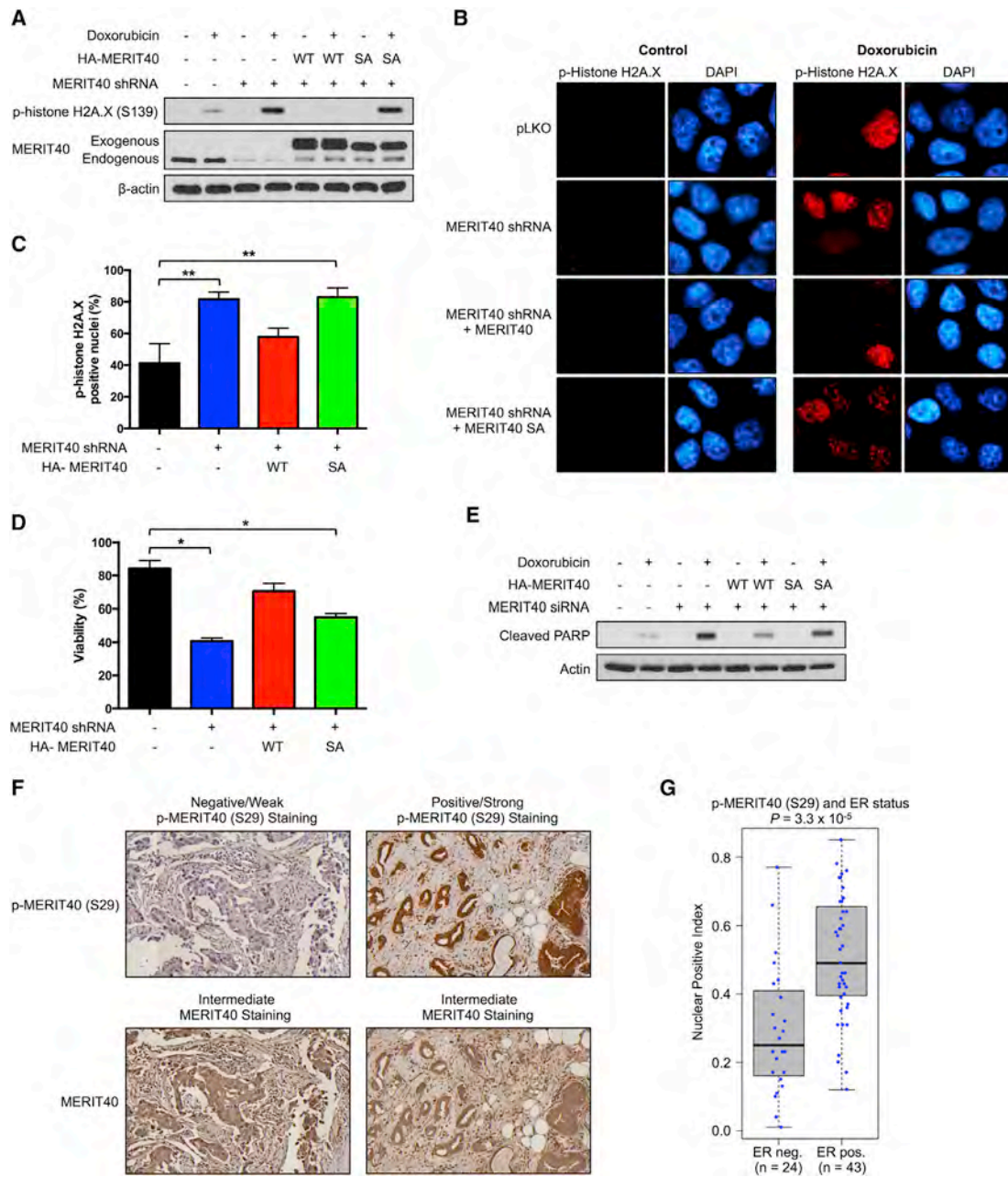


Figure 4. Contribution of MERIT40 Phosphorylation toward the Resolution of DNA Damage and Cell Survival following Doxorubicin Exposure and Detection of Phospho-MERIT40 in Human Breast Tumors

(A) T47D cells were infected with empty vector or a MERIT40 shRNA construct and empty vector, WT MERIT40, or MERIT40 S29A mutant expression constructs. Cells were exposed to 0.5 μ M doxorubicin for 1 hr, washed with PBS, and incubated in fresh media for an additional 6 hr. See also Figure S2.

