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14. ABSTRACT <u>Epigenetic regulation of tumor cell sensitivity to chemotherapy.</u> The overall thrust of this project has been to establish that genetic and pharmacologic inhibition of NURF will be effective in sensitizing breast tumor cells to chemotherapy, through both direct (cell autonomous) and indirect (cell non-autonomous, immune mediated) pathways. To this end, we have shown that suppression of the bromodomain PHD finger transcription factor subunit (BPTF) of the nucleosome remodeling factor NURF enhances sensitivity of the 4T1 murine breast tumor cell line to the topoisomerase II poisons, doxorubicin and etoposide as well as the microtubule, poison, paclitaxel. These effects demonstrated selectivity against the tumor cells for doxorubicin and etoposide, but not paclitaxel, as similar sensitization was not observed for the topoisomerase II poisons in embryonic stem cells. Sensitization to doxorubicin and etoposide with BPTF knockdown was associated with increased DNA damage, topoisomerase II crosslinking and autophagy. However, there was no concomitant increase in apoptosis or senescence. Sensitization to doxorubicin was confirmed in animals bearing the syngeneic 4T1 breast tumor cells using both genetic and pharmacologic inhibition of BPTF. The sensitization appeared to be autophagy-dependent. Finally, treatment of 4T1 and MDA-MB231 breast cancer cells with the selective BPTF bromodomain inhibitor, AU1, recapitulates genetic BPTF inhibition, including in vivo sensitization to doxorubicin, increased topoisomerase II DNA crosslinks and increased DNA damage. These studies suggest that BPTF could potentially be targeted with small molecule inhibitors to enhance the effectiveness of topo-II targeted cancer chemotherapeutic drugs.					
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Table of Contents

1. Introduction..... 1

2. Keywords..... 1

3. Accomplishments..... 1

4. Limitations..... 3

5. Impact.....4

6. Changes/Problems.....4

7. Products.....5

8. Special Reporting Requirements.....6

9. Appendices.....7

INTRODUCTION The goals of this research project are to develop a novel therapeutic strategy for improving the treatment of triple negative breast cancer (TNBC). To this end, the collaboration between the Landry and Gewirtz laboratories has focused on both the cell autonomous and cell non-autonomous impact(s) of suppression of the epigenetic modulator, NURF, to enhance the response to chemotherapeutic drugs, specifically doxorubicin. Our preliminary data strongly suggested that suppression of NURF activity through silencing of the BPTF subunit promoted enhanced autophagy that could directly mediate cell killing *in vitro* and promote an antitumor immune response to breast tumor cells. Much of the data that has been generated appears in the attached manuscript that has been submitted for publication. We will cite these data in this progress report, as well as other unpublished data that will be part of a second manuscript which is in preparation for publication, and indicate how these findings relate to the approved Statement of Work (SOW).

KEYWORDS NURF; BPTF; doxorubicin; autophagy; apoptosis;

ACCOMPLISHMENTS The major goals of this project were to 1. Define immune cells required for antitumor activity to NURF depleted tumors treated with doxorubicin. 2. Characterize the cell autonomous and non-cell autonomous effects of combined NURF inhibition and doxorubicin treatment. 3. Determine the role of autophagy in doxorubicin induced DAMP secretion and immunogenicity of NURF depleted cells. 4. Define NURF regulated mechanisms for sensitization to doxorubicin-induced DNA damage.

Our research groups have made significant progress in Year 1 primarily relating to goals 2 and 4, as indicated by the data described below, and submitted for publication.

Figure 1 (see submitted manuscript in Appendix) builds upon our initial findings that BPTF knockdown (KD) sensitizes 4T1 breast tumor cells to doxorubicin and etoposide - both agents act primarily through inhibition of topoisomerase II. We also observe sensitization to paclitaxel, but not to ionizing radiation, cisplatin, 5-fluorouracil or azacytidine. These data, generated through analysis of clonogenic survival, indicate that the sensitization is not uniformly applicable to all classes of drugs currently or potentially used in the treatment of breast cancer. Furthermore, it appears that sensitization to paclitaxel may prove to have a different mechanistic basis than for doxorubicin and etoposide. Of particular relevance are the experiments shown in **Figure 1E** utilizing embryonic stem cells as a model for normal tissue toxicity. These cells were not sensitized by BPTF knockdown, in support of the potential selectivity of this therapeutic strategy. However, we do observe sensitization to paclitaxel, and for this reason, paclitaxel studies were not pursued, except in isolated cases. *Sensitivity studies in the tumor cells were performed in both the Gewirtz and Landry laboratories. Studies in the embryonic stem cells were performed in the Landry laboratory.*

Figure 2 presents experiments designed to probe the basis for sensitization via BPTF inhibition. **Figure 2A** shows that two 4T1 cell lines in which BPTF was suppressed exhibit increased DNA damage, as measured by gamma H2AX, upon exposure to either doxorubicin or etoposide, but not to radiation or paclitaxel. These observations appear to be consistent with the lack of sensitization to radiation and, as mentioned above, to a different mechanism of sensitization to doxorubicin and etoposide than for paclitaxel. **Figures 2B** and **2C** support and extend these findings in terms of demonstrating increased cross-linking of the topoisomerase II enzymes, which leads to increased DNA damage, which is the molecular target of doxorubicin and etoposide. These experiments relate to **Specific Aim 1, Major Task 2**, Characterize the cell autonomous (and cell non-autonomous) effects of combined NURF inhibition and doxorubicin treatment. Studies of DNA damage were performed in both the Landry and Gewirtz laboratories. *Studies relating to DNA cross-linking were performed in the laboratory of our collaborator, Dr. Yves Pommier at the NCI, using materials supplied by the Landry laboratory.*

Additional studies relating to **Specific Aim 1, Major Task 2** are presented in **Figure 3**. Here we assess the nature of the sensitization to doxorubicin, etoposide and paclitaxel in terms of apoptosis, senescence and autophagy. The autophagy studies are also relevant to **Specific Aim 2, Major Task 1**, Define NURF regulated mechanisms for sensitization to doxorubicin induced DNA damage. Here we demonstrate that there is a relatively modest (but statistically significant) increase in apoptosis (assessed by evaluating Annexin V positivity via flow cytometry) induced by etoposide and paclitaxel

with BPTF knockdown, but not for doxorubicin, strongly suggesting that apoptosis does not account for the observed sensitization in cell culture. No increase in senescence was observed for doxorubicin, etoposide or paclitaxel, unequivocally ruling out senescence as a mechanism for sensitization. However, as shown in **Figure 3C**, there is a clear increase in the extent of autophagy by doxorubicin and etoposide, but not paclitaxel, again distinguishing sensitization to paclitaxel from sensitization to the topoisomerase II poisons. These findings were confirmed by additional assays for autophagy, specifically the colocalization of lysosomes and mitochondria (a key substrate of the autophagic process) in **Figure 3D**, Western blotting for the conversion of the autophagic membrane protein LC3 from forms I to II, a central element of the autophagy process. Of particular importance are the studies presented in **Figure 3F**, clearly demonstrating that sensitization to doxorubicin is lost when autophagy is genetically blocked with knockdown of the key autophagy protein, ATG5. The studies relating to autophagy are a component of **Specific Aim 2, Subtask 1**, Define the nature of autophagy that sensitizes NURF depleted cells to doxorubicin. *Studies relating to apoptosis, senescence and autophagy were performed jointly by the Gewirtz and Landry laboratories.*

Figure 4 presents data that are critical to the first part of **Major Aim 3, Milestone #2**, essentially to determine the contribution of autophagy to the effects of doxorubicin on tumor growth (and enhanced immune activity to NURF depleted breast tumor cells). Studies relating to the second component of this milestone are in progress. In this series of experiments, 4T1 mouse breast tumor cells that were either competent or deficient in BPTF expression were implanted into mice. **Figure 4B** indicates that at the dose of doxorubicin administered (5mg/kg administered at days 5, 12 and 19), the drug exhibited only modest effects on tumor growth. In contrast, the BPTF KD cells demonstrated pronounced interference with tumor growth, essentially replicating and reinforcing the findings in cell culture. These experiments further indicate that the effects of BPTF KD are maintained in an immune competent environment. **Figure 4E** shows that when autophagy is blunted by knockdown of the ATG5 gene, doxorubicin effectiveness is enhanced, consistent with doxorubicin-induced autophagy being cytoprotective in this *in vivo* experimental model. Furthermore, in comparing **Figures C and F**, it is evident that blunting of autophagy interferes with the chemo-sensitization resulting from BPTF KD, further supporting the conclusion that promotion of autophagy is, in large part, responsible for the enhanced response to doxorubicin in cells where NURF function has been suppressed. *Studies in which BPTF KD tumors were implanted in mice were performed largely in the Landry laboratory but with assistance from the Gewirtz laboratory where autophagy was inhibited.*

Figure 5 addresses the goals set by **Major Task 2, Milestone #2**, which was to determine the cell autonomous cytotoxic effects and the non-cell autonomous immune cell effects of tumor cell AU1 treatment and doxorubicin exposure. Data is presented both in cell culture and in tumor bearing animals. The cell culture studies presented in **Figure 5C** show that the BPTF inhibitor, AU1, sensitizes the 4T1 breast tumor cells to doxorubicin in a clonogenic survival assay. **Figure 5D** is essentially a negative control experiment demonstrating that AU1 and BPTF KD have identical functions, as no sensitization to doxorubicin will occur once BPTF has been genetically silenced. **Figures 5E and 5F**, respectively, recapitulate the observations made with genetic silencing of BPTF in that the AU1 compound enhances DNA damage (measured by levels of gamma H2AX, a standard marker of DNA double strand breaks) and increased autophagy. **Figure 5G** shows that AU increases DNA-Topoisomerase II complex formation similarly to BPTF KD, but that this effect is lost in BPTF KD cells. Again, this confirms that the AU1 drug and BPTF KD have the same effect in the tumor cells. *Studies in cell culture (cell autonomous effects) were performed jointly by the Landry and Gewirtz laboratories.*

Figure 5I through **Figure 5M** address the latter part of **Major Task 2, Milestone #2**, presenting data in tumor bearing animals. **Figure 5I** and **Figure 5J** demonstrate that AU1 enhances the antitumor effects of doxorubicin, recapitulating the outcome of the studies with genetic silencing of BPTF (**Figure 4**). The studies in **Figure 5K** and **Figure 5L** are relevant to the latter section of **Major Task 2, Milestone #3**, Determine the ...cell non-autonomous immune cell effects of tumor cell AU1 treatment and doxorubicin exposure as well as **Major Task 3, Milestone #2**, Determine the importance of autophagy to the effects of doxorubicin on tumor growth...to NURF depleted breast tumor cells. Basically, the silencing of ATG5 eliminates the sensitization to doxorubicin by AU1, again reinforcing that AU1 has the identical targeting impact as with BPTF KD.

Finally, comparing the last sets of images on **Figure 5M**, it is apparent that the combination of doxorubicin and AU1 promotes extensive apoptosis in the breast tumors implanted in the mice (based on staining of the apoptosis- indicating protein, cleaved caspase 3), whereas the apoptosis is essentially eliminated when autophagy is suppressed by genetic silencing of the autophagy regulatory protein, ATG5. Again, this implicates autophagy in sensitization to chemotherapy by targeting of BPTF. *Studies in tumor bearing animals were performed largely by the Landry laboratory but with assistance by the Gewirtz laboratory. The Gewirtz laboratory also contributed to the silencing of the autophagy regulatory gene, ATG5.*

All of the above studies were performed in the 4T1 breast tumor cell line, which is a prerequisite for transplantation of tumors into an immunocompetent animal where the role of the immune system in chemosensitization can be assessed. In order to extend these studies to a human breast tumor cell lines, the studies in **Figure 6** were performed using triple negative MDA-MB231 breast tumor cells. The studies in **Figures 6A** and **Figure 6B** demonstrate sensitization to doxorubicin, paclitaxel and etoposide, although the effect with etoposide is quite modest. **Figure 6C** and **Figure 6D** reiterate the finding that the pharmacologic NURF inhibitor, AU1, like genetic silencing of BPTF, sensitizes the breast tumor cells to doxorubicin and paclitaxel. **Figure 6D** further implicates increased DNA damage in the observed sensitization to doxorubicin, whereas **Figure 6E** supports the conclusion generated in the studies with 4T1 cells, that sensitization is also associated with an increase in drug-induced autophagy. The studies in **Figure 6F** again confirm the necessity for autophagy in chemosensitization by pharmacologic BPTF/NURF inhibition, as genetic silencing of ATG5 abrogates the sensitization to doxorubicin. **Figure 6G** and **Figure 6H** further establish the involvement of autophagy in sensitization to doxorubicin, as the impact of BPTF KD is entirely abrogated with silencing of ATG5. **Figures 6I** and **Figure 6J** utilize breast tumor cell spheroids, a 3-dimensional model system, to confirm the necessity of functional autophagy for sensitization and the promotion of apoptosis as a critical element of the sensitization process. These studies contribute to **Milestone #2 of Major Task 3**, to Determine the cell autonomous cytotoxic effects ...of tumor cell AU1 treatment and doxorubicin exposure. *The studies in tumor organoids were performed in the Landry laboratory. All of the other studies were performed jointly by the Gewirtz and Landry laboratories.*

Limitations Encountered in Year 1 of the Project

Here it should be noted that our university was largely in a state of lockdown for three months in the first part of this year, severely limiting the capacity to perform laboratory research. Dr. Liliya Tyutyunyk-Massey, a student in Dr. Gewirtz's laboratory that worked in this project, completed her degree in December of 2019, and left the institution. Furthermore, the university instituted a hiring freeze and prevented international students from joining our programs. Nevertheless, significant progress has been made in achieving the goals of this project with the assistance of Mr. Ryan Finnegan, who joined Dr. Gewirtz's laboratory prior to the lockdown.

