Knockout of DROSHA increases the sensitivity of HCT116 cells to apoptosis in response to actinomycin-D treatment

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral affairs in partial fulfillment of the requirements for the degree of

Master of science

in

Biology

Carleton University
Ottawa, Ontario

Abstract

MicroRNAs are short non-coding RNAs that function as sequence-directed post-transcriptional inhibitors of gene expression. The cellular response to ten drugs including actinomycin-D (ACT-D) was examined in a genetically modified HCT116 colon cancer cell line with a deletion in the gene encoding a critical miRNA processing enzyme called *DROSHA*. We found that the DROSHA null subline was more susceptible than the parental cells expressing wild-type DROSHA to apoptosis induced by several drugs, most prominently ACT-D. This increase in susceptibility to apoptosis was characterized by increased DNA fragmentation, increased caspase 3/7 activity, and loss of membrane integrity. The increased susceptibility to apoptosis was not associated with differences in DNA synthesis, RNA synthesis, protein synthesis, metabolic activity, p53 response or the induction of replicative senescence. Our results suggest that these cell lines are equally sensitive to the direct effects of ACT-D but these DROSHA null cells are more sensitive to apoptosis induced by a subset of drugs exemplified by ACT-D.

Acknowledgements

I would like to thank my supervisor Dr. Bruce McKay for his unwavering support and substantial contributions to my learning during the past two years. My appreciation for his patience and wisdom throughout my research experience cannot be understated. I extend my thanks to Carleton University as well for allowing me the opportunity to complete my Masters research in the Department of Biology. I would also like to thank the members of my advisory committee Dr. William Willmore and Dr. Marc Ekker for their guidance. Furthermore, I would like to thank Erin van Zyl for sharing her invaluable knowledge and experience in the lab with me. Erin has been an exceptional teacher and friend throughout my entire time working with her. My great appreciation also goes out to all current and previous students in the McKay lab who I have worked with, thank you for making the lab an excellent workplace that I looked forward to coming into each day. Finally, I would like to thank my family, friends, and partner for all their support and encouragement throughout my research. I would not be where I am today without those closest to me and I am profoundly grateful for it.

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1. Introduction

1.1 MicroRNA function

The regulation of gene expression is a fundamental process with major implications for the health and survival of all organisms. Our understanding of how gene expression is regulated is constantly evolving. Several mechanisms that impact gene expression are currently recognized including regulation at the transcriptional, post-transcriptional, and post-translational levels (Buccitelli & Selbach, 2020). Transcriptional regulation can involve transcription factors, chromatin modifications, and epigenetic changes, all of which directly affect the transcription of RNA from genomic DNA (Buccitelli & Selbach, 2020; Chen & Rajewsky, 2007). Post-translational control of gene expression usually involves protein-protein interactions or modifications that alter the outcome of various cellular processes (Tahmasebi et al., 2018). Post-transcriptional regulation of gene expression is particularly complex. The processing of mRNA transcripts by alternative splicing and modifications like polyadenylation can affect how genes are expressed (Bentley, 2014). Similarly, RNA binding proteins and interactions with non-coding RNAs can alter mRNA localization and rate of decay (Chen & Rajewsky, 2007; Hentze et al., 2018). In recent years, the importance of non-coding RNAs (ncRNAs) in regulating gene expression has become unequivocal. Some ncRNAs that regulate gene expression include small-interfering RNAs (siRNA), long non-coding RNAs (lncRNA), piwi-interacting RNAs (piRNAs), and microRNAs (miRNA) (Kaikkonen et al., 2011). ncRNAs like miRNAs are reportedly important to health and are being considered in the development of novel therapeutic strategies that may impact development, life expectancy and quality of life.

Micro RNAs (miRNAs) are small ncRNAs (~22 nucleotides long) that play an important role in regulating post-transcriptional gene expression by modulating the stability and translation of specific mRNAs (Friedman et al., 2009). In other words, miRNAs function to attenuate gene expression (Figure 1) in a wide variety of important cellular processes including differentiation, apoptosis, and stress responses (Abdelfattah et al., 2014).

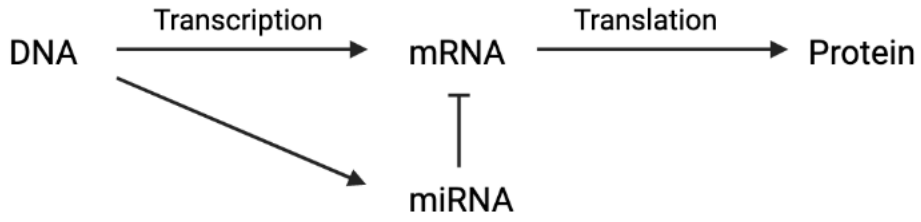

Figure 1. miRNAs downregulate gene expression by transiently or permanently preventing the translation of mRNAs into proteins. Created using BioRender.com.

Similar to endogenous small interfering RNAs (siRNAs), miRNAs bind to competent mRNA transcripts to interfere with downstream translation (Bartel, 2004). Target binding by miRNAs requires partial complementarity between the miRNA 5' seed sequence and the 3'-untranslated region (UTR) of the mRNA transcript. The 5' seed sequence of the miRNA refers to the nucleotides (nt) at positions 2-8 at the 5'-end of a mature miRNA (Schmittgen, 2008). Approximately 80% of miRNA-target interactions occur through a perfect or near perfect (~1nt mismatch) binding with the 5' seed sequence (Grosswendt et al., 2014). Micro RNAs act to downregulate gene expression in two distinct ways, direct mRNA cleavage and translational repression by blocking translational initiation (Schmittgen, 2008). Recent studies using ribosome profiling in mammalian cells suggest that miRNAs initially elicit an inhibitory effect on gene expression through

translational repression, however the majority of miRNA-mediated repression occurs later through mRNA decay (Eichhorn et al., 2014).

It has been estimated that more than 50% of human genes are subject to miRNA regulation (Friedman et al., 2009). In humans, the predicted number of functional mature miRNAs range from 1,500 to 2,500 while there are over 22,000 recognized protein-coding genes (Kehl et al., 2017; Naeli et al., 2022). Given that the number of genes vastly outnumbers the number of miRNAs it is not surprising that a single miRNA can regulate numerous mRNAs. Interestingly, a single gene can also be regulated by more than one miRNA (Gebert & MacRae, 2019). MicroRNAs regulate the expression of many genes important for healthy cellular function and as such, when dysregulated, abnormal miRNA expression can be implicated in disease including but not limited to diabetes (Ling et al., 2009), neurological disease (Provost, 2010), cardiovascular disease (Cheng & Zhang, 2010), and cancers (Iorio & Croce, 2012).

1.2 Canonical microRNA processing

MicroRNA genes are often found within intronic and intergenic regions of DNA where they are transcribed by RNA polymerase II into primary miRNAs (pri-miRNAs) with hairpin-like structures (Gregory et al., 2004; Lee et al., 2004). RNA polymerase III is sometimes required for the transcription of a few select pri-miRNAs however the majority are predicted to be transcripts of RNA polymerase II (Ohler et al., 2004). Pri-miRNA transcripts can range from hundreds of base-pairs (bp) in length to over 10 kilobases (kb) (Bartel, 2004; Saini et al., 2007). The secondary structure of pri-miRNAs is of particular importance. This structure contains relatively long sections of single stranded RNA flanking double stranded RNA stem-loops (Auyeung et al., 2013). Pri-miRNA hairpin

structures are recognized and processed in the nucleus by the microprocessor complex which is composed of the ribonuclease III enzyme DROSHA and its RNA binding cofactor, DiGeorge Critical Region 8 (DGCR8) (Gregory et al., 2004). First, pri-miRNA transcripts form a pre-cleavage complex with DGCR8 by binding the base of the hairpin loop structure at the junction of single and double stranded RNA (SD junction). DROSHA then cleaves the pri-miRNA substrate at the base of the stem loop, about 11bp away from the SD junction (Lee, Han, et al., 2006). This produces a smaller ~70 nucleotide (nt) stem loop intermediate RNA fragment known as the precursor miRNA (pre-miRNAs) with a characteristic RNase III ~2 nt 3' overhang (Bartel, 2004).

Pre-miRNAs are then actively exported from the nucleus using Ran-GTP and Exportin 5 (XPO5), a nuclear export protein (Wu et al., 2018). In the cytoplasm, pre-miRNAs interact with another ribonuclease III enzyme, DICER, which recognizes the double stranded portion of the RNA stem loop (Rossi, 2005). The pre-miRNA is cleaved by DICER in cooperation with a complex of proteins composed of the HSP90 chaperone protein, TRBP (transactivation response element RNA-binding protein), and protein activator of PKR (PACT) (Michlewski & Cáceres, 2019). Cleavage by DICER occurs around two helical turns from the base of the stem loop to yield ~22-nt long miRNA duplexes with a ~2 nt 3' overhang (Bartel, 2004). TRBP and PACT are not required for cleavage however they stabilize DICER and promote cleavage fidelity (Fukunaga et al., 2012; Lee, Hur, et al., 2006).

The miRNA duplexes produced consist of 3p and 5p strands which can both take part in RNA silencing. These strands are derived from the 3' and 5' ends of the hairpin respectively (Medley et al., 2021). A protein called argonaute (AGO) is required for

miRNA-directed cleavage of target transcripts. There are four isoforms of AGO1-4 in mammals however argonaute-2 (AGO2) is the prominent form involved in miRNA gene silencing (Gebert & MacRae, 2019). The miRNA duplex is then loaded onto AGO in an ATP-dependent manor using HSC70/HSP90 chaperone proteins which forms the RNA-induced silencing complex (RISC) (Iwasaki et al., 2010). In the context of a specific miRNA-mRNA relationship, one strand is known as the guide strand (miR) while the other is referred to as the passenger strand (miR*). Either the 3p or 5p derived miRNA strand can be the functional guide strand. Depending on the miRNA, the relative abundance of 3p and 5p miRNAs can be equal or preferential to one strand over another (Medley et al., 2021). AGO removes the passenger strand by unwinding the miRNA duplex while the guide/mature miRNA remains as part of the mature RNA-induced silencing complex (RISC) (Kobayashi & Tomari, 2016). The passenger miRNA strand is usually degraded however in some cases it also can be integrated into the RISC complex at a lesser frequency (Gregory et al., 2005). The mature miRNA with the RISC (miRISC) binds to both partially and fully complementarity mRNA targets through the conserved 5' seed sequence (Schmittgen, 2008).

Target gene expression is reduced in one of two ways, direct mRNA cleavage and translational repression (Djuranovic et al., 2011). The factors that dictate which type of miRNA silencing occurs are dependent upon the type of organism, developmental stage, and environmental conditions (Naeli et al., 2022). The exact mechanisms of translational repression are still unclear however there is some consensus that the predominant form of miRNA mediated repression is through inhibition of translation initiation. Translational initiation requires mRNA 5' cap binding by eukaryotic initiation factor 4E (eIF4E) (Naeli

et al., 2022). This process is obstructed through miRNA activity thereby preventing downstream ribosome assembly and translation elongation. mRNAs that are repressed from translation congregate into processing bodies (P-Bodies) where both transient gene silencing and mRNA decay occur (Humphreys et al., 2005; Pillai et al., 2005). Although translational repression of target genes is well documented as the initial form of gene silencing, the majority of miRNA-mediated repression in mammalian cells occurs later through mRNA decay (Eichhorn et al., 2014). Recent evidence suggests that miRNA-mediated cleavage occurs on the mRNA across from and between 10^{th} and 11^{th} nucleotides from the 5' end of the miRNA (Elbashir et al., 2001; Hansen et al., 2011). Through this process, mature canonical miRNAs are able to target and reduce the stability and translation of competent mRNA transcripts (Figure 2).

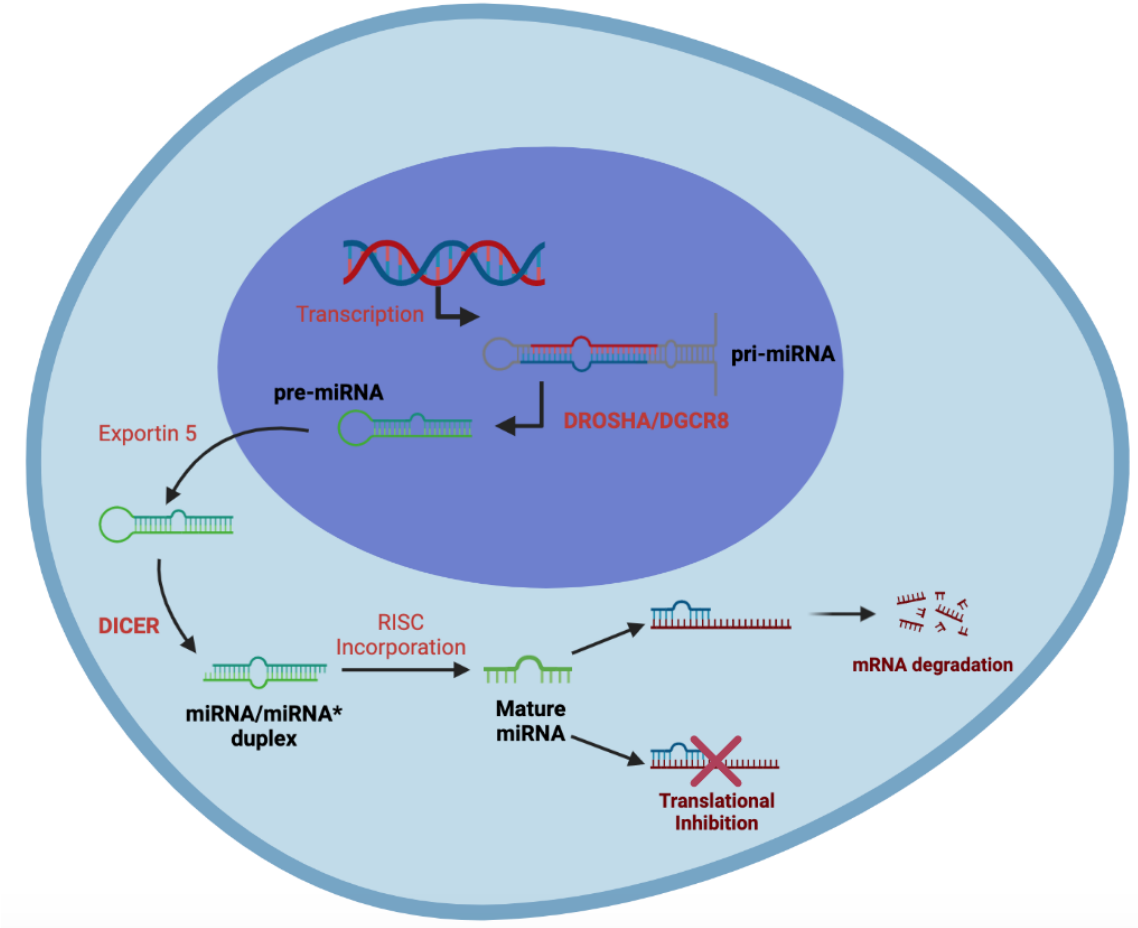

Figure 2. Canonical miRNA biogenesis pathway. MicroRNAs are transcribed and subsequently processed by the DROSHA/DGCR8 in the nucleus. They are exported into the cytoplasm by XPO5 and cleaved by DICER and loaded onto the RNA-induced silencing complex (RISC). Mature miRNAs guide the RISC complex to competent mRNA transcripts for destabilization and/or degradation. Created using BioRender.com.

