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The document was well written. The abstract, introduction, and results were excellent. Presentation of the data could have been better for the western blots. The blots contained markings on the image itself, The original image should be labelled outside of the blot. I like that you have put thought into the implications of the work. Good luck in the future.

Final Grade Submitted for BIOC 4908 (Letter): A*

Knockout of DROSHA Increases Sensitivity of HCT 116 Cells to Apoptosis Induced by Pladienolide B and DRB

By

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Bachelor of Science- Biochemistry (Hons)

Abstract

MicroRNAs (miRNAs) are short non-coding RNAs involved in post-transcriptional regulation by gene silencing that play important roles in drugs responses. DROSHA is a nuclease that is required to produce miRNAs, so DROSHA null cells fail to produce miRNAs. DROSHA null HCT 116 colon cancer cells were used to determine the role of DROSHA and miRNAs in the cellular response to two drugs that inhibit RNA synthesis and pre-mRNA splicing, 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), and pladienolide (PB), respectively. It was found that the DROSHA-null cell line was more sensitive to cell death induced by both drugs relative to its parental control. This was characterized by increased DNA fragmentation and cell death induced by DRB but not PB could be inhibited with a broad range caspase inhibitor (ZVAD). The rescue of DRB treated Dro -/- cells suggest that lack of miRNA system leads to apoptosis through a caspase dependant pathway and that this effect is agent-specific.

Acknowledgements

I would like to thank my supervisor, Dr. Bruce McKay for his support and contribution to my learning during the past year. I appreciated his guidance in allowing to better myself in pursuing scientific research. I would also like to thank my parents and members of the McKay lab for contributing to an unforgettable work environment and for also teaching me all the skills that were the groundwork for all my future work. I would also like to thank my colleagues who made this experience more fun when shared.

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1 Introduction

1.1 microRNA function

Regulation of gene expression is a process fundamental to the maintenance of proper homeostasis and information exchange between a cell's environment and its action. Throughout the history of molecular biology, several mechanisms of regulations have been recognized, each at a critical interface (Buccitelli & Selbach, 2020). Transcriptional regulation involves chromatin modification, recruitment of transcription factors and epigenetic modifications affecting transcription of mRNA from genomic DNA. Post-translational regulation involves protein-protein interactions, or side chain modifications that alter protein function and role in cellular fate. Post-transcriptional regulation is studied in most details to unravel its inherent complexity. Processes like poly-adenylation and alternative splicing of mRNA transcription directly affect turnover to residue chains from genetic information (Bentley, 2014). Other methods of modulating mRNA translation are through interaction of transcripts with RNA binding proteins and non-coding RNAs. These have direct effects on mRNA localization and rate of decay. ncRNAs involved in regulating gene expression are siRNAs (small-interfering RNAs), lncRNAs (long non-coding RNAs), piRNAs (piwi-interacting RNAs), and miRNAs (microRNAs) (Kaikkonen et al., 2011). miRNAs are being studies as means to provide novel therapeutic strategies.

MicroRNAs (miRNAs) are small ncRNAs (~ 22 nt long) essential to post-transcriptional regulation of gene expression. They impact the stability and translation of mRNA through a negative feedback system, attenuating gene expression (Friedman et al., 2009). They stabilize the information transfer from genomic DNA to functional protein ensuring proper balance at the transcriptome-proteome interface. They confer that stability to numerous control systems,

spanning memory consolidation and learning in neurones, sleep patterns and dysregulations, embryonic development and cellular differentiation, apoptosis, and response to cellular stressors (Abdelfattah et
al., 2014). Similar to siRNAs, they bind to competent mRNA transcripts to repress translation (Bartel, 2004). Target binding by miRNAs require partial complementarity between the miRNA 5' seed sequence and the 3'-untranslated region (UTR) of the mRNA transcript. The seed sequence refers to the nt 2-8 of the 5' end of a mature miRNA transcript (Schmittgen, 2008). 80% of miRNA-mRNA target interaction occur with perfect or near perfect complementarity (~1 nt mismatch). The mechanisms of downregulation gene expression include direct mRNA cleavage or repressing translation by blocking translation initiation. Interestingly, Ribosome Profiling elucidated that miRNA initially inhibit gene translation through repression and the majority of miRNA related gene expression depression occur through mRNA decay (Eichhorn et al., 2014).

It is estimated that more than 50% of human genes are subjected to miRNA regulation. In humans, the ratio of miRNA to protein-coding genes as about 2000 to 22, 000 (Kehl et al., 2017; Naeli et al., 2022). With the number of genes vastly outnumbering the number of miRNAs, it follows that a single miRNA can regulate numerous mRNAs. Also, a single gene can also regulate more than one miRNA. Considering their previously mentioned span of stability induction, they form a most intricate network (Gebert & MacRae, 2019).

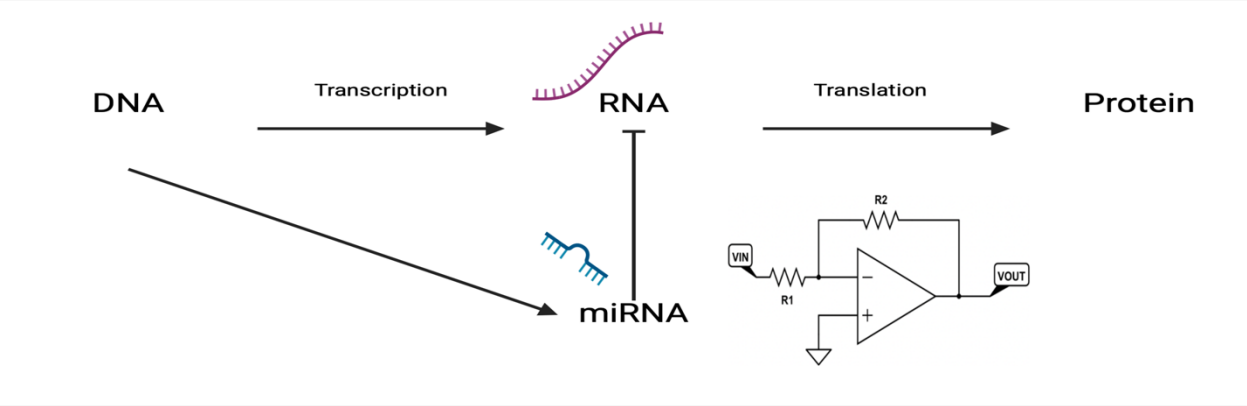

Figure 1: miRNAs downregulate expression by transiently or permanently preventing the translation of mRNA into proteins. It is a negative-feedback stabilizing system for the central dogma.

1.2 Canonical miRNA Processing

miRNA genes are found between coding genes, throughout the genome, where they are transcribed by RNA Polymerase II into primary miRNAs (pri-miRNAs) with a looped. hairpin-like structures (Gregory et al., 2004; Lee et al., 2004). Although the majority are transcribed by RNAPII, a few of them require RNAPIII (Ohler et al., 2004). Pri-miRNA transcripts can range from 100s to 10, 000 bp in length (Bartel, 2004; Saini et al., 2007) . They are made of dsRNA forming an open loop at the top. There are ssRNA flanking both sides of the stem-loop structure (Auyeung et al., 2013). Pri-miRNA hairpin structures are recognized and processed in the nucleus the ribonuclease III enzyme DROSHA and its RNA binding co-factor DiGeorge Critical Region 8 (DGCR8), forming the microprocessor complex (Gregory et al., 2004). DROSHA recognizes the pri-miRNA and process it by cleaving the flanking ssRNAs. Accurate cleaving is produced as DGCR8 binds to the junction just between dsRNA of the stem-loop and the ssRNA. Cleavage occurs approximately 11 bp downstream of the junction. The result is precursor miRNA (pre-miRNA) with a 2 nt, 3' extension (Bartel, 2004).

The pre-miRNA is then exported to the cytoplasm via Exportin-5 (XPO5) through the nuclear membrane. There, it is processed by DICER, another RNase III enzyme. DICER, in cooperation

with accessory proteins (HSP90, TRBP, PACT), cleave the hairpin approximately two helical turns from its base. This forms a miRNA duplex. The miRNA duplex consists of two strands: the 5p strand and the 3p strand, from each side of the hairpin (Medley et al., 2021). The choice is dependent on thermodynamic properties.