In addition, in order to publish papers regularly, we are required to perform the experiments in Year 1 slightly out of sequence from the submitted SOW (see descriptions above). This will likely occur again in Year 2, where some experiments which were proposed in Year 1 (**flow cytometry - Aim1, Major Task2, Subtask 2**) will be somewhat delayed before they can be completed, while studies slated to be completed in Year 3 (vaccination studies which will monitor the nature of the CD8 T cell response) will be completed early and be included in the manuscript in progress.

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

The project provided for the training and professional development of Dr. Liliya Tyutyunyk-Massey, a PhD candidate in Dr. Gewirtz's laboratory. Currently, Mr. Ryan Finnegan is training for his Ph.D. working on this project.

How were the results disseminated to communities of interest?

Due to the Coronavirus pandemic, the meeting of the American Association for Cancer Research was canceled, and we were unable to present our data at this forum.

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

Preliminary data (not included in the Accomplishments section) suggest that the autophagy that appears to be central to chemosensitization when NURF (BPTF) is inhibited actually takes the form of mitophagy, where the mitochondria are the primary organelle that is degraded by the cell. A primary focus of our cell culture work will revolve around the characterization of mitophagy and its role in chemosensitization. Additional efforts will further investigate the nature of the autophagy/mitophagy with regard to its function in this system (i.e. whether it is cytoprotective or cytotoxic).

4. IMPACT:

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

Epigenetic regulation of tumor cell sensitivity to chemotherapy. These studies indicate that pharmacologic inhibition of NURF (e.g. AU1) may be effective in sensitizing breast tumor cells to chemotherapy, through both direct (cell autonomous) and indirect (cell non-autonomous, immune mediated) pathways.

What was the impact on other disciplines?

The results presented in the Accomplishments section have broad impacts to the field of **epigenetics** in that they characterize the nuclear functions for the NURF complex in chemosensitization, which has not been recognized previously.

This work also has impact to the field of **immunotherapy**, strongly suggesting that inhibiting NURF is a novel means to enhance the immunogenicity of breast tumor cells, which has implications for the use of immunogenic modulators.

What was the impact on technology transfer?

None to report

What was the impact on society beyond science and technology?

None to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Actual or anticipated problems or delays and actions or plans to resolve them:

A three-month shutdown of the university coupled with a hiring freeze significantly delayed our research efforts.

Changes that had a significant impact on expenditures

Due to the shutdown, the fact that Dr. Tyutyunyk-Massey completed her degree work and the hiring freeze, expenditure of some of the grant funds has been delayed.

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

None to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Papers

One manuscript has been submitted to Molecular Cancer Research and a second manuscript is currently under development.

Grant Submissions

DOD Breast Cancer Program (2020)

BC200069 Full Application: Targeting breast cancer senescence as a therapeutic strategy to prevent or delay disease recurrence

Presentations

- **Website(s) or other Internet site(s)**
- **Technologies or techniques**
- **Inventions, patent applications, and/or licenses**
- **Other Products**

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Dr. David Gewirtz. Principal Investigator; person-months worked - 12

Ms. Liliya Tyutyunyk-Massey; Ph.D. candidate in Dr. Gewirtz's laboratory; person-months worked -5

Mr. Ryan Finnegan, Ph.D. candidate in Dr. Gewirtz's laboratory; person-months worked – 12

The person months worked include the 3-month shutdown period.

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

No

What other organizations were involved as partners?

The laboratory of Dr. Yves Pommier at the National Cancer Institute performed collaborative studies relating to the cross linking of topoisomerase II with DNA.

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS:

The Accomplishments section clearly lists the contributions of both laboratories. Because the studies are so tightly integrated, many of the experiments reflect the input and involvements of both laboratories.

9. **APPENDICES:** Manuscript submitted to Molecular Cancer Therapeutics

Medical Center

In the tradition of the Medical College of Virginia

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Dear Senior Editor,

Please find attached our manuscript entitled "Autophagy Dependent Sensitization of Triple Negative Breast Cancer Models to Topoisomerase II Poisons by Inhibition of The Nucleosome Remodeling Factor." as a first-time submission for a research article in *Cancer Research*.

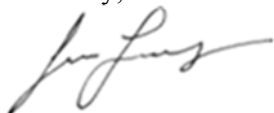
Epigenetic factors are key regulators of cancer specific biology and as such are widely recognized as high potential drug targets for therapeutics. Several recent reports have shown the epigenetic regulator the nucleosome remodeling factor (NURF) is overexpressed in a wide variety of cancers resulting in a more aggressive cancer with a poorer prognosis. We and others have shown that NURF has key functions in regulating cancer cell growth and cancer cell immunogenicity which could explain these observations.

In this manuscript we continue our work on NURF to demonstrate that NURF in both mouse and human breast cancer models suppresses the anticancer effects of several chemotherapies. Key observations from our study include...

- 1.) NURF suppresses the ability of topoisomerase poisons from creating DNA damaging topoisomerase crosslinks.
- 2.) NURF promotes a protective form of therapy-induced autophagy. Inhibiting NURF significantly improves the toxic effects of chemotherapies by preventing a protective form of autophagy.
- 3.) NURF suppresses therapy-induced mitophagy (a form of autophagy specific to mitochondria). To our knowledge this the first epigenetic regulator to have these functions.
- 4.) We show for the first time that NURF can be inhibited pharmacologically for therapeutic benefit using mouse tumor models. When published these studies will stimulate additional efforts developing cancer therapeutics targeting the NURF complex.

These key observations from our study span several disciplines of cancer research including cancer epigenetics, the cell autonomous responses to chemotherapies (DNA damage and autophagy (including mitophagy)) and the characterization of a novel cancer therapeutics. As such we believe that this manuscript will be of general interest to cancer researchers and appeal to the broad readership of *Cancer Research*.

Sincerely,



Joseph Landry, PhD
Associate Professor
Department of Human and Molecular Genetics
Virginia Commonwealth University School of Medicine

Autophagy Dependent Sensitization of Triple Negative Breast Cancer Models to Topoisomerase II Poisons by Inhibition of The Nucleosome Remodeling Factor.

Tyutyunyk-Massey, Liliya²; Sun, Yilun³; Dao, Nga¹; Ngo, Hannah¹; Dammalapati, Mallika¹; Vaidyanathan, Ashish¹; Singh, Manjulata¹; Haqqani, Syed¹; Haueis, Joshua¹; Ryan Finnegan²; Xiaoyan, Deng⁵; Kirberger, Steve⁴; Bos Paula⁶; Bandyopadhyay⁵, Dipankar; Pomerantz, William⁴; Pommier, Yves³; Gewirtz, David²; and Landry, Joseph, William^{1#}.

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Key Words: Chromatin Remodeling, NURF, Doxorubicin, Topoisomerase 2, Autophagy

Running Title: NURF Regulates Therapy-Induced Autophagy

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ABSTRACT

Epigenetic regulators can modulate the effects of cancer therapeutics. To further these observations, we discovered that the bromodomain PHD finger transcription factor subunit (BPTF) of the nucleosome remodeling factor (NURF) suppresses sensitivity of the 4T1 breast tumor cell line to doxorubicin, etoposide and paclitaxel. BPTF functions in suppressing the cytotoxicity of doxorubicin and etoposide, but not paclitaxel, and may be selective to cancer cells as similar suppression was not observed in embryonic stem cells. Sensitization to doxorubicin and etoposide with BPTF knockdown (KD) was associated with increased DNA damage, topoisomerase II (Top2) crosslinking and autophagy; however, there was no concomitant increase in apoptosis or senescence. Sensitization to doxorubicin was confirmed *in vivo* with the syngeneic 4T1 breast tumor model using both genetic and pharmacological inhibition of BPTF. The effects of BPTF inhibition *in vivo* are autophagy dependent, based on genetic autophagy inhibition. Finally, treatment of 4T1 and MDA-MB-231 breast cancer cells with the selective BPTF bromodomain inhibitor, AU1, recapitulates genetic BPTF inhibition, including *in vitro* sensitization to doxorubicin, increased Top2-DNA crosslinks and DNA damage. Taken together, these studies demonstrate that BPTF functions to suppress the antitumor activity of doxorubicin and etoposide, preventing the resolution of Top2 crosslinking and associated autophagy. These studies suggest that BPTF could potentially be targeted with small molecule inhibitors to enhance the effectiveness of Top2-targeted cancer chemotherapeutic drugs.

INTRODUCTION

Epigenetic regulators play a prominent role in cancer-specific biology , and, as a result, have been targeted to develop novel cancer therapies (1). Several laboratories, including ours, have discovered that the chromatin remodeling complex nucleosome remodeling factor (NURF) suppresses cancer growth using *in vitro* and *in vivo* models (2-7). NURF is a prototypical chromatin remodeling complex, catalyzing the ATP-dependent sliding of nucleosome positions in chromatin with profound effects on gene expression (8).

Doxorubicin and etoposide are widely effective chemotherapeutic agents that act by targeting topoisomerase II (Top2) and generating reactive oxygen species (ROS). Mammalian Top2 resolves DNA topological issues such as supercoils and catenanes by cleaving the DNA duplex, generating the catalytic intermediate Top2 cleavage complex (Top2cc) (9). Doxorubicin and etoposide induce cellular damage primarily through the stabilization of Top2cc (hence they are termed Top2 poisons), which interfere with DNA metabolism and, if left unrepaired, lead to cell death (10). To survive, cancer cells in part activate autophagy to consume and recycle damaged macromolecules and generate energy. Targeting autophagy pharmacologically to sensitize tumor cells to doxorubicin has been demonstrated with a variety of cell line models (11).

Autophagy is a well characterized cellular process induced by cancer cells after exposure to cytotoxic therapies (12). Autophagy is regulated by a well characterized pathway requiring 30 or more autophagy-related proteins (ATGs), which leads to the sequestering

of select cargos into the autophagosome. The autophagosome fuses with a lysosome where the low pH and degradative enzymes digest the cytoplasmic components to micromolecules, thereby providing nutrients to promote survival (12). Autophagy occurs in many forms including macroautophagy, microautophagy, chaperone-mediated autophagy, mitophagy and lipophagy. These processes differ in the type of cargo targeted for degradation by fusion with the lysosome. In the case of mitophagy defective mitochondria are targeted for degradation which improves cell homeostasis in times of metabolic stress (13).

In this study, we examined whether NURF regulates sensitivity to commonly used anticancer agents by inhibiting its essential BPTF subunit. From our studies we identified agents that BPTF suppresses the therapeutic effects of Top2 poisons through effects on Top2-induced DNA damage and appeared to require functional autophagy.

RESULTS

BPTF Suppresses the Toxicity of Select Therapies to 4T1 Breast Cancer Cells.

To create a NURF inhibited 4T1 cell line BPTF was targeted for KD because it is unique and essential to the function of the NURF complex (**Fig. 1A**)(14). Western blotting confirmed that BPTF shRNAs depleted BPTF, but not the SNF2 and pRbAp48 subunits, in 4T1 cells (**Fig. 1B**). BPTF KD 4T1 cells were assayed for sensitivity to 7 chemotherapeutic drugs and ionizing radiation through clonogenic survival assays. These studies indicated that BPTF KD sensitized 4T1 cells to the Top2 poisons doxorubicin and

etoposide, the microtubule poison paclitaxel, the histone deacetylase inhibitor (HDACi) panobinostat, but did not sensitize the cells to the DNA damaging agents ionizing radiation or cisplatin, the DNA methyltransferase inhibitor (DNMTi) azacytidine, or the nucleoside analog 5-flurouracil (**Fig. 1C**).

To examine the selectivity of BPTF as a suppressor of chemotherapeutic drug sensitivity for cancer cells, we also assayed BPTF KO embryonic stem cells (ESCs) as a normal tissue model (**Fig. 1D**) (15). These studies demonstrated that the sensitization to doxorubicin, etoposide, and panobinostat observed in the 4T1 cells did not extend to the ESC (**Fig. 1E**). We did observe sensitization of BPTF KO ESCs to paclitaxel, which served as a rationale for not pursuing additional studies with this drug. Instead, we focused our follow-up studies on doxorubicin and etoposide, as these drugs are widely utilized (10), have similar mechanisms of action, largely by targeting Top2, and in the case of doxorubicin, by also generating reactive oxygen species (ROS) (16). To test the possibility that drug accumulation and/or efflux might explain sensitivity, we measured and observed equivalent accumulation of doxorubicin (**Fig. S1A, B**), as well as the accumulation and efflux of pump substrates rhodamine 6G (17) and hoechst 33342 (18) (**Fig. S1C-H**) in these cells suggesting that drug accumulation or efflux is not regulated by BPTF.

BPTF Promotes the Repair of Doxorubicin- and Etoposide-Stabilized Top2 Cleavage Complexes in 4T1 cells.

Measurement of ROS resulting from doxorubicin treatment of BPTF KD and wild-type (WT) 4T1 cells using DCFH-DA (19), showed similar levels of cellular ROS (**Fig. S2A**). These results, combined with an inability of ROS scavenger's glutathione and N-acetylcysteine to affect the toxicity of doxorubicin to BPTF KD 4T1 cells (**Fig. S2B**), suggest that BPTF does not regulate doxorubicin-induced accumulation of ROS.

Additional studies demonstrated that BPTF KD 4T1 cells showed equivalent sensitivity to the Top1 poison camptothecin (**Fig. S2C**) and to the Top2 catalytic inhibitor ICRF-187 (**Fig. S2D**), suggesting that BPTF suppresses toxicity selectively to Top2 poisons. Unlike doxorubicin and etoposide, which trap Top2cc, ICRF-187 inhibits Top2 by blocking its ATP hydrolysis activity (20). Consequently, it is plausible that BPTF could regulate sensitivity to doxorubicin and etoposide, at least in part, by regulating the resolution of Top2 crosslinking, enhancing DNA double-strand breaks (DSBs). Consistent with this hypothesis, flow cytometric analysis showed increased DNA-damage marker, γ H2AX (21), after exposure to doxorubicin and etoposide but not paclitaxel or radiation in BPTF KD cells (**Fig. 2A**); which was supported by quantitative Western blotting (**Fig. S2E-G**).