1.3 Non-canonical microRNA processing

Only certain miRNAs are capable of circumventing the typical pathway of miRNA biogenesis, these are known as non-canonical miRNAs. Non-canonical miRNAs are produced independent of processing by one or both of the ribonuclease III enzymes DROSHA and DICER (Abdelfattah et al., 2014). In experimental models exhibiting disruption of DROSHA and/or DICER, miRNAs produced via the canonical pathway cannot be produced while many non-canonical miRNAs will persist (Kim et al., 2016).

DROSHA/DGCR8 independent miRNAs include mirtrons, small nucleolar RNA-derived miRNAs (snoRNAs), miRNAs from endogenous short hairpin RNAs, and miRNAs from tRNAs (Abdelfattah et al., 2014) (Figure 3). Mirtrons are similar to canonical miRNAs however they are always transcribed from intronic sequences and processed by spliceosomes in the nucleus rather than DROSHA/DGCR8. Although they contain short hairpin structures, mirtrons are too short for recognition by the microprocessor complex and therefore are exported from the nucleus and directly cleaved by DICER in the cytoplasm (Berezikov et al., 2007). snoRNAs are small noncoding RNAs found in the nucleolus of numerous organisms that have been found to interact with AGO1-4 and interfere with the translation and stability of mRNAs (Stavast & Erkeland, 2019). While some snoRNAs have been suggested to follow canonical miRNA biogenesis, others have been identified as DROSHA/DGCR8-indepenent and only require DICER for their production (Abdelfattah et al., 2014). Some siRNAs have also been discovered that are able to fold into pre-miRNA-like hairpin structures. These siRNAs, referred to as short hairpin RNAs (shRNAs) or 5'-capped pre-miRNAs, lack the conserved RNA sequence flanking the pri-miRNA hairpin required for microprocessor complex binding (Babiarz et al., 2008). For this reason, shRNAs are produced in a DROSHA/DGCR8 independent but DICER-dependent manor. Finally, some non-canonical miRNAs are derived from the transfer RNA (tRNA) maturation pathway. These RNA fragments are released from tRNA-processing enzymes and can act as substrates for DICER cleavage through their clover-leaf structure (Abdelfattah et al., 2014). Cleavage by DICER produces tRNA-derived RNA (tDR) fragments, some of which can associate with AGO to direct RNA silencing similar to canonical miRNAs (Stavast & Erkeland, 2019).

In the absence of DICER, the vast majority of canonical miRNA processing is prevented however some DICER-independent non-canonical miRNAs persist (Kim et al., 2016). One unique miRNA has been annotated that is known to be produced independent of DICER. Human miR-451 is similar to 5'-capped pre-miRNAs however it is processed in the nucleus by DROSHA/DGCR8 but is too short to act as a DICER substrate and therefore is loaded directly onto AGO (Cheloufi et al., 2010). Finally, some other less well-described non-canonical miRNAs exist that can be produced in the absence of DGCR8, Exportin-5, DICER and AGO2. These biomolecules, known as splicing-independent mirtron-like miRNAs (simtrons) are uniquely DROSHA-dependent but independent of all other major contributors to canonical miRNA processing (Havens et al., 2012). Two recognized simtrons, miR-1225 and miR-1228, are known to interact with the RISC complex and, like other types of miRNA, play a role in RNA silencing (Havens et al., 2012).

The degree to which non-canonical miRNAs contribute to healthy cellular function is still unclear. It is known that loss of canonical miRNA processing through disruption of DROSHA or DICER ribonucleases causes drastic changes in cellular phenotype (Abdelfattah et al., 2014). Embryonic lethality has been observed in knockout mice that are unable to produce functional DROSHA, DGCR8, DICER, or AGO2 (Park et al., 2010). Even tissue-specific conditional knockouts *in vivo* are often fatal, like in the case of DROSHA disruption in T-Cells which leads to lethal inflammatory disease (Chong et al., 2008). Considering these findings, it is clear that non-canonical miRNA activity alone is insufficient to maintain healthy cellular function. For this reason, only *in vitro* models can be used to study the global constitutive loss of canonical miRNAs. A DROSHA null cell

line was generated at the Center for RNA Research in Seoul, Korea using RNA-guided Cas9 endonucleases to knockout the RNase IIIa domain of DROSHA ribonuclease (Kim et al., 2016). In the generation of this knockout cell line, it was found that deletion of DROSHA prevented the production of 96.5% of the miRNAs detected in the parental (Wild-Type) cells. Some non-canonical miRNAs produced in the DROSHA null cell line were identified to be 5' capped pre-RNAs and mirtrons produced independently from DROSHA (Kim et al., 2016). This provided evidence helping to confirm that DROSHA is essential in the production of canonical miRNAs and that in its absence, some non-canonical miRNAs persist.

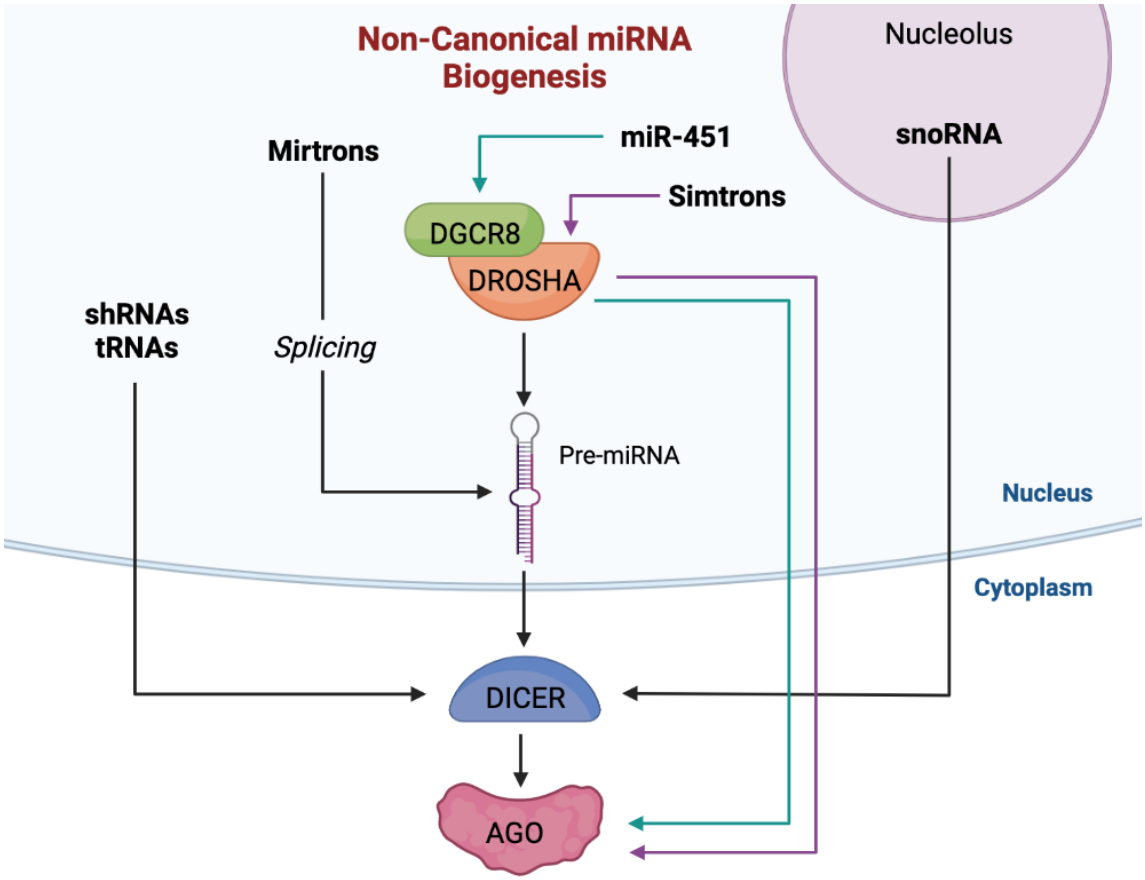

Figure 3. Non-canonical pathways of miRNA processing. DROSHA-independent miRNAs include mirtrons, shRNAs, snoRNAs, and tRNAs. DICER-independent miRNAs include miR-451 and simtrons. Created using BioRender.com.

1.4 MicroRNAs in drug responses

It has been estimated that more than 50% of human genes are subject to regulation by miRNAs. For this reason, it is not surprising that miRNA activity affects the expression of genes associated with stress responses to pharmacological agents (Rukov & Shomron, 2011). Response to drug treatment depends on many cellular processes including membrane transport, drug breakdown/metabolism, and the ability for a drug to interact with its target (Nikolaou et al., 2018). Recent studies have suggested that cancer cells can adopt resistance to chemical agents by altering miRNA expression targeting membrane transport proteins like ATP-binding cassette (ABC) transporters (Chen et al., 2015). Examples like this highlight the importance of miRNAs as regulators of the cellular microenvironment. It has been demonstrated that ABCB1 (MDR1), a multidrug-resistance transporter protein involved in the cellular export of numerous agents, is negatively regulated by miR-873 (Wu et al., 2016). These studies have suggested that the dysregulation of ABCB1 by altered miR-873 expression may promote a multidrug-resistance phenotype conferring resistance to paclitaxel in ovarian cancer cells (Wu et al., 2016). The broad scope of pharmacological agents that can be transported by ABCB1 suggests that miRNA activity likely affects resistance to more than just paclitaxel. Cisplatin-resistance in MCF-7 breast cancer cells has been partially attributed to reduced expression of miR-345 and miR-7 which target the *ABCC1* gene (Pogribny et al., 2010). Actinomycin-D (ACT-D), an antibiotic with deleterious effects on transcription and DNA synthesis, has been reported to use ABCB1 in addition ABCC1 and ABCC2 for efflux from the cell (Hill et al., 2013; Liu et al., 2016). It therefore may be a reasonable prediction that miRNA activity also affects cellular responses to ACT-D.

The examples listed above outline the ways in which miRNAs can affect intracellular pharmacokinetic processes like drug transport. However, studies grouping gene ontology have suggested that miRNAs play a much more significant role in regulating the biological response to drugs (Rukov & Shomron, 2011). Previous studies have shown that miRNA activity may affect therapeutic responses to anti-cancer drugs and other pharmacological agents. For example, miRNAs miR-106 and miR-150 which function to regulate *RB1* and *TP53* genes respectively, were shown to reduce the sensitivity of A549 cells to cisplatin-induced apoptosis (Wang et al., 2010). In this example, DNA damage caused by cisplatin would normally promote cell cycle arrest and apoptosis through p53 as well as p21 and RB downstream (Gonzalez et al., 2001). In conditions with excess miR-106 and miR-150 activity the levels of available *TP53* and *RB1* transcripts are insufficient for cisplatin alone to induce apoptosis (Wang et al., 2010). See *Rukov and Shomron 2011* for a compiled list of studies where specific miRNAs were implicated in variable cellular responses to different chemical agents.

1.5 Types of cell death

Although it seems counter-intuitive cell death is a process that is imperative to the healthy function of multicellular life. The clearance and replacement of old and/or damaged cells prevents numerous life-threatening diseases like cancers which develop due to the uncontrolled growth and division of cells usually due to genetic dysregulation and/or mutation (D'Arcy, 2019). Two major subtypes of cell death are apoptosis and necrosis. A third process, known as autophagy, has also been suggested as an addition type of cell death (Hotchkiss et al., 2009). These distinct modes of cell death differ in the way that they are induced, their biomolecular signals and the consequential morphological changes that

occur as a result of these processes (Galluzzi et al., 2007). Apoptosis and autophagy are two different types of programmed cell death which means they are controlled at the genetic level (Conradt, 2009). In contrast, necrosis is usually considered non-programmed type of cell death, however it its guided at a lower level by some pre-programmed mechanisms (Ouyang et al., 2012).

Apoptosis is one of the most well-known and important modes of cell death. The trademark morphological features of cells undergoing apoptosis are decreased size of the cell, condensation of chromatin, nuclear fragmentation, and plasma-membrane bleb formation (Doonan & Cotter, 2008). An important distinction between apoptosis and necrosis is that membrane integrity is preserved until the late stages of apoptosis while it is lost earlier in the process of necrosis (Doonan & Cotter, 2008; Nishimura & Lemasters, 2001). The two major pathways of apoptosis are the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Figure 4). These are separate but convergent processes that are activated in distinct ways however both require the activation of cysteine-proteases known as caspases, to act as effectors of programmed cell death (Elmore, 2007).

The death receptor (DR) pathway is triggered when members of the tumor necrosis factor (TNF) superfamily interact with "death receptors" on the surface of the cell (Sayers, 2011). Multiple DRs and DR-ligands exist, for example the TNF superfamily includes the ligand TNF which binds to TNF receptor 1 (TNFR1). Another similar example is CD95 (FAS) which is a DR that is ligated by the CD95-ligand (FAS-L) (Newton et al., 1998). In this process, stimulation of death receptors by DR ligands leads to the recruitment of procaspase 8 monomers to the cytosolic domain of the receptor. A multiprotein complex

is assembled in the cytosol called the death-inducing signal complex (DISC) (D'Arcy, 2019). Procaspase 8 associates with the DISC through adaptor proteins like FAS-associated death domain (FADD). The accumulation of procaspase 8 at the DISC promotes dimerization of procaspase 8 leading to activation of caspase 8 (Peter & Krammer, 2003). Caspase 8 activates downstream effectors of apoptosis like the executioner caspases 3, 6, and 7 (Sayers, 2011).