The duplex is loaded onto an Argonaute (AGO) protein forming the core of the RNA-induced silencing complex (RISC)

Following loading, the passenger strand is degraded, while the guide strand remains bound to AGO.

The RISC-miRNA complex identifies target mRNAs through sequence complementarity, particularly via the conserved 5' seed region of the miRNA. Upon binding, miRNAs either cause direct cleavage of the target mRNA or by repressing its translation. The choice of mechanism is context-dependent sensitive to cellular environment, species, and other such factors (Naeli et al., 2022).

The dampening of translation is thought to be blocking binding of the eukaryotic initiation factor eIF4E to the 5' cap structure of target mRNAs, preventing recruitment of ribosomes or inhibiting elongation (Naeli et al., 2022). While translational inhibition is often the initial mode of repression, long-term gene silencing in mammalian cells is predominantly mediated through mRNA destabilization and decay (Eichhorn et al., 2014).

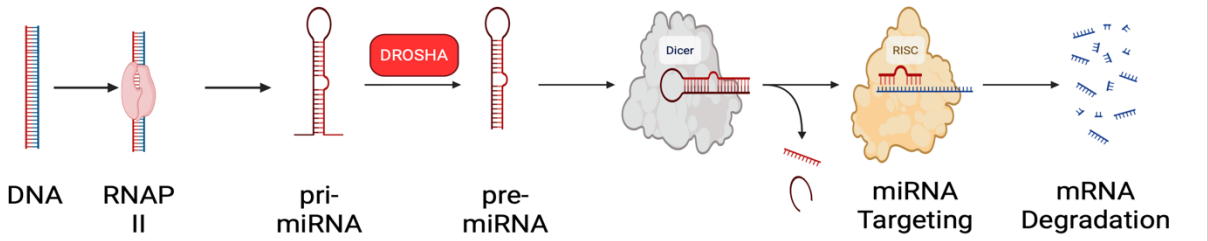

Figure 2: Canonical miRNA biogenesis pathway. MicroRNAs are transcribed from DNA to primary miRNA by RNAP II and processed by the DROSHA/DGCR8 to pre-miRNAs in the nucleus. They are exported into the cytoplasm, cleaved by DICER to keep only one guide strand, and loaded onto the RNA-induced silencing complex (RISC). Mature miRNAs guide the RISC complex to competent mRNA transcripts for destabilization and/or degradation.

1.3 non-Canonical miRNA Processing

While most microRNAs (miRNAs) follow the canonical biogenesis pathway involving the sequential action of the RNase III enzymes DROSHA and DICER, a subset of miRNAs bypasses one or both of these processing steps. These are referred to as non-canonical miRNAs (Abdelfattah et al., 2014). Non-canonical miRNAs can be generated independently of DROSHA, DICER, or both, and they are often detectable in experimental models where canonical processing is genetically disrupted.

Several classes of DROSHA/DGCR8-independent miRNAs have been identified, including mirtrons, snoRNA, miRNAs, short hairpin RNAs (shRNAs), and tRNA-derived fragments (tDRs) (Abdelfattah et al., 2014). Mirtrons originate from intronic sequences and bypass Microprocessor cleavage. Instead, they are processed by the spliceosome to form shorter hairpin structures, allowing them to escape DROSHA recognition, transported to the cytoplasm, where they are cleaved by DICER to form miRNAs (Berezikov et al., 2007).

Small nucleolar RNAs (snoRNAs), involved in rRNA modification, have also been shown to interact with Argonaute proteins and regulate mRNA translation. While some snoRNAs require DROSHA, some are independent, however, still requiring DICER for their maturation. (Stavast & Erkeland, 2019; Abdelfattah et al., 2014).

Although the absence of DICER abolishes most canonical miRNA biogenesis, a few DICER-independent miRNAs remain. The most well-characterized example is miR-451, which is processed by DROSHA/DGCR8 in the nucleus but is too short to serve as a substrate for DICER. Instead, it is directly loaded onto AGO. Another rare class, the simtrons (splicing-independent mirtron-like miRNAs), are uniquely dependent on DROSHA but do not require DGCR8, Exportin-5, DICER, or AGO2. Simtrons such as miR-1225 and miR-1228 have been shown to function in RISC-mediated gene silencing (Havens et al., 2012).

Despite the redundancy of the numerous possible combinations of these alternative processing routes, the functional contribution of non-canonical miRNAs to normal cellular physiology remains incompletely understood. Knock out of DROSHA, DGCR8, DICER, or AGO results in severe phenotypes, including embryonic lethality in knockout mice (Park et al., 2010) and fatal inflammatory disease in DROSHA K/O in T-cells (Chong et al., 2008). These observations present the essential role of canonical miRNA pathways in maintaining cellular homeostasis, while showing the unsatisfactory compensation of non-canonical miRNA biogenesis.

Studies on the consequences of canonical miRNA loss, a DROSHA-null cell line was developed using CRISPR-Cas9–mediated deletion of the RNase IIIa domain of DROSHA (Kim et al., 2016). 96.5% of miRNAs present in wild-type cells were unavailable in the absence of DROSHA. Some non-canonical miRNAs persisted, including 5'-capped pre-miRNAs and mirtrons that do not require DROSHA processing.

1.4 miRNAs in Drug Response

It is thought that more than 50% of human genes are under miRNA regulation. It is also reasonable to suppose that the locus of miRNA regulation also covers genes related to cellular stress events (Rukov & Shomron, 2011). Artificial induction of cellular stress in target cells is

the founding philosophy of chemotherapy. Drug treatments are prescribed to achieve these effects. As reaction to this disruption in cellular homeostasis, miRNA can either accelerate the cell to apoptosis or stabilize the compromised cell such that it maintains survival and proliferation. There is evidence that miRNA contributes to both.

The buffer against intra-cellular chaos can occur at different levels for damage induced by drugs. The alteration of cross-membrane transport can determine the fate of a cell based on its intake or rejection of toxic compounds. Transporter proteins are known targets of the miRNA regulatory network. Decreasing gene expression of a transport protein ensures that there are less paths for the drug to get into the cell to cause its fatal effects. Other miRNAs might regulate the genes that code for the enzyme that functionalize and conjugate drugs to convert it into its active form. Other mode of action includes upregulation of protein to trigger drug breakdown or defunctionalisation such that its interaction with its target cellular component is impaired. Since miRNA is a negative regulator, this would be achieved through decreasing basal miRNA transcript to produce a higher amount of these enzymes. Other mode of regulation might be targeting efflux transporters such that the drugs are flushed out of the cell before it has had its effects. These are all means that the cell might escape poisoning by decreasing the interaction of the drug with the cell. Other means of regulation could also involve after the damage has been done; if an organelle, apparatus, or cellular structure is damaged causing distress in the intracellular environment, miRNA might be able to help alleviate this tension, by decreasing rate of reaction within cells so that repair can occur, providing alternative means of fulfilling the function, or even shutdown a pathway if it is auxiliary to cell survival and its maintenance cause more damage. Other more concrete means of preserving cellular life in chaotic intracellular environment, is to dampen the canonical response to irreversible damage. It can mitigate the

signal which would trigger apoptosis and result in cell survival. Ans similarly for the opposite side, miRNA can also help accelerate the cells towards apoptosis in events of cellular stress.

1.5 miRNA in Cancer Cells

Research has presented conclusions the intricate and multifaceted nature of miRNAs. Cancer cells can adopt resistance to chemical agents by altering the expression of target membrane protein like ATP-binding-cassette (ABC) transporters, enhancing efflux of drugs (Chen et al., 2015). MicroRNAs (miRNAs) have emerged as key regulators of cellular drug response, particularly through their influence on genes involved in drug transport and apoptosis. One well-characterized example involves miR-873, which negatively regulates the expression of ABCB1 (also known as MDR1), a multidrug-resistance transporter protein that facilitates the flushing out of chemotherapeutic agents. Dysregulation of miR-873 expression has been shown to enhance ABCB1 activity, causing paclitaxel resistance in ovarian cancer cells (Wu et al., 2016). Given the wide array of pharmacological compounds transported by ABCB1, miRNA-mediated regulation likely extends beyond paclitaxel to impact resistance to other drugs as well. Similarly, reduced expression of miR-345 and miR-7, which target the ABCC1 gene, also caused cisplatin resistance in MCF-7 breast cancer cells (Pogribny et al., 2010).

