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Understanding the Role of Apoptotic DNase DFFB in Cancer Acquired Drug Resistance

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by

David Adrian Guinto Gervasio

Committee in charge:

Professor Matthew Hangauer, Chair
Professor Douglass Forbes, Co-Chair
Professor Gulcin Pekkurnaz

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The Thesis of David Adrian Guinto Gervasio is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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Figure 5 is coauthored with Gervasio, David and Williams, August. The thesis author was the primary author of this figure.

ABSTRACT OF THE THESIS

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by

David Adrian Guinto Gervasio

Master of Science in Biology

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Professor Matthew Hangauer, Chair
Professor Douglass Forbes, Co-Chair

Acquired resistance to cancer therapy is a formidable obstacle preventing cancer treatments from fully curing patients. Across multiple cancer types, analysis of tumor shrinkage in response to treatment reveals a residual, quiescent, and drug tolerant cancer cell population termed cancer persister cells. Persister cells initially survive drug treatment through reversible, non-genetic mechanisms but subsequently acquire resistance-conferring mutations and regrow to seed

the emergence of drug resistant tumors. The mechanisms by which persister cells acquire mutations are unknown. Here, we investigate our hypothesis that persister cells experience drug stress-induced sublethal apoptotic signaling which results in activation of apoptotic DNases. These DNases, which normally serve to fragment chromosomal DNA during apoptosis, instead promote DNA damage and mutagenesis within surviving persister cells allowing for regrowth into drug-tolerant expanded persister cell (DTEP) colonies. We observed that during extended treatment with targeted therapies, persister cells require apoptotic DNase (DFFB) to regrow into drug-resistant proliferating cells. Consistent with the hypothesis that persister cells are mutationally active, we also observed that persister cells exhibit elevated levels of DNA damage. This DNA damage was absent in DFFB KO persister cells, suggesting that DFFB activation is required to induce DNA damage in persister cells. These findings reveal that sublethal apoptotic signaling and activation of DFFB may play a role in persister cell mutagenesis and tumor relapse.

INTRODUCTION

Cancer relapse often occurs in patients who have initially responded to cancer treatment (Glickman & Sawyers, 2012). How initially drug-sensitive tumors become drug-resistant is one of the most important questions in cancer research because acquired resistance to cancer therapy prevents effective cancer treatments from providing durable responses (Camidge et al., 2014; Lim et al., 2018; Oxnard, 2016). It is unknown whether these drug resistant cells preexist prior to treatment or emerge during treatment; however, recent studies of acquired resistance to cancer therapy point to the latter (Ye et al., 2013). Cell culture models aimed at identifying drug resistance mutations in cancer have revealed a small subpopulation of drug tolerant cancer cells, termed persister cells, that have been observed in multiple cancer types (Sharma et al., 2010). Importantly, studies have directly revealed that persister cells can acquire drug-resistance conferring mutations in vitro (Hata et al., 2016; Ramirez et al., 2016).

Persister cells utilize non-mutational mechanisms to enter a quiescent and drug tolerant cell state through poorly understood epigenetic mechanisms (Sharma et al., 2010). The drug tolerant state of persister cells is reversible as evidenced by re-sensitization to drug following a drug holiday, mirroring retreatment responses observed following drug holidays in cancer patients (Hangauer et al., 2017; Kurata et al., 2004; Sharma et al., 2010). Analysis of the epigenetic state of persister cells has revealed that the chromatin altering enzyme, histone deacetylase KDM5, is required for persister cell formation and survival though the precise functional chromatin alterations underlying this effect are unknown (Sharma et al., 2010). It has also been discovered that histone deacetylase inhibitors selectively kill persister cells (Sharma et al., 2010). Neither KDM5 nor HDAC inhibition is adequate to fully eliminate persister cells, however. Importantly, a fraction of persister cells can exit the reversible drug tolerant state, acquire resistance-conferring

mutations not present in the primary tumor, re-enter the cell cycle, and emerge as irreversibly drug tolerant expanded persisters (DTEPs) through unknown mechanisms (Hata et al., 2016; Ramirez et al., 2016; Romano et al., 2013; Sharma et al., 2010).

The process by which persister cells enter a quiescent state and obtain drug resistance-conferring mutations to seed regrowth of genetically resistant cells in reminiscent of bacterial and fungal acquired resistance to antibiotics and antifungal agents. For example, in various bacteria and fungi, exposure to antibiotic stress triggers DNA damage, halts cell proliferation, and upregulates error-prone polymerases (Fitzgerald et al., 2017; Layton & Foster, 2003). This microbial stress response allows for populations to increase their genetic diversity and allow for subpopulations to obtain genetic resistance. It would be interesting to determine if this ancient stress response strategy has been retained in cancer persister cells to obtain resistance conferring mutations.