The mitochondrial pathway of apoptosis is induced by endogenous signals produced by the cell in response to stressors like reactive oxygen species (ROS), DNA damage, the unfolded protein response (UPR) and other pro-apoptotic stimuli (Loreto et al., 2014; Pandey et al., 2019). The fine balance between pro-apoptotic and anti-apoptotic signals from members of the BCL2 protein family regulates the mitochondrial pathway of apoptosis (Singh et al., 2019). BCL2 protein family members are identified by the presence of a BCL2 homology protein domain which facilitates various protein-protein interactions to aid in this regulatory process (Adams & Cory, 2007). This protein family is made up of both pro-survival and pro-apoptotic members (Hernández Borrero & El-Deiry, 2021). Pro-survival members of this family like BCL2 and BCL-XL typically have up to four BCL2 homology domains. Pro-apoptotic proteins in the BCL2 homology family like BAX and BAK have three BCL2 homology regions while others like PUMA, NOXA, BIM, and BID contain a BCL2 homology 3 (BH3) domain (Hotchkiss et al., 2009). Different stress stimuli act through activation of these BH3 domain containing proteins to induce apoptosis. For example, BIM induces apoptosis in the absence of sufficient levels of growth factors while PUMA is essential for inducing apoptosis following DNA damage (Bouillet et al., 1999; Jeffers et al., 2003). BH3-containing proteins bind to pro-survival BCL2 family members

like BCL2 to inhibit their function. In normal conditions, BCL2 binds to and prevents the activity of pro-apoptotic proteins like BAX. Inhibition of anti-apoptotic BCL2 proteins by BH3-containing proteins like PUMA leads to the release pro-apoptotic proteins like BAX and BAK (Kim et al., 2006). These proteins increase mitochondrial outer membrane permeability by oligomerizing into pores that increase membrane permeability (Cheng et al., 2001). Permeability of the mitochondrial outer membrane leads to the release of pro-apoptotic proteins like cytochrome c and other apoptogenic proteins like SMAC/DIABLO from the intermembrane space of the mitochondria into the cytoplasm. SMAC/DIABLO interferes with cytosolic inhibitors of apoptosis proteins (IAPs), promoting further caspase activation and reinforcement of the apoptotic signalling cascade (Goldstein et al., 2005; Strasser, 2005). Cytochrome c activates caspase-9 through the adaptor protein apoptotic protease activation factor 1 (APAF1). Cytochrome c binds to monomeric APAF1s inducing a conformational change that allows homo-oligomerization of APAF1 into a complex called the apoptosome. The apoptosome converts procaspase 9 into caspase 9 which promotes downstream signals and effectors of apoptosis (D'Arcy, 2019).

The death receptor and mitochondrial pathways of apoptosis converge at the step where the executioner caspases 3, 6, and 7 are activated by caspase 8 (death receptor pathway) and caspase 9 (mitochondrial pathway) (Strasser, 2005). Caspases 3, 6, and 7 are proteases that act as effectors of apoptosis by cleaving many cellular proteins as well as activating DNase enzymes in the nucleus that degrade genomic DNA (Hotchkiss et al., 2009). The pathway of apoptosis that occurs depends upon several factors like the state of cell cycle regulators and the type and degree of stress stimuli (Vermeulen et al., 2003). In

some cases, both of major apoptotic pathways can be simultaneously activated in response to multiple stress stimuli (Hughes et al., 2008).

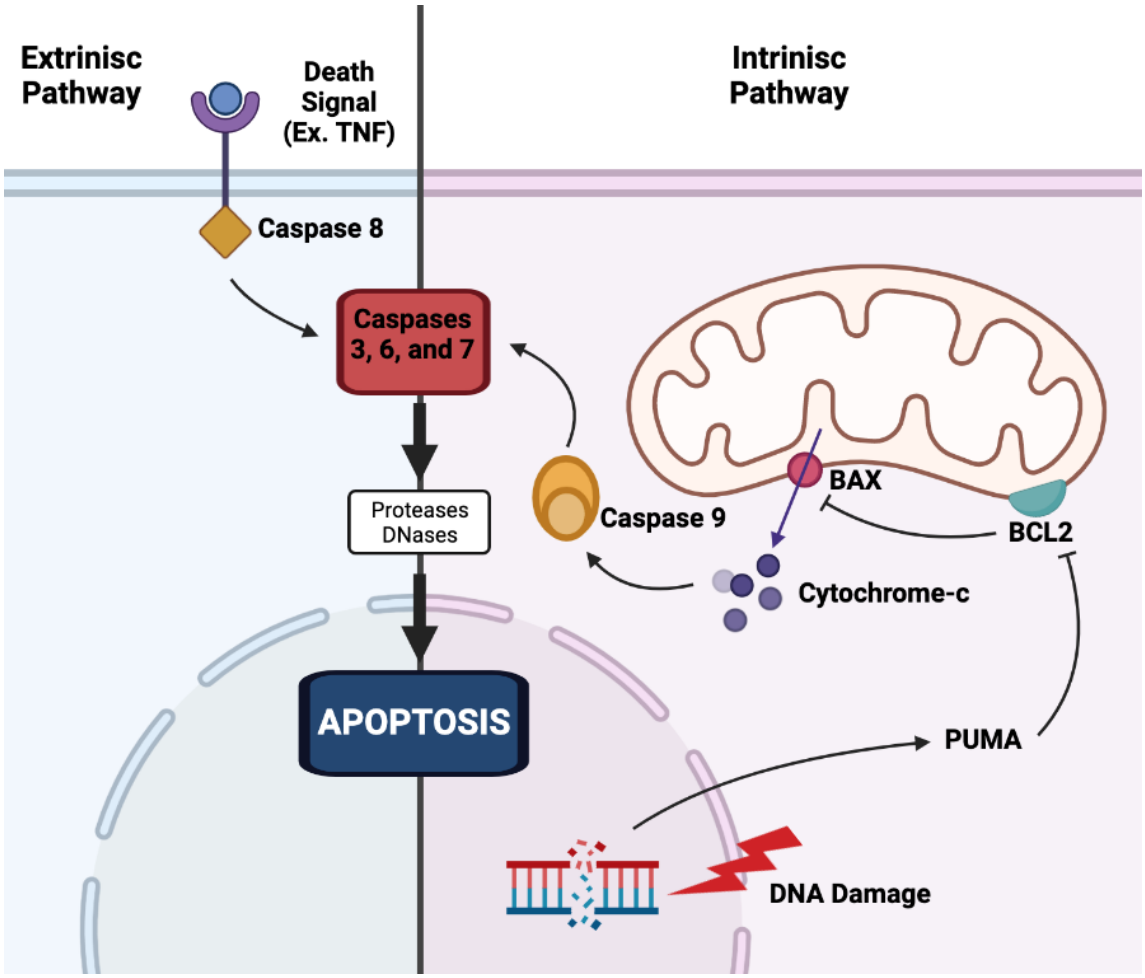

Figure 4. The two major pathways of apoptosis. The death receptor pathway (extrinsic) is activated by cell surface death receptors and the mitochondrial pathway (intrinsic) is activated by stressors like DNA damage that lead to proapoptotic signalling at the mitochondria. Apoptosis is activated through these distinct pathways which promote apoptosis through the activation of initiator (caspase 8 and 9) and executioner caspases (caspase 3, 6, and 7). Created using BioRender.com.

Necrosis is another form of cell death that is often identified by morphological changes like cell and organelle swelling, loss of membrane integrity, and activation of inflammatory responses (Doonan & Cotter, 2008). It is typically caused by severe cellular

damage resulting from hypoxia, radiation, chemical agents, and/or extreme temperature coupled with the exhaustion of ATP sources (D'Arcy, 2019; Proskuryakov et al., 2003). The spillage of proteolytic enzymes, like cathepsins, into the cytoplasm from ruptured lysosomes is a major cause of cellular damage during necrosis. Processes leading to necrosis often involve ROS, calcium ions, and proteins like poly-ADP-ribose polymerase (PARP) which promote deleterious activity of proteolytic enzymes (Hotchkiss et al., 2009; Zong & Thompson, 2006). One hallmark feature of necrosis is an increase in intracellular calcium ion concentration. In necrosis, the integrity of the cell membrane is compromised which causes an influx of extracellular ions like calcium and fluids (Doonan & Cotter, 2008; Zong & Thompson, 2006). This results in hypertonicity and the characteristic cell and organelle swelling associated with necrosis. In addition to swelling, the increased calcium concentration in the cell activates effectors of necrosis like intracellular Ca^{2+}-dependent proteases. One type of Ca^{2+}-dependent proteases known as calpains destroy cellular components like the cytoskeleton, the plasma membrane, transporters, and other important proteins (Bano et al., 2005; Zong & Thompson, 2006). In response to severe DNA damage caused by ROS or other damaging agents, the enzyme PARP facilitates DNA repair in an ATP-dependent manor (Zong & Thompson, 2006). Usually, in programmed cell death like apoptosis, PARP is cleaved and consequently inactivated in order to protect the cells limited sources of ATP (Proskuryakov et al., 2003). Highly stressed cells can only undergo apoptosis in the presence of ATP while in the absence of ATP, cell death proceeds by necrosis. Following severe oxidative stress and/or DNA damage PARP is activated leading to depletion of ATP and induction of necrosis (Los et al., 2002; Proskuryakov et al., 2003). Necrosis can also be activated by the ligation of extracellular signals to special

receptors on the surface of the cell, for example high concentrations of TNF (Laster et al., 1988). *In vivo* necrotic cells release damage-associated molecular-pattern (DAMP) molecules into the circulatory system which activate innate immune defence responses (Hotchkiss et al., 2009; Zong & Thompson, 2006).

Autophagy describes a crucial process where cells break down and recycle overabundant and/or damaged macromolecules and organelles (Levine & Deretic, 2007). It is an adaptive process that is usually not lethal and is activated in response to low levels of stress like nutrient scarcity. This process is important for metabolic efficiency in low nutrient conditions and for the clearance of misfolded proteins and pathogens (Levine & Deretic, 2007). It is controversial whether autophagy alone is a mode of cell death however it is suggested that in conditions where nutrient scarcity is prolonged, the absence of autophagic substrates may lead to a type of autophagy-associated or autophagic cell death (ACD) (Hotchkiss et al., 2009; Kroemer et al., 2009). There is a general consensus that while autophagy may not independently cause cell death, major features of autophagy often either precede or accompany other modes of cell death (Kroemer & Levine, 2008). In other words, autophagy likely contributes to cell death rather than acting as the sole facilitator. The predominant feature that characterizes ACD is an elevated number autophagy-associated double membraned structures called autophagosomes (Jung et al., 2020). These structures are formed in the major pathway of autophagy, known as macroautophagy. The autophagosome encloses the materials to be degraded before fusing with the lysosome. In the lysosome, acidic hydrolases breakdown the material into smaller substrates which are recycled for later use in the assembly new biomolecules (D'Arcy, 2019). Two other forms of autophagy exist which differ from macroautophagy in the ways that materials destined

for degradation are delivered to lysosomes: microautophagy and chaperone-mediated autophagy. Microautophagy refers to the process by which the membrane of the lysosome engulfs materials destined for degradation (Li et al., 2012). Chaperone-mediated autophagy requires heat-shock cognate proteins which facilitate the transport of autophagic cargo (Dice, 2007). Previous research into autophagy-associated cell death has indicated that the disruption of autophagy-associated genes promotes cell death rather than survival suggesting that autophagy exists primarily to promote cell survival (Hotchkiss et al., 2009). This is complimented by clinical evidence which reports that autophagy can foster resistance to cell death induced by DNA-damaging agents (Sui et al., 2013).

1.6 p53 structure and function

The p53 tumor suppressor is a protein that plays crucial roles in protecting the integrity of DNA in the cell, thus it has been termed "the guardian of the genome". Its importance in maintaining healthy cellular function cannot be understated as the gene encoding p53, *TP53*, is mutated in more than 50% of all cancers (Lain & Lane, 2003). p53 acts as a transcription factor and is involved in a complex biomolecular network responding to a variety of stressors including DNA damage, activation of oncogenes, and replication stress. When cells are subjected to one of these stressors, p53 can interact with other proteins and/or be post-translationally modified to control the transcription of response genes (Kastenhuber & Lowe, 2017).

The p53 protein structure is made up of five major domains: the transactivation domain (TAD), proline-rich domain (PRD), DNA-binding domain (DBD), tetramerization domain (TD), and a regulatory domain (Harris, 1996). The TAD is comprised of TAD1 and TAD2 which bind to different cofactors to facilitate a variety of stress-induced p53

signals. The TAD region is important for binding MDM2, a major negative regulator of p53 activity (Brady et al., 2011; Lin et al., 1994). The PRD of p53 aids in DNA binding which is particularly important to p53 as a transcription factor (Walker & Levine, 1996). The DBD, as its name suggests, is also involved in p53 DNA binding. A p53 response element of target genes is recognized by the DBD of p53 allowing it to function as a transcription factor (Hernández Borrero & El-Deiry, 2021). The TD allows for oligomerization of four p53 proteins into a tetramer which facilitates p53-protein interactions and DNA binding. The p53 oligomer has slight conformational changes that aid in molecular interactions like ubiquitination of p53 by MDM2 which requires this tetrameric state (Halazonetis & Kandil, 1993; Maki, 1999). Finally, the C-terminus regulatory domain of p53 blocks its own DBD, preventing p53 transcriptional activity. Cellular stress signals lead to modification of this regulatory domain by acetylation or phosphorylation, which activates p53, allowing its DBD to bind p53 response elements (Hupp et al., 1992; Liu et al., 2004). Nuclear export and localization signals are also found on this C-terminus regulatory domain. These signals are important to allow p53 to act as a transcription factor in the nucleus as well as its proteasomal degradation in the cytosol following ubiquitination by MDM2 (Hernández Borrero & El-Deiry, 2021; Lohrum et al., 2001).