While these examples emphasize the role of miRNAs in regulating drug flushing, other findings indicated that miRNAs exert influence over intracellular action of drug and target, the pharmacodynamics, (Rukov & Shomron, 2011). miR-106 and miR-150 have been shown to reduce the sensitivity of A549 lung cancer cells to cisplatin-induced apoptosis by downregulating RB1 and TP53, respectively (Wang et al., 2010). Cisplatin DNA damage

activates p53, causing cell cycle arrest and apoptosis through p21 and RB. However, elevated levels of miR-106 and miR-150 diminish the availability of TP53 and RB1 transcripts, impairing the apoptotic response even in the presence of cisplatin (Gonzalez et al., 2001; Wang et al., 2010).

Furthermore, despite the seemingly oncogenic nature of miRs, some are reported to also present tumor suppressor activity. miR-34a, a direct transcriptional target of p53, induces apoptosis through other intermediates like *BCL2*, *CDK6*, and *MET*. The let-7 miRNAs are critical in maintaining cellular differentiation and preventing uncontrolled proliferation by downregulating oncogenes like *RAS*, *MYC*, and *HMGA2*. miR-15a and miR-16-1 also promote apoptosis and reduce tumor viability by targeting *BCL2* and *CCND1*. In colon and breast cancer models, miR-143 and miR-145 have been shown to inhibit proliferation and invasion through suppression of *KRAS*, *ERK5*, and *c-Myc*. Together, these miRNAs demonstrate the capacity of endogenous small RNAs to act as regulators of tumor-suppressive networks by modulating oncogenes, cell cycle regulators, apoptosis pathways, and epigenetic modifiers.

These findings demonstrate that miRNAs influence drug resistance not only by modulating efflux transporter expression but also by regulating key genes involved in the cellular stress response and apoptosis. *Rukov and Shomron* (2011), provided a list of miRNAs associated with variable therapeutic responses, underscoring the complexity and clinical significance of miRNA-mediated drug resistance mechanisms.

1.6 Critical Stressor Points- Transcription Stress

Cells rely on coordination of transcription and translation to make proteins. Cancer cells are more demanding on that system as they require constant provision of protein to sustain proliferation. Choosing a weak point in attempts to mitigate that proliferation confronts us with a choice between these two important processes. Stressing transcription in cells can prove to be more lethal in cancer cells due to its placement in the central dogma. If transcription fails, the whole chain collapses and results in chaotic internal environment by catastrophic failure.

5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) is a chemical compound able to inhibit transcription elongation in RNA Polymerase II, the enzyme responsible for producing nascent mRNA transcripts. Cyclin Dependent Kinase 9 (CDK9) and Cyclin T1 form the P-TEF-1 which phosphorylate the C-terminal domain on RNAP II to allow elongation. Treatment of cells with DRB leads to inhibition of CDK9, failure of the P-TEF-1 to form and abolition of elongation. This has been shown to trigger apoptosis in leukemic cells and colon carcinoma cells. The cell cycle arrest and apoptosis occur through p53 and is independent of inhibition of other CDKs by DRB (*Poele et.al, 1999)*.

Alternative means of triggering transcription stress is through spliceosome inhibition. Pladienolide B (PB) is a polyketide isolated from *Streptomyces platensis* in Japanese soil. It binds and inhibits the SF3B1 complex within the human spliceosome. The damage to splicing machinery result in misfolded proteins, intron retention, exon skipping, and incompetent mRNA transcripts losing information for protein synthesis. This activates a suite in stress responses within cells which eventually lead to apoptosis or death by exhaustion/necrosis due to unmet energetic demands (*Makhafola et.al, 2020)*.

1.7 Types of Cell Death

The primary modes of cell death include apoptosis, necrosis, and, under certain conditions, autophagy-associated cell death (ACD). Apoptosis, a genetically regulated and non-inflammatory form of programmed cell death, is characterized by cell shrinkage, chromatin, nuclear fragmentation, and membrane blebbing, while maintaining membrane integrity until late in the process (Doonan & Cotter, 2008). It occurs via two pathways: the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway, both of which converge on the activation of effector caspases such as caspase 3, -6, and -7 (Elmore, 2007).

The extrinsic pathway is initiated by the binding of death ligands—such as TNF and FAS-L—to their corresponding death receptors (e.g., TNFR1 and CD95/FAS), triggering formation of the death-inducing signaling complex (DISC). This complex recruit and activates procaspase-8 via adaptor proteins such as FADD, leading to downstream activation of executioner caspases (Sayers, 2011; Peter & Krammer, 2003).

The intrinsic pathway is activated in response to intracellular stressors such as oxidative damage, unfolded protein accumulation, or genotoxic insults. The mitochondrial response is regulated by the BCL2 protein family, which includes both survival-enhancing, BCL2, BCL-XL and pro-apoptotic, BAX, BAK, and the BH3-only BIM, PUMA, and NOXA (Adams & Cory, 2007; Hernández Borrero & El-Deiry, 2021). Stress-activated BH3-only proteins antagonize anti-apoptotic BCL2 and cause oligomerization of BAX and BAK on the mitochondrial outer membrane, leading to membrane permeabilization. This event results in the cytosolic release of cytochrome c and SMAC/DIABLO, which respectively activate APAF1-caspase-9 apoptosome

assembly and neutralize inhibitors of apoptosis proteins (IAPs), (Goldstein et al., 2005; D'Arcy, 2019).

In contrast to apoptosis, necrosis is an unregulated, pathological form of cell death, marked by early membrane rupture, organelle damage, and inflammatory responses. It is typically triggered by external stresses like ischemia, radiation, chemical insults, or decrease in ATP (Proskuryakov et al., 2003). During necrosis, loss of membrane integrity causes influx of calcium ions, which activate destructive calcium-dependent enzymes such as calpains. Lysosomal rupture and reactive oxygen species (ROS) production also contribute to cell damage (Zong & Thompson, 2006). Necrotic cells also release damage-associated molecular patterns (DAMPs) that trigger innate immune responses (Hotchkiss et al., 2009).

Autophagy, unlike apoptosis or necrosis, is a cellular survival mechanism wherein cytoplasmic components, including damaged organelles and protein aggregates, are degraded, and recycled via lysosomal pathways (Levine & Deretic, 2007). This process is critical during nutrient deprivation and stress and contributes to metabolic balance and protein quality control. Autophagy involves the formation of double-membraned vesicles known as autophagosomes, which engulf cytoplasmic material including organelles and foreign objects, and fuse with lysosomes for degradation (D'Arcy, 2019). Inhibition of Autophagy has been shown to make cells more susceptible to death (Hotchkiss et al., 2009). Although a mechanism essential for proper cellular function, excessive autophagy can lead to autophagy-associated cell death (ACD) under prolonged stress conditions. ACD is characterized by the accumulation of autophagosomes and often co-occurs with, or facilitates, apoptosis and necrosis (Kroemer &

Levine, 2008). Studies on cancer therapy indicates that autophagy may contribute to resistance to apoptosis, following treatment with DNA-damaging agents (Sui et al., 2013).

2 Rational, Hypothesis, and Objective

2.1 Rationale and Hypothesis

Previous studies at McKay labs demonstrated sensitivity of colon carcinoma cells to ACT-D transcription stressor in absence of microRNA processing system. It is supposed that this effect is not unique to ACT-D which prevents elongation by linking with DNA; but also, to RNAP II elongation inhibition by DRB and also spliceosome inhibition by PB. The determination of the precise mechanism of miRNA protection is out of the scope of this project, nor the individual miRNAs involved. The previous method determined the death to be apoptotic in nature by use of a Caspase 3/7 Assay.

We hypothesise that loss of DROSHA and subsequently miRNA will lead to increased sensitivity to transcription stress by DRB and PB in HCT 116 cells relative to WT. We furthermore suppose that these deaths are apoptotic in nature.

2.2 Objective

The desired outcome of this experiment was to evaluate the role of miRNA network in promoting or inhibiting cancer growth. Previous studies only revealed impact of singular miRs on cancer proliferation. The net effect of the miRNA buffer system was still in question. It is unclear by analysing only singular parts, as miRs can act both for and against cancer growth. Furthermore, its intricate regulation network spanning from drug uptake, excretion to mitigation of damage leads to an unclear image on whether they act as stabilizers to the chaotic internal

environment of cancer cells, protecting them from apoptosis, or they lead to a global systemic benefit of killing cancer cells. We wished to elucidate that query.