(B) T47D cells were infected with empty vector or a MERIT40 shRNA construct and empty vector, WT MERIT40, or MERIT40 S29A mutant expression constructs. Cells were exposed to 0.5 μ M doxorubicin for 1 hr, washed with PBS, and incubated in fresh media for an additional 6 hr. Phosphorylation of histone H2A.X was monitored by immunofluorescence.

(C) The percentage of cells with nuclear p-Histone H2A.X foci was quantified by counting 200 cells in each treatment condition (t test, $**p < 0.01$).

(D) MCF10A cells were transfected with control or MERIT40 small interfering RNA (siRNA) constructs and empty vector, WT MERIT40, or MERIT40 S29A mutant expression constructs. Cells were exposed to 0.2 μ M doxorubicin for 48 hr. Cell viability is expressed as a percentage of viability observed in untreated cells (t test, $*p < 0.05$).

(E) MCF10A cells were transfected with control or MERIT40 siRNA constructs and empty vector, WT MERIT40, or MERIT40 S29A mutant expression constructs. Cells were exposed to 0.2 μ M doxorubicin for 24 hr.

(F) Detection of p-MERIT40 and MERIT40 in invasive breast tumor tissue samples by IHC is shown.

(G) Relationship between p-MERIT40 IHC staining in human breast tumor samples and ER status is shown.

to DNA damage. Specifically, we show that MERIT40 phosphorylation at Ser29 facilitates the association of MERIT40 with the ubiquitin-binding protein Rap80. Furthermore, we show that MERIT40 phosphorylation promotes nuclear Rap80 focus formation. Rap80 is a critical scaffold protein in the BRCA1-A complex required to relocate and target the remaining subunits of the BRCA1-A complex to sites of DNA damage (Bian et al., 2012; Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007; Wu et al., 2012). Future studies to investigate the effect of MERIT40 phosphorylation in facilitating the association of MERIT40 with additional components of the BRCA1-A complex are warranted. Importantly, we also demonstrate that MERIT40 phosphorylation promotes DNA damage repair and contributes to cell survival following doxorubicin exposure. We therefore propose that inhibition of MERIT40 phosphorylation and disruption of DNA damage repair contributes to the efficacy of combination therapy with PI3K/Akt inhibitors and doxorubicin.

Our study also identifies the PARP family member TNKS as a component of the BRCA1-A complex. We demonstrate that MERIT40 interacts with TNKS and furthermore that MERIT40 phosphorylation promotes the association of MERIT40 with TNKS. TNKS previously has been implicated in the regulation of DNA damage repair and has been shown to influence stability of the catalytic subunit of DNA-PK via poly-ADP-ribosylation (PARsylation) (Dregalla et al., 2010). In addition, PARP1 has been shown to PARsylate BRCA1 and thereby maintain stability of the BRCA1-A complex (Hu et al., 2014). The ability of TNKS to influence DNA damage repair via PARsylation of DNA-PK, BRCA1, or additional BRCA1-A complex components clearly merits further investigation. In addition, it will be interesting to evaluate the contribution of MERIT40 phosphorylation to DNA damage-induced protein PARsylation.

Molecular markers that might predict the efficacy of combination therapy with PI3K/Akt pathway inhibitors and cytotoxic agents, like doxorubicin, are lacking. Another interesting observation arising in this study is that phospho-MERIT40 staining is associated with ER status in invasive breast tumor tissue samples. It should be noted that activating mutations in the *PIK3CA* gene occur in a significant proportion of ER-positive breast cancers (Cancer Genome Atlas Network, 2012). Our results suggest that MERIT40 phosphorylation could be used as a biomarker in ER-positive breast cancer patients to predict sensitivity to traditional cytotoxic chemotherapy agents, and they indicate that patients with high levels of phospho-MERIT40 would likely benefit from combination therapy with Akt inhibitors and doxorubicin.