Activation of p53 signals can promote DNA repair, reduced cell cycle progression, replicative senescence, and apoptosis (Smith et al., 2003) (Figure 5). The p53 protein functions in a negative feedback loop with MDM2, its negative regulator. In normal conditions, MDM2 is bound to p53 preventing activation, however in stressed conditions p53 is phosphorylated and the interaction between MDM2 and p53 is weakened, leading

to the liberation and activation of p53 (Lin et al., 1994; Teufel et al., 2009). DNA damage is detected through telangiectasia-muted (ATM) or ataxia telangiectasia and Rad3-related (ATR) kinases which act as sensors of genomic stress. ATM/ATR activity promotes DNA damage responses (DDR) by phosphorylating inactive p53-MDM2 protein complexes leading to the release and subsequent activation of p53 (Mijit et al., 2020). Active p53 acts as a transcription factor to promote the expression of a wide array of genes including *MDM2* (Cabrita et al., 2017). MDM2 proteins are then translated, completing the negative feedback loop as MDM2 interacts with p53 to reduce its activity. p53 is also able to activate cell cycle arrest in response to stress through cell cycle checkpoints (Cabrita et al., 2016). In the face of stress like DNA damage, p53 activity leads to a reduction in cell cycle progression by promoting transcription of *CDKN1A*, a downstream target gene encoding the $p21^{WAF1}$ (p21) protein. p21 is a cyclin-dependent kinase inhibitor (CKI) that is important for inducing cell cycle arrest in response to signals from p53 (Cabrita et al., 2016). It primarily does this by interacting with the cyclin-dependent kinase (CDK) complexes like cyclin E/CDK2 and cyclin D/CDK4 to promote G_1 interphase arrest by blocking the phosphorylation of the retinoblastoma protein (RB). Unphosphorylated RB is sequesters E2F transcription factors preventing the transcription of genes required for cell cycle progression (Hernández Borrero & El-Deiry, 2021; Stewart et al., 1999). p21 can also promote a G_2/M phase arrest by blocking the cyclin B/CDK1 complex, however this is less frequent than the G_1 arrest (Dash & El-Deiry, 2005). Other ways that p53 can contribute to cell cycle arrest include transactivation of proliferating cell nuclear antigen (PCNA), a protein important for DNA synthesis and repair. PCNA can interact with other p53 activated proteins like p21 and GADD45 thereby interfering with DNA replication

(Xu & Morris, 1999). p53 regulated transcription of growth arrest and DNA damage inducible genes (GADD) like *GADD45* can also promote cell cycle arrest in both G_1/S and G_2/M phase (Kastan et al., 1992; Wang et al., 1999).

DNA damage can be caused by numerous conditions including but not limited to radiation, chemical agents, and oxidative stress (Hotchkiss et al., 2009). In stressed conditions, p53 first halts cell cycle progression as previously described to prevent the accumulation of damage before promoting DNA repair, replicative senescence, or apoptosis (Helton & Chen, 2007). The fate of the cell depends on the level of damage present in the cell. In low levels of stress, p53 can promote cell cycle arrest and DNA repair mechanisms to allow for recovery from mild stress stimuli. Numerous DNA repair pathways can be activated or facilitated by p53 including nucleotide excision repair (NER), mismatch repair (MMR), base excision repair (BER), homologous recombination (HR), and non-homologous end-joining (NHEJ) (Helton & Chen, 2007). Although p53 can regulate DNA repair independent of transcriptional activity it also can induce transcription of DNA repair proteins like DDB2, XPC and PCNA. The production of DNA repair associated genes promotes the assembly of DNA repair machinery and subsequent repair processes (Helton & Chen, 2007; Hernández Borrero & El-Deiry, 2021).

To protect the integrity of DNA during high levels of genomic stress p53 can also direct a process called replicative senescence. Senescence is an irreversible type of cell cycle arrest that occurs in aged cells that have undergone numerous divisions leading to DNA damage like the shortening of telomeres (Mijit et al., 2020). Various other stress factors can also promote what is referred to as stress-induced premature senescence (SIPS) like oncogene activation, DNA damage, and oxidative damage (van Deursen, 2014).

Senescent phenotype can be dependent on cell type, however it is often characterized by morphological changes like cellular enlargement, expression of senescence-associated B-galactosidase, and resistance to apoptosis (Itahana et al., 2001). High levels of DNA damage can be caused by both endogenous and exogenous factors like ROS and pharmacological agents. When damage is too severe for the cell to re-initiate cell cycle progression, replicative senescence is induced through ATM/ATR which activate p53 by liberating it from MDM2 (Hu et al., 2012). Other studies have shown that numerous pathways also exist by which p53 can be activated to promote replicative senescence even in the absence of DDR initiators (Jung et al., 2019). This highlights the importance of p53 in facilitating induced-senescent phenotypes. In response to stress, p53 plays a role in the initiation of replicative senescence through p21. The inhibitory effects of p21 by CDK inhibition initiate cell cycle arrest which is one of the hallmark features of senescence (Itahana et al., 2001). In addition to this, p21 activity inhibits apoptosis which is often mediated through p53 by preventing the activity of pro-apoptotic biomolecules like caspases (Yosef et al., 2017). While p21 plays a major role in initiating p53-induced senescence, the senescent phenotype is preserved through the CDK inhibitor p16 and members of the retinoblastoma protein (RB) family (Itahana et al., 2001). P16 inhibits CDK4-6 thereby preventing RB phosphorylation. Unphosphorylated RB remains bound to transcription factors like E2F proteins preventing the production of proteins required for cell cycle progression like cyclin E and A (Bringold & Serrano, 2000). Through this process p16 aids in maintaining senescent phenotype by hindering RB function thus sustaining the cell cycle arrest initiated by p53 and p21 activity (Mijit et al., 2020).

In high levels of stress where DNA damage is too severe to repair, p53 will promote cell death by transcribing the genes responsible for apoptosis. p53-mediated apoptosis can occur through both the extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway is simply promoted through augmented transcription of cell surface death receptors like FAS and TRAIL receptors (DR4/DR5) (Helton & Chen, 2007; Maelfait & Beyaert, 2008). In response to severe DNA damage p53 promotes the intrinsic apoptotic pathway through the BCL2 family of proteins. BH family members play important roles in apoptosis stimulated by oncogene activation and/or genotoxic damage, with the major regulator being p53, the guardian of the genome (Elmore, 2007). p53 transcriptional activity transactivates pro-apoptotic BAX, NOXA, and PUMA which in turn interfere with anti-apoptotic BCL2 family members. In addition to this, following translocation to the mitochondria p53 can directly interact with BCL2 family members like BAX can promote their homo-oligomerization which contributes to mitochondrial outer membrane permeabilization (Chipuk et al., 2004; Helton & Chen, 2007). p53 transcriptional activity and interactions with the BCL2 protein family both lead to the liberation of cytochrome c from the mitochondria. Cytochrome c activates caspase 9 to promote apoptosis via the intrinsic pathway apoptosis (D'Arcy, 2019). The extrinsic apoptotic pathway converges with the intrinsic pathway after caspase 8 is activated downstream of death receptors (Strasser, 2005). Caspases 3, 6, and 7 are proteases that act as effectors of apoptosis by cleaving many cellular proteins and activating DNase enzymes in the nucleus to degrade genomic DNA (Hotchkiss et al., 2009).

Many pharmacological agents are known to produce DNA damage and the subsequent activation of programmed cell death via a p53-dependent signalling cascade

(Ljungman et al., 1999). Actinomycin-D (ACT-D) is an antibiotic that inhibits transcription by intercalating DNA base pairs, blocking the progression of RNA polymerases (Liu et al., 2016). ACT-D has been reported to induce DNA damage, increase p53 protein levels and promote G_1-phase arrest (Kastan et al., 1991).

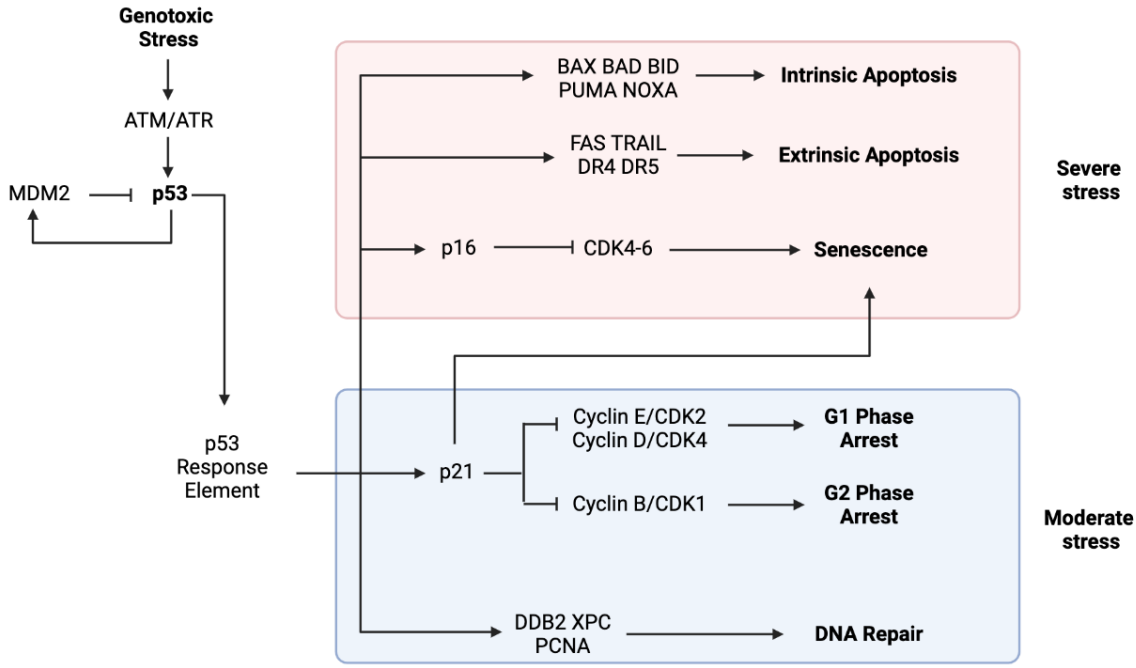

Figure 5. p53 transactivates different processes in response to genotoxic stress. p53 is negatively regulated by MDM2. Genotoxic stress activates p53 through ATM/ATR thereby promoting p53 response element genes. Under mild stress DDB2/XPC/PCNA are activated to promote DNA repair and p21 reduces cell cycle progression. Severe stress promotes replicative senescence through p21/p16 and pro-apoptotic signals through PUMA/NOXA/BID/BAD/BAX and FAS/TRAIL/DR4/DR5. Created using BioRender.com.

1.7 p53 and microRNAs

Patterns of transcriptional activity by p53 involve a complex network of feedback loops and signalling cascades that promote and stabilize effectors of stress-induced p53 responses. MicroRNAs are important post-transcriptional regulators of gene expression that significantly contribute to regulating p53 activity (Liao et al., 2014). As such, they

play a role in modulating many of the major cellular processes that are mediated by p53 like cell cycle arrest and apoptosis. p53-miRNA regulatory interactions are considerably extensive and complex however a subset of these important interactions is given here (Figure 6). The miR-34 family of miRNAs are upregulated by p53 in order to inhibit the production of proteins involved in cell cycle progression and cell survival, for example CDK4, CDK6, and BCL2 (Hermeking, 2012). The inhibition of these CDKs prevents the phosphorylation of RB protein leading to reduced cell cycle progression (Stewart et al., 1999). By inhibiting the production of the pro-survival BCL2 protein, the miR-34 family also elicits a pro-apoptotic effect (Kim et al., 2006). Other miRNAs like miR-192 and miR-215 are specifically activated by p53 following DNA damage-associated stress. Similar to the miR-34 family, these miRNAs act to downregulate the expression of proteins involved in cell cycle progression and survival like RB1 a crucial transcriptional regulator, and PIM1 a ser/thr kinase (Feng et al., 2011; Georges et al., 2008; Knudsen & Vasioukhin, 2010). miRNA-directed translational repression and/or degradation of these transcripts therefore promotes cell cycle arrest and susceptibility to cell death. miR-205 and miR-107 are two additional miRNAs involved in negative regulation of the cell cycle. miR-205 inhibits E2F1 thereby blocking the production of important substrates for cell cycle progression like cyclin E and A (Bringold & Serrano, 2000; Piovan et al., 2012). miR-107 targets CDK6, similar to the miR-34 family, to promote arrest in the G_1/S phase of the cell cycle (Böhlig et al., 2011; Hernández Borrero & El-Deiry, 2021).

Given the complexity of the p53 network, it is not surprising the p53 itself is regulated by p53-induced miRNAs (Figure 6). It does this partially through miR-34a, miR-143, miR-145, miR-192, miR-194, and miR-215 all of which act to reduce expression of

MDM2 the negative regulator of p53, and MDMX a homolog of MDM2 (Mandke et al., 2012; Pichiorri et al., 2010; Shadfan et al., 2012; Zhang et al., 2013). By repressing and degrading MDM2 transcripts through miRNAs, p53 promotes its own activity in a positive feedback loop. At least 20 p53-induced miRNAs are recognized that directly regulate p53 in a negative feedback loop. Some of these miRNAs include miR-125b, miR-504, miR-33, miR-380-5p, miR-25, miR-30d, miR-214, miR,-375, miR-1285, and miR-3151(Liu et al., 2017). All of these miRNAs have been reported to bind to the 3' UTR of p53 mRNA and interfere with its translation.

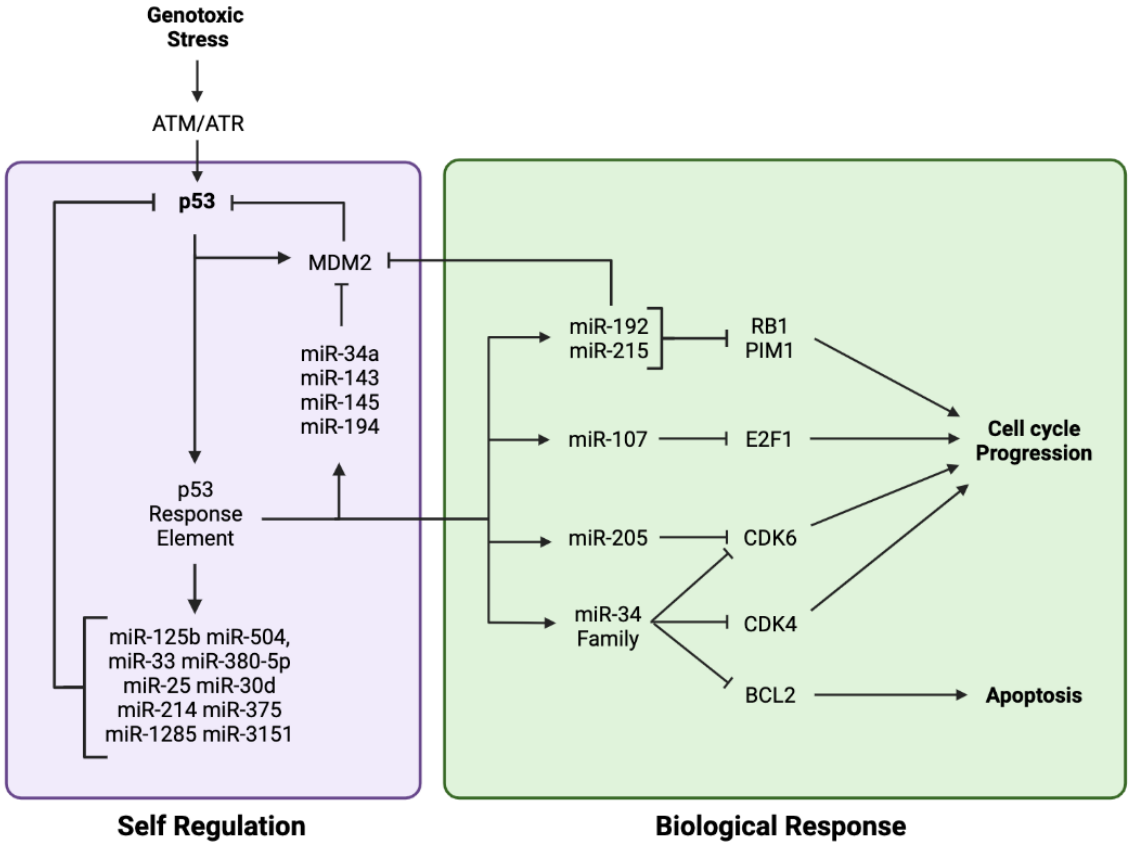

Figure 6. p53 transactivates many miRNAs with a variety of effects. p53-induced miRNAs can contribute to regulation of cell cycle progression and apoptosis. p53-induced miRNAs also regulate p53 itself both directly and indirectly through inhibition of MDM2, the major negative regulator of p53. Created using BioRender.com.