3 Methods

3.1 Cell Culture

Wild-type (Parental) and DROSHA null (Dro) HCT116 cells obtained from the Korean Collection for Type Cultures (KCTC) and were grown in McCoy's 5A cell culture medium (+1.5 mM L-Glutamine, +2.2 g/L sodium bicarbonate) (Hyclone, Logan, UT) supplemented with 9% heat inactivated newborn calf serum (NBCS) (Gibco, Auckland, NZ) and 3% fetal bovine serum (FBS) (Gibco, Grand Island, NY) with 90 units/mL penicillin and 90 µg/mL streptomycin antibiotics (Gibco, Grand Island, NY). All cell lines were grown in an incubator at 37 °C with 5% CO2.

3.2 Drug Treatment

Prior to all experiments, cells were seeded in 6 well plates as a density of 90, 000 cells/well in fresh growth medium at least 48h before all drug treatments. For all following drugs, the stock solution was prepared in DMSO (Sigma-Aldrich, Cat#D2650, St. Louis, MO). DRB (Sigma-Aldrich, Cat#D1916, St. Louis, MO) was prepared at a stock of 100 mM for experimental concentrations of 25, 50, 100 µM. Pladienolide B (Merck Millipore, Cat#530196, St. Louis, MO) was prepared at a stock of 100 µM for experimental concentrations 25, 50, and 100 nM. All experiments were treated with a No Drug (ND) and a DMSO vehicle control.

Following that, the experiment was replicated for each drug each with the 2 highest concentrations (50, 100 µM for DRB and 50, 100 nM for PB). For each concentration of each

drug, the cells were treated with and without ZVAD-fmk Broad Caspase Inhibitor (Merck Millipore, Cat#219007, St. Louis, MO) for a stock of 100 μM in DMSO and an experimental concentration of 25 μM. ND and DMSO controls were also included for all experiments.

3.3 Sub-G1 Assay

Cells were seeded in 6 well plates at a density of 90,000 cells per well. 24 hours later, media was replaced with fresh media containing vehicle control or pharmacological 31agents being tested. 48 hours following treatment, adherent and detached cells were collected, rinsed with PBS, and fixed in 70% ethanol at -20°C for at least 24 hours. The fixed cells were collected by centrifugation, washed with PBS, and stained in 20 μg/mL of propidium iodide (PI) (Sigma-Aldrich, Cat#P4170, St. Louis, MO) in PBS (+50μg/mL RNaseA, Bio Basic, Cat#RB0473, Markham, ON) for a minimum of 30 minutes. An Accuri™ C6 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ) was used to measure the relative level of PI fluorescence (FL2) in cells from each sample. This allowed the ratios of cells with sub-G1, G1, S, and G2 levels of DNA content to be evaluated using DB C Sampler Software (BD Biosciences, Franklin Lakes, NJ).

Cells undergoing apoptosis fragment their DNA, leading to lower PI staining. Cells with less that 2C DNA were considered non-viable. Statistical analysis was performed using a 2-way ANOVA test on GraphPad Prism Software. P-values represented the “column factor” which compared the % of cells with sub-G1 DNA content in parental and DROSHA null HCT116 cells. Differences with $p<0.05$ were considered statistically significant.

3.4 Immunoblot Assay

Cells were seeded in 6-well plates at a density of 500,000 cells per well. 24 hours later, proteins were collected using a cell scraper in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Cat#PI89901, Ottawa, CA) sonicated and quantified using the Bio-Rad Protein Assay (Bio-Rad, Cat#5000006, Hercules, CA) according to the manufacturers protocol. Absorbance was measured at 595 nm using a BioTek Cytation 3 microplate reader (Fisher Scientific, Cat#CYT3V, Ottawa, CA) and data interpreted using the Gen5 Software (Fisher Scientific, Cat#CYT3V, Ottawa, CA). Sample protein concentrations were determined using a standard curve generated using the absorbance of bovine serum albumin (BSA) protein standards with known concentration.

Protein samples of equal amounts (50 µg) were prepared in dH2O-Protein mixture, with 10X dichlorodiphenyltrichloroethane (DTT) (Invitrogen, Cat#NP0009, Carlsbad, CA) reducing reagent and 4X NuPAGE LDS sample buffer (Invitrogen, Cat#NP0007, Carlsbad, CA) before incubation at 70 °C for 10 minutes. Protein samples and the MagicMark XP Western Standard (Invitrogen, Cat#LC5602, Carlsbad, CA) protein ladder were all loaded into a 4-12% NuPAGE Bis-Tris gel (Invitrogen, Cat#NW04120BOX, Carlsbad, CA) and resolved by gel electrophoresis at 200V in MOPS-SDS running buffer (50nM MOPS, 50mM Tris Base, 0.1% SDS, 1mM EDTA) for 30 minutes.

After the proteins separated, they were transferred onto a nitrocellulose membrane using 1X NuPAGE transfer buffer (Life Technologies, NP0006-1, Carlsbad, CA) with 10% methanol. After the transfer, proteins on the nitrocellulose membrane were stained with 1.3mg/mL Ponceau S (Sigma-Aldrich, Cat#P3504, St. Louis, MO) (+1% acetic acid) to successful. Protein transfer

and even loading. The membrane was then placed in a blocking solution of 5% milk powder in TBST (50nM Tris, 150mM NaCl, 0.1% Tween 20, pH 7.6) for one hour and then incubated overnight in a target specific primary antibody: rabbit anti-Drosha, or mouse anti-Actin (Cell Signaling Technology, D30F3 3410S, Danvers, MA).

Membranes were then washed (4x for 5 minutes) in TBST before incubation in the appropriate goat anti-rabbit, or goat anti-mouse (Abcam, Cat#6721, Cambridge, UK) horseradish peroxidase-conjugated secondary antibody, 1:20,000 goat-anti-rabbit, 1: 10,000 for goat anti-mouse, for two hours. Membranes were again washed in TBST (4x 5 mins + 1x 10 mins) before chemiluminescent imaging using Clarity Western ECL Substrate (Bio-Rad, Cat#170-5060, Hercules, CA) and the Fusion FX-5XT imager with VisionCapt Fusion 3 Mega software v16.11 (Vilber Lourmat, Marne-la-Vallee, FR).

4 Results

4.1 DROSHA null cells displayed increased cell death with PB and DRB treatment

DRB and PB, both mechanistically different transcription stressors were chosen from a set of readily available drugs in the lab to determine whether DROSHA null cells exhibited differential susceptibility to drug induced cell death. DRB operates by preventing phosphorylation of the carboxy-terminal domain of the large subunit of RNA polymerase by blocking P-TEFb, a cyclin dependant kinase. This results in inhibition of transcription elongation. PB binds and inhibits the SF3B1 complex within the human spliceosome, resulting in aberrant mRNA transcript.

The dose-response relationship of these various drugs was compared between parental HCT116 colorectal carcinoma cells and genetically modified HCT116 cells lacking the ribonuclease DROSHA. Cells undergoing apoptosis fragment their DNA leading to a decrease in Propidium Iodide (PI) staining, which provide a useful opportunity to measure the sensitivity of the drug treated cells to drug-induced cell death. Initial assessment of each cell line's sensitivity in response to these drugs was determined by one-parameter flow cytometric analysis using PI to determine the percentage of cells with less than 2C DNA content (Sub-G1 Assay). Cells with less than 2C (G1) DNA content were considered non-viable. We found that loss of DROSHA had agent-specific effects on cell sensitivity to apoptosis in HCT116 cells treated with DRB and PB. Although the trends of the dose response curve were different, exposure of DROSHA null cells to either DRB or PB led to a significant increase in the proportion of cells with sub-G1 DNA content. This was not uniform across all concentrations of drugs but represented what appears to be different dose-response relationships. For these drugs, the percentage of parental HCT116 cells with sub-G1 DNA content plateaued with increasing drug concentration while the proportion of DROSHA null cells with sub-G1 continued to increase at higher concentrations.