Coincident to increased γ H2AX abundance, we observed that BPTF KD resulted in increased doxorubicin- and etoposide-stabilized Top2cc. Using an *in vivo* complex of enzyme (ICE) assay (22), enhancement of Top2 α and Top2 β cc was observed in doxorubicin- and etoposide-treated BPTF KD cells compared to control cells (**Fig. 2B-D**). Western blotting confirmed that the elevation in Top2cc levels was not a result of BPTF-

regulated Top2 protein expression (**Fig. S2H**). To determine whether the observed Top2cc elevation in BPTF KD cells was a result of enhanced Top2 chromatin attachment, we performed chromatin fractionation studies. We found that BPTF KD did not alter the levels of chromatin-bound Top2 α and Top2 β both in the absence and presence of doxorubicin, suggesting that BPTF does not affect the Top2 chromatin localization, and that the increase in Top2cc levels in BPTF cells likely resulted from Top2cc repair deficiency (**Fig. S2I**). Consistent with BPTF's role in regulating doxorubicin-induced DNA damage, immunofluorescence imaging showed that BPTF is nuclear prior to and after doxorubicin treatment (**Fig. S2J**). Taken together, these results support a model where BPTF resides in the nucleus and regulates the resolution of drug-induced Top2cc to promote the repair of Top2-linked DSBs.

Influence of BPTF on Doxorubicin and Etoposide Induced Apoptosis, Senescence, and Autophagy

An analysis of apoptosis by flow cytometric measurement of annexinV/7AAD staining (23) showed BPTF KD only modestly altered the extent of doxorubicin, etoposide, and paclitaxel induced apoptosis (**Fig. 3A**) and is unlikely to contribute to sensitization. Quantification of senescence by measuring SA- β -galactosidase activity using C₁₂FDG (24) showed that doxorubicin, etoposide, and paclitaxel all induced cellular senescence (**Fig. 3B**). However, the overall extent of therapy-induced senescence was unaltered in the BPTF KD cells (**Fig. 3B**), indicating that sensitization is not a consequence of increased senescence.

It is well established that a primary response to anticancer chemotherapeutic drugs and radiation is autophagy (25). Flow cytometric and microscopy-based quantification of autophagy by acridine orange (AO) staining (26) shows a substantial increase in doxorubicin and etoposide-induced autophagy with BPTF KD (**Fig. 3C**, **Fig. S3A**), whereas BPTF does not appear to regulate paclitaxel induced autophagy (**Fig. 3C**). In addition to the increase in AO staining the use of fluorescent dyes which selectively label lysosomes and mitochondria shows a substantial increase in co-localization in BPTF KD cells treated with doxorubicin and etoposide but not paclitaxel (**Fig. 3D**, **Fig. S3B**). The co-localization of mitochondria with lysosomes is characteristic of mitophagy, an autophagy promoted process which selectively targets defective mitochondria (27). Another critical measure of autophagy is the conversion of LC3B from LC3B-I to LC3B-II (28). Western blot analysis of LC3B demonstrated increased LC3B conversion (Ratio LC3B-II/LC3B-I) with in the 4T1 BPTF KD cells prior to doxorubicin treatment (**Fig. 3E**). These results are consistent with an overall increase in autophagy, including the selective process of mitophagy.

To further interrogate the potential involvement of autophagy in the sensitization of breast cancer cells to doxorubicin by BPTF KD, we depleted ATG5 by shRNA in the WT and BPTF KD 4T1 cell lines (**Fig. S3C**). Evaluation of LC3B conversion confirmed that ATG5 KD compromised the capacity of 4T1 cells to undergo autophagy, as measured by a decreased ratio of LC3-II to LC3-I (**Fig. 3E**). Using *in vitro* clonogenic survival assays, we

further determined that the autophagy induced by doxorubicin was nonprotective in the 4T1 cells *in vitro*, as reported previously (29). This conclusion is based on the observation that ATG5 KD failed to further sensitize the WT and BPTF KD 4T1 cells to doxorubicin exposure (**Fig. 3F**).

A Requirement for Autophagy in the Sensitization of 4T1 Cells to Doxorubicin in Tumor-Bearing Animals

To determine whether the chemosensitization observed in cell culture by BPTF KD would also be observed *in vivo*, we surgically implanted WT and BPTF KD 4T1 cells into the 4th mammary fat pad of syngeneic BALB/c mice. Consistent with previous results (2), we did not observe any significant differences in BPTF KD 4T1 tumor growth in untreated mice (**Fig. 4A**). The tumor-bearing mice received 3 administrations of 5 mg/kg of doxorubicin at 7-day intervals, a dose that has minimal effects on the growth of WT 4T1 breast tumors (**Fig. 4B**). However, the doxorubicin treatments resulted in significant reductions in BPTF KD 4T1 tumor volume over the entire time period (**Fig. 4C**). These results replicate the cell culture findings demonstrating that BPTF KD 4T1 cells are more sensitive to doxorubicin and, by extension, that BPTF suppresses the antitumor effects of doxorubicin.

In mice, WT or BPTF KD 4T1 cells in combination with ATG5 KD grew similarly as tumors (**Fig. 4D**). In contrast to the clonogenic survival assays, doxorubicin treatment of mice bearing WT 4T1 tumors with ATG5 KD demonstrated that the sensitization to doxorubicin was modestly improved when autophagy was inhibited, demonstrating that autophagy is

modestly protective to WT 4T1 tumors *in vivo* (**Fig. 4E**). Strikingly, the improvement in tumor sensitization to doxorubicin with BPTF KD was largely lost when combined with ATG5 KD (**Fig. 4F**). These results suggest that functional autophagy is required for sensitization by BPTF KD *in vivo*.

The BPTF Bromodomain Inhibitor AU1 Sensitizes 4T1 Cells to Select Therapies

To implement the clinical significance of the above findings we tested if BPTF is a druggable target using a specific inhibitor of the BPTF bromodomain identified previously as AU1 (**Fig. 5A,B**) (30). Pretreatment of 4T1 cells with AU1 resulted in increased sensitivity to doxorubicin (**Fig. 5C**) but not radiation (**Fig. S4A**), as was the case for the BPTF KD cells (**Fig. 1C**). Interestingly, AU1 treatment did not recapitulate the sensitization to paclitaxel (**Fig. S4B**) observed for BPTF KD (**Fig. 1C**) suggesting that AU1, and inhibition of the bromodomain, may not affect all BPTF's functions.

Using BPTF KD2 because it is the most efficient KD (**Fig. 1B**), we further demonstrate that AU1 treatment of BPTF KD 4T1 cells did not further sensitize the cells to doxorubicin, which is consistent with AU1 inhibiting BPTF (**Fig. 5D**). Further evidence that AU1 targets BPTF consistent with the fact that, like BPTF KD, the combination of AU1 and doxorubicin resulted in increased DNA damage as determined by flow cytometric measurement of γ H2AX staining (**Fig. 5E**), AO staining (**Fig. 5F**) and doxorubicin and etoposide-trapped induced Top2cc (**Fig. S4C, Fig. 5G**). AU1 treatment of BPTF KD cells did not further increase doxorubicin- and etoposide-induced Top2cc, consistent with similar outcomes

with BPTF KD (**Fig. 2B-D**), further suggesting that AU1 has specificity for BPTF (**Fig. S4C, Fig. 5G**).

Consistent with our observations using BPTF KD 4T1 cells *in vitro* (**Fig. 3F**), ATG5 KD in combination with AU1 treatment did not alter the sensitivity of 4T1 cells to doxorubicin (**Fig. 5H**). However *in vivo* treatment of established WT 4T1 tumors with AU1 resulted in a significant reduction of tumor weight when combined with doxorubicin, which confirms our previous experiments using genetic BPTF KD (**Fig. 5I, J**). Intratumoral injections of AU1 were used for these studies because AU1 has poor pharmacokinetics (31). Identical experiments using ATG5 KD tumors revealed reduced sensitization to doxorubicin with AU1 treatments, supporting a role for autophagy in the enhanced therapeutic benefits (**Fig. 5K, L**). Histological analysis of tumors treated with combinations of doxorubicin and AU1 showed an increase in the necrotic portion of the tumor, which stained positive for cleaved caspase 3 (**Fig. S4D**). A further analysis of the non-necrotic portions of the tumors also showed an increase in cleaved caspase 3-positive cells with the combined treatment (**Fig. 5M**). These enhancements of cleaved caspase 3 with combined AU1 and doxorubicin treatment were not observed in the ATG5 KD tumors (**Fig. 5M**), which is consistent with the dependence of sensitization by BPTF KD on functional autophagy shown in Figure 4.

BPTF Suppresses the Sensitivity of the Human MDA-MB-231 TNBC Cell Line to Chemotherapies

To determine whether NURF suppresses the sensitivity of human TNBC to chemotherapeutic agents, we generated control and BPTF KD MDA-MB-231 cells by lentivirus transduction (**Fig. 6A**). As with 4T1 cells, BPTF KD MDA-MB-231 cells showed increased sensitivity to doxorubicin, etoposide, and paclitaxel (**Fig. 6B**). Also, as was the case with the mouse 4T1 TNBC model, AU1 treatment of MDA-MB-231 cells resulted in enhanced sensitivity to doxorubicin, but not paclitaxel (**Fig. 6C**). The ability of AU1 to recapitulate the sensitization of TNBC specifically to doxorubicin but not paclitaxel was conserved. As was the case in the mouse 4T1 model, flow cytometric analysis of γ H2AX staining indicated that BPTF significantly suppresses doxorubicin-induced DNA damage in MDA-MB-231 cells (**Fig. 6D**), and also is necessary for doxorubicin-induced autophagy, as suggested by increased overlap of lysosomes and mitochondria, an indicator of mitophagy (**Fig. 6E**). As previously published, autophagy is protective in the WT MDA-MB-231 cells (comparing WT and WT/ATG5 KD cells treated with doxorubicin)(**Fig. S4F**) (32), in contrast to being nonprotective in the 4T1 cells (**Fig. 3F**). Autophagy appeared to be required for the sensitization of AU1-treated MDA-MB-231 cells to doxorubicin (**Fig. 6F**), as sensitization from AU1 and doxorubicin treatment was abrogated in ATG5 KD cells. As with AU1 treatment, sensitization to doxorubicin was also observed in BPTF KD MDA-MB-231 cells, and this sensitization required ATG5 (**Fig. 6G, H**). The requirement for autophagy in the sensitization of BPTF KD MDA-MB-231 cells to doxorubicin was also observed after the cells were aggregated into spheroids (**Fig. 6I**). Annexin V/7AAD staining demonstrated that apoptosis was increased in doxorubicin-treated BPTF KD MDA-MB-231 cells as compared to WT MDA-MB-231 cells, and this increase was reduced with ATG5 KD (**Fig. 6J**). Taken together, these results demonstrate that

inhibition of BPTF can improve the response of breast cancer cells to Top2-targeted chemotherapeutic agents and implicate autophagy in sensitization.

DISCUSSION

Epigenetic changes to the cancer genome that promote cancer-specific biology are reversible (33) and, as such, have been pursued as therapeutic targets (1). In contrast to DNMTs and HDACs, which have been targeted for decades, inhibitors of chromatin remodeling enzymes are just being developed (34). In this study, we aimed to determine how the chromatin remodeling complex NURF functions to regulate the sensitivity of breast cancer cells to chemotherapeutic drugs and radiation. By targeting BPTF, the unique and essential subunit of NURF (14), we show that NURF selectively suppresses the effects of chromatin-targeted chemotherapies doxorubicin, etoposide, and panobinostat. These results are consistent with independent studies in tissue culture showing that BPTF KD sensitizes to cisplatin and 5-fluorouracil (3) and to HDAC inhibitors (7). They are also consistent with similar studies with the NuRD remodeling complex and daunorubicin, cytarabine (35) and docetaxel (36), and SWI/SNF, which suppresses sensitivity to a large number of chemotherapies (37). Hence there is growing evidence that inhibiting chromatin remodeling complexes sensitizes multiple cancer types to broad classes of chemotherapeutic agents.

One important mechanism by which BPTF suppresses doxorubicin and etoposide toxicity is inhibition of Top2cc. Both BPTF and Top2 localizes to regions of open chromatin including promoters, enhancers and CTCF binding sites making their direct functional

interaction plausible (38, 39). Equivalent Top2 chromatin association after doxorubicin exposure with BPTF KD suggests it is unlikely that BPTF regulates access of Top2 to the DNA. The elevated levels of Top2cc suggest that BPTF participates in the repair of Top2cc possibly including TDP2, which has specific functions in resolving trapped Top2cc (40). It is unlikely that BPTF regulates DSB repair pathways as radiation- and doxorubicin-induced DNA damage use the Mre11 repair pathways and the defects in DNA damage repair were selectively observed with doxorubicin-induced DNA damage and not to radiation treated cells.

An unanticipated outcome from this study is that the sensitization of BPTF KD cells to chemotherapeutic agents requires autophagy. Despite the induction of both senescence and apoptosis with doxorubicin-induced DNA damage (41), BPTF selectively affects autophagy as shown by increased AO staining, mitophagy and LC3B conversion. This could be the result of BPTF regulating autophagy control points including the Ulk1 (42), Bcl2/Beclin1 (43), or by regulating the expression of autophagy components (44) or lysosome biogenesis (45). The latter is plausible as many autophagy and lysosome biogenesis genes are regulated by epigenetic regulators (reviewed extensively in (44)). Changes in the autophagy pathway may not necessarily influence cell autonomous toxicity but could selectively contribute to the loss of protective autophagy observed in tumors (a non-cell autonomous effect) as we observed for the 4T1 tumors. Possible non-cell autonomous effects could involve changes in autophagy-regulated angiogenesis (46), sensitization to therapies in a hypoxic tumor environment (47), a therapy-induced

antitumor immune response through inflammatory cytokines (48), or by inducing autophagic cell death (49).