In summary, our study identifies a mechanism by which the PI3K/Akt pathway mediates DNA repair in response to chemotherapy exposure, and it indicates that combining PI3K/Akt inhibitors with doxorubicin may constitute a successful strategy to overcome chemotherapy resistance in breast cancer, at least in part by disrupting the phosphorylation of MERIT40.

EXPERIMENTAL PROCEDURES

Immunoblotting

Cells were washed with PBS and lysed in RIPA buffer. Lysates were resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose membrane (Bio-Rad) followed by immunoblotting.

Sulforhodamine B Assay

Cell viability was monitored using the sulforhodamine B (SRB) assay. Adherent cells were fixed by the addition of 12.5% (w/v) trichloroacetic acid and incubation at 4°C for 1 hr. Wells were washed with water and cells were stained by the addition of SRB solution (0.5% [w/v] SRB and 1% acetic acid). Wells were washed twice with 1% acetic acid and allowed to dry at room temperature. SRB was solubilized with 10 mmol/l Tris (pH 10.5) and absorbance at 510 nm was measured.

In Vitro Kinase Assay

MSCV-HA-Flag-MERIT40 or MSCV-HA-Flag-MERIT40 Ser29Ala MERIT40 was immunoprecipitated from cell extracts and incubated with 500 ng of recombinant Akt1, Akt2, or Akt3 (Sigma-Aldrich) in a kinase buffer containing 250 μ mol/l cold ATP for 1 hr at 30°C. Eluates were resolved by SDS-PAGE.

Immunoprecipitation

Nuclear extracts were prepared by high salt extraction. For whole-cell lysate preparation, cells were washed with PBS and lysed in EBC lysis buffer. Lysates were incubated with 1–2 μ g antibody overnight at 4°C followed by incubation with protein A/G Sepharose beads (Amersham Biosciences). Immune complexes were washed with NETN buffer and eluted by incubation for 5 min at 95°C in SDS-PAGE sample buffer. Eluates were resolved by SDS-PAGE.

Immunofluorescence

Cells plated on coverslips were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100, and blocked with 1% BSA in 20 mmol/l Tris-HCl (pH 7.5) for 20 min. Coverslips were then incubated with the appropriate antibodies. After washing twice with PBS, coverslips were mounted with Prolong Gold antifade reagent containing DAPI (Life Technologies). Images of cells were acquired using a fluorescence microscope (Nikon Eclipse Ti) and digital image analysis software (NIS-Elements, Nikon).

Tissue Microarrays and Image Analysis of Immunohistochemistry

Two tissue microarrays containing breast tissue specimens, from the archives of the Department of Pathology at the BIDMC, were constructed as previously described (Elouli et al., 2014). Immunohistochemistry staining was done for total MERIT40 and p-MERIT40 Ser29. Computational image analysis of protein expression was performed using Definiens TissueStudio 3.6.1 to yield the intensity of nuclear expression of p-MERIT40 Ser 29 and MERIT 40 in the cancer epithelium. The proportion of positively staining epithelial nuclei was recorded for each core and reported as the Nuclear Positive Index.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.05.004>.

AUTHOR CONTRIBUTIONS

K.K.B. and A.T. designed the experiments and wrote the paper. K.K.B. performed all the experiments. K.K.B., L.M.-K., A.H.B., and A.T. analyzed and interpreted the data.