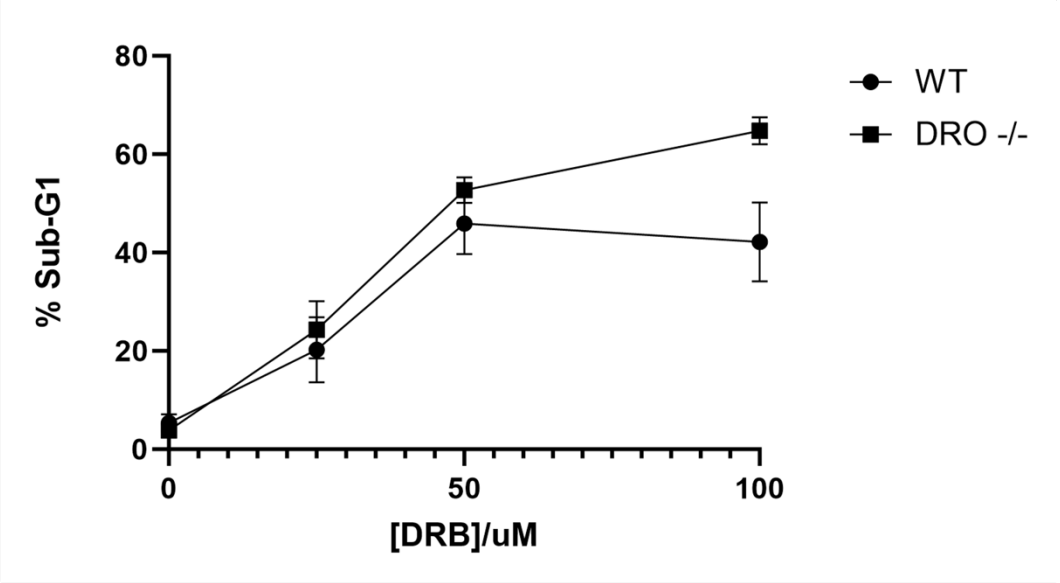

Figure 3: One Parameter Flow Cytometric Analysis of Sub-G1 DNA. HCT 116 WT and null cells were exposed to DRB at concentrations of 25, 50 and 100 uM for 48 hours, Controls were ND and DMSO. For WT, ND was at 5.37 ±3.00, DMSO was at 4.37 ± 2.9, 25 uM was at 20.23 ± 11.4, 50 uM was at 49.9 ± 10.8, and 100 uM was at 42.2 ± 14. For Dro -/-, the corresponding values were at 3.83 ± 1.79, 4.3 ± 3.4, 24.3 ± 10.1, 52.7 ± 4.5, 64.7 ± 4.7. These represent the (Average ± Standard deviation) across a minimum of 3 biological replicates. Indicated on the graph are the average values ± SEM as error bars. Apoptosis was quantified as the number of cells with less than 2C DNA content. The sensitivity of cell lines were compared by 2-way ANOVA using GraphPad Prism Software. The p-values obtained were as follows: p=0.0378 for resolving difference between cell lines and p<0.0001 for the resolution of difference between drug treatment. No significant interaction was reported.

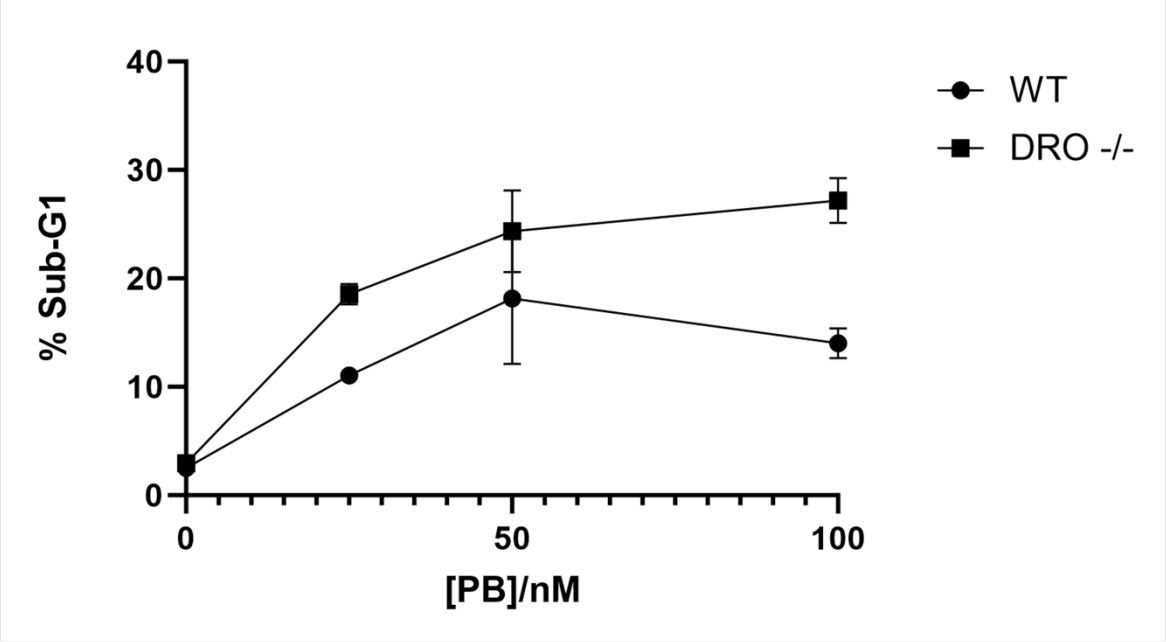

Figure 4: One Parameter Flow Cytometric Analysis of Sub-G1 DNA. HCT 116 WT and null cells were exposed to PB at concentrations of 25, 50 and 100 nM for 48 hours, Controls were ND and DMSO. For WT, ND was 2.5 ± 0.6, DMSO was at 2.8 ± 1.1, 25 nM was at 11.0 ± 1.08, 50 nM was at 18.1 ± 10.5, and 100 uM was at 14.0 ± 2.4. For Dro -/-, the corresponding values were at 2.9 ± 1.2, 2.1 + 0.4, 18.5 ± 1.6, 24.4 ± 6.5, 27.2 ± 3.6. These represent the (Average ± Standard deviation) across a minimum of 3 biological replicates. Indicated on the graph are the average values ± SEM as error bars. Apoptosis was quantified as the number of cells with less than 2C DNA content. The sensitivity of cell lines were compared by 2-way ANOVA using GraphPad Prism Software. The p-values obtained were as follows: p=0.0025 for resolving difference between cell lines and p<0.0001 for the resolution of difference between drug treatment. No significant interaction was reported.

4.2 Caspase Inhibitor Rescues DROSHA null cells from DRB induced cell death

z-VAD-fmk is an irreversible, cell-permeant, pan-caspase inhibitor. Apoptosis is mode of regulated cell death that can be initiated by 2 pathways: It proceeds via two main pathways: the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway, both of which converge on the activation of effector caspases such as caspase 3, -6, and -7. Apoptosis can still proceed independent of caspase, but the action of these proteins represents the canonical means of apoptosis. Blocking caspase action represents inhibition of apoptosis by its primary mechanism.

We found that across both concentrations of 50 and 100 μM DRB, both parental and WT cells irrespective of ZVAD treatment displayed increased cell death relative to ND and DMSO control. Between with and without ZVAD treatment, the parental cell line displayed no significant change in cell death. This was across both tested concentrations. Within the same concentration, as obtained in the previous experiment, DROSHA null cells again displayed more death relative to parental cell line. When comparing DROSHA null cells with and without ZVAD treatments, there is a significant decrease in cell death with ZVAD rescue. The decrease in death was lower than the baseline WT death. These were common for both tested concentrations.