1.8 Rationale and hypothesis

Post-transcriptional regulation of gene expression occurs in several ways from mRNA processing to altering of the expression and degradation of mRNAs by RNA binding proteins and non-coding RNAs like miRNAs (Buccitelli & Selbach, 2020). An emerging field in bioinformatics termed miRNA pharmacogenomics attempts to decipher the importance of specific miRNAs in cellular responses to pharmacological agents (Rukov & Shomron, 2011). These recent studies have highlighted the importance of miRNAs as post-transcriptional regulators of gene expression. Previous studies using experimental approaches have examined the role of individual miRNAs on stress and DNA damage responses. However, one miRNA almost never describes the whole picture, and the net effect of miRNAs is often unclear in specific cellular responses. Multiple miRNAs can cooperate in order to fine-tune gene expression by promoting a specific process and simultaneously inhibiting the inhibitors of that process (Bracken et al., 2016). To add to this, singular miRNAs have the capacity to directly regulate multiple genes as well as indirectly by targeting the transcripts of other regulatory molecules like transcription factors (Bracken et al., 2016). As we have learned more about these complex regulatory networks it has become increasingly clear that considering only singular miRNA-mRNA relationships is insufficient to understanding how gene expression is altered in responses to stress. To our knowledge the effect of disrupting miRNA processing altogether on cellular drug responses has not been examined. As such, the focus of this research was to assess implications of a total disruption of canonical miRNA processing in the context of drug responses. To do this we chose to use a cell line carrying a deletion in the *DROSHA* gene to determine the overall contribution of miRNAs to drug responses *in vitro*. We

hypothesized that the absence of DROSHA would affect cellular responses to pharmacological agents but given the literature we could not predict either the direction of the effect or the relevant agents.

1.9 Objective

The objective of this study was to evaluate whether the disruption of the ribonuclease III enzyme DROSHA affects cellular responses to pharmacological agents. We used the HCT116 human colon cancer cell line in addition to a genetically modified HCT116 subline lacking functional DROSHA to measure differential sensitivity, direct effects on common biochemical processes, and the p53 response following treatment with the DNA damage inducing agent actinomycin-D. The goal of this work was to determine if and to what extent the loss of canonical miRNA processing affects cellular responses to pharmacological agents.

2. Methods

2.1 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). Normal fibroblasts (GM00038) were obtained from Coriell Repositories (Camden, NJ) and normal neonatal forskin fibroblasts expressing human telomerase (NFhTrt) were obtained from Mats Ljungman (University of Michigan) (O'Hagan & Ljungman, 2004). GM00038 (GM38) and NFhTrt fibroblasts were grown in DMEM media (+4.5g/L D-Glucose, +L-Glutamine, +110 mg/L Sodium Pyruvate) (Gibco, Bleiswijk, NL) supplemented with 10% FBS, 90 units/mL penicillin and 90 μg/mL streptomycin antibiotics. All cell lines were grown in an incubator at 37°C with 5% CO_2.

2.2 Drug Treatment

For all initial sub-G_1 drug screening experiments, drug names, stock solutions, and experimental concentrations prepared are given in Table 1. Prior to all experiments cells were seeded in fresh growth medium at least 24 hours before all drug treatments. For all other experiments cell cultures were treated with a no drug (ND) dH_2O vehicle control, 10, 25, 50, 100, and/or 250nM actinomycin-D (ACT-D) (Sigma-Aldrich, Cat#A9415, St. Louis, MO) prepared using a 20μM ACT-D in dH_2O stock solution diluted in McCoy's 5A medium.

Drug Name	Stock Solution (Concentration/Diluent)	Experimental concentrations
Doxorubicin (Sigma-Aldrich, Cat#D1515, St. Louis, MO)	86.2μM in dH_2O	0.1, 0.25, 0.5, and 1.0 μM
Paclitaxel (Sigma-Aldrich, Cat#T7191, St. Louis, MO)	Stock #1: 1171μM in methanol Stock #2: 2μM in dH_2O	1, 2, 5, 10, and 25nM
Vincristine (Sigma-Aldrich, Cat#V8879, St. Louis, MO)	Stock #1: 1mM in dH_2O Stock #2: 2μM in dH_2O	2, 5, 10, 25, and100nM
Actinomycin-D (Sigma-Aldrich, Cat#A9415, St. Louis, MO)	Stock #1: 797μM in dH_2O (1mg/mL) Stock #2: 20μM in dH_2O	10, 25, 50, 100, and 250nM
DRB (Sigma-Aldrich, Cat#D1916, St. Louis, MO)	50mM in DMSO	25, 50, and 100μM
Cisplatin (Sigma-Aldrich, St. Louis, MO)	5mM in dH_2O	5, 10 and 20μM
Cytochalasin-D (Sigma-Aldrich, Cat#C8273, St. Louis, MO)	2.55mM in dH_2O	1, 2.5, and 10μM
Hydroxyurea (Sigma-Aldrich, Cat#H8627, St. Louis, MO)	Stock #1: 657mM in dH_2O (50mg/mL) Stock #2: 100mM in dH_2O	0.2, 0.5, 1.0, 2.0, and 5.0mM
Mimosine (Sigma-Aldrich, Cat#M0253, St. Louis, MO)	10mM in dH_2O	100, 250, 500, and 1000μM
Cyclohexamide (Sigma-Aldrich, Cat#C7698, St. Louis, MO)	3.55mM in dH_2O	10, 20, and 30μM

Table 1. Drug preparation information for screening experiments. Information includes drug names, stock solutions (concentration/diluents), and experimental concentrations prepared.

2.3 Sub-G_1 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

agents 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, rinsed 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). 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 proportion of cells with sub-G_1, G_1, S, and G_2 levels of DNA content to be determined. Cells undergoing apoptosis fragment their DNA leading to a decrease in 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-G_1 DNA content in parental and DROSHA null HCT116 cells. Differences with $p<0.05$ were considered statistically significant.

2.4 Caspase 3/7 assay

The CellEvent™ Caspase 3/7 Green Flow Cytometry Assay Kit (Invitrogen, C10427, Eugene, ORE) was used to measure caspase activation in HCT116 P and DROSHA null cells treated for 24 hours with either a dH_2O vehicle control (ND) or 10, 25, 50, 100, or 250nM ACT-D. This kit uses an inactive fluorescent nucleic acid binding dye that when cleaved by activated caspase -3 or -7 emits a fluorescent signal detectable by flow cytometry. Adherent and detached cells were collected and resuspended in PBS. Then CellEvent™ caspase 3/7 green detection reagent was added and allowed to incubate at 37°C for 25 minutes before SYTOX™ AADvanced™ Dead cell stain was added and allowed to incubate for an additional 5 minutes. Samples were analyzed using a BD Accuri

C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) to measure relative caspase activity by CellEvent™ caspase 3/7 green detection reagent fluorescence (511/533nm). Late apoptotic and necrotic cell death was measured by SYTOX™ AADvanced™ Dead cell stain fluorescence (546/647nm). Relative caspase activity was calculated for both parental and DROSHA null HCT116 cells by normalizing absorbance readings to the vehicle control.

2.5 Propidium iodide dye exclusion assay

For PI dye exclusion assays, HCT116 P and DROSHA null cells were seeded at a density of $9.0x10^4$ cells per well of a 6-well plate. After 24 hours, the media was replaced with fresh media containing a dH_2O vehicle control or 10, 25, 50, 100, or 250nM ACT-D. At both 24H and 48H following treatment, adherent and detached cells were collected, resuspended in PBS, and stained in 20 μg/ml of PI in PBS (+50μg/mL RNaseA, Bio Basic, Cat#RB0473, Markham, ON). A BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) was used to measure the relative proportion of PI positive cells from each sample. This type of live cell flow cytometry allowed the proportion of viable and non-viable cells to be quantified because PI is only able to enter cells that have lost membrane integrity. Membranes become permeable to PI during necrosis and late apoptosis (Harrison & Vickers, 1990; Hotchkiss et al., 2009).

2.6 Immunoblot Analysis

Cells were seeded in 6-well plates at a density of 180,000 cells per well. 24 hours later cells were treated with either a dH_2O vehicle controls, 50nM, or 250nM actinomycin-D for 8 and 24 hours. Proteins were collected using a cell scraper in 1% SDS (BioShop, Cat#SDS001.500, Burlington, ON) in dH_2O with protease inhibitor cocktail (Sigma-

Aldrich, Roche cOmplete ULTRA Protease Inhibitor Tablets, Cat#05892791001, St. Louis, MO), sonicated and quantified using the Bio-Rad Protein Assay (Bio-Rad, Cat#5000006, Hercules, CA) according to the manufacturers protocol. Absorbance was measured using an iMARK microplate reader (Bio-Rad, Hercules, CA) and the MPM6 software v6.3 (Bio-Rad, Hercules, 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 were prepared in dH_2O supplemented with 10X dichlorodiphenyltrichloroethane (DTT) (Invitrogen, Cat#NP0009, Carlsbad, CA) reducing reagent and 4X NuPAGE LDS sample buffer (Invitrogen, Cat#NP0007, Carlsbad, CA) before incubating at 70°C for 10 minutes. Protein samples and either the Precision Plus™ Dual Color Standards (Bio-Rad, Cat#161-0374, Hercules, CA) or the MagicMark™ XP Western Standard (Invitrogen, Cat#LC5602, Carlsbad, CA) protein ladder were 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). After the proteins were 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 ensure even protein 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: mouse anti-MDM2 (Santa Cruz Biotechnology, SMP14

sc-965, Dallas, TX), mouse anti-p21 (Calbiochem, Ab-1 OP64, Burlington, MA), mouse anti-Noxa (Calbiochem, 114C307 OP180, Burlington, MA), mouse anti-βactin (Sigma-Aldrich, A5316, St. Louis, MO), rabbit anti-Puma (Calbiochem, Ab-1 PC686, Burlington, MA) rabbit anti-Drosha (Cell Signalling Technology, D30F3 3410S, Danvers, MA) or rabbit anti-p53 (Santa Cruz Biotechnology, FL-393 sc-6243, Dallas, TX). Membranes were then washed (4x for 5 minutes) in TBST before incubation in the appropriate goat anti-mouse (Abcam, Cat#6789, Cambridge, UK) or goat anti-rabbit (Abcam, Cat#6721, Cambridge, UK) horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution in TBST for goat-anti-mouse; 1:20,000 for goat-anti-rabbit) 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 3Mega software v16.11 (Vilber Lourmat, Marne-la-Vallee, FR). Densitometric analysis of western blots was done using ImageJ software v.1.5.3.

2.7 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Prior to RNA isolation, cells were seeded in 6-well plates at a density of $1.8x10^5$ cells/well. 24 hours later, cells were treated with a dH_2O vehicle control, 50nM or 250nM actinomycin-D for 8 and 24 hours. RNA was collected using the EZ-10 DNAaway RNA Miniprep kit (Bio Basic, Cat#BS88136, Burlington, ON) according to the manufacturers protocol. Isolated RNA samples were quantified using a DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, DE). An equal amount of RNA sample was then reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Cat#4368814, Waltham, MA) according to the manufacturers

protocol. qRT-PCR was performed using the StepOnePlus Real-Time System (Applied Biosystems™, Waltham, MA) and the StepOne Software v2.3 (Applied Biosystems™, Waltham, MA). Reactions were prepared using 10µL of SensiFAST™ SYBR Hi-ROX Mix (BioLine, FroggaBio Inc., Trononto, ON) SYBR green, 4.2µL of RNAse free water, 4.2µL of cDNA, and 0.8µL of both forward and reverse primers each (Table 2). The cycle parameters were as follows: 2 minutes (min) at 95°C followed by 40 cycles of 5 seconds (sec) at 95°C, 15 sec at annealing temperature, and 1 min at 72°C. Annealing temperatures used were at least 5°C less than the lowest melting temperature (Tm) primer set.

Primer	**Forward Sequence (5'-3')**	**Reverse Sequence (5'-3')**
GAPDH	AGC CAC ATC GCT CAG ACA	GCC CAA TAC GAC CAA ATC
TP53 (Guo et al., 2021)	CAG CAC ATG ACG GAG GTT GT	TCA TCC AAA TAC TCC ACA CGC
CDKN1A	GGA GAC TCT CAG GGT CGA AA	GCT TCC TCT TGG AGA TCA G
MDM2	CCA TGA TCT ACA GGA ACT TGG TAG TA	TCA CTC ACA GAT GTA CCT GAG TCC
PUMA (BBC3)	AAA TCT CGG AAG AGG GAG GA	ATC TAC AGC GCA TAT ACA G

Table 2. Primers sequences used for gene expression analysis by qRT-PCR.