Like bacteria and fungi, persister cells acquire mutations allowing regrowth into DTEPs. Therefore, DTEPs differ genetically from persister cells and exhibit non-reversible drug tolerance. As a result, DTEPs are not susceptible to drug re-sensitization or histone deacetylase inhibitors likely because their drug tolerance is no longer controlled by chromatin modifications (Sharma et al., 2010). Individual DTEP colonies formed from single-cell derived NSCLC persister cells in cell culture can harbor diverse drug resistance mechanisms and mutations; many mutations of which are also found in relapsed tumors of cancer patients (Hata et al., 2016; Ramirez et al., 2016; Romano et al., 2013). These data suggest that the development of resistance through the persister state is a common route by which acquired drug resistance may occur in patients (Hata et al., 2016). Not only is this finding relevant to the discovery of drug-resistance mechanisms, it also is consistent in the clinical setting which patients develop resistance after years of initial effective

therapy (Oxnard, 2016). The development of diverse drug resistance mechanisms results in the heterogeneity of relapsed cancer cell populations and adds to the difficulty of further anti-cancer treatment (Dagogo-Jack & Shaw, 2018). Although researchers have identified persister cells' transformation into DTEPs in a wide array of cancer types, the mechanisms by which persister cells acquire mutations are unknown. Because of the above findings, persister cells have received increased attention for their roles in acquired drug resistance (Oxnard, 2016).

Here, we wish to explore the central and critical question: How do persister cells acquire mutations? Previous research on cancer mutagenesis details how genetic mutations can occur following errors in DNA replication; however, persister cells are arrested in a non-dividing, quiescent state and therefore do not actively replicate their DNA (Gaillard et al., 2015; Sharma et al., 2010). It is also believed that DNA mutations in cancer can be a result of exposure to reactive oxygen species (ROS); however, persister cells also do not consistently exhibit increased levels of ROS (Hangauer et al., 2017; Jeggo et al., 2016). Therefore; replication mistakes and increased exposure to ROS and other DNA damaging agents are unlikely to contribute to persister cell mutagenesis.

A possible hint as to how genetic mutations can occur in the absence of DNA replication or exposure to increased ROS levels follows a recently discovered phenomenon in non-persister cell types (Ichim et al., 2015; Tang et al., 2012). The mechanism describes how cells pushed to the brink of death from a variety of stresses, including drug stress, experience sublethal apoptotic signaling but avoid death allowing them to recover and proliferate (Tang et al., 2012). Initiation of apoptotic responses were confirmed by the presence of late-stage apoptosis hallmarks such as mitochondrial permeabilization, caspase-3 activation, and apoptotic DNase-mediated DNA fragmentation, each of which were previously thought to be part of an irreversible path to cell

death (Tang et al., 2012). Cells that recover from apoptosis can acquire genetic mutations through chromosomal breakage, chromosomal loss, and unrepaired DNA damage as a result of the initial apoptotic response (Tang et al., 2012). Therefore, these cells have the potential for higher tumorigenicity (Tang et al., 2012). Related studies on the reversible apoptotic response reveals how low levels of mitochondrial permeabilization may play a role in non-lethal apoptotic pathway activation (Ichim et al., 2015).

Mitochondrial outer membrane permeabilization (MOMP), a hallmark of apoptosis, was previously thought to lead to rapid cell death; however, it was recently discovered that MOMP can be activated in a small number of mitochondria without killing the cell. The limited mitochondrial permeabilization in drug-stressed cells has been termed minority MOMP. Minority MOMP causes cells' mitochondria to release small amounts of cytochrome c into the cytosol which activates apoptotic caspase signaling and the activation of downstream DNases (Ichim et al., 2015). As part of the apoptotic program, activated mitochondrial DNases (ENDOG and AIF) are translocated to the nucleus (Arnoult et al., 2003). Caspase-activated DNase DFFB is also activated by cleavage of its inhibitor (ICAD) by caspase 3 (Xinjian Liu et al., 2015; Xuesong Liu et al., 1997; Sakahira et al., 1998). Activation of these DNases normally triggers DNA fragmentation followed by cell death during apoptosis (Sakahira et al., 1998). However, sublethal activation of caspases following minority MOMP is associated with increased genomic instability and tumor initiation as a result of unrepaired DNA damage without cell death (Ichim et al., 2015; Tang et al., 2012). These findings focused on the impact that this phenomenon may have on tumor initiation. Rather than resistance mutations arising through DNA replication errors or exposure to mutagens, we hypothesize that persister cells also experience drug stress-induced sublethal apoptotic signaling which results in the activation of apoptotic DNases. These DNases may promote DNA damage

and mutagenesis leading to the acquisition of drug resistance-conferring mutations that allow surviving persister cells to regrow into drug-tolerant expanded persister cell colonies.

Here we determine the role of apoptotic DNase DFFB in persister cell mutagenesis and acquired drug resistance by utilizing CRISPR gene knockout, cell viability assays, and immunoblotting techniques. Our results reveal a potential therapeutic target to prevent the emergence of drug resistant tumors by preventing the acquisition of resistance conferring mutations and regrowth in cancer persister cells.

MATERIALS AND METHODS

Cell culture. EGFR-mutant non-small-cell lung cancer PC9 (Altschuler Lab) was cultured in RPMI-1640 medium supplemented with penicillin, streptomycin and 5% FBS. BRAF-mutant A375 melanoma (ATCC) cells were cultured in DMEM supplemented with penicillin, streptomycin and 10% FBS. Each cell line was maintained in a 5% CO2 atmosphere at 37 °C.