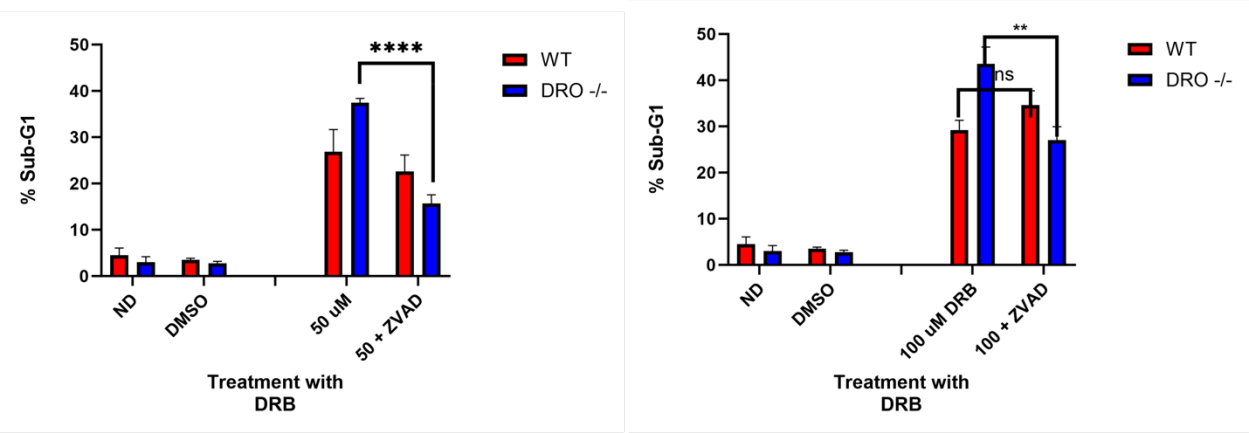

Figure 5: One parameter Flow Cytometric Analysis of Sub-G1 DNA content across WT, and Dro -/- cell lines. Apoptosis was quantified as cells with less than 2C DNA content. Controls were ND and DMSO, and treatment were 50 or 100 uM DRB, with or without ZVAD caspase inhibitor. ND and DMSO controls. The plots represent the average values across 3 biological replicates and ± SEM. The following values also represent the same. ND for WT was at 4.53 ± 1.53, DMSO was at 3.47 ± 0.38, Corresponding Dro -/- values were at 3.30 ±1.15, and 2.77 ± 0.42. For the 50-uM treatment, WT values were at 26.9 ± 4.8 without ZVAD and 22.6 ± 3.5 with ZVAD. For the Dro -/- cell line, values were at 37.4 ±0.96 without ZVAD and 15.7 ± 1.9 with ZVAD. There was no significant difference within WT, with and without ZVAD. The difference in Dro -/- cell line was significant between with and without ZVAD ($p < 0.0001$). For the 100-uM treatment, WT values were at 29.2 ± 2.1 without ZVAD and 34.6 ± 3.1 with ZVAD. For the Dro -/- cell line, values were at 43.6 ± 3.6 without ZVAD, and 27 ± 2.9 with ZVAD. Within WT, there was no significant difference with and without ZVAD and for the Dro -/- population, there was significant difference ($p = 0.0016$). Difference between cell lines, were computed individually for each concentration with multiple unpaired t-tests and yielded no significance. T-test is more vulnerable to noise inherent of flow cytometry. All treatments were significantly different from controls ($p < 0.0001$). The statistical responses were computed by a 2-Way ANOVA and post-hoc Tukey Test and multiple t-tests using GraphPad Prism Software.

4.3 PB induced death is Caspase independent

Across both tested concentrations of 50 and 100 nM PB, treatments at both concentrations displayed increased cell death relative to ND and DMSO control. No significant difference was also noted across the WT treatments with and without ZVAD at both concentrations. No significance was also detected across the treatment with and without ZVAD for DROSHA null across both concentrations. High variability within biological replicates obscured any statistical signal power to allow for recovery of significant difference.

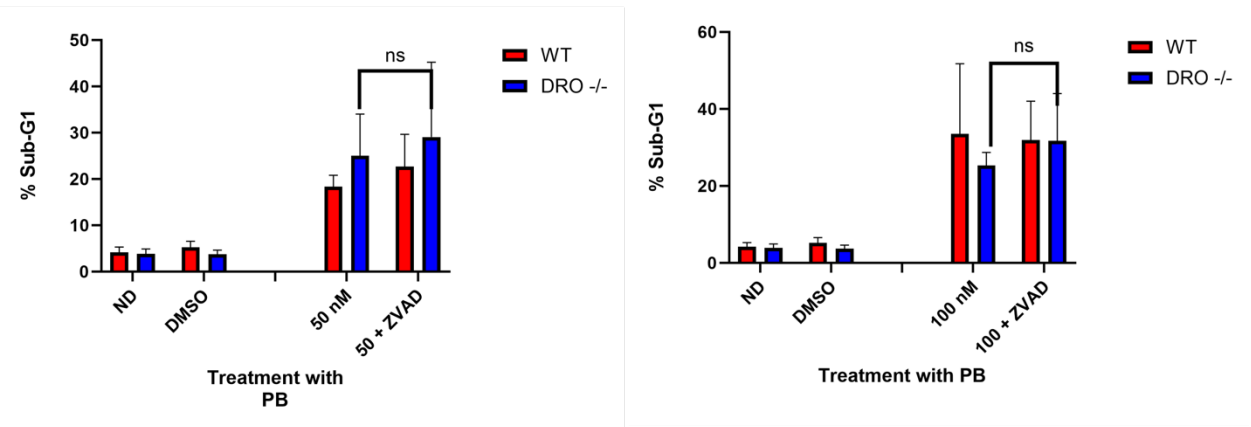

Figure 6: One parameter Flow Cytometric Analysis of Sub-G1 DNA content across WT, and Dro -/- cell lines. Apoptosis was quantified as cells with less than 2C DNA content. Controls were ND and DMSO, and treatment were 50 or 100 nM PB, with or without ZVAD caspase inhibitor. ND and DMSO controls. The plots represent the average values across 3 biological replicates and ± SEM. The following values also represent the same. For WT, ND was at 4.17 ± 1.1, DMSO was 5.23 ± 1.3. For Dro -/-, ND was 3.87 ± 1.0, DMSO was 3.7 ± 0.9. For the 50 nM treatment, WT produced 18.4 ± 2.5 without ZVAD and 22.7 ± 7.0 with ZVAD. For Dro -/-, we obtained 25.0 ± 9.0 without ZVAD and 29.1 ± 16.2 with ZVAD. For the 100 nM treatment, WT produced values at 33.5 ± 18.0 without ZVAD and 31.9 ± 10.0 with ZVAD. Dro -/- produced 25.3 ± 3.4 without ZVAD and 31.7 ± 12.3 with ZVAD. Two Way ANOVA yielded no significant difference between treatment, owing potentially to the very large noise within replicates overpowering statistical signal between each sample. Difference between cell lines, were computed individually for each concentration with multiple t-tests and yielded no significance. T-test is more vulnerable to noise inherent of flow cytometry. All treatments were significantly different from controls ($p < 0.0001$). The statistical responses were computed by a 2-Way ANOVA and post-hoc Tukey Test and multiple t-tests using GraphPad Prism Software.

4.4 Immunoblotting confirms absence of DROSHA in null cell line

To confirm that the DROSHA null cells obtained from the KCTC repository was effectively null, DROSHA levels were assessed by immunoblotting. As expected, DROSHA was readily detectable in parental HCT116 cells but appeared to be absent in the DROSHA null cells. Previous work in our lab measured the expression of representative canonical miRNAs in these cell lines and confirmed that canonical miRNA expression was significantly reduced in DROSHA null cells compared to their parental cell line (Browning et al., submitted for publication). Therefore, this cell line is DROSHA-deficient and exhibits the anticipated defect in canonical miRNA processing.