2.8 DNA replication assay: BrdU Staining

Cells were seeded at a density of $1.8x10^5$ cells/well in 6-well plates 48 hours before collection. 3 hours before collection the media was replaced with fresh media containing a dH_2O vehicle control or one of the ACT-D concentrations being tested. 2 hours following treatment, the media was replaced again with freshly prepared drug media containing 30µM 5-Bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich, Cat#B5002, St. Louis, MO). After 1 hour to allow for BrdU incorporation during DNA synthesis, cells were collected,

washed with PBS, and fixed overnight in 70% ethanol at -20°C. After fixation, cells were washed with PBS and incubated in a new PBS solution containing 50μg/mL RNaseA for 30 min at 37°C. Cells were washed with PBS again and resuspended in ice cold 0.1 N HCl + 0.7% Triton X-100 (Millipore Sigma, Cat#648466, Indianapolis, USA) for 15 mins. Cells were washed again with PBS, resuspended in dH_2O, placed in a boiling water bath for 15 mins, and then placed on ice for another 15 minutes. The cells were resuspended in PBS + 0.5% Tween 20 (Sigma-Aldrich, Cat#P9416, St. Louis, MO) before being resuspended in an FITC conjugated mouse anti-BrdU antibody (BD Pharmingen, Cat#556028, Franklin Lakes, NJ) in a 1:1000 dilution in HBT solution (PBS +4.7% FBS, +0.47% Tween 20) and incubated at room temperature for 30 mins in the dark. A final wash in HBT solution was followed by data analysis using the Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) which can detect the green-fluorescent signal emitted by the FITC-labelled BrdU incorporated into the DNA of replicating cells. The percentage of BrdU positive cells (percentage BrdU+) could then be calculated which indicates the level of DNA synthesis activity in treated cells relative to the vehicle control.

2.9 Transcription assay

We adapted the Click-iT® RNA Imaging Kit (Invitrogen™, C10329, Eugene, ORE) for a flow cytometric analysis of nascent RNA transcription in DROSHA null and parental HCT116 cells following treatment with ACT-D. $4.0x10^4$ cells/well were seeded in 48-well plates, 48 hours before collection. 3 hours before collection the media was replaced with fresh media containing a dH_2O vehicle control or one of the ACT-D concentrations being tested. 1 hour before collection, the media was once again replaced with new media containing the original ACT-D treatment concentration in addition to 1mM

ethynyl uridine (EU). EU is a ribonucleotide homolog used to label nascent RNA when investigating transcriptional activity. After waiting 1 hour to allow for fluorescent label incorporation during transcription, cells were collected and fixed using 3.7% formaldehyde in PBS for 15 mins at room temperature. The fixed cells were then washed with PBS and resuspended in 0.5% Triton® X-100 in PBS for 15 minutes at room temperature. The cells were washed again with PBS before being incubated in a Click-iT® reaction cocktail (prepared according to the manufacturers protocol) for 30 mins in the dark to fluorescently label EU incorporated into newly synthesized RNA. The samples were then washed once with Click-iT® reaction rinse buffer, washed twice more with PBS, and finally resuspended in 100µL PBS in a 96 well plate. Samples were analyzed using an Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) which can detect the green-fluorescent signal emitted by EU label incorporated into nascent transcripts. By normalizing green (FL1) fluorescence of ACT-D treated samples to respective dH_2O vehicle controls of each cell line, relative FL1 mean can be calculated, indicating relative transcriptional activity of drug treated cells compared to vehicle controls.

2.10 Translation assay

We adapted the Click-iT® HPG Alexa Fluor 488 Protein Synthesis Assay Kit (Invitrogen™, C10428, Eugene, ORE) for a flow cytometric analysis of nascent protein synthesis in DROSHA null and parental HCT116 cells following treatment with ACT-D. $4.0x10^4$ cells/well were seeded in 48-well plates, 48 hours before collection. 3 hours before collection the media was replaced with fresh media containing a dH_2O vehicle control or one of the ACT-D concentrations being tested. 30 minutes before collection, the media was once again replaced with fresh methionine-free DMEM containing the original ACT-D

treatments in addition to 50μM L-homopropargylglycine (HPG). HPG is a methionine analog used to label nascently translated polypeptides when assessing translational activity. After waiting 30 minutes to allow for HPG incorporation during translation, cells were collected and fixed using 3.7% formaldehyde in PBS for 15 mins at room temperature. The fixed cells were then washed with PBS and resuspended in 0.5% Triton® X-100 in PBS for 20 minutes at room temperature. The cells were washed again with PBS before being incubated in a Click-iT® reaction cocktail (prepared according to the manufacturers protocol) for 30 mins in the dark to fluorescently label HPG incorporated into newly translated proteins. The samples were then washed once with Click-iT® reaction rinse buffer, washed twice more with PBS, and finally resuspended in 100μL PBS in a 96 well plate. Samples were analyzed using an Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) which can detect the green-fluorescent signal emitted by HPG labels incorporated into nascent polypeptides. By normalizing green (FL1) fluorescence of ACT-D treated samples to respective dH_2O vehicle controls of each cell line, relative FL1 mean can be calculated, indicating relative translational activity of drug treated cells compared to vehicle controls.

2.11 MTT assay

The Vybrant® MTT Cell Proliferaction Assay Kit (Invitrogen, V-13154, Eugene, ORE) was used to measure relative metabolic activity in parental and DROSHA null HCT116 cells following ACT-D treatments. Briefly, cells were seeded $1.8x10^4$ cells/well in a 96-well plate in triplicates and allowed 24 hours to adhere. A negative control triplicate containing only growth media and no cells was included. Cells were then treated with either dH_2O as a vehicle control in untreated (ND) controls or 10, 25, 50, 100, or 250nM ACT-D

for 48 hours. The media was replaced with 100µL of fresh growth medium and 10µL of 12mM MTT stock solution was then added into each well of the 96-well plate and incubated 4 hours at 37°C. Following MTT reduction, DMSO (EMD Millipore, Cat#317275, Billerica, MA) was added to dissolve formazan crystals in a 2:1 ratio of DMSO to growth media. After 10 minutes, the absorbance of each sample was measured at 540nm using a Cytation 5 imaging reader (BioTek, Winooski, VT). Relative metabolic activity was calculated for both parental and DROSHA null HCT116 cells by subtracting the absorbance of the negative control and normalizing absorbance readings to the vehicle control.

2.12 Senescence Associated β-Galactosidase (SA-β-Gal) Staining Assay

Late passage (~25-30) GM00038 (GM38) primary fibroblasts were used as a positive control for senescence. The human telomerase expressing immortalized primary fibroblast cell line NFhTrt was used as negative control for senescence. $2.0x10^5$ GM38 cells and $7.5x10^5$ NFhTrt cells were seeded in individual wells of a 6-well plate on glass coverslips 96 hours before fixation and staining. Concurrently, Parental and DROSHA null HCT116 cells were seeded at a density of $5.0x10^4$ cells/well in a 6-well plate on glass coverslips. HCT116 were seeded 48 hours before treatment with a dH_2O vehicle control, 50nM, or 250nM ACT-D. 48 hours after these ACT-D treatments, coverslips for all four cell lines were rinsed with PBS and fixed for 10 minutes in 4% formaldehyde (Fisher Chemical, Cat#F79-1, Fair Lawn, NJ). Following a second PBS wash, coverslips were incubated at 37°C in freshly prepared SA-β-Gal mix: 40mM citric acid/sodium phosphate, 5mM potassium ferricyanide, 5mM potassium ferrocyanide (Fisher Scientific), 150mM NaCl (BioShop, Burlington, ON), 2mM $MgCl_2$, and 1mg/mL X-gal in dH_2O, pH 5.5; for

12 hours (All chemicals were purchased from Bio Basic, Markham, ON unless specified otherwise). The coverslips were then washed with PBS, air-dried, and mounted onto glass microscope slides with UltraCruz® Mounting Medium (Santa Cruz Biotechnology, sc-24941, Dallas, TX). An Axio Imager.M2 (Zeiss, Oberkochen, DE) microscope and AxioVision software v4.8 were used to take images. Random images of slides were captured using a random number generator to assign X/Y coordinates as the microscope stage locator graduations. Senescent cells appeared blue while normal cells were unstained. Stained and unstained cells in each image were counted by two volunteers blind to the experimental design. The average proportion of blue stained cells from two independent counts was found and the mean proportion of blue stained cells from three independent experiments was calculated.

3. Results

3.1 Initial screen to identify potential effects of miRNA processing on drug-induced apoptosis

We chose 10 mechanistically different drugs readily available in the lab to determine whether DROSHA null cells exhibited differential susceptibility to drug-induced apoptosis. The modes of action and general effects of each drug are given in Table 3. 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. To confirm that the DROSHA null cells obtained from the KCTC repository were 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 (Figure 7). 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.

Drug Name	**Mode of Action**	**General Effects**
Doxorubicin (Tacar et al., 2013)	1) Binds to enzymes associated with DNA (ex. Topoisomerase I/II) leading to DNA damage 2) Intercalates in DNA base pairs, blocking DNA/RNA polymerases	1) Failed DNA repair leads to arrest in G_1 and/or G_2 phases of the cell cycle and apoptosis 2) Inhibition of DNA replication and RNA transcription
Paclitaxel (Weaver, 2014)	Promotes microtubule polymerization/stabilization	Inhibition of mitosis, cell cycle arrest occurs in metaphase

	preventing proper spindle assembly	
Vincristine (Morris & Fornier, 2008)	Interacts with B-tubulin subunit preventing microtubule polymerization	Inhibition of chromosomal separation leading to cell cycle arrest in metaphase
Actinomycin-D (Liu et al., 2016; Ljungman et al., 1999)	Antibiotic that intercalates in DNA base pairs, blocking RNA polymerases. It can also interfere with topoisomerases	Inhibition of transcription and induction of DNA damage
DRB (Zorio & Bentley, 2001)	Prevents phosphorylation of the carboxy-terminal domain of the large subunit of RNA polymerase by blocking P-TEFb, a cyclin-dependent kinase	Inhibits RNA polymerase II transcription elongation
Cisplatin (Dasari & Tchounwou, 2014; Furuta et al., 2002)	Crosslinks DNA purine bases, interference with DNA repair, transcription and replication	Inhibits transcription and DNA replication leading to induction of apoptosis
Cytochalasin-D (Trendowski, 2015)	Blocks actin polymerization by binding to filamentous actin	Inhibition of microfilament activity leading to inhibition of cell division and apoptosis
Hydroxyurea (Agrawal et al., 2014)	Inhibitor of ribonucleotide reductase	Inhibition of DNA synthesis/replication, arresting cells in G_1/S phase
Mimosine (Mosca et al., 1995; Park et al., 2012)	Amino acid thought to disrupt ribonucleotide reductase	Inhibitor of DNA synthesis/replication, arresting cells in G_1/S phase
Cyclohexamide (Schneider-Poetsch et al., 2010)	Binds to the ribosome to block translation elongation	Inhibitor of protein synthesis

Table 3. Mode of action and effects of drugs used in screening experiments. Initial screening was performed by the one-parameter flow cytometric analysis of cell sensitivity by sub-G_1 assay.

Cells undergoing apoptosis fragment their DNA leading to a decrease in propidium iodide (PI) staining which provides a useful opportunity to measure the sensitivity of drug

treated cells to apoptosis (Kajstura et al., 2007; Zhang & Xu, 2000). Initial assessment of each cell lines' 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-G_1 assay) (Vanzyl et al., 2018). Cells with less than 2C (G_1) DNA content were considered non-viable. We found that loss of DROSHA expression has agent specific effects on cell sensitivity to apoptosis in HCT116 cells treated with the drugs tested (Figure 8 and 9). We did not detect any effect of DROSHA deletion on the sensitivity of cells to apoptosis induced by cisplatin, cytochalasin-D, paclitaxel, cycloheximide, or hydroxyurea measured this way (Figure 8A-E). In contrast, exposure of DROSHA null HCT116 cells to actinomycin-D, vincristine, doxorubicin, DRB, and mimosine treatments led to a significant increase in the proportion of cells with sub-G_1 DNA content, compared to the parental cell line (Figure 9A-E). This was not uniform across all concentrations of drug but represented what appears to be different dose response relationships. For these drugs, the percentage of parental HCT116 cells with sub-G_1 DNA content plateaued with increasing drug concentration while the proportion of DROSHA null cells with sub-G_1 continued to increase at higher concentrations. Therefore, DROSHA deficient cells appeared to be more sensitive to apoptosis induced by 5 of the 10 drugs tested.

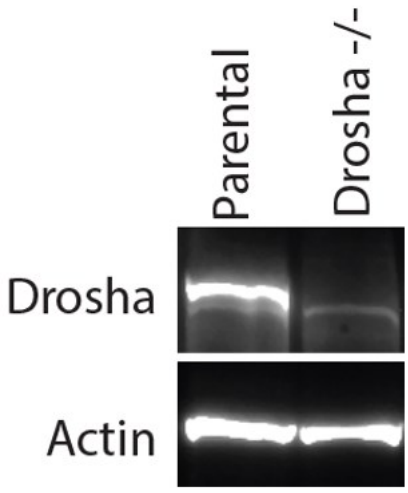

Figure 7 Validation of DROSHA null HCT116 cell line. Western blots were used to show the expression of DROSHA, and Actin (loading control) proteins in both parental and DROSHA null (Drosha-/-) HCT116 cells in normal conditions.