Persister cell derivation. Persister cells were derived from treatment of EGFR-mutant non-small-cell lung cancer PC9 with 2.5 μM erlotinib for at least 9 days or treatment of BRAF-mutant A375 melanoma cells with 0.25 μM dabrafenib and 25 nM trametinib for at least 14 days with fresh drug added every 3 days, unless otherwise stated.

Drug tolerant expanded persister cell derivation. Drug tolerant expanded persister cells were derived from treatment of EGFR-mutant non-small-cell lung cancer PC9 with 2.5 μM erlotinib for at least 5 weeks or treatment of BRAF-mutant A375 melanoma cells with 0.25 μM dabrafenib and 25 nM trametinib for at least 7 weeks with fresh drug added every 3 days.

Cell viability assay. 500 cells per well were plated in 12-well plates and allowed to adhere for 24 hours. Cells were then treated to derive persister cells. Cell viability was assessed using CellTiter Glo (Promega) after derivation of PC9 and A375 persister cells. P values calculated using a two-tailed t test; $p < 0.05$ is significant.

Quantifying DTEP colonies. Biological triplicate plates of DTEPs were derived with the protocol described above. Colonies and colony sizes were counted by hand through visual inspection under microscope. P values calculated using a two-tailed t test; $p < 0.05$ is significant.

Chemicals. Erlotinib hydrochloride and dabrafenib were purchased Selleck Chemicals. Trametinib was purchased from ApexBio. Q-VD-OPh was purchased from MedKoo Biosciences.

Etoposide was purchased from Research Products International. All chemicals were stored as stock solutions in DMSO (Thermo Scientific).

CRISPR-mediated DFFB deletion. CRISPR-mediated editing was previously performed by the UC San Francisco Cell and Genome Engineering Core following a previously published protocol (Hangauer et al., 2017). A375 DFFB knockout clones KO1 and KO2 were both generated using the same sgRNA 5'-CAGCCCGAGGAAGTTCGGCG-3'. PC9 DFFB knockout clones KO1 and KO2 were both generated using the same sgRNA 5'-GCTCCGTGCCATCCTCGTAC-3'. All KO clones were confirmed by western blot.

Immunoblotting. Cancer persister and DTEP cell were derived in 10 or 15 cm plates. Cells were then washed with PBS and lysed using RIPA buffer (Thermo Scientific) supplemented with Phosphatase inhibitor (Thermo Scientific) and protease inhibitor (Thermo Scientific). Lysates were centrifuged at 13,000g at 4 ℃ for 5 min, and the protein concentration of the supernatant was determined using the Pierce BCA Protein Assay Kit. Lysates were mixed with sample buffer (Thermo Scientific) and denatured at 70℃ for 10 min. Samples were separated by SDS–PAGE (NuPage 4–12% Bis-Tris Gel, Life Technologies), run with Chameleon 700 Pre-stained Protein Ladder, and transferred to a nitrocellulose membrane using an iBlot system (Life Technologies). Membranes were blocked with 5% BSA for 1 h at room temperature, and then incubated with primary antibody at 4 ℃ overnight. LICOR secondary antibodies were then incubated with the membrane for 1 h at room temperature, and the membrane was imaged using the LICOR Odyssey Imaging System. β-Tubulin levels were measured as a loading control. Antibodies commercial sources were: γH2AX (Cell Signaling Technology, #9718); β-Tubulin (Invitrogen, BTR7); LICOR IRDye 680RD (LICOR); DFFB (LifeSpan Biosciences, LS-C64703)

Differential Expression Analysis. A375 parental and persister cells were prepared for single cell RNA-sequencing using 10x Genomics Single Cell Protocols for sequencing on 10x Genomics Chromium System. Data analyses were performed using the Seurat (v3.1) R package.

RESULTS

We sought to identify a mechanism by which persister cells acquire mutations to regrow and seed the emergence of drug resistant tumors (Figure 1a). We focused our work on the BRAF-mutant melanoma cancer line A375 and EGFR-mutant non-small-cell lung cancer line PC9. Upon treatment for 15 or 9 days, respectively, with cytotoxic concentrations of targeted chemotherapy drugs clinically used for BRAF mutant melanoma (BRAF inhibitor dabrafenib and MEK inhibitor trametinib) and EGFR mutant non-small cell lung cancer (EGFR inhibitor erlotinib), reveals a small population of surviving persister cells (Figure 1b, c). Extended treatment of persister cells with targeted chemotherapy drug for 4 or more weeks results in the emergence of a small number of actively dividing drug tolerant expanded persister cell (DTEP) colonies (Figure 1b, c). These cell culture models recapitulate the process of acquired drug resistance.

To test our hypothesis that persister cell regrowth into DTEPs depend on apoptotic DNases that promote DNA damage and mutagenesis, we obtained CRISPR-mediated DFFB knockout A375 and PC9 cells (Figure 2a). If persister cells require apoptotic DNases to promote mutagenesis, then we would expect that DFFB knockout cells cannot acquire resistance conferring mutations allowing for regrowth. We confirmed that DFFB knockout does not drastically affect persister formation or survival in both cancer lines (Figure 2b-e). However, while DFFB knockout does not affect persister cell viability, we observed that during extended treatment with targeted therapies, DFFB KO persister cells are unable to regrow into drug tolerant expanded persister (DTEP) colonies in both our cancer cell models (Figure 2f, g). Following at least 5 consecutive weeks of targeted cancer therapy, no colonies greater than 25 cells formed from DFFB knockout persister cells (Figure 2f, g).