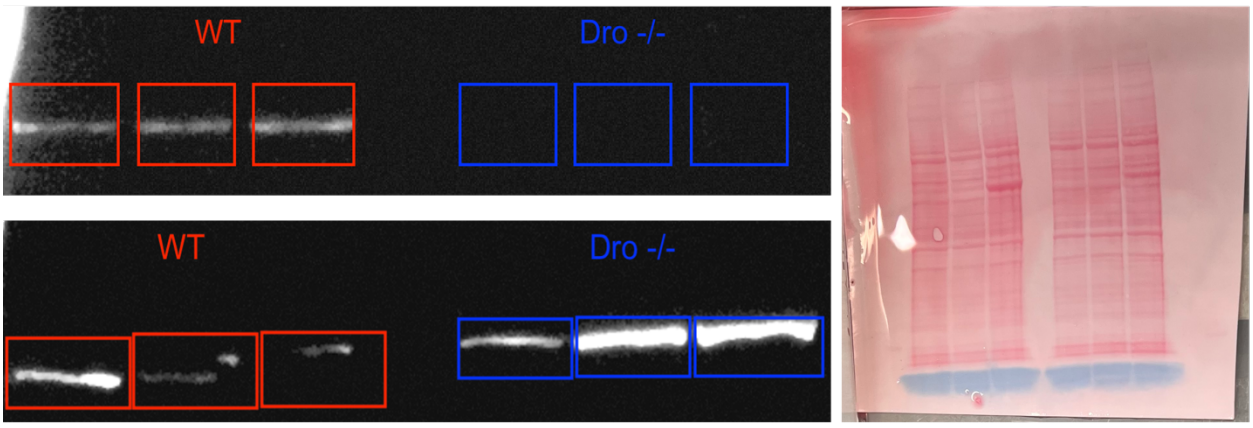

Figure 7: Validation of DROSHA null and parental HCT 116 cell lines in 3 biological replicates. Western Blots were used to show the expression of DROSHA in parental and absence in null cell line (Top Left) and Actin (Bottom Left) as loading Control in both cell lines. The images come from different membranes from the same biological replicates. Ponceau S Stain of the membrane is also included as proof of even loading.

5 Discussion

5.1 Summary

The result indicate that transcription stress induces Apoptosis in colorectal carcinoma cells (*In Virto*).

The chosen cell line was HCT 116 and validation of DROSHA knockout was confirmed through western blot.

Two mechanisms of stress were chosen: treatment with DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole), causing failure at elongation on RNAP II, and PB (Pladienolide B), causing inhibition of the spliceosome, resulting in incompetent mRNA transcripts.
Apoptosis was characterized as DNA fragmentation using PI staining and flow cytometry through a sub-G1 assay.

Throughout the concentration range, the WT cells peaked in death and that effect decreased as the concentration of drugs increased while death kept increasing in Dro -/- cells as concentration increased.

We also experimented with ZVAD-fmk, a pan-caspase inhibitor. With both DRB and PB treatments, each at 2 concentrations.

In WT cells, we again noted that treatment with drugs induced more death relative to control. This death was not reversed with ZVAD treatment with no significant difference between with and without ZVAD treatment. Both were at higher deaths relative to control.

In Dro -/- cells, we again noted higher death relative to control but also to WT. The cells were rescued through ZVAD treatment with a noticeable decrease in apoptosis with significant difference between with and without ZVAD treatment. The cell death did not decrease back to control values. They were lower compared WT values.

These trends were observed for DRB at concentrations of 50 and 100 uM.

We failed to replicate those trends for PB. There was no significant difference between with and without ZVAD treatments in either cell line. Statistical significance could also not be recovered from control and treatments. We observed very large intra-replicate noise, weakening the statistical signal power despite graphs showing a definitive increase in death with PB treatment.

5.2 Interpretation

DNA fragmentation is most characteristic of apoptosis compared to other modes of cell death like necrosis or autophagy. Caspase-3 cleaves Caspase-activated DNAse which cause digestion of DNA into short, small fragments. The result is a DNA ladder pattern of ~ 200 bp with regular intervals, when a gel is run. DNA cleavage can still occur due to internal chaos during necrosis and autophagy. The pathway is not triggered to the same replicability in other modes of cell

death. On a gel, this results in a smeared pattern. Permeabilization of cells through EtOH fixing while also preserving ratios of DNA content allows PI to faithfully assess DNA fragmentation status within a sample. Measuring a hallmark of apoptosis provides a solid base for a confident and faithful assessment.

Recording DNA fragmentation (and potentially apoptosis) after treatment with either PB or DRB demonstrate the effectiveness of transcriptional stress on the colon carcinoma cells. This is a trivial observation given the importance of transcription elongation and splicing to cells irrespective of it being cancerous or not. DRB had an overall higher lethality compared to PB, owing to the higher concentrations employed for the same number of cells seeded. For the response of WT cells under each of the drugs, each at different doses, the responses were significantly different indication dose dependence of the action of the drug on the apoptosis of the cell. For the WT cell line under both drugs, maximum response was noted at the 50 μM of DRB and 50 nM of PB concentrations. Past this, there was a decrease in the recorded amount of apoptosis, indicating a dose optimum for the treatment. The decrease in response could be potentially attributed to a protective cellular mechanism which triggers under more abrupt shock like very strong cytotoxic exposure. This could be senescence or another pathway to quickly expunge the cells of the drugs, which activated only under critical stress. It could also be rapid shutdown of transcription leading to failure to produce the required apoptotic genes leaving cells to die by autophagy or necroptosis, which are not captured in a sub-G1 Assay.

We can gauge deeper understanding when we study that in relation to another parameter. For example, presence and absence of miRNA buffering system. Most interestingly, while our

hypothesis expected more death in the DROSHA null cell line due lack of passive stabilization, and that we also obtained such results for both drugs across the whole concentrations, something unexpected was also recorded. At higher concentrations while the WT cells had their supposed protective mechanism engaged and the death decreased, the Dro -/- apoptosis count kept increasing hinting towards miRNA being involved in some way in that observed salvation.

Also, since we were targeting transcription and miRNA rely on the same mechanism to form, we went with the assumption that the miRNA buffer system is constant and has a longer life span persisting even after disruption. We seeded cells at a density of 90,000 and waited a minimum of 48 hours before treatment to ensure that the miRNA system was still operating and recovered from potential shocks during seeding. The lack of significant difference between ND and DMSO vehicle control reveal that the cytotoxic effect of DMSO is irrelevant, and all significance are derived only through the drug action without interference from the drug vehicle. All our noted effects would indicate the safeguarding effect of the buffer system until its dissolution due to lack of maintenance. It is to note that cancer cells in their free environment do not face this issue and the effect of the buffer system on promoting survivability might be more important *in-vivo* due to its ability to be maintained.

The follow-up experiment was to evaluate caspase activity in the previous experiment. The two highest drug concentration was chosen for better resolution as the difference they present is more important compared to the lower concentration. Again, we noted the same effect with the DRB treatment, the ratios of death were preserved without ZVAD-fmk. When treated with broad caspase inhibitor, DNA fragmentation is abolished due to inhibition of Caspase -3 and failure to

trigger the Caspase-activated DNAse. Caspase inhibitors have minimal effect on the trigger of senescence, this is the role of p21; caspase are primarily executioners of apoptosis.

In WT cells, treatment with DRB caused more death as seen previously too, compared to the untreated controls. There was no significant change in death when treated with ZVAD. This was unexpected as we originally thought that the DNA fragmentation recorded was due to apoptosis and it means that this is occurring independent of caspase. In Dro -/- cells, we again recorded more death relative to both control and treated WT. However, when treated with ZVAD, the amount of death remained higher than control, but it decreased below the WT treatment death baseline. The caspase inhibitor has rescued the cells from caspase dependent apoptosis in the Dro -/- cell line.

These findings would suggest that caspase activation is the dominant mode of death pathway in absence of miRNAs. The decrease below the baseline WT death reveals the intricate role of miRNAs, some which were absent in the Dro -/- cell line could have contributed to cell death via other means in the WT cell line. The lack of these particular ones allowed Dro -/- ones to be rescued even more in presence of ZVAD. These provide a clear demonstration of both the tumour suppressor and oncogenic activity of miRNAs.

To restate the above findings more coherently, we found miRNA to not only protect HCT 116 cells from caspase-derived death, but also promote a slight shift towards non-caspase death modes. The bulk of death in both WT and Dro -/- that remained higher than control despite ZVAD treatment could be attributed to a non-caspase death that also fragments DNA. These

could be necroptosis or parthanatos. It could also be exhaustion of cells which managed to survive the effects of the drug but were killed due to other systems failing.

Regrettably, the same observations could not be recovered for the PB treatment with ZVAD. Although the plots indicate more death relative to control, we obtained no statistically significant difference between controls any of the treatments. This was owing to the large variation within biological replicates as could be seen from the very large error bars. F = MSB/MSW, with the within variation increasing, the F-statistic, quantifying statistical difference signal power, decreases yielding lower certainty in rejecting null hypothesis due to obscuration of signal for difference. This is partially due to time constraint in ability to replicate experiments and technical difficulties with the flow cytometer during the experimental period with PB. DRB samples were ran first followed by PB, this large variation in the PB reads could be due to debris within the flow cytometer interfering with the signal, although this was not noted for the first experiment set.