ACKNOWLEDGMENTS

The authors thank the histology core at the BIDMC for their technical support for IHC; Steven Elledge for providing MSCV-HA-Flag-MERIT40; Frank Sicherer for providing pLP-dmyc-TNKS2; and members of the A.T. laboratory for discussions. This work was supported in part by grants from the Department of Defense Breast Cancer Research Program (BC110900, A.T.), a John Gavin Post-doctoral Fellowship from the Genesis Oncology Trust of New Zealand (K.K.B.), and the National Library of Medicine of the NIH (K22LM011931, A.H.B.).

Received: November 4, 2014

Revised: March 12, 2015

Accepted: May 1, 2015

Published: May 28, 2015

REFERENCES

- Antoniou, A.C., Wang, X., Fredericksen, Z.S., McGuffog, L., Tarrell, R., Sinilnikova, O.M., Healey, S., Morrison, J., Kartsonaki, C., Lesnick, T., et al.; EMBRACE; GEMO Study Collaborators; HEBON; kConFab; SWE-BRCA; MOD SQUAD; GENICA (2010). A locus on 19p13 modifies risk of breast cancer in BRCA1 mutation carriers and is associated with hormone receptor-negative breast cancer in the general population. *Nat. Genet.* **42**, 885–892.
- Baselga, J. (2011). Targeting the phosphoinositide-3 (PI3) kinase pathway in breast cancer. *Oncologist* **16** (Suppl 1), 12–19.
- Bezler, M., Hengstler, J.G., and Ullrich, A. (2012). Inhibition of doxorubicin-induced HER3-PI3K-AKT signalling enhances apoptosis of ovarian cancer cells. *Mol. Oncol.* **6**, 516–529.
- Bian, C., Wu, R., Cho, K., and Yu, X. (2012). Loss of BRCA1-A complex function in RAP80 null tumor cells. *PLoS ONE* **7**, e40406.
- Bolton, K.L., Tyrer, J., Song, H., Ramus, S.J., Notaridou, M., Jones, C., Sher, T., Gentry-Maharaj, A., Wozniak, E., Tsai, Y.Y., et al.; Australian Ovarian Cancer Study Group; Australian Cancer Study (Ovarian Cancer); Ovarian Cancer Association Consortium (2010). Common variants at 19p13 are associated with susceptibility to ovarian cancer. *Nat. Genet.* **42**, 880–884.
- Bozulic, L., Surucu, B., Hynx, D., and Hemmings, B.A. (2008). PKBalpha/Akt1 acts downstream of DNA-PK in the DNA double-strand break response and promotes survival. *Mol. Cell* **30**, 203–213.
- Cancer Genome Atlas Network (2012). Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61–70.
- Courtney, K.D., Corcoran, R.B., and Engelman, J.A. (2010). The PI3K pathway as drug target in human cancer. *J. Clin. Oncol.* **28**, 1075–1083.
- Dienstmann, R., Rodon, J., Serra, V., and Tabernero, J. (2014). Picking the point of inhibition: a comparative review of PI3K/AKT/mTOR pathway inhibitors. *Mol. Cancer Ther.* **13**, 1021–1031.
- Dregalla, R.C., Zhou, J., Idarte, R.R., Battaglia, C.L., Liber, H.L., and Bailey, S.M. (2010). Regulatory roles of tankyrase 1 at telomeres and in DNA repair: suppression of T-SCE and stabilization of DNA-PKcs. *Aging (Albany, N.Y. Online)* **2**, 691–708.
- Elloul, S., Kedrin, D., Knoblauch, N.W., Beck, A.H., and Toker, A. (2014). The adherens junction protein afadin is an AKT substrate that regulates breast cancer cell migration. *Mol. Cancer Res.* **12**, 464–476.
- Feng, L., Huang, J., and Chen, J. (2009). MERIT40 facilitates BRCA1 localization and DNA damage repair. *Genes Dev.* **23**, 719–728.
- Guettler, S., LaRose, J., Petsalaki, E., Gish, G., Scotter, A., Pawson, T., Rottapel, R., and Sicheri, F. (2011). Structural basis and sequence rules for substrate recognition by Tankyrase explain the basis for cherubism disease. *Cell* **147**, 1340–1354.