Consistent with our hypothesis that persister cells activate apoptotic DNases that promote DNA damage and mutagenesis, we also observed that persister cells exhibit elevated levels of DNA damage marker γH2AX (Figure 3a, b). Furthermore, consistent with DTEPs evading drug stress due to acquisition of resistance mutations, preliminary results indicate that DTEPs exhibit lower levels of γH2AX than persister cells, but higher than untreated parental cells (data not shown). In contrast to wild type persister cells, DNA damage marker γH2AX was absent in DFFB knockout persister cells (Figure 3c). To further confirm that γH2AX signal in drug tolerant cells is a result of DNase mediated DNA damage, we treated DTEP cells with pan-caspase inhibitor Q-VD-OPh which resulted in decreased γH2AX signal (Figure 3d). These data support the hypothesis that apoptotic caspase signaling activated DFFB in persister cells, and that DFFB activity is required for DNA damage and mutagenesis leading to acquisition of resistance mutations and DTEP formation.

Following our experiments on the effects of DFFB in persister cells, we utilized single cell RNA-sequencing in order to identify potential genes and pathways unique to persister cells and DTEPs. Preliminary analysis of the scRNAseq data revealed distinct populations of parental, persister, and DTEP cells, wherein persisters and DTEPs clustered closer to each other than to parental cells, confirming the difference in transcriptional activity between these cancer cell states (Figure 5a). We also observed a small population of cycling persister cells that can be distinguished from quiescent persister cells (Figure 5b). These rich data will be explored further in the future to characterize changes that occur during formation of persister cells and expansion into DTEPs.

DISCUSSION

Our data reveal that during extended treatment with targeted therapies, persister cells require the activation of apoptotic DNase DFFB to promote persister cell mutagenesis and regrowth (Figure 2f, g). The activation of DFFB in the absence of cell death suggests that persister cells may require sublethal apoptotic signaling in the form of mitochondrial outer membrane permeabilization (MOMP). Recent studies have identified that cells under stress may exhibit sublethal activation of apoptotic DNases through minority MOMP (Gong et al., 2019; Ichim et al., 2015; Tang et al., 2012). Therefore, we propose that persister cells activate DFFB through similar mechanisms to induce DNA damage and promote mutagenesis required for the acquisition of drug resistance mutations and tumor relapse.

To explore the role of DFFB in persister cell mutagenesis and regrowth we investigated DNA damage as a marker for mutational activity. The presence of DNA damage in persister cells has previously been reported; however, no studies have revealed which processes contribute to increased DNA damage in persister cells (Raha et al., 2014; Russo et al., 2019; Sharma et al., 2010). Analysis of DNA damage in our persister cell models reveal that DNA damage is significantly inhibited in DFFB knockout persister cells (Figure 3a). This suggests that DNA damage in persister cells is a result of activated DFFB. Furthermore, by blocking the activation of DFFB using pan-caspase inhibitor Q-VD-OPh, we were able to inhibit the induction of DNA damage in drug tolerant expanded persister cells (Figure 3c). Recent studies have identified an upregulation of error-prone polymerases and downregulation of DNA repair enzymes in persister cells, which in combination with our findings may present a mechanism for how mutations are introduced in persister cells (Russo et al., 2019). Our results indicate that DFFB mediated DNA

damage may be an important precursor for the acquisition of drug-resistance mutations in persister cells.

It is possible that DNA damage in persister cells is not exclusively induced by the activation of DFFB as persister cells may also utilize other apoptotic DNases such as ENDOG and AIF, both which are directly released from the mitochondria during apoptosis (Arnoult et al., 2003). Therefore, investigating the activation of ENDOG and AIF in persister cells may be useful to determine if DNA damage is also induced by other apoptotic DNases.

Using the results of these experiments, we have proposed a potential mechanism persister cells may use to introduce resistance conferring mutations and regrow into drug tolerant expanded persister cells (Figure 4). Our model proposes that persister cells exhibit sublethal levels of MOMP resulting in apoptotic signaling which includes cytochrome c release, caspase 3 activation, and subsequently DFFB activation which leads to increased DNA damage. This proposed mechanism may allow persister cells to become DTEPs.

To further characterize the persister cell state and transition into DTEPs, we are currently utilizing differential expression analysis of parental, persister, and DTEP cell scRNAseq of our model cancer cell lines A375 and PC9. Preliminary results reveal distinct cell populations separating parental, persister, and DTEP cells (Figure 5a). Interestingly, analysis of cell cycle reveals that a majority of persister cells are arrested in G1, a non-dividing stage of the cell cycle; however, there are a subset of persister cells which have re-entered the cell cycle and cluster close to DTEP cells (Figure 5b). Following our model, we hypothesize these cycling cells, which have re-entered the cell cycle, have acquired mutations through sublethal apoptotic signaling and have begun to transition into the DTEP state where they can regrow without apoptotic stress. Further analysis of this data can potentially provide new information about biological processes and

molecular functions that are unique to persister and DTEP cells as well as provide us with potential therapeutic targets to prevent persister cell mutagenesis.