The Western Blot validated the Knockout of DROSHA, although a second membrane was generated from the same biological replicate, an actin loading control was required and this was obtained from a second membrane owing to time constraint. This explains why the bands are not exactly the same. Also in the actin control, some bands appeared as faded. This was most likely during transfer process and is potentially technical in origin due to experimenters still being familiarized with western Blot. A ponceau S Stain control was included as substitution.

5.3 Literature Cross-Reference

The transcription competence of HCT 116 prior to drug treatment has been well established in literature. For example (*Cattaneo et.al, 2006)* studied a polymorphism in the E-cadherin promoter by making use of the luciferase assay to successfully establish observations. Also, previous work in McKay lab utilized HCT 116 as reporter cell lines for XBP1 and ATF4. All of these require functional transcription system. The successful use of HCT 116 for these experiments prove the viability of transcription in the cell line used in our experiment.

The same was true for the splicing competence of the spliceosome of parental HCT 116. (*Makhafola et.al, 2020)* utilized Pladienolide B as control for the interruption of splicing in HCT 116 cells. Without treatments, and splicing functional, hnRNPB1 has exon retention which allows for BcL-XL to prevent mitochondrial membrane permeabilization. With splicing interrupted, the isoform switches to hnRNPA2 which had exon skipping and in unable to allow for proper splicing of BcL2L1, causing BcL-XS and subsequent membrane permeabilization and apoptosis in HCT 116 cells. This study both concluded the transcription competence and viability of PB to target the spliceosome.

As for the competence of DRB to inhibit transcription elongation, (*Poele et.al, 1999)* showed DRB blocked RNA synthesis in LS174T, HC29, and SW28 CRC cell lines through monitoring [5,6-^{3}H] Uridine inclusion. These all have functional transcription apparatus similar to HCT116. Furthermore, the paper also showed that DRB triggered p53 accumulation in all 3 cells with only those with functional p53 leading to apoptosis.

For the drug ranges chosen, numerous studies made mention of an optimal dose of DRB having an IC_{50} of 35-50 μM. They also emphasized the low potency of DRB. (*Myriam et.al, 2003)* concluded DRB to be a low potency drug alone but to have important synergistic effects when treated with TNF-α. Also, previous work at McKay lab screened a set of drugs for further experiment. Actinomycin-D was chosen over DRB as even if both showed significant response, ACT-D showed greater effect. PB was already established by (*Makhafola et.al, 2020)* to be potent even at lower doses (0-10 nM), with apoptosis confirmed by immunochemistry and viability assay.

The presence of a drug optimum and increased protection beyond a certain concentration is characteristic of non-linear responses found throughout biology. ACT-D, Vincristine, Doxorubicin, DRB and L-Mimosine all showed similar response in previous works in McKay Lab (*G. Sharpe, 2022*). We managed to replicate the previous result with DRB and a similar effect with PB together with the undampened acceleration in death with Dro -/-, not observed in WT.

This non-monotonic, hormesis response was explained by (*Owen et.al, 2014)* to be originating from the tendency of the drug to aggregate at high doses, leading to decreased potency. Another study by (*Andrea et.al, 1997*) showed DRB treatment managed to stabilize p53 in fibroblasts but failed to induce apoptosis. This would require functional transcriptional apparatus to produce effectors like PUMA, BAX, and Fas. Intense transcriptional stress prevents this.

There have been numerous articles citing the involvement of miRNA in delaying apoptosis; they are known as oncomiRNAs. (*Karakatsanis et.al, 2011*) found miR-21,-221 to be associated with poor prognosis. miR-21 negatively targets PTEN which brakes PI3K-AKT pathway, allowing for energy allocation to cancerous cells. miR 17/92 cluster, a family of miRNAs involved in cell cycle acceleration is frequently found over-expressed in lymphomas. miR-19 was found to also antagonize PTEN, and activating Akt-mTOR pathway, also promoting cell growth (*Olive et.al, 2009*). It makes sense removing such miRs would allow cancerous cell to deplete in energy and proliferative potential. Tumor-supressor miRNAs have also been found to exist. Knocking these out could explain why DRO -/- under caspase inhibition had lower death that WT cells. miR-137 is a known regulator of ferroptosis, a non-apoptotic cell death (*Luo et.al, 2018*), miR-155 has been shown to promote necroptosis (*Young et.al, 2018*). Absence of these miRNAs could have provided a survival advantage against non-caspase death as observed.

Other studies by (*Yang et.al, 2015*) also found p38 MAPK pathway to be activated in cellular stress, causing phosphorylation of DROSHA, weakening binding with DGCR8, and decreasing the efficiency of miRNA while also leading to DROSHA degradation. This study agrees with our current observation of miRNA providing stability against cell death by protecting against stress.

5.4 Future Directions

The interpretation of the ZVAD-PB experiment proved to be unreliable due to high noise owing to technical issues with equipment and time constraint. Re-running the experiment is most likely to provide data more aligned with the previously mentioned observations and showing PB to induce apoptosis too.

We had issues in differentiating between necroptosis and apoptosis through the sub-G1 assay. Running extracted DNA on a gel could help elucidate this matter. Apoptosis involves regular fragmentation of DNA and that would appear as a ladder-like pattern with regularly spaced bands on the gel while necroptosis yields randomly sized strands which would be seen as a smeared band. This would help to know if the DNA fragmentation is truly caspase-independent occurring through necroptosis or another means.

We also lacked a ZVAD-fmk control for our experiments, replicating the experiments with such control would allow us to assess necroptosis promotion of ZVAD over apoptosis and if it has a significant impact.

More detailed studies could be made concerning the viability of cells using MTT assay, evaluation of p53 and p21 levels following DRB treatment to understand how the cells respond to the damage induced by DRB at a more precise level. This could be done in HCT 116 cells or record apoptosis in HCT 116 cells with a mutant p53 and see if we get the same response. The same study could also be repeated in other cell lines like HeLa and U2OS to monitor the universality of the response to DRB and PB.

Following treatment with DRB/PB and without drugs, extractions from WT and Dro -/- could be sent for RNASeq to evaluate the genes involved in the response we observed.

Further studies could look into the non-linear dose response of the drug and the existence of an optimum. Experiments could be ran on a smaller scale, around the optima of 50 μM DRB to see if the same bell-shaped pattern is obtained. Also, western blots targeting apoptotic proteins could be made at the different concentrations to see if the protective effect is due to the failure of transcription to produce apoptotic genes.

The western blot validation could be repeated such that the same membrane is treated with both anti-DROSHA and anti-Actin, allowing for densitometric analysis and semi-quantitative proof of DROSHA knock out in our cell line

6 Conclusions

Our experiment confirmed and demonstrated the protective effect miRNAs had on cancer cells following induction of transcription stress by DRB and PB. We also saw that miRNAs not only protect from caspase-dependent death, but its presence also favoured non-caspase dependent death when treated with DRB.

This experiment added another layer to the already known protection provided by miRNAs. We also managed to show that while individual studies each showed how one miRNA was for or against cancer growth, the system as a whole leaned more towards cancer growth while also retaining traces of cancer inhibition.

This study would hopefully provide slightly better understanding of the complexity of biological systems and how stabilizers like miRNAs can be hijacked by a disease and hopefully translate to therapeutic advancements to strip the disease of one of its stolen armours.

While our model was *in-vitro*, it could provide a stable core that other clinical experiments can build on to attack the disease in a different way.

For more intellectual and philosophical pursuits, miRNAs are involved in highly delicate processes in important tissues. In the brain, they are involved in memory consolidation and synaptic connection/disconnection during learning and also in embryo development requiring precise formation and death of tissues. It seems counter-intuitive that a messy process would be favoured over a clean one.

Apoptosis, even if precise, clean, and valuable, is an energy expensive process, the suppression of caspase dependent pathways and slight promotion of non-caspase pathways provide a less costly way of making small adjustment to fit the needs of the organisms. Apoptosis evolved prior to miRNA control system. This might have been a way for nature to be more efficient. In diseases like cancer, this efficient tool turns against the organism, by understanding the body better, we can better solve its issues while also admiring the paradoxical state of nature where complexity, simplicity, fragility, and toughness all co-exist.

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