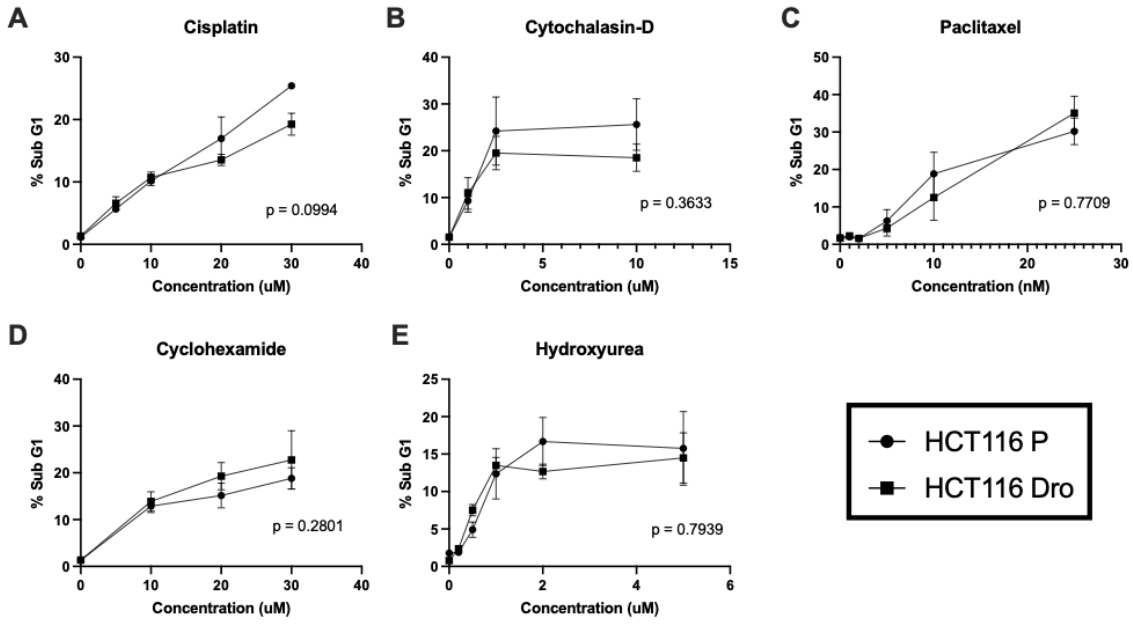

Figure 8. One parameter flow-cytometric analysis of sub-G_1 DNA content for drugs that produced no differences in two cell lines. HCT116 parental (P) and DROSHA null (Dro) cell lines were exposed to drugs at the indicated concentrations for 48 hours. Apoptosis was measured by the fraction of cells with less than 2C DNA content. Each value represents the mean +/- SEM determined from a minimum of 3 independent experiments. The sensitivity of cell lines was compared statistically by 2-way ANOVA using Graphpad Prism software and p-values are shown in each panel.

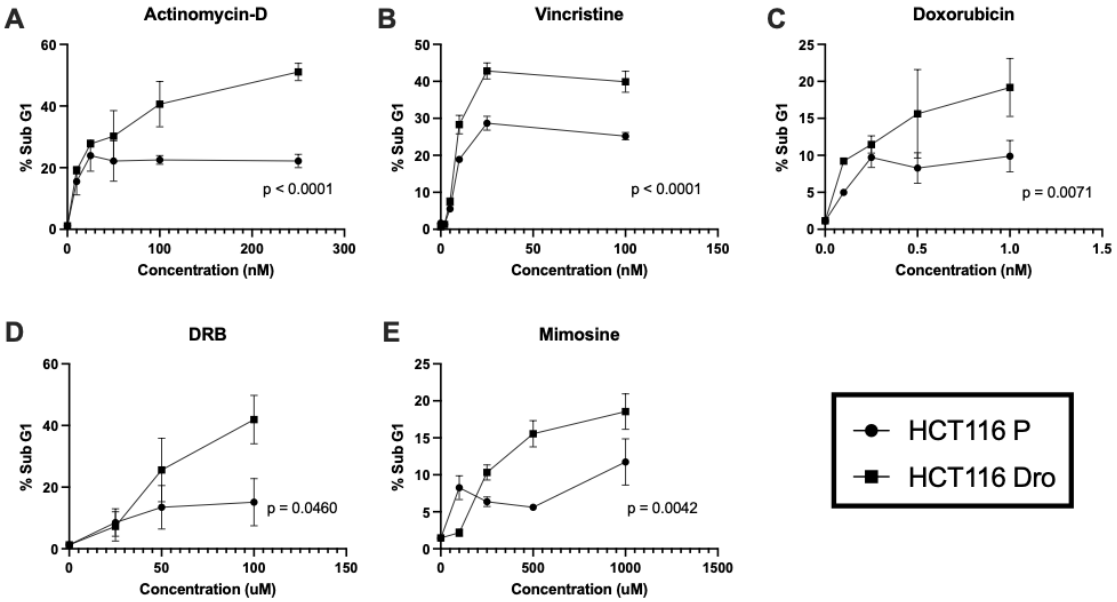

Figure 9. One parameter flow-cytometric analysis of sub-G_1 DNA content for drugs with differential effects in two cell lines. HCT116 parental (P) and DROSHA null (Dro) cell lines were exposed to drugs at the indicated concentrations for 48 hours. Apoptosis was measured by the fraction of cells with less than 2C DNA content. Each value represents the mean +/- SEM determined from a minimum of 3 independent experiments. The sensitivity of cell lines was compared statistically by 2-way ANOVA using Graphpad Prism software and p-values are shown in each panel.

3.2 DROSHA-deficient cells are more sensitive to actinomycin-D-induced apoptosis

From this initial drug screening, actinomycin-D (ACT-D) was chosen as the candidate drug to focus on in further study because it exhibited the largest, and among the most consistent, differences between cell lines (Figure 9A). The sub-G_1 assay relies upon the fragmentation of DNA to measure apoptotic cell death but DNA fragmentation may also be present in other forms of cell death like necrosis (Riccardi & Nicoletti, 2006). For this reason, we needed to confirm through independent methods that the disruption of DROSHA sensitizes cells to apoptosis induced by ACT-D.

The presence/activity of upstream effectors of apoptosis like cysteine proteases (caspases) presents another useful indicator of apoptotic activity. These proteins form a

cascade of proteolytic activity that can be triggered in response to various stresses. Caspase-3 and -7 are known as executioner caspases that are common to apoptosis induced by many agents (Elmore, 2007). When active, they function to cleave a variety of substrates in the cell leading to many of the biomolecular changes associated with apoptosis including but not limited to DNA fragmentation (Carrasco et al., 2003). Caspase 3/7 activity assays are specific to apoptotic cell death (D'Arcy, 2019; Maelfait & Beyaert, 2008; Yuan et al., 2016). Here we found that ACT-D increased caspase 3/7 activity less than two-fold in the parental HCT116 cells however, this increase quickly plateaued (Figure 10). In contrast, caspase 3/7 increased continuously in the DROSHA null cell line to more than four-fold (Figure 10). Caspase 3/7 activity was significantly higher in the DROSHA null HCT116 cells across the range of ACT-D doses tested. This confirms that DROSHA null HCT116 cells exhibit elevated sensitivity to apoptosis compared to the parental cell line following ACT-D treatment.

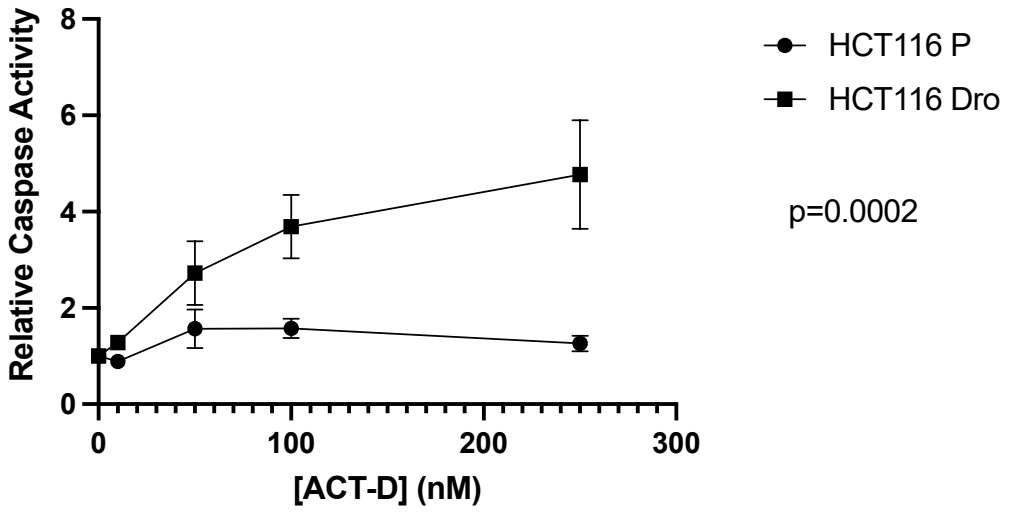

Figure 10. Flow cytometric analysis of relative caspase (3/7) activity. Caspase 3/7 were measured using fluorescent dye activated by caspase 3/7 activity in response to 24-hour ACT-D treatments in parental (P) and DROSHA (Dro) null HCT116 cells. Each value represents the mean relative caspase activity +/- SEM determined from a minimum of 3 independent experiments. Relative caspase activity of the two cell lines was compared statistically by 2-way ANOVA using Graphpad Prism software and the p-value is shown in the panel.

Live cells have intact plasma membranes which are selective of the types of molecules able to pass into and out of the cell (Adan et al., 2016). The integrity of the plasma membrane may be lost in necrosis and late apoptosis (Elmore, 2007). Dye exclusion assays take advantage of changes in plasma membrane integrity during cell death to measure cell sensitivity. Propidium iodide (PI) is a fluorescent DNA stain that can be used in dye exclusion assays as it is only able to enter the membrane of dead cells to reach the nucleus (Strober, 2015). The proportion of cells with reduced membrane integrity can be measured by flow cytometry through detection of fluorescent signals (FL2) which is increased in dead cells that have been stained with PI. We found that both parental and DROSHA null HCT116 cells increased PI uptake with corresponding increases in ACT-D. After 24-hour treatments the DROSHA null HCT116 cells exhibited a significantly

higher relative proportion of PI-penetrated cells than the parental cells (Figure 11A). At 48H, the pattern observed was similar except that the parental HCT116 cells were especially sensitive to a concentration of 50nM ACT-D for unknown reasons (Figure 11B). These results appear to compliment the findings from the sub-G_1 and caspase assays suggesting that DROSHA null HCT116 cells are uniquely sensitive to apoptosis induced by ACT-D, especially at the highest dose tested (250nM).

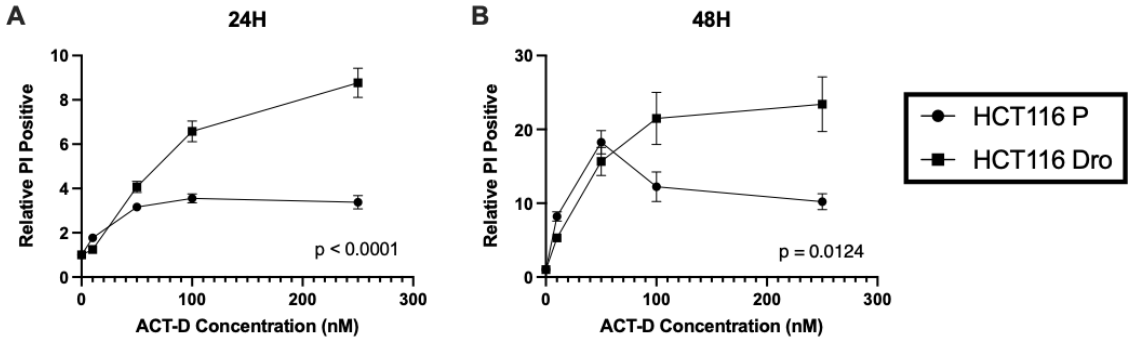

Figure 11. Flow cytometric analysis of membrane integrity. A PI dye exclusion assay was performed in parental (P) and DROSHA null (Dro) HCT116 cells treated for (A) 24-hours or (B) 48-hours with ACT-D. Loss of membrane integrity is associated with increased red fluorescence (FL2 channel) which allowed detection of PI positive and negative cell populations by flow cytometry. Each value represents the mean relative proportion of PI positive cells (Relative PI positive) +/- SEM determined from a minimum of 3 independent experiments. Relative PI positive of the two cell lines was compared statistically by 2-way ANOVA using Graphpad Prism software and p-values are shown in the panel.

3.3 Loss of DROSHA doesn't affect actinomycin-D-induced p53 responses

Actinomycin-D has been reported to activate p53 and both G_1-phase arrest and apoptosis in cancer cell lines (Kastan et al., 1991; Liu et al., 2016). HCT116 cells have also been reported to undergo drug induced apoptosis in a p53-dependent manner (Blagosklonny & El-Deiry, 1998). To ensure that our cell lines behaved in a manner consistent with previous literature, we assessed the role of p53 in ACT-D-induced cell death in parental and DROSHA null HCT116 cell lines. The expression of p53 was assessed by western blotting in both cell lines following treatment with the highest concentration of ACT-D tested (250nM). This is where the largest and most significant disparity in cell sensitivity was observed. The level of p53 increased similarly in both cell lines after 8-hours and remained elevated to at least 24-hours. Therefore, there was no clear difference in p53 levels that could explain the difference in sensitivity to ACT-D induced apoptosis. The p53 protein is a transcription factor that regulates the expression of a large number of transcripts and their encoded proteins (Hernández Borrero & El-Deiry, 2021). We sought to determine if known targets of p53 (MDM2, p21, and PUMA) were differentially induced between cell lines under these conditions. Unexpectedly, these proteins are not induced at 8-hours and were downregulated by 24-hours despite increased p53 expression (Figure 12). This indicates that following this cytotoxic dose of ACT-D, a p53 response is not activated at the protein level in either the presence or absence of DROSHA.