In summary, drug-tolerant persister cells have recently gained attention for their possible role in acquired drug resistance which may seed the emergence of drug resistant tumors (Oxnard, 2016). Here we have shown that apoptotic DNase DFFB is required for persister cell regrowth and the induction of DNA damage in persister cells which may allow for the acquisition of mutations. We also propose a model for how persister cells exhibit sublethal levels of apoptotic signaling to transform into DTEPs. These results support our hypothesis that cancer persister cells require sublethal levels of apoptotic signaling as a mechanism to introduce resistance conferring mutations and allow for tumor regrowth.

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FIGURES

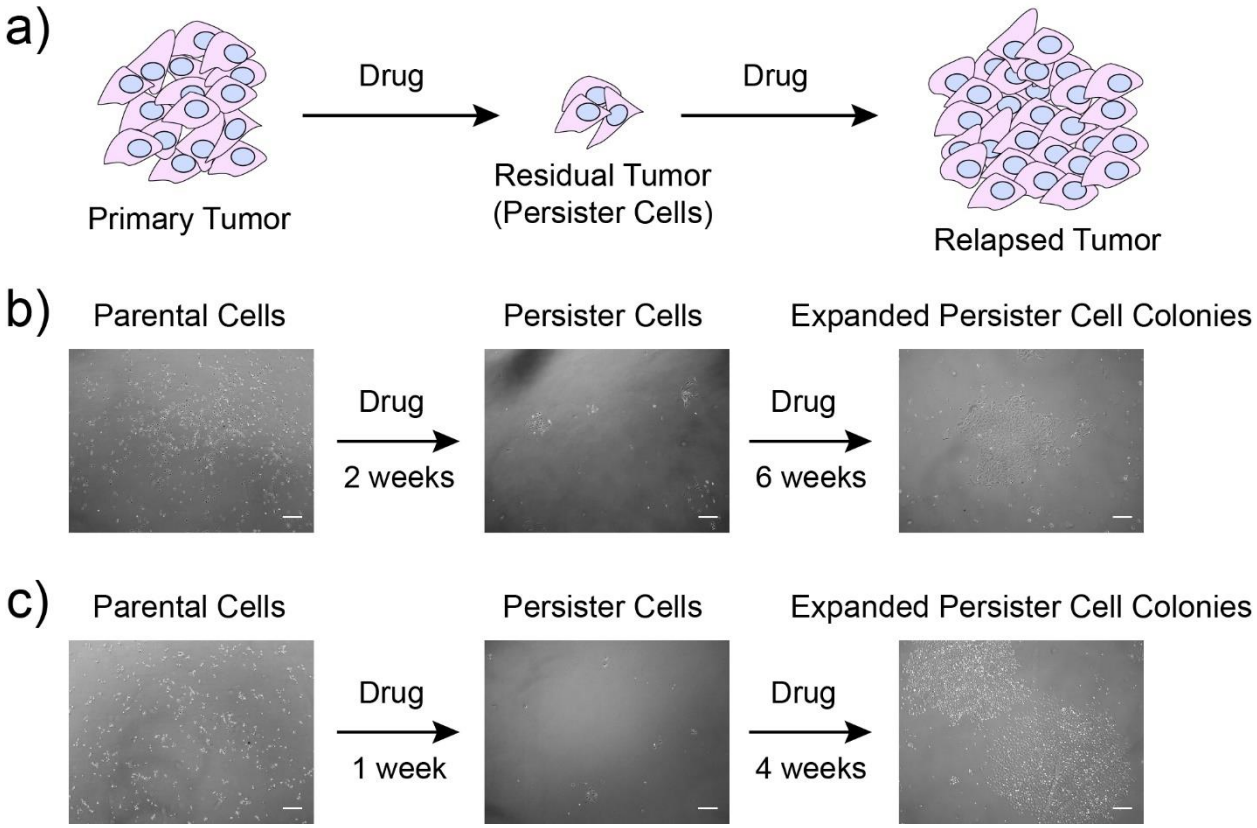

Figure 1. Schematic and generation of persister cells. a) Cartoon depicting acquired drug resistance. **b)** Cell culture model of acquired drug resistance in A375 melanoma cells. **c)** Cell culture model of acquired drug resistance in PC9 adenocarcinoma cells. A375 melanoma cells were treated with 0.25 μM dabrafenib and 25 nM trametinib. PC9 adenocarcinoma cells were treated with 2.5 μM erlotinib. Scale bar = 10 μm.