The BPTF bromodomain inhibitor AU1 has been shown previously to inhibit the growth of the K562 human cancer cells in culture (31). This present report extends these observations by showing that AU1 has anti-cancer activity when used in combination with Top2 inhibitors using *in vitro* and *in vivo* tumor models. These results are different from those observed when using the SWI/SNF bromodomain inhibitor ADAADiN, which functions as a broad sensitizer to chemotherapeutic agents by suppressing ABC transporter expression (37). Unexpectedly, the BPTF inhibitor AU1 does not sensitize to paclitaxel, for which we showed sensitization for BPTF KD. These results suggest that specifically inhibiting the BPTF bromodomain, the target for AU1 (30), may only inhibit a subset of BPTFs function in sensitization to chemotherapeutic agents. Functions in regulating BPTF sensitization to paclitaxel could occur through other functional domains in BPTF including the C-terminal PHD finger, which is well known to interact with H3K4me3 the N-terminal PHD finger, or through interaction surfaces important for transcription factor binding (14).

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METHODS

Cell Culture

Wild type (WT) and BPTF knockdown (KD) (described in (15)) 4T1 (Gift from Fred Miller, Wayne State University), MDA-MB-231 (ATCC, Cat# HTB-26™) and HEK 293T (ATCC, Cat# CRL-3216) were cultured in complete media (CM) (DMEM, 10% fetal bovine serum [FBS], 2 mM glutamine, and penicillin/streptomycin). Embryonic stem (ES) cells were maintained as described previously (50).

ShRNAs were introduced into MDA-MB-231 cells using the lentiviral pLVTHM system. pLVTHM plasmids Ctrl-sh1, Bptf-sh1, Bptf-sh2 are available at Addgene as stock numbers 83046, 83277, 83278, respectively. Transduced cells were selected with 0.5 µg/ml puromycin after 48 hours and subsequently grown in as described above.

Atg5 shRNA was introduced into the WT and BPTF KD 4T1 cells using the MISSION pLKO.1 lentivirus system (Millipore-Sigma). Viruses were concentrated by ultracentrifugation as described previously and used immediately to transduce WT and BPTF KD 4T1 cells lines without selection. Atg5 KD was confirmed 72 hrs. post transduction by Western blotting.

Mycoplasma contamination was tested and confirmed to be negative every year using Universal Mycoplasma Detection Kit (ATCC, Cat# 30-1012K).

Clonogenic Survival Assays

Cells were plated into 6 well plates and the following morning cells were treated with chemotherapy or radiation. Cells were treated for 24 hrs. after which they were washed and cultured in fresh media. Colonies were stained with crystal violet and counted. The fraction viable after treatment was calculated by the ratio # colonies treated / # colonies untreated.

Drug Accumulation and Efflux Assays

To measure doxorubicin accumulation cells were treated with 1 µg/ml doxorubicin for 1 hr. Cells were washed, dispersed from culture, washed twice in FACS buffer (1% FBS, hanks balanced salt solution [HBSS]), resuspended and analyzed. To measure rhodamine 6G accumulation, cells were treated with 1 µM rhodamine 6G for 3 hrs. and processed as described above. Efflux was measured by washing cells twice with fresh media after and culturing for 24 hours. Media was changed once during the 24 hrs. Hoechst 33258 was performed similarly including 1 hr. accumulation of 15 µM and 24 hr. efflux.

Determination of DNA Damage.

Cells were treated with drugs for 24 hours. After drug removal cells were collected, washed with PBS and centrifuged. Pellets were suspended in 90% ice cold methanol fixed and permeabilized for 30 min at RT. Then cells were washed in PBS again, suspended in 2% BSA and blocked for 30 min on shaker. γ H2AX conjugated antibody (BD Pharmingen, Cat#560445) was added 1:100 and cells were stained for 1.5 hours on shaker protected from light. Cells were washed and analyzed.

Determination of Cell Apoptosis

Apoptosis was monitored by Annexin V-FITC/PI staining (BD Biosciences) according to the manufacturer's instructions. Samples were analyzed by BD FACSCanto II and BD FACSDiva software at Virginia Commonwealth University Flow Cytometry Core Facility as described previously.

Fluorescent Staining

Acridine Orange - After exposure to chemotherapy or radiation, and recovery, cells were stained with 1 μ g/ml acridine orange (Invitrogen, Cat# A3568) at 37°C for 25 min and then washed with PBS. Cells were observed under an inverted fluorescence microscope and images taken at 20X magnification. For quantification of autophagy, cells were collected with trypsin and washed with PBS and analyzed by flow cytometry.

LysoTracker and MitoTracker - Cells were seeded in 6-well plates on coverslips. After exposure to chemotherapy or radiation, cells were stained with 100 nM LysoTracker Red DND-99 (Invitrogen, L7528) at 37°C for 30 min and then 100 nM MitoTracker Green FM (Invitrogen, M7514) for an additional 30 min. Cells were observed under a fluorescence microscope and images taken at 100X magnification. Overlapping LysoTracker positive and MitoTracker positive organelles were determined by image overlay.

β -Galactosidase - To quantify β -Gal positive senescent cells, after exposure to Doxorubicin, cells were treated with Bafilomycin (100 nM) for 1 h to achieve lysosomal alkalization and stained with C₁₂FDG (Invitrogen, Cat#D2893) (10 μ M) fluorescent β -Galactosidase substrate for 2 h at 37 °C. After incubation, cells were collected, washed, suspended in PBS and analyzed by flow cytometry. All experimental procedures were performed with cells protected from light.

Tumor Studies

BALB/cJ female mice 6-8 weeks of age weighting ~ 20 g (Jackson Laboratory) were housed under aseptic barrier conditions as approved by Virginia Commonwealth University IACUC animal protocol AD10000017. 5×10^4 4T1 cells were surgically transplanted into the fourth mammary fat pad. Tumors were measured by caliper measurements 3 days/week starting from days 7 post transplantation. Doxorubicin at 5 mg/kg was given once a week.

Spheroid Assays

Alamar blue assay (Thermo Scientific, Rockford, USA, Cat # DAL1025) was done with the MDA-MB-231-spheroids seeded on 96 well spheroids plate (Corning). Spheroids were treated with 1 μ M doxorubicin for 4 days and 10% Alamar blue in media was used

to develop the purple color for 24 hrs and the fluorescence was measured on a microplate reader. A similar assay in parallel was done for the untreated cells. The percentage (%) difference of the live cells between control and treated was calculated.

Top2cc Measurements

Top2ccs were isolated and detected using in vivo complex of enzyme (ICE) assay as previously described (22). Briefly, cells were lysed to reduce the viscosity of DNA and layered onto CsCl solution (150% w/v), followed by centrifugation. The resulting pellet containing was dissolved in TE buffer. 2 µg of DNA is applied per slot on the blotting apparatus and was subsequently subjected to immunoblotting with Top2α (Abcam, Cat# ab52934) and Top2β (Novus Biologicals, Cat# NB-100-40842). Quantitative slot blotting was performed using Pierce Super Signal ELC Femto detection reagent (Thermo Fisher, Cat# 37075) with the Bio-Rad ChemiDoc® System. Top2cc signals were quantified by densitometric analysis using ImageJ.

After 20 min doxorubicin (100 µM) treatment, 4T1 cells were subjected to subcellular fractionation to isolate chromatin using Subcellular Protein Fractionation Kit (Thermo Fisher, cat# 78840) following manufacturer's instructions for Western blotting to assess the chromatin fraction of Top2α and β.

Western Blotting

Total proteins from cell cultures was extracted by TRI Reagent (Millipore-Sigma, Cat# T9424) using manufactures suggested protocol. Protein pellets were dissolved in 8M urea and 1% SDS at 65 C then quantified using the DC protein assay (BioRad, Cat# 5000112) and a BSA standard. Proteins were resolved by SDS-PAGE, transferred to PVDF using as described previously (50). Primary antibodies: anti-BPTF (Millipore-Sigma, ABE24), anti-SNF2L (Santa Cruz, sc-79088), pRbAp46/48 (Santa Cruz, sc-33170), Top2α (Abcam, ab52934), Top2β (Novus Biologicals, NB-100-40842), LC3B (Novus, NB100-2220) ATG5 (Cell Signaling, 12994S), anti-Cyclophilin B (Thermo Fisher, PA1-027A), anti-GAPDH (Cell Signaling, 2118), then anti-mouse or rabbit HRP secondary (Cell Signaling, 7074S). Qualitative Western blotting was done using film. Quantitative Western blotting was performed using Pierce Super Signal ELC Femto detection reagent (Thermo Fisher, 37075) with the LI-COR Odyssey® Fc system. Loading was determined by Cyclophilin B or GAPDH.

Statistics

Excel was used to calculate statistical differences between groups using a paired two-tailed student's t-test. When required T-test p-values were adjusted using the FDR method in R software for all figures involving multiple comparisons'.

Figure Legends

Figure 1.) The Epigenetic Regulator BPTF Protects 4T1 Cancer Cells from Select Cytotoxic Chemotherapies. (A) Cartoon showing the 3 subunit (BPTF, SNF2 and RbAp48) NURF complex interacting with a transcription factor (TF) and remodeling nucleosome positioning (blurred nucleosome). (B) Western blotting for BPTF, SNF2L and

pRbAp48 from 4T1 cells expressing control (WT) or 2 independent BPTF knockdown (KD1 or KD2) shRNAs. Cyclophilin B (CypB) is the loading control. (C) Fraction of 4T1 cells with clonogenic potential after 24-hour exposure to 50 nM doxorubicin, 500 nM etoposide, 5 Gy radiation, 50 nM paclitaxel, 500 nM cisplatin, 5 μ M 5-fluorouracil, 20 μ M azacytidine or 100 nM panobinostat. (D) Western blotting for BPTF from WT or BPTF knockout (KO) mouse embryonic stem cells (ESC). Cyclophilin B (CypB) is the loading control. (E) Fraction of ESC with clonogenic potential after 24-hour exposure to 5 nM doxorubicin, 50 nM etoposide, 25 nM panobinostat or 12.5 nM paclitaxel. Panels C and E = N>3 biological replicates, * = FDR < 0.05.

Figure 2.) BPTF Protects 4T1 Cells from Doxorubicin and Etoposide Induced DNA Damage. (A) Percentage of cells positive for γ H2AX staining after 48 hours exposure to 50 nM doxorubicin, 500 nM etoposide, 50 nM paclitaxel or 5 Gy radiation. (B) Measurement of TOP2 α or TOP2 β crosslinking in 4T1 cells exposed to 10 μ M doxorubicin or 10 μ M etoposide using in-vivo complex of enzyme (ICE) assay. (C) Quantitation of TOP2 α ICE assay results from panel B. (D) Quantitation of TOP2 β ICE assay results from panel B. Panels A, C, D = N>3 biological replicates, * = FDR < 0.05.

Figure 3.) BPTF Regulates the Cell Autonomous Response of 4T1 Cells to Therapy. (A-C) Measurement of annexinV positive (A), β -galactosidase positive (C₁₂FDG fluorescence) (B), or acridine orange (AO) positive (C) WT and BPTF KD 4T1 cells by flow cytometry after exposure to 50 nM doxorubicin, 500 nM etoposide or 50 nM paclitaxel for 48 hours followed by 48 hours of recovery. (D) Percentage of LysoTracker positive organelles which overlap with Mitotracker positive organelles as determined by fluorescence microscopy after 48 hours treatment as described in panels A-C followed by 72 hours recovery. (E) Western blotting for LC3B in 4T1 cells in the absence or presence of an ATG5 KD shRNA with or without 50 nM doxorubicin for 48 hours followed by 24 hours recovery. Cyclophilin B (CypB) is the loading control. (F) Fraction of cells with clonogenic potential after 24-hour exposure to 50 nM doxorubicin. Panels A, B, C, F = N>3 biological replicates, * = FDR < 0.05. Panel D = 10 cells counted per condition, * = FDR < 0.05.

Figure 4.) Therapy Induced Tumor Growth Control of 4T1 Tumors Requires BPTF Regulated Autophagy. (A) Growth of surgically transplanted orthotropic WT and BPTF KD 4T1 cells in BALB/c mice measured by tumor volume. (B, C) Same as in A. Cohorts of mice were untreated or treated with 3 doses of 5 mg/kg doxorubicin at days 5, 12 and 19. (D) Growth of surgically transplanted orthotropic ATG5 KD or ATG5 KD + BPTF KD 4T1 cells in BALB/c mice measured by tumor volume. (E, F) Same as in D. Cohorts of mice were untreated or treated with 3 doses of 5 mg/kg doxorubicin at days 5, 12 and 19. In all plots N>3 biological replicates, * = pValue < 0.05.

Figure 5.) BPTF Inhibition in 4T1 Cells Using the Selective BPTF Bromodomain Inhibitor AU1 Recapitulates Effects from BPTF KD. (A) Cartoon showing the BPTF bromodomain. (B) AU1 structure. (C) Clonogenic potential of cells after 24-hour exposure to 10 μ M AU1 alone or in combination with 50 nM doxorubicin. (D) Clonogenic potential cells after 24-hour exposure to 10 μ M AU1 alone or in combination with 50 nM

doxorubicin. (E) Mean florescent intensity (MFI) for γ H2AX staining in cells after 48 hours exposure to 10 μ M AU1 alone or in combination with 50 nM doxorubicin. (F) Measurement of acridine orange (AO) positive cells by flow cytometry after exposure to 10 μ M AU1 with or without 50 nM doxorubicin for 48 hours followed by 48 hours of recovery. (G) Quantitation of TOP2 α and TOP2 β ICE assay results from Fig. S4C. (H) Fraction of cells with clonogenic potential after 24-hour exposure to 10 μ M AU1 alone or in combination with 50 nM doxorubicin. (I, J) Weight of 4T1 tumors harvested from mice at 30 days from BALB/c mice. Tumors were treated with intratumor injections of AU1 (10 μ l @ 10 mM in DMSO) and/or 5 mg/kg doxorubicin at days 7, 14 and 21. (K, L) Weight of ATG5 KD 4T1 tumors harvested from mice at 30 days from BALB/c mice. Tumors were treated with intratumor injections of AU1 and/or 5 mg/kg doxorubicin at days 7, 14 and 21. (M) Immune staining for cleaved caspase 3 on tumors from panels I-L. Scale bar is 50 μ m. Caspase 3 staining intensity of 5-10 fields by ImageJ analysis indicated below images. Panels C-G = N>3 biological replicates, * = FDR < 0.05. Panels H-K = N>6 tumors, * = pValue < 0.05.