- Hu, X., Kim, J.A., Castillo, A., Huang, M., Liu, J., and Wang, B. (2011). NBA1/MERIT40 and BRE interaction is required for the integrity of two distinct deubiquitinating enzyme BRCC36-containing complexes. *J. Biol. Chem.* **286**, 11734–11745.
- Hu, Y., Petit, S.A., Ficarro, S.B., Toomire, K.J., Xie, A., Lim, E., Cao, S.A., Park, E., Eck, M.J., Scully, R., et al. (2014). PARP1-driven poly-ADP-ribosylation regulates BRCA1 function in homologous recombination-mediated DNA repair. *Cancer Discov.* **4**, 1430–1447.
- Isakoff, S.J., Engelman, J.A., Irie, H.Y., Luo, J., Brachmann, S.M., Pearline, R.V., Cantley, L.C., and Brugge, J.S. (2005). Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. *Cancer Res.* **65**, 10992–11000.
- Kim, H., Chen, J., and Yu, X. (2007). Ubiquitin-binding protein RAP80 mediates BRCA1-dependent DNA damage response. *Science* **316**, 1202–1205.
- Manning, B.D., and Cantley, L.C. (2007). AKT/PKB signaling: navigating downstream. *Cell* **129**, 1261–1274.
- Moritz, A., Li, Y., Guo, A., Villén, J., Wang, Y., MacNeill, J., Kornhauser, J., Sprott, K., Zhou, J., Possemato, A., et al. (2010). Akt-RSK-S6 kinase signaling networks activated by oncogenic receptor tyrosine kinases. *Sci. Signal.* **3**, ra64.
- Papilomata, E., and O'Regan, R. (2014). The PI3K/AKT/mTOR pathway in breast cancer: targets, trials and biomarkers. *Ther. Adv. Med. Oncol.* **6**, 154–166.
- Rodon, J., Dienstmann, R., Serra, V., and Tabernero, J. (2013). Development of PI3K inhibitors: lessons learned from early clinical trials. *Nat. Rev. Clin. Oncol.* **10**, 143–153.
- Shao, G., Patterson-Fortin, J., Messick, T.E., Feng, D., Shanbhag, N., Wang, Y., and Greenberg, R.A. (2009). MERIT40 controls BRCA1-Rap80 complex integrity and recruitment to DNA double-strand breaks. *Genes Dev.* **23**, 740–754.
- Sobhian, B., Shao, G., Lilli, D.R., Culhane, A.C., Moreau, L.A., Xia, B., Livingston, D.M., and Greenberg, R.A. (2007). RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. *Science* **316**, 1198–1202.
- Tewey, K.M., Rowe, T.C., Yang, L., Halligan, B.D., and Liu, L.F. (1984). Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* **226**, 466–468.
- Wallin, J.J., Guan, J., Prior, W.W., Edgar, K.A., Kassees, R., Sampath, D., Belvin, M., and Friedman, L.S. (2010). Nuclear phospho-Akt increase predicts synergy of PI3K inhibition and doxorubicin in breast and ovarian cancer. *Sci. Transl. Med.* **2**, 48ra66.
- Wang, B., Matsuoka, S., Ballif, B.A., Zhang, D., Smogorzewska, A., Gygi, S.P., and Elledge, S.J. (2007). Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. *Science* **316**, 1194–1198.
- Wang, B., Hurov, K., Hofmann, K., and Elledge, S.J. (2009). NBA1, a new player in the Brca1 A complex, is required for DNA damage resistance and checkpoint control. *Genes Dev.* **23**, 729–739.
- Wu, J., Liu, C., Chen, J., and Yu, X. (2012). RAP80 protein is important for genomic stability and is required for stabilizing BRCA1-A complex at DNA damage sites in vivo. *J. Biol. Chem.* **287**, 22919–22926.
- Xu, N., Lao, Y., Zhang, Y., and Gillespie, D.A. (2012). Akt: a double-edged sword in cell proliferation and genome stability. *J. Oncol.* **2012**, 951724.