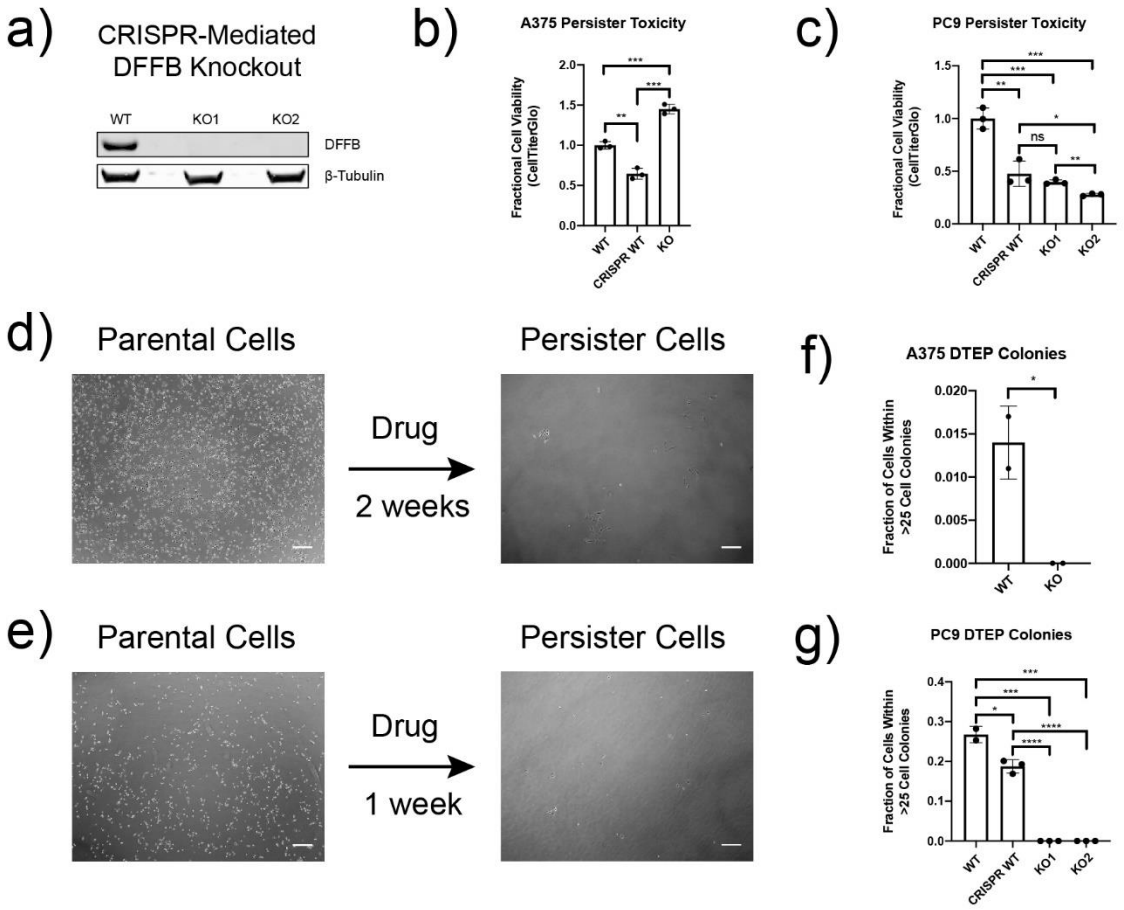

Figure 2. Persister cells require apoptotic DNase DFFB to relapse. a) CRISPR knockout of apoptotic DNase DFFB in two A375 cell lines. **b)** A375 DFFB KO does not negatively affect persister cell viability. **c)** PC9 DFFB KO does not strongly negatively affect persister cell viability. **d)** Cell culture of A375 DFFB KO cells depicting the formation of persister cells. **e)** Cell culture model of PC9 DFFB KO cells depicting the formation of persister cells. **f)** A375 DFFB KO persister cells are unable to regrow into drug resistant expanded persister cell colonies. **g)** PC9 DFFB KO persister cells are unable to regrow into drug resistant expanded persister cell colonies. A375 melanoma cells were treated with 0.25 μM dabrafenib and 25 nM trametinib. PC9 lung cancer cells were treated with 2.5 μM erlotinib. CRISPR WT cells refers to control cells placed through the CRISPR editing and single cell clonal population derivation process, but which were not successfully edited. P values calculated using a two-tailed t test; * is $p < 0.05$, ns is not significant $p > 0.05$. Scale bar = 10 μm.