Figure 6.) BPTF Similarly Functions in Human MDA-MB-231 Breast Cancer to Suppress Toxicity of Doxorubicin and Paclitaxel. (A) Western blotting for BPTF from cells expressing control (WT) or 2 independent BPTF KD1 or KD2 shRNAs. Cyclophilin B (CypB) is the loading control. (B) Fraction of cells with clonogenic potential after 24-hour exposure to 20 nM doxorubicin, 600 nM etoposide or 200 nM paclitaxel. (C) Fraction of cells with clonogenic potential after 24-hour exposure to 10 μ M AU1 alone or in combination with 20 nM doxorubicin or 200 nM paclitaxel. (D) Mean florescent intensity (MFI) of cells for γ H2AX staining after 48 hours exposure to 50 nM doxorubicin. (E) Percentage of Lysotracker positive organelles which overlap with Mitotracker positive organelles as determined by fluorescence microscopy after 48 hours 50 nM doxorubicin treatment followed by 48 hours recovery. (F) Fraction of cells with clonogenic potential after exposure to 10 mM AU1 with or without 20 nM doxorubicin. (G, H) Fraction of cells with clonogenic potential after 24-hour exposure to 10 μ M AU1 alone or in combination with 20 nM doxorubicin. (I) Fraction of viable cells in spheroids at 4 days after 24-hour exposure to 20 nM doxorubicin. (J) Measurement of annexinV positive cells by flow cytometry after exposure to 50 nM doxorubicin for 48 hours followed by 96 hours of recovery. Panels C-H = N>3 biological replicates, * = FDR < 0.05. Panel I = N>3 biological replicates, * = FDR < 0.1. Panel J * = pValue < 0.05.

Supplemental Figure 1.) BPTF Suppresses Sensitivity to Select Chemotherapies Independently of Efflux Pump Activity. (A,B) (A) Representative flow cytometry traces and (B) aggregate mean fluorescent intensity (MFI) values for WT and BPTF KD 4T1 cells treated doxorubicin. (C) Representative flow cytometry traces for WT and BPTF KD 4T1 cells treated with rhodamine 6G then allowed to efflux for 24 hours. (D) Representative flow cytometry traces for WT and BPTF KD 4T1 cells treated 1 hour with 15 μ M hoechst 33342 then allowed to efflux for 24 hours. (E-H) Aggregate normalized MFI values of (E) rhodamine 6G accumulation then (G) 24 hour efflux. Aggregate normalized MFI values of (F) hoechst 33342 accumulation then (H) 24 hour efflux. Panels B, E-H = N>3 biological replicates.

Supplemental Figure 2.) NURF Selectively Suppresses Toxicity to Topoisomerase 2 Poisons and Improves their DNA Damaging Effects. (A) Percentage of WT or BPTF KD 4T1 cells positive for DCFH-DA staining with or without 48 hours exposure to 50 nM doxorubicin. (B) Fraction of WT or BPTF KD 4T1 cells with clonogenic potential after 24 hour exposure to 50 μ M glutathione or 25 μ M N-acetylcystine with or without 50 nM doxorubicin. (C) Fraction of WT or BPTF KD 4T1 cells with clonogenic potential after 24-hour exposure to 50 nM camptothecin. (D) Fraction of WT or BPTF KD 4T1 cells with clonogenic potential after 24-hour exposure to 20 μ M ICRF-187. (E-G) Western blotting for γ H2AX from WT or BPTF KD 4T1 cells after 48 hours treatment with (E) 50 nM doxorubicin, (F) 500 nM etoposide or (G) 30 minutes after 5 Gy radiation exposure. Cyclophilin B (CypB) is the loading control. (H) Western blotting for Top2 alpha and Top2 beta from WT or BPTF KD 4T1 cells. GAPDH is the loading control. (I) Top2 association by chromatin fractionation after 20 min exposure to 100 μ M doxorubicin. Western blotting for Top2 alpha and beta were subsequently performed. Histone H3 is the loading control. (J) Representative microscopy images of WT or BPTF KD 4T1 cells untreated or treated with 50 nM doxorubicin for 48 hours stained with anti-BPTF antibodies. Phase contrast images and DAPI counter stain of nucleus is shown. Scale bar represents 20 μ m. Panels A-D = N>3 biological replicates.

Supplemental Figure 3.) Validation of Autophagy by Microscopy and ATG5 KD in 4T1 Cells. (A) Representative microscopy images of WT or BPTF KD 4T1 cells stained with acridine orange after 48 hours treatment with 50 nM doxorubicin or 500 nM etoposide followed by 48 hours recovery. (B) Representative microscopy images of LysoTracker Red and Mitotracker Green stained WT and BPTF KD 4T1 cells after 48 hours treatment with 50 nM doxorubicin, 500 nM etoposide or 50 nM paclitaxel followed by 72 hours recovery. Scale bar is 20 μ m. (C) Western blotting for BPTF and ATG5 from WT or BPTF KD 4T1 cells in the absence or presence of an ATG5 KD shRNA. Cyclophilin B (CypB) is the loading control.

Supplemental Figure 4.) Selective BPTF Bromdomain Inhibitor AU1 Recapitulates Effects from BPTF KD. (A, B) Fraction of WT 4T1 cells with clonogenic potential of cells treated with 10 μ M AU1 alone or in combination with (A) 5 Gy radiation or (B) after 24-hour exposure to 50 nM paclitaxel. (C) Measurement of TOP2 α or TOP2 β crosslinking in WT or BPTF KD 4T1 cells exposed to 10 μ M doxorubicin or 10 μ M etoposide using ICE assay. (D) Analysis of paraffin sections stained with hematoxylin and eosin stain or immune stained for cleaved caspase 3. Quantification of % surface area of the tumor positive for cleaved caspase 3 by Image J is shown as inset. Scale bar is 5 mm. (E) Western blotting for ATG5 from MDA-MB-231 cells expressing control (WT) or ATG5 knockdown (KD) shRNAs. Cyclophilin B (CypB) is the loading control. Panels A, B = N>3 biological replicates.

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Figure 1

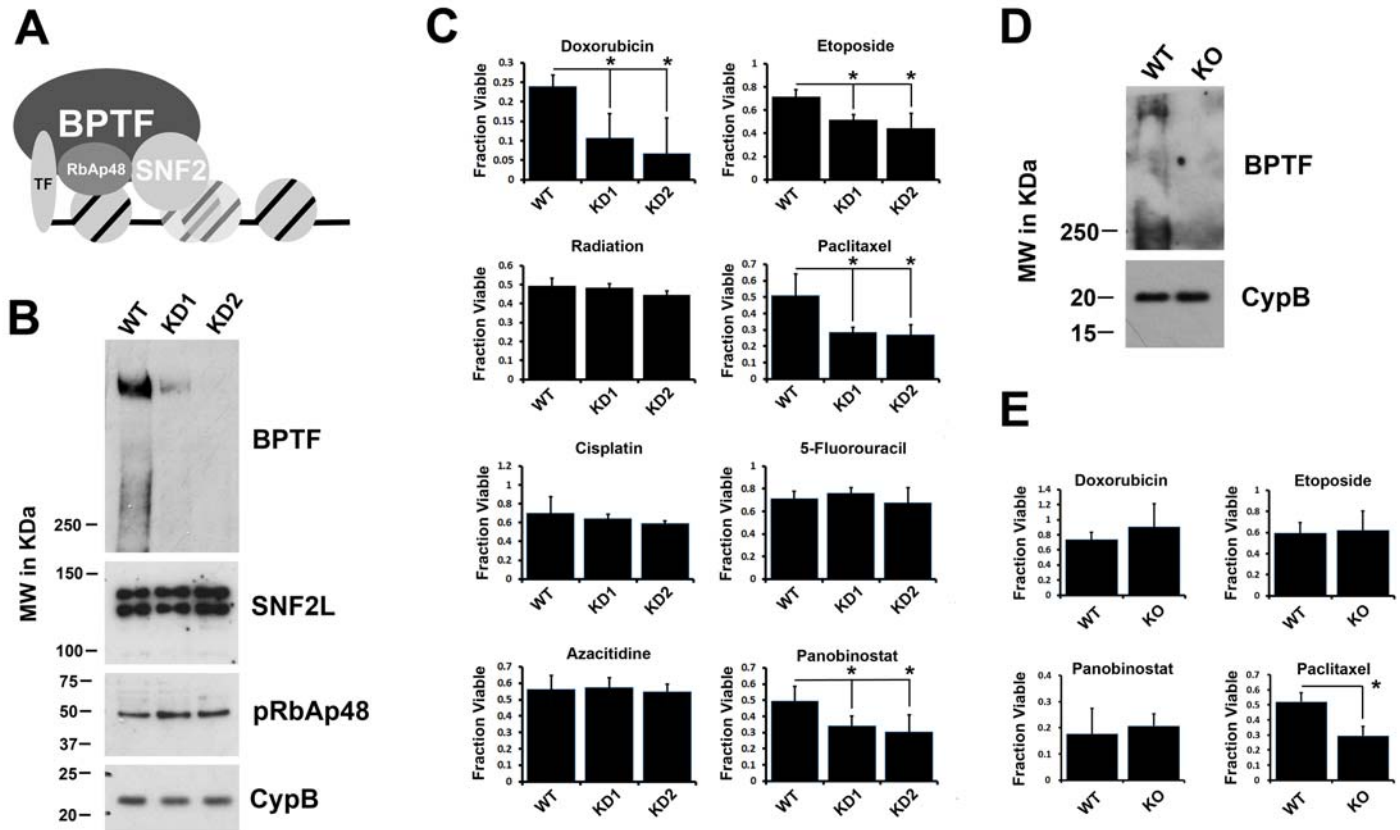


Figure 1.) The Epigenetic Regulator BPTF Protects 4T1 Cancer Cells from Select Cytotoxic Chemotherapies. (A) Cartoon showing the 3 subunit (BPTF, SNF2 and RbAp48) NURF complex interacting with a transcription factor (TF) and remodeling nucleosome positioning (blurred nucleosome). (B) Western blotting for BPTF, SNF2L and pRbAp48 from 4T1 cells expressing control (WT) or 2 independent BPTF knockdown (KD1 or KD2) shRNAs. Cyclophilin B (CypB) is the loading control. (C) Fraction of 4T1 cells with clonogenic potential after 24-hour exposure to 50 nM doxorubicin, 500 nM etoposide, 5 Gy radiation, 50 nM paclitaxel, 500 nM cisplatin, 5 μ M 5-fluorouracil, 20 μ M azacytidine or 100 nM panobinostat. (D) Western blotting for BPTF from WT or BPTF knockout (KO) mouse embryonic stem cells (ES). Cyclophilin B (CypB) is the loading control. (E) Fraction of ES with clonogenic potential after 24-hour exposure to 5 nM doxorubicin, 50 nM etoposide, 25 nM panobinostat or 12.5 nM paclitaxel. Panels C and E = N>3 biological replicates, * = FDR < 0.05.