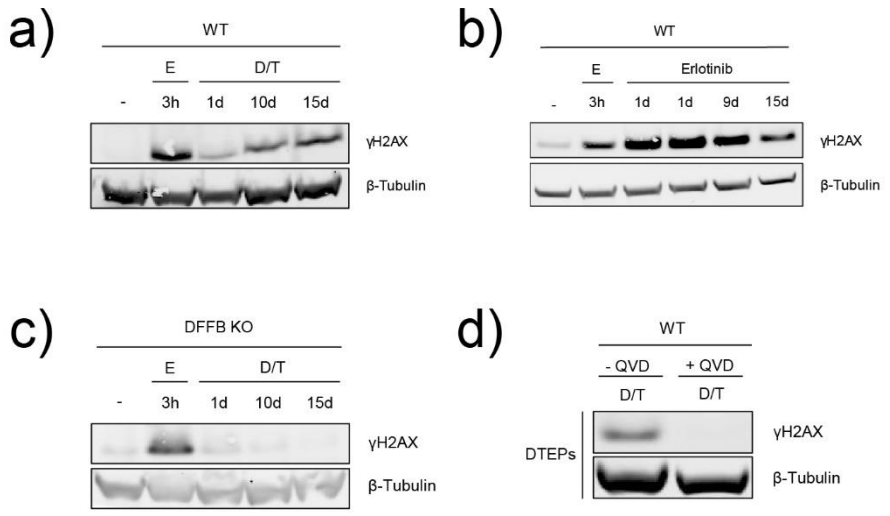

Figure 3. Apoptotic DNase DFFB is required to induce DNA damage in persister cells. **a)** Western blot analysis indicating increased γH2AX expression, a marker of DNA damage, in A375 persister cells (15 days drug treatment) **b)** γH2AX expression is increased in PC9 persister cells (9 days drug treatment). **c)** γH2AX expression is absent in A375 DFFB KO persister cells **d)** Western blot analysis indicating decreased γH2AX expression in A375 DTEPs co-treated with 10 μM pan-caspase inhibitor Q-VD-OPh. A375 cells were either left untreated, treated with 100 μM etoposide for 3 hours, or treated with 0.25 μM dabrafenib and 25 nM trametinib for one to 15 days and assessed at several time points (3h, 1d, 10d, 15d) during treatment. PC9 cells were either left untreated, treated with 100 μM etoposide for 3 hours, or treated with 2.5 μM erlotinib for one to 15 days and assessed at several time points (3h, 1d, 9d, 15d) during treatment. Note that at day 15, PC9 cells have begun to form DTEPs while at day 9 PC9 cells remain purely quiescent persister cells. Etoposide used as positive control for DNA damage. β-Tubulin used as loading control. E is 100 μM etoposide; D/T is 0.25 μM dabrafenib and 25 nM trametinib; QVD is 10 μM Q-VD-OPh; h is hours; d is days.

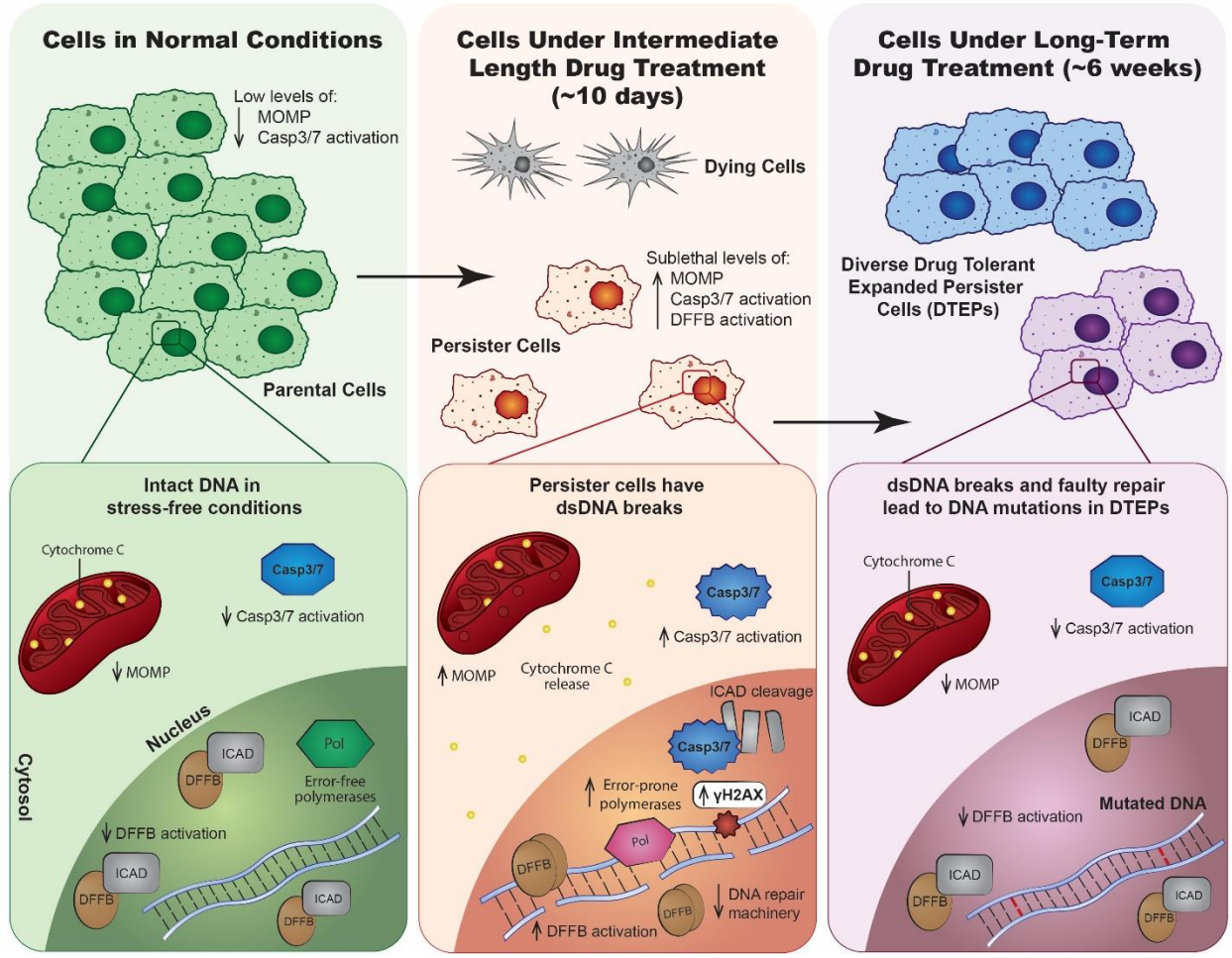

Figure 4. Cartoon depicting the function of DFFB in persister cell mutagenesis. Our proposed mechanism of DFFB mediated persister cell mutagenesis is depicted. Upon release of cytochrome c from the mitochondria during intermediate length drug treatment (~10 days), caspases 3 and 7 cleave the inhibitor of DFFB (ICAD) which results in DFFB mediated DNA damage and increased expression of DNA damage marker γH2AX. Faulty repair following DNA damage by DFFB leads to DNA mutations in DTEPs.

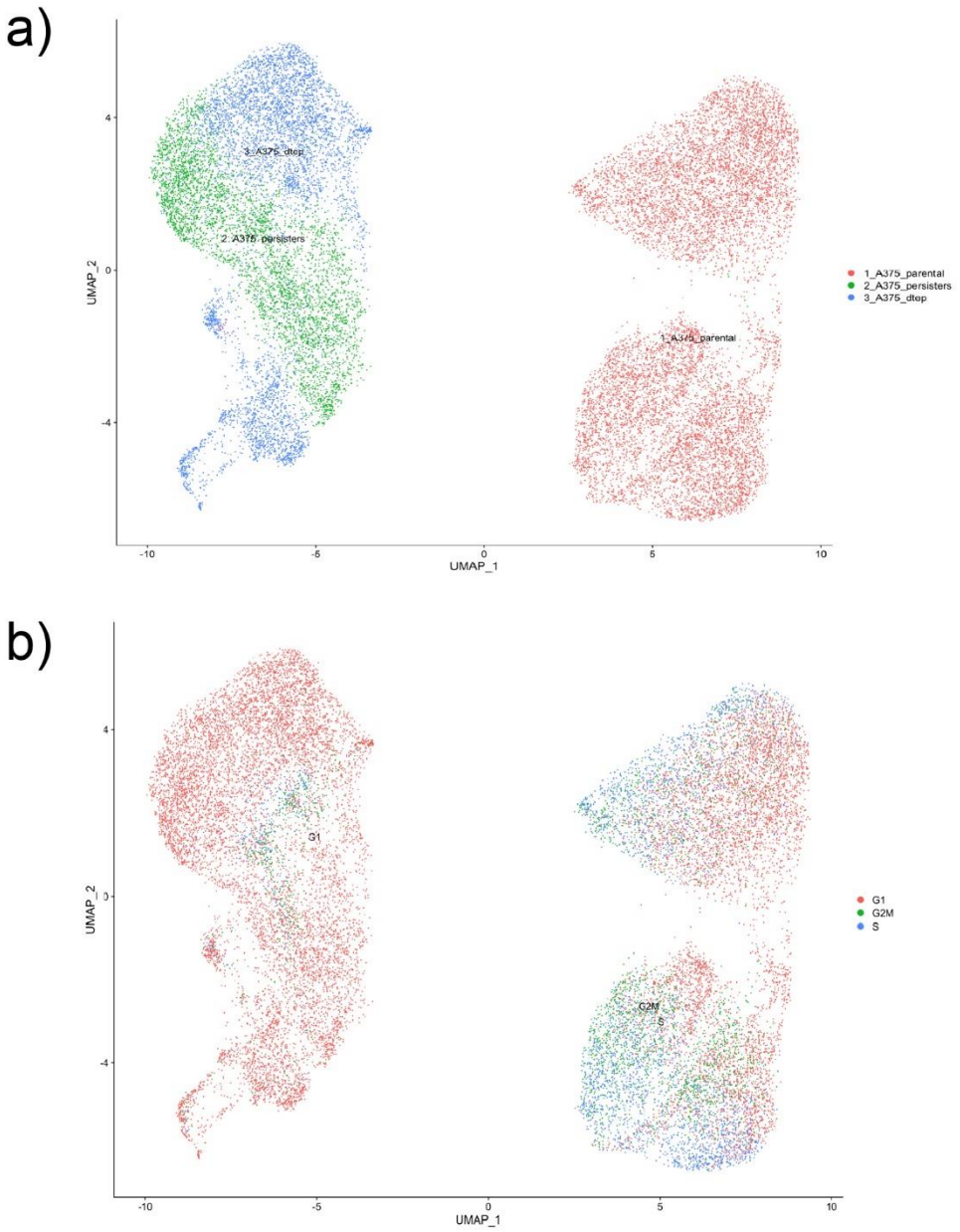

Figure 5. Single Cell RNA-sequencing UMAPs of A375 parental, persister, and DTEP cells. a) Colors represent cell type. Red is parental; green is persister; blue is DTEP. b) Colors represent cell cycle stage. Red is G1; green is G2M; blue is S. A majority of persister cells are arrested in G1 with a subpopulation of persister cells re-entering the cell cycle.

Figure 5 is coauthored with Gervasio, David and Williams, August. The thesis author was the primary author of this figure.