Figure 2

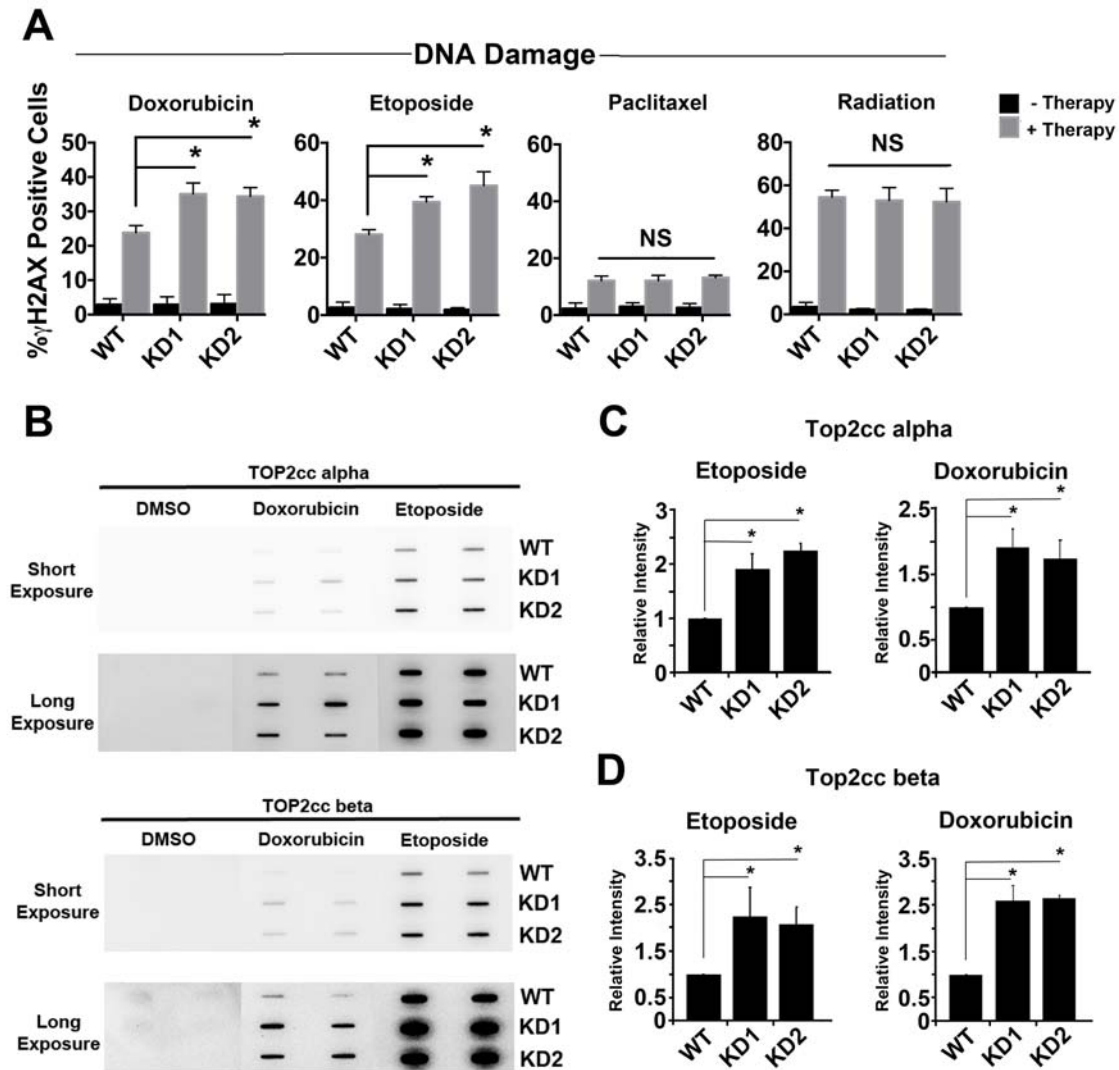


Figure 2.) BPTF Protects 4T1 Cells from Doxorubicin and Etoposide Induced DNA Damage. (A) Percentage of cells positive for γ H2AX staining after 48 hours exposure to 50 nM doxorubicin, 500 nM etoposide, 50 nM paclitaxel or 5 Gy radiation. (B) Measurement of TOP2 α or TOP2 β crosslinking in 4T1 cells exposed to 10 μ M doxorubicin or 10 μ M etoposide using in-vivo complex of enzyme (ICE) assay. (C) Quantitation of TOP2 α ICE assay results from panel B. (D) Quantitation of TOP2 β ICE assay results from panel B. Panels A, C, D = N>3 biological replicates, * = FDR < 0.05.

Figure 3

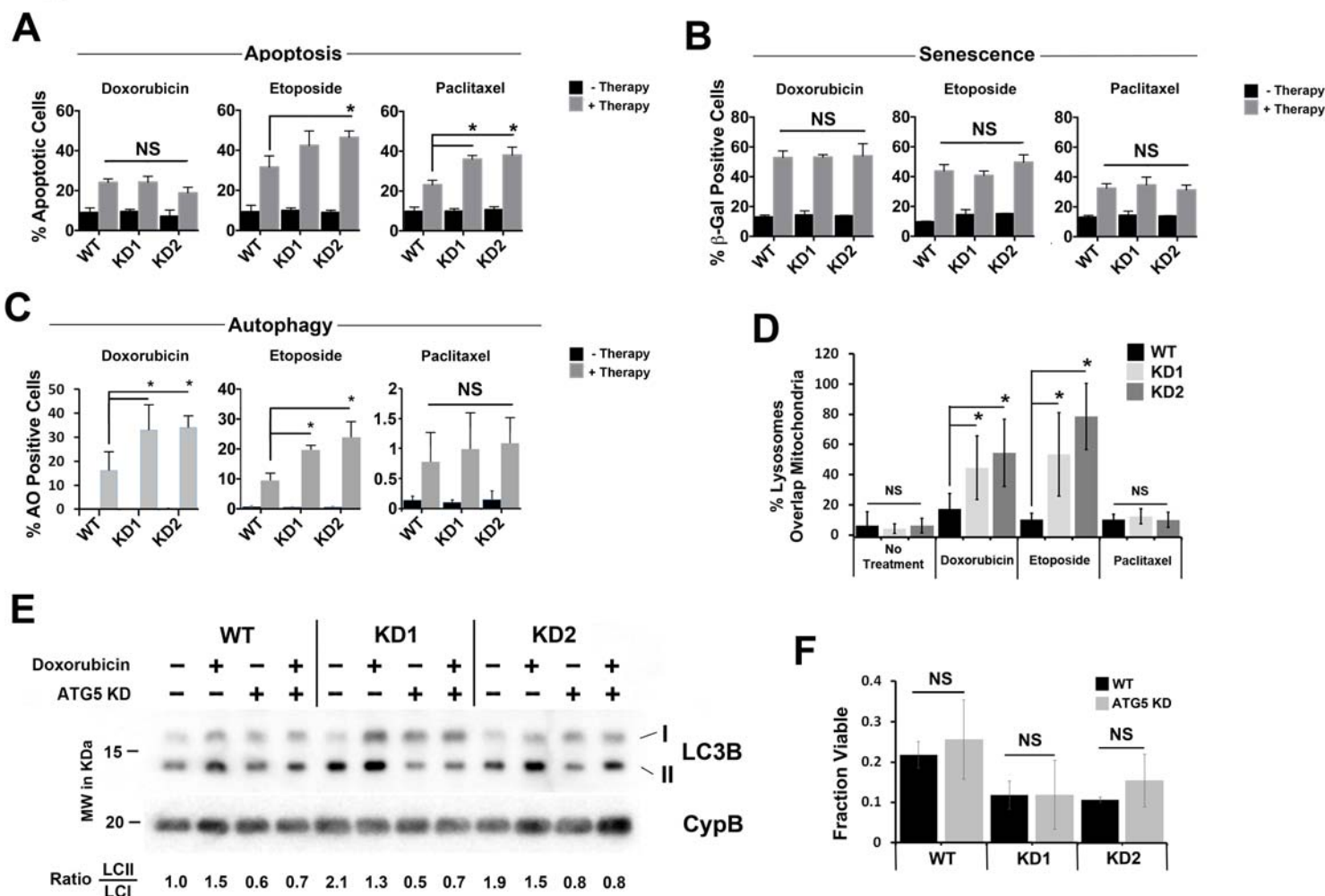


Figure 3.) BPTF Regulates the Cell Autonomous Response of 4T1 Cells to Therapy. (A-C) Measurement of annexinV positive (A), β -galactosidase positive (C12FDG fluorescence) (B), or acridine orange (AO) positive (C) WT and BPTF KD 4T1 cells by flow cytometry after exposure to 50 nM doxorubicin, 500 nM etoposide or 50 nM paclitaxel for 48 hours followed by 48 hours of recovery. (D) Percentage of Lyso-tracker positive organelles which overlap with Mitotracker positive organelles as determined by fluorescence microscopy after 48 hours treatment as described in panels A-C followed by 72 hours recovery. (E) Western blotting for LC3B in 4T1 cells in the absence or presence of an ATG5 KD shRNA with or without 50 nM doxorubicin for 48 hours followed by 24 hours recovery. Cyclophilin B (CypB) is the loading control. (F) Fraction of cells with clonogenic potential after 24-hour exposure to 50 nM doxorubicin. Panels A, B, C, F = N>3 biological replicates, * = FDR < 0.05. Panel D = 10 cells counted per condition, * = FDR < 0.05.

Figure 4

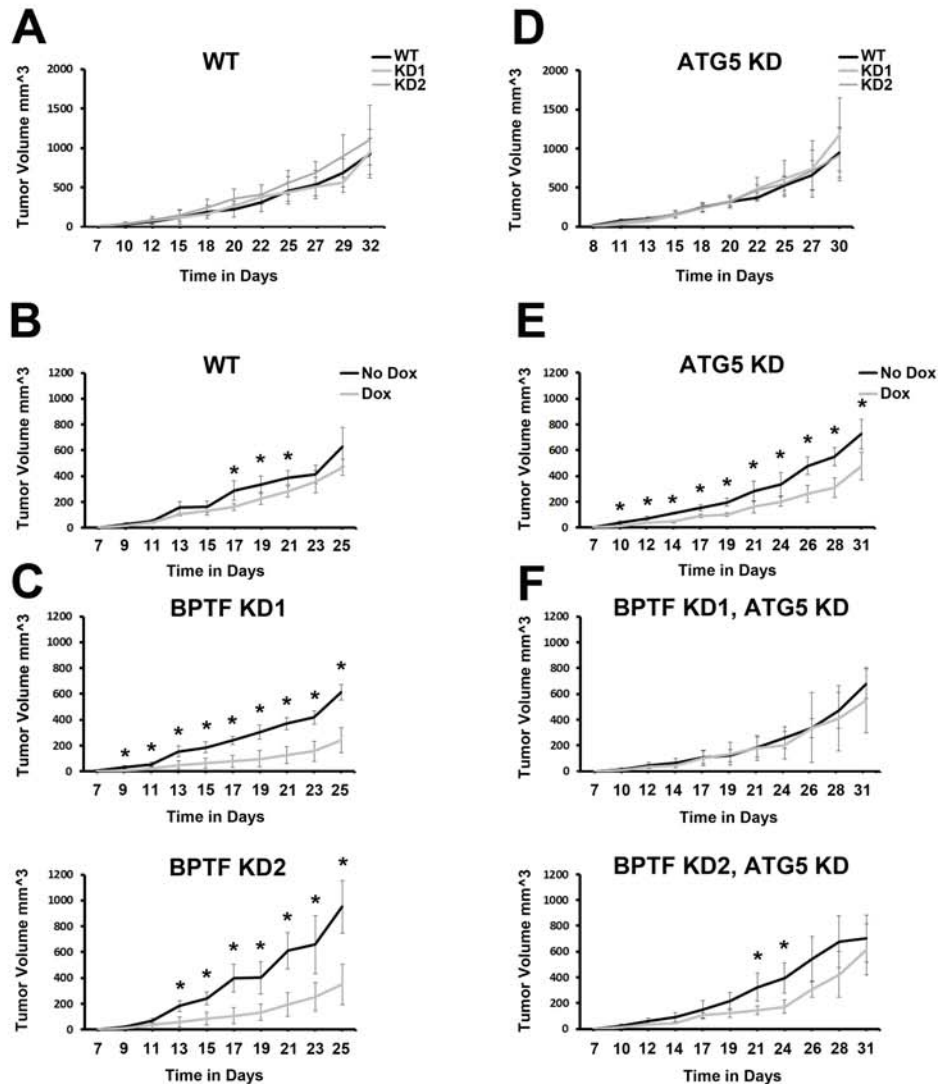


Figure 4.) Therapy Induced Tumor Growth Control of 4T1 Tumors Requires BPTF Regulated Autophagy. (A) Growth of surgically transplanted orthotropic WT and BPTF KD 4T1 cells in BALB/c mice measured by tumor volume. (B, C) Same as in A. Cohorts of mice were untreated or treated with 3 doses of 5 mg/kg doxorubicin at days 5, 12 and 19. (D) Growth of surgically transplanted orthotropic ATG5 KD or ATG5 KD + BPTF KD 4T1 cells in BALB/c mice measured by tumor volume. (E, F) Same as in D. Cohorts of mice were untreated or treated with 3 doses of 5 mg/kg doxorubicin at days 5, 12 and 19. In all plots N>3 biological replicates, * = pValue < 0.05.

Figure 5

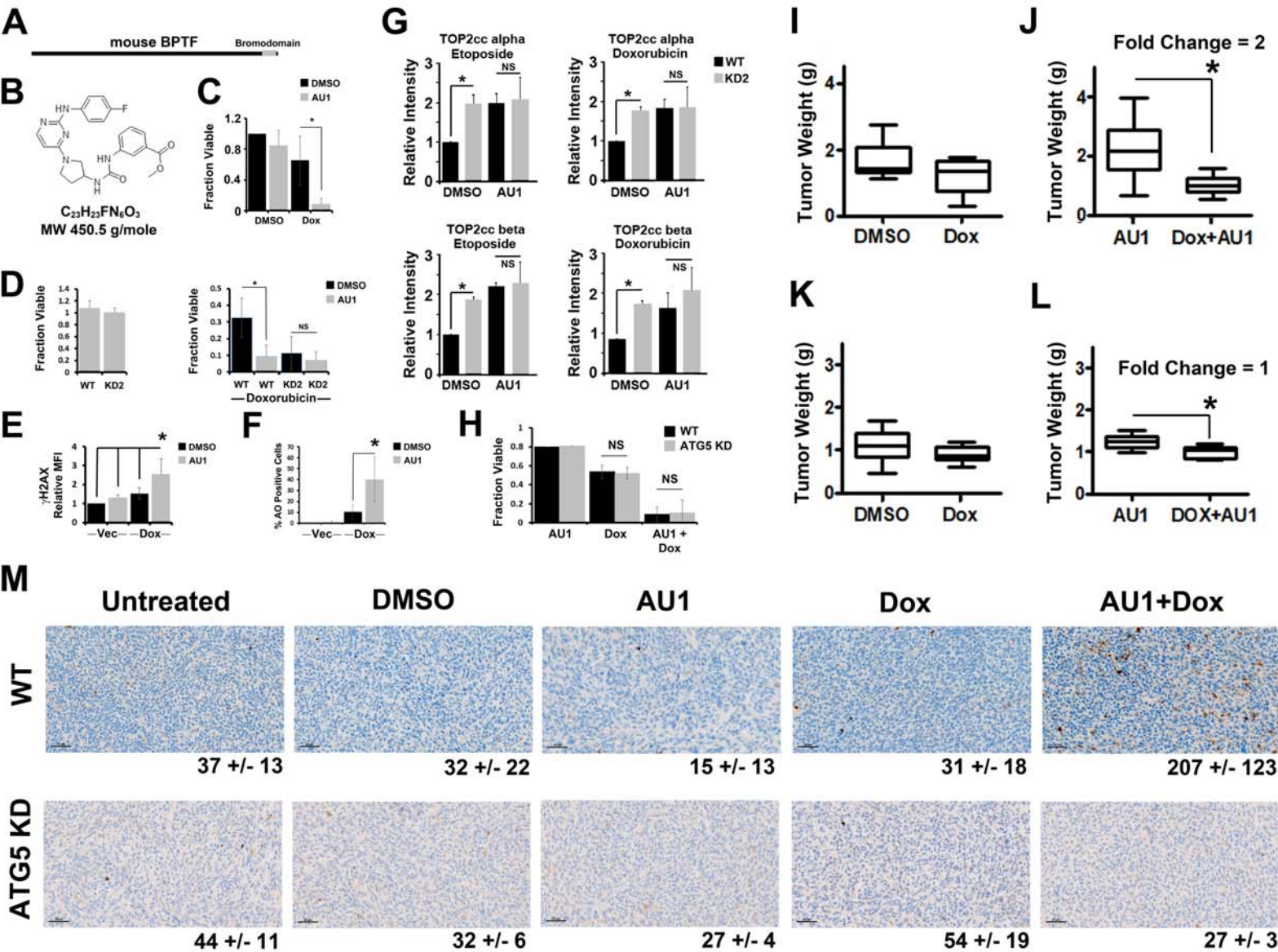


Figure 5.) BPTF Inhibition in 4T1 Cells Using the Selective BPTF Bromodomain Inhibitor AU1 Recapitulates Effects from BPTF KD. (A) Cartoon showing the BPTF bromodomain. (B) AU1 structure. (C) Clonogenic potential of cells after 24-hour exposure to 10 μ M AU1 alone or in combination with 50 nM doxorubicin. (D) Clonogenic potential cells after 24-hour exposure to 10 μ M AU1 alone or in combination with 50 nM doxorubicin. (E) Mean florescent intensity (MFI) for γ H2AX staining in cells after 48 hours exposure to 10 μ M AU1 alone or in combination with 50 nM doxorubicin. (F) Measurement of acridine orange (AO) positive cells by flow cytometry after exposure to 10 μ M AU1 with or without 50 nM doxorubicin for 48 hours followed by 48 hours of recovery. (G) Quantitation of TOP2 α and TOP2 β ICE assay results from Fig. S4C. (H) Fraction of cells with clonogenic potential after 24-hour exposure to 10 μ M AU1 alone or in combination with 50 nM doxorubicin. (I, J) Weight of 4T1 tumors harvested from mice at 30 days from BALB/c mice. Tumors were treated with intratumor injections of AU1 (10 μ l @ 10 mM in DMSO) and/or 5 mg/kg doxorubicin at days 7, 14 and 21. (K, L) Weight of ATG5 KD 4T1 tumors harvested from mice at 30 days from BALB/c mice. Tumors were treated with intratumor injections of AU1 and/or 5 mg/kg doxorubicin at days 7, 14 and 21. (M) Immune staining for cleaved caspase 3 on tumors from panels I-L. Scale bar is 50 μ m. Caspase 3 staining intensity of 5-10 fields by ImageJ analysis indicated below images. Panels C-G = N>3 biological replicates, * = FDR < 0.05. Panels H-K = N>6 tumors, * = pValue < 0.05.

Figure 6

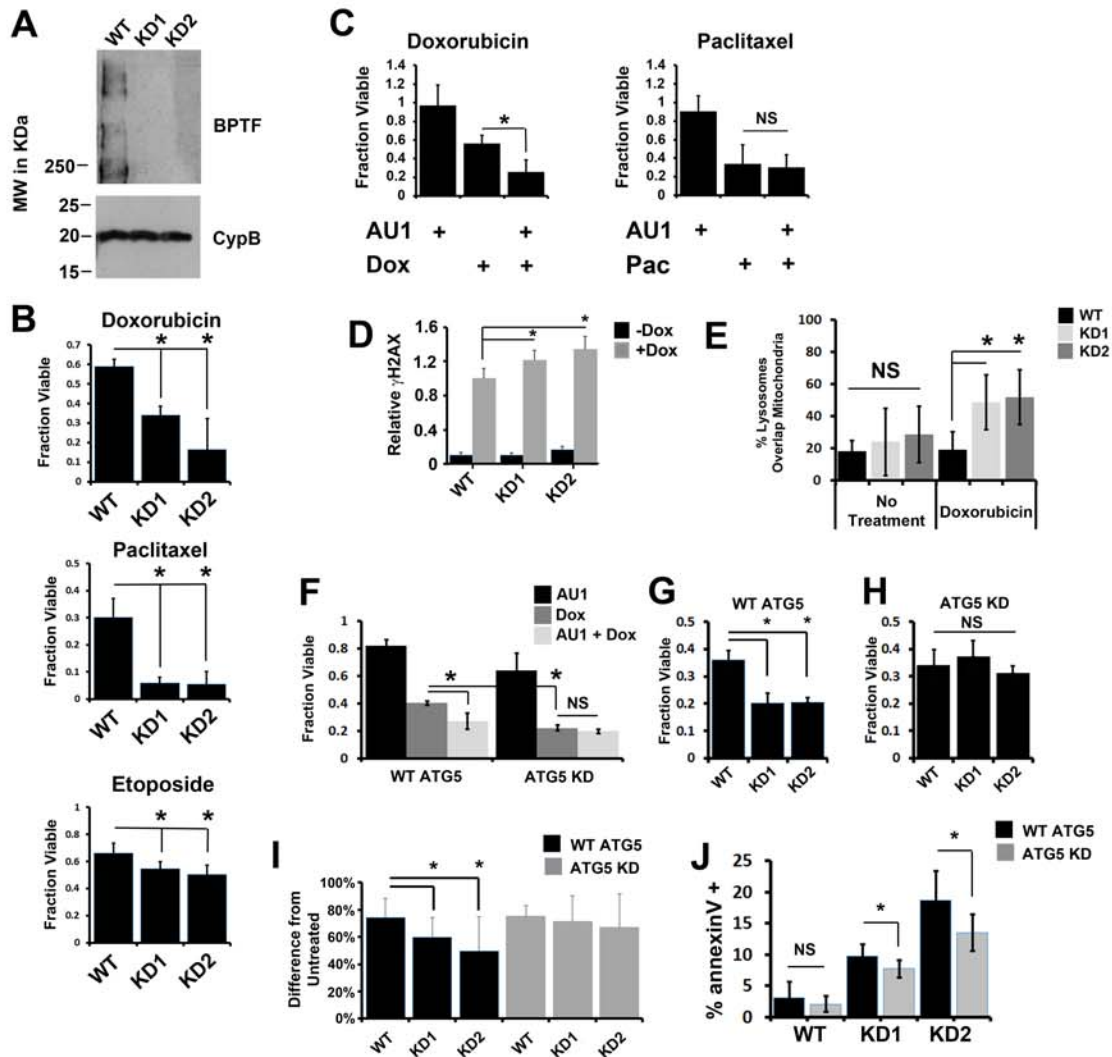
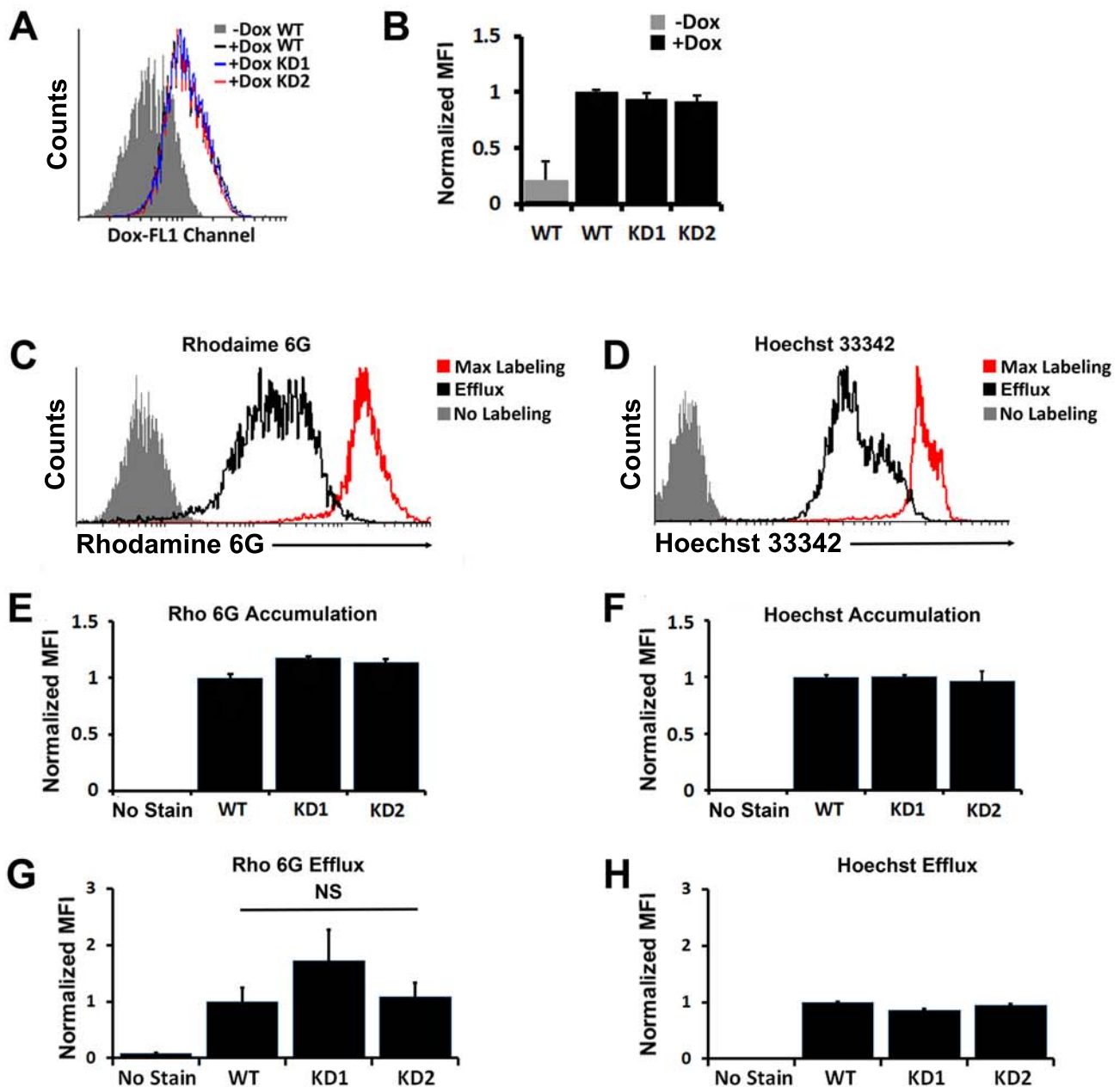


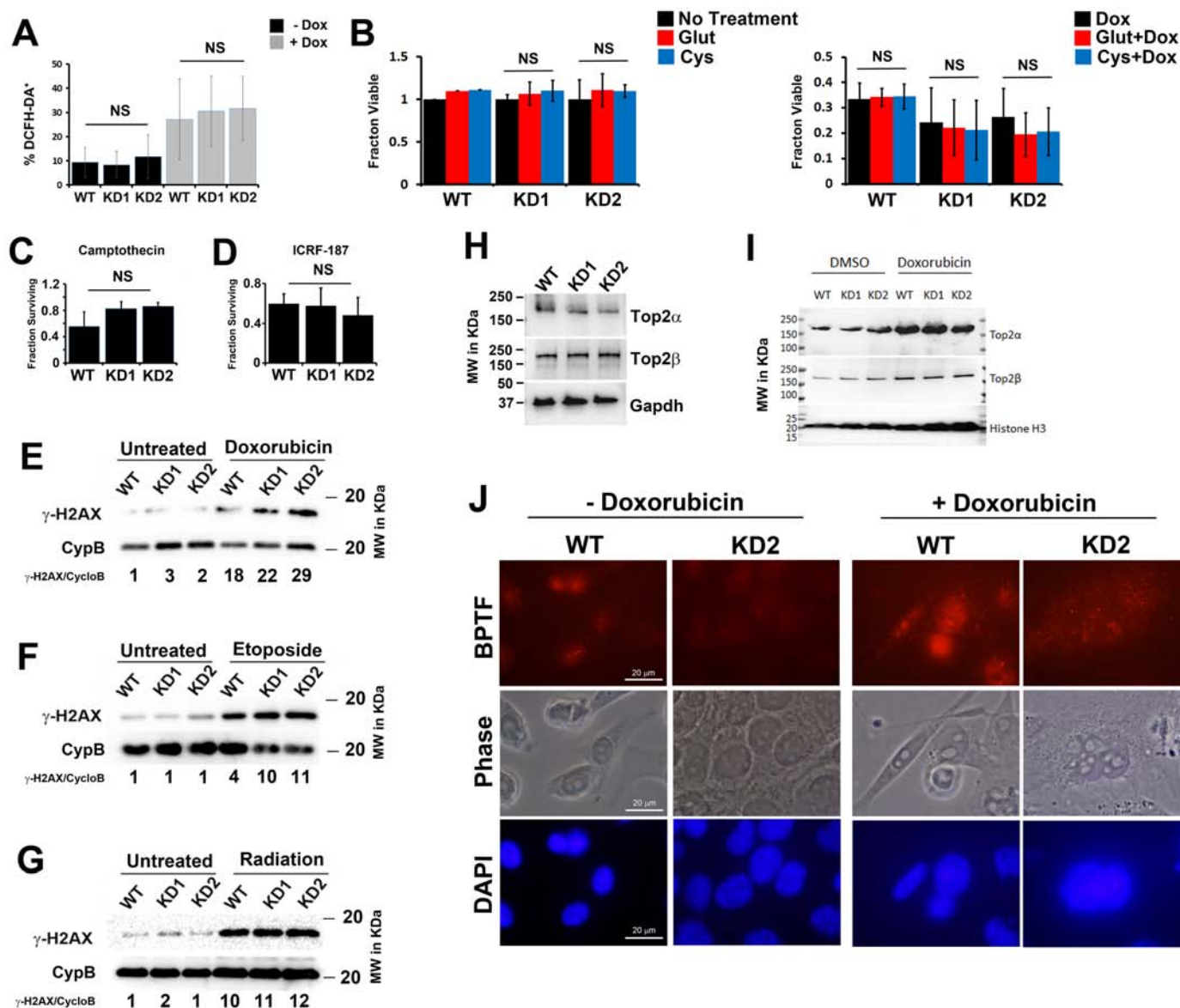
Figure 6.) BPTF Similarly Functions in Human MDA-MB-231 Breast Cancer to Suppress Toxicity of Doxorubicin and Paclitaxel. (A) Western blotting for BPTF from cells expressing control (WT) or 2 independent BPTF KD1 or KD2 shRNAs. Cyclophilin B (CypB) is the loading control. (B) Fraction of cells with clonogenic potential after 24-hour exposure to 20 nM doxorubicin, 600 nM etoposide or 200 nM paclitaxel. (C) Fraction of cells with clonogenic potential after 24-hour exposure to 10 μ M AU1 alone or in combination with 20 nM doxorubicin or 200 nM paclitaxel. (D) Mean florescent intensity (MFI) of cells for γ H2AX staining after 48 hours exposure to 50 nM doxorubicin. (E) Percentage of Lysotracker positive organelles which overlap with Mitotracker positive organelles as determined by fluorescence microscopy after 48 hours 50 nM doxorubicin treatment followed by 48 hours recovery. (F) Fraction of cells with clonogenic potential after exposure to 10 mM AU1 with or without 20 nM doxorubicin. (G, H) Fraction of cells with clonogenic potential after 24-hour exposure to 10 μ M AU1 alone or in combination with 20 nM doxorubicin. (I) Fraction of viable cells in spheroids at 4 days after 24-hour exposure to 20 nM doxorubicin. (J) Measurement of annexinV positive cells by flow cytometry after exposure to 50 nM doxorubicin for 48 hours followed by 96 hours of recovery. Panels C-H = N>3 biological replicates, * = FDR < 0.05. Panel I = N>3 biological replicates, * = FDR < 0.1. Panel J * = pValue < 0.05.

Supplemental Figure 1



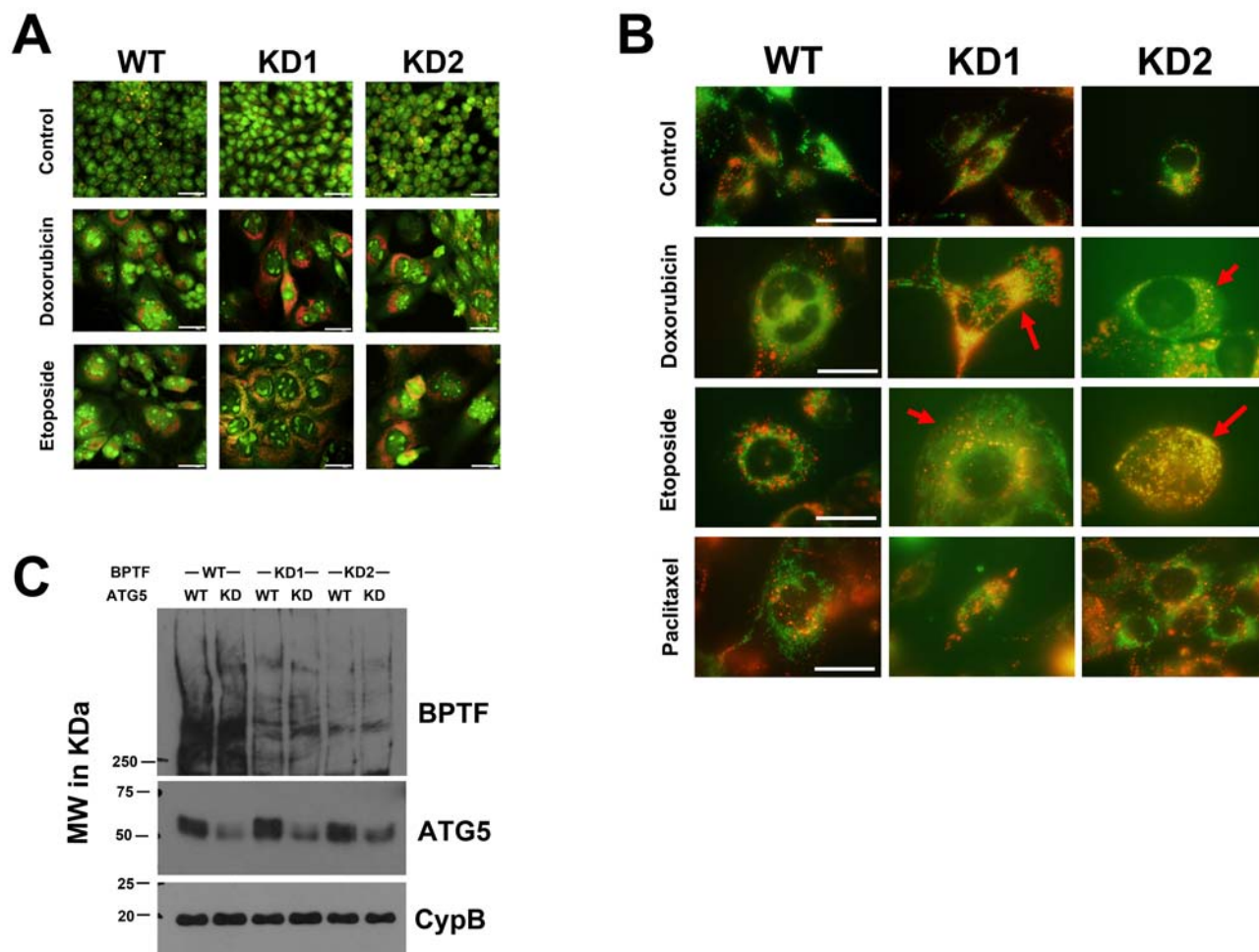
Supplemental Figure 1.) BPTF Suppresses Sensitivity to Select Chemotherapies Independently of Efflux Pump Activity. (A,B) (A) Representative flow cytometry traces and (B) aggregate mean fluorescent intensity (MFI) values for WT and BPTF KD 4T1 cells treated doxorubicin. (C) Representative flow cytometry traces for WT and BPTF KD 4T1 cells treated with rhodamine 6G then allowed to efflux for 24 hours. (D) Representative flow cytometry traces for WT and BPTF KD 4T1 cells treated 1 hour with 15 μ M hoechst 33342 then allowed to efflux for 24 hours. (E-H) Aggregate normalized MFI values of (E) rhodamine 6G accumulation then (G) 24 hour efflux. Aggregate normalized MFI values of (F) hoechst 33342 accumulation then (H) 24 hour efflux. Panels B, E-H = N>3 biological replicates.

Supplemental Figure 2



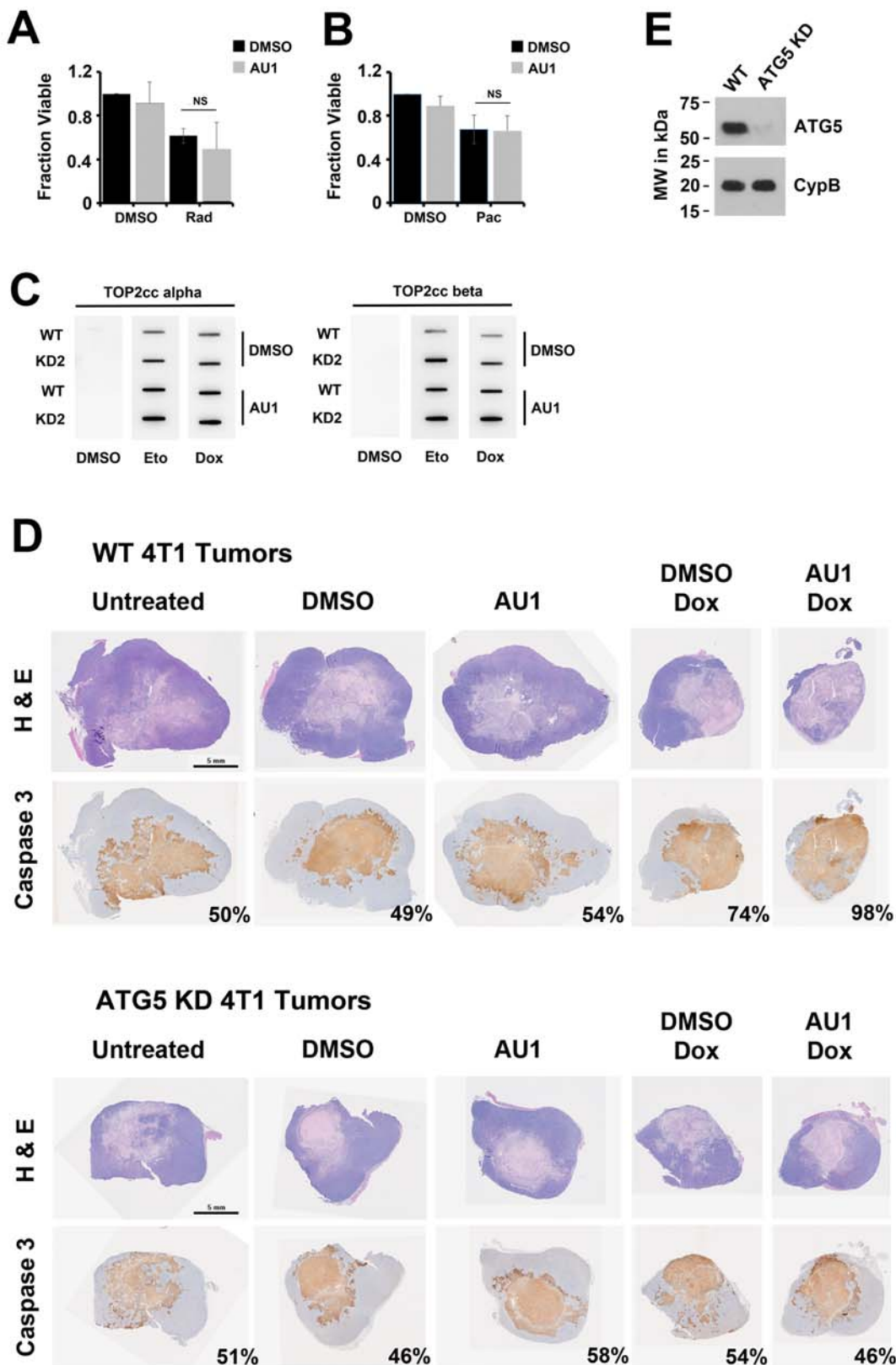
Supplemental Figure 2.) NURF Selectively Suppresses Toxicity to Topoisomerase 2 Poisons and Improves their DNA Damaging Effects. (A) Percentage of WT or BPTF KD 4T1 cells positive for DCFH-DA staining with or without 48 hours exposure to 50 nM doxorubicin. (B) Fraction of WT or BPTF KD 4T1 cells with clonogenic potential after 24 hour exposure to 50 μ M glutathione or 25 μ M N-acetylcystine with or without 50 nM doxorubicin. (C) Fraction of WT or BPTF KD 4T1 cells with clonogenic potential after 24-hour exposure to 50 nM camptothecin. (D) Fraction of WT or BPTF KD 4T1 cells with clonogenic potential after 24-hour exposure to 20 μ M ICRF-187. (E-G) Western blotting for γ H2AX from WT or BPTF KD 4T1 cells after 48 hours treatment with (E) 50 nM doxorubicin, (F) 500 nM etoposide or (G) 30 minutes after 5 Gy radiation exposure. Cyclophilin B (CypB) is the loading control. (H) Western blotting for Top2 alpha and Top2 beta from WT or BPTF KD 4T1 cells. GAPDH is the loading control. (I) Top2 association by chromatin fractionation after 20 min exposure to 100 μ M doxorubicin. Western blotting for Top2 alpha and beta were subsequently performed. Histone H3 is the loading control. (J) Representative microscopy images of WT or BPTF KD 4T1 cells untreated or treated with 50 nM doxorubicin for 48 hours stained with anti-BPTF antibodies. Phase contrast images and DAPI counter stain of nucleus is shown. Scale bar represents 20 μ m. Panels A-D = N>3 biological replicates.

Supplemental Figure 3



Supplemental Figure 3.) Validation of Autophagy by Microscopy and ATG5 KD in 4T1 Cells. (A) Representative microscopy images of WT or BPTF KD 4T1 cells stained with acridine orange after 48 hours treatment with 50 nM doxorubicin or 500 nM etoposide followed by 48 hours recovery. (B) Representative microscopy images of Lysotracker Red and Mitotracker Green stained WT and BPTF KD 4T1 cells after 48 hours treatment with 50 nM doxorubicin, 500 nM etoposide or 50 nM paclitaxel followed by 72 hours recovery. Scale bar is 20 μ m. (C) Western blotting for BPTF and ATG5 from WT or BPTF KD 4T1 cells in the absence or presence of an ATG5 KD shRNA. Cyclophilin B (CypB) is the loading control.

Supplemental Figure 4



Supplemental Figure 4.) Selective BPTF Bromdomain Inhibitor AU1 Recapitulates Effects from BPTF KD. (A, B) Fraction of WT 4T1 cells with clonogenic potential of cells treated with 10 μ M AU1 alone or in combination with (A) 5 Gy radiation or (B) after 24-hour exposure to 50 nM paclitaxel. (C) Measurement of TOP2 α or TOP2 β crosslinking in WT or BPTF KD 4T1 cells exposed to 10 μ M doxorubicin or 10 μ M etoposide using ICE assay. (D) Analysis of paraffin sections stained with hematoxylin and eosin stain or immune stained for cleaved caspase 3. Quantification of % surface area of the tumor positive for cleaved caspase 3 by Image J is shown as inset. Scale bar is 5 mm. (E) Western blotting for ATG5 from MDA-MB-231 cells expressing control (WT) or ATG5 knockdown (KD) shRNAs. Cyclophilin B (CypB) is the loading control. Panels A, B = N>3 biological replicates.