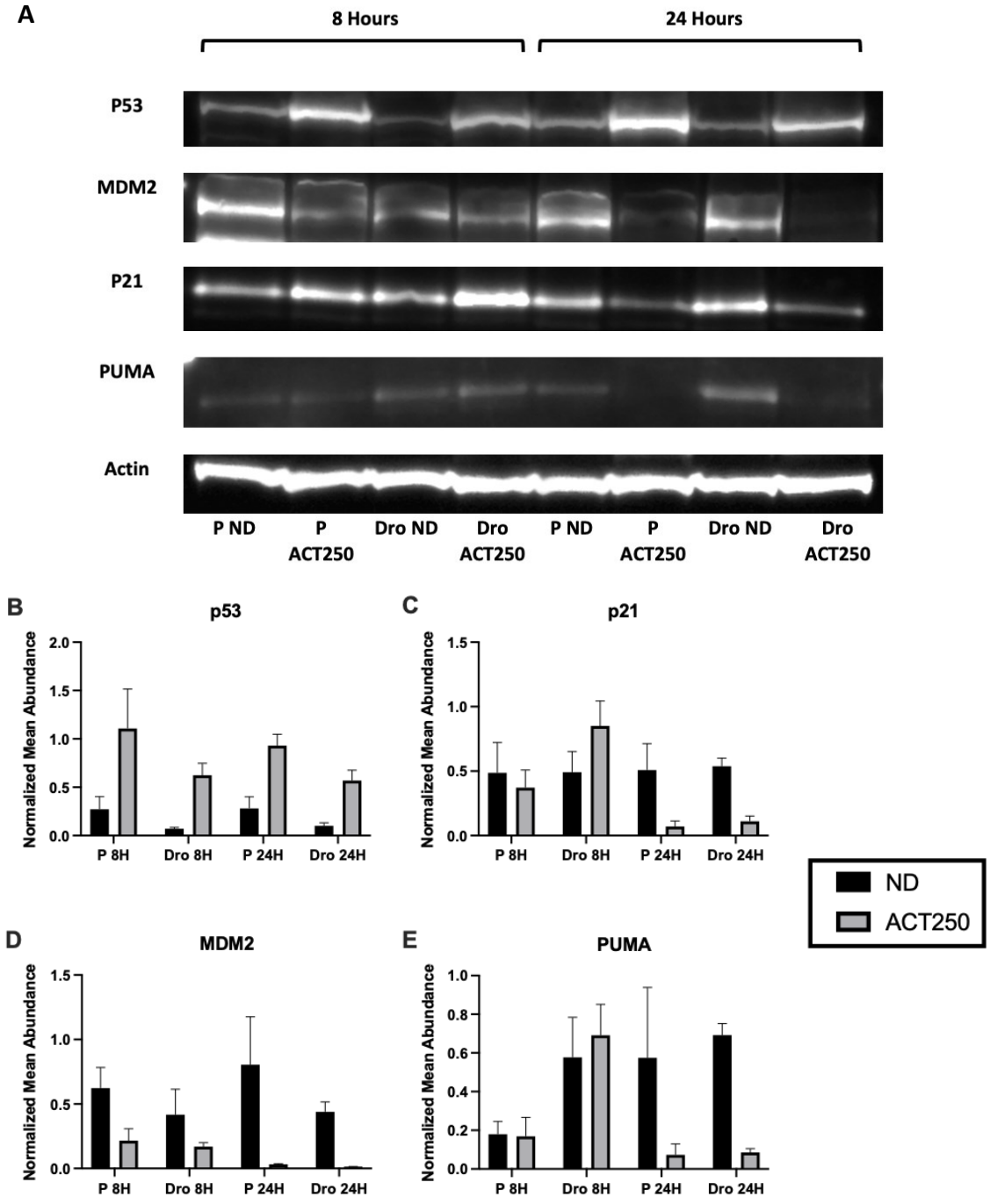

Figure 12. p53 response-related protein expression. A) Western blots were used to show the expression of p53, MDM2, p21, PUMA, and Actin (loading control) proteins in both parental (P) and DROSHA null (Dro) HCT116 cells treated for 8 and 24 hours with 250nM ACT-D (ACT250) or a vehicle control (ND). B-E) Quantification of western blots showing mean abundance of p53, p21 MDM2, and PUMA normalized to Actin. Error bars represent +/- SEM from three independent experiments quantified using ImageJ Software.

The lack of p53-induced protein expression was unexpected, so we also measured the induction of *MDM2*, *CDKN1A* (p21), and *BBC3* (PUMA) mRNAs by qRT-PCR under the same conditions. Much like the pattern of each of these proteins, their transcripts were not induced at 8-hours and were downregulated by 24-hours (Figure 13). *BBC3* (PUMA) expression was reduced after 8- and 24-hours in parental cells but only after 8-hour treatments in the DROSHA null cell line. After 24-hour treatments, *BBC3* (PUMA) expression was highly variable but appeared to be unchanged or slightly increased (Figure 13E). The reason for this difference is not known however, one might predict that the high level of apoptosis observed in DROSHA null cells may select for a subpopulation of cells with elevated levels of *BBC3* (PUMA). Decreases in the mRNA levels of *CDKN1A*, *MDM2*, and *PUMA* (*BBC3*) were consistent with changes in the protein levels of p21, MDM2, and PUMA under the same conditions (Figure 12). Together these findings indicate that 250nM ACT-D treatment failed to induce typical p53 transcriptional activity in the presence and absence of DROSHA despite increases in p53 protein levels. It has been previously reported that very high concentrations of actinomycin-D (>200nM) can be too high to induce a conventional p53 response because ACT-D is an intercalating agent that blocks transcription (Ljungman et al., 1999). Considering this, cytotoxic levels of DNA intercalation by ACT-D may have repressed *CDKN1A* (p21), *MDM2*, *BBC3* (PUMA) mRNAs, preventing the induction of a conventional p53 response in both cell lines. Importantly, this argues that the difference in sensitivity to apoptosis between cell lines cannot be explained by differences in the p53-transcriptional response.

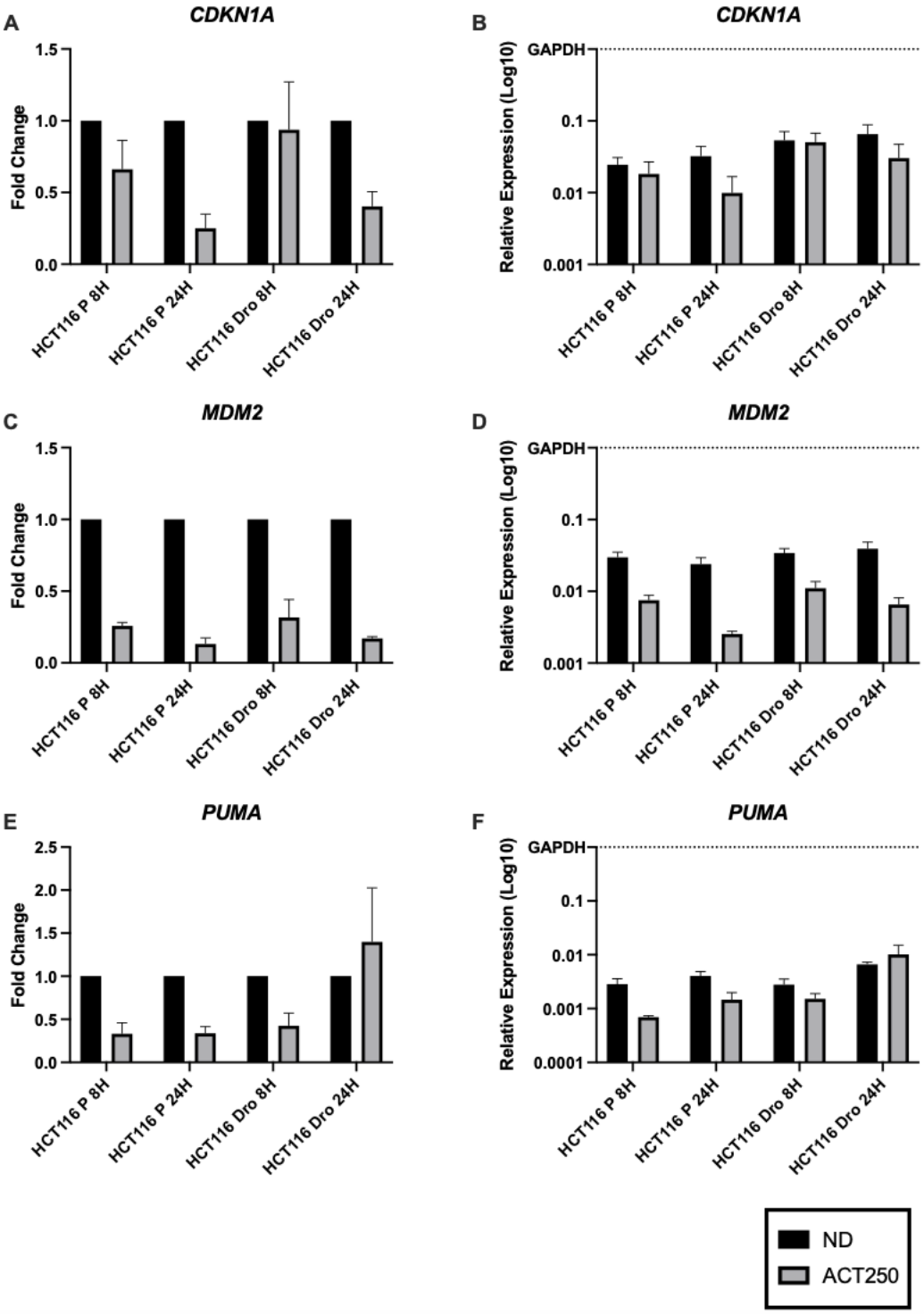

Figure 13. Gene expression analysis of p53 related genes following 250nM ACT-D (ACT250) treatments. HCT116 parental (P) and DROSHA null (Dro) cells were treated with ACT-D for 8- and 24-hours before RNA was isolated. Gene expression was measured by qRT-PCR. A, C, E) Levels of gene expression are given as fold-change relative to untreated (ND) samples. B, D, F) Levels of gene expression are shown relative to GAPDH on a Log10 scale. Error bars represent +/- SEM from four independent experiments.

To assess the p53 response following less severe transcriptional stress, p53, MDM2, p21, and PUMA levels were examined following treatment with 50nM ACT-D. In both cell lines, the expression of p53 response-related proteins was increased equally. PUMA protein levels were similar in the presence and absence of DROSHA. For both 8- and 24-hour ACT-D treatments PUMA appeared to be unchanged or slightly increased (Figure 14). As expected, p53, MDM2 and p21 protein levels were all induced following exposure to 50nM ACT-D for both 8- and 24-hour treatments (Figure 14). This increase was also similar in both cell lines. These results suggest that this lower concentration of ACT-D led to a more typical p53 response at the protein level. Interestingly the presence/absence of DROSHA had no effect on the activation of this p53 response at the protein level.

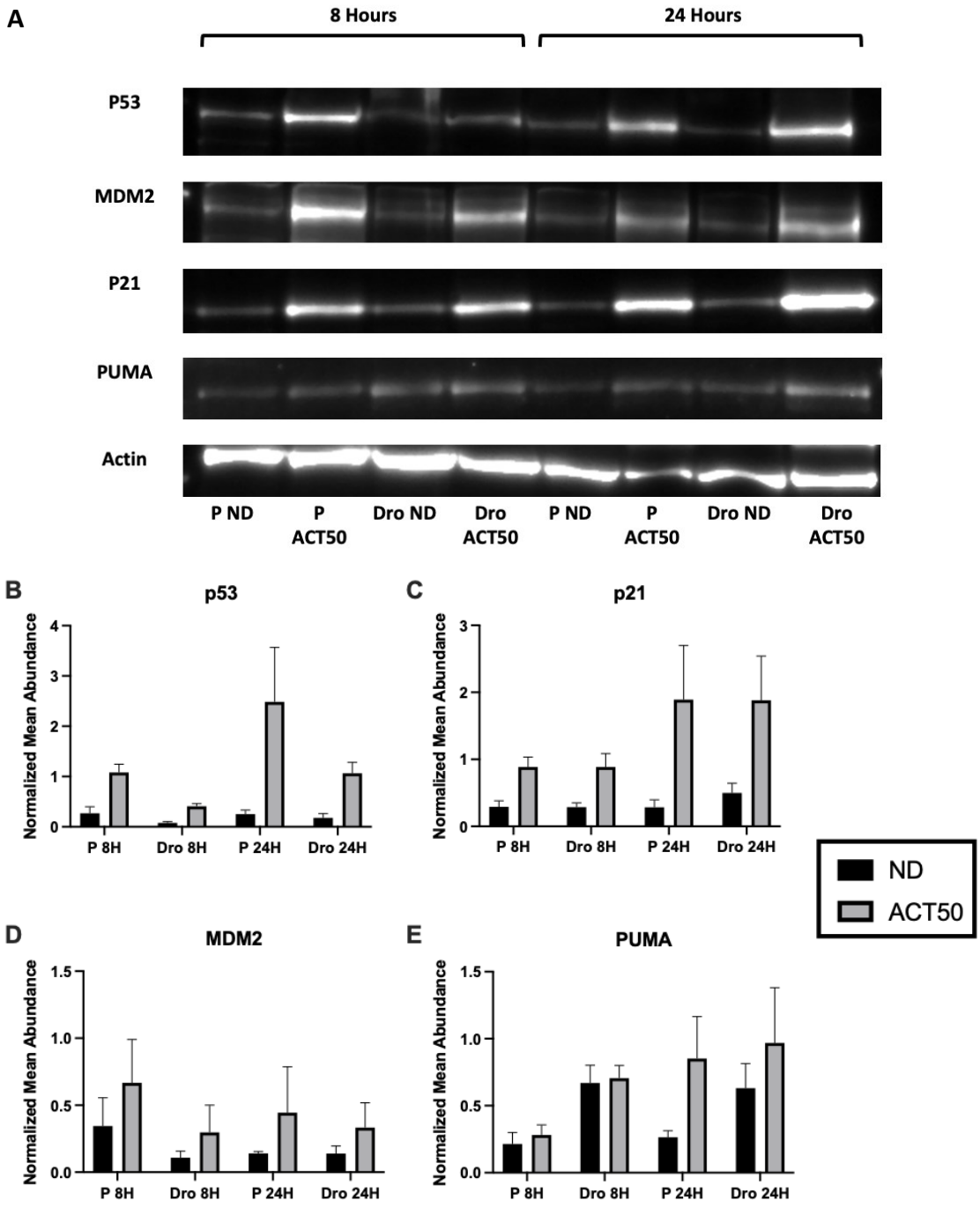

Figure 14. p53 response-related protein expression. A) Western blots were used to show the expression of p53, MDM2, p21, PUMA, and Actin (loading control) proteins in both parental (P) and DROSHA null (Dro) HCT116 cells treated for 8 and 24 hours with 50nM ACT-D (ACT50) or a vehicle control (ND). B-E) Quantification of western blots showing mean abundance of p53, p21 MDM2, and PUMA normalized to Actin. Error bars represent +/- SEM from three independent experiments quantified using ImageJ Software.

The expression of genes that are transcriptionally regulated by p53 (*CDKN1A*, *MDM2*, and *BBC3*) were also measured by qRT-PCR following 50nM ACT-D treatments. We found that the expression of p53-inducible genes in parental and DROSHA null HCT116 cells was similar following this lower concentration ACT-D treatment (Figure 15). The expression of *CDKN1A*, *MDM2*, and *BBC3* (PUMA) mRNAs generally appeared to increase in both parental and DROSHA null HCT116 cells following 8- and 24-hour ACT-D treatments (Figure 15). These results generally correlate with p53-response related protein levels where we saw increases in both p21 and MDM2, but PUMA did not change appreciably or consistently (Figure 14). Overall, 50nM ACT-D treatments appeared to induce a more typical p53 transcriptional response than was observed with 250nM treatments. The major finding from this data was that no differences were observed in p53-transcriptional activity or protein levels in the presence or absence of DROSHA. Therefore, differential sensitivity of these cell lines to ACT-D-induced apoptosis cannot be readily explained by differences in the p53 response caused by loss of miRNA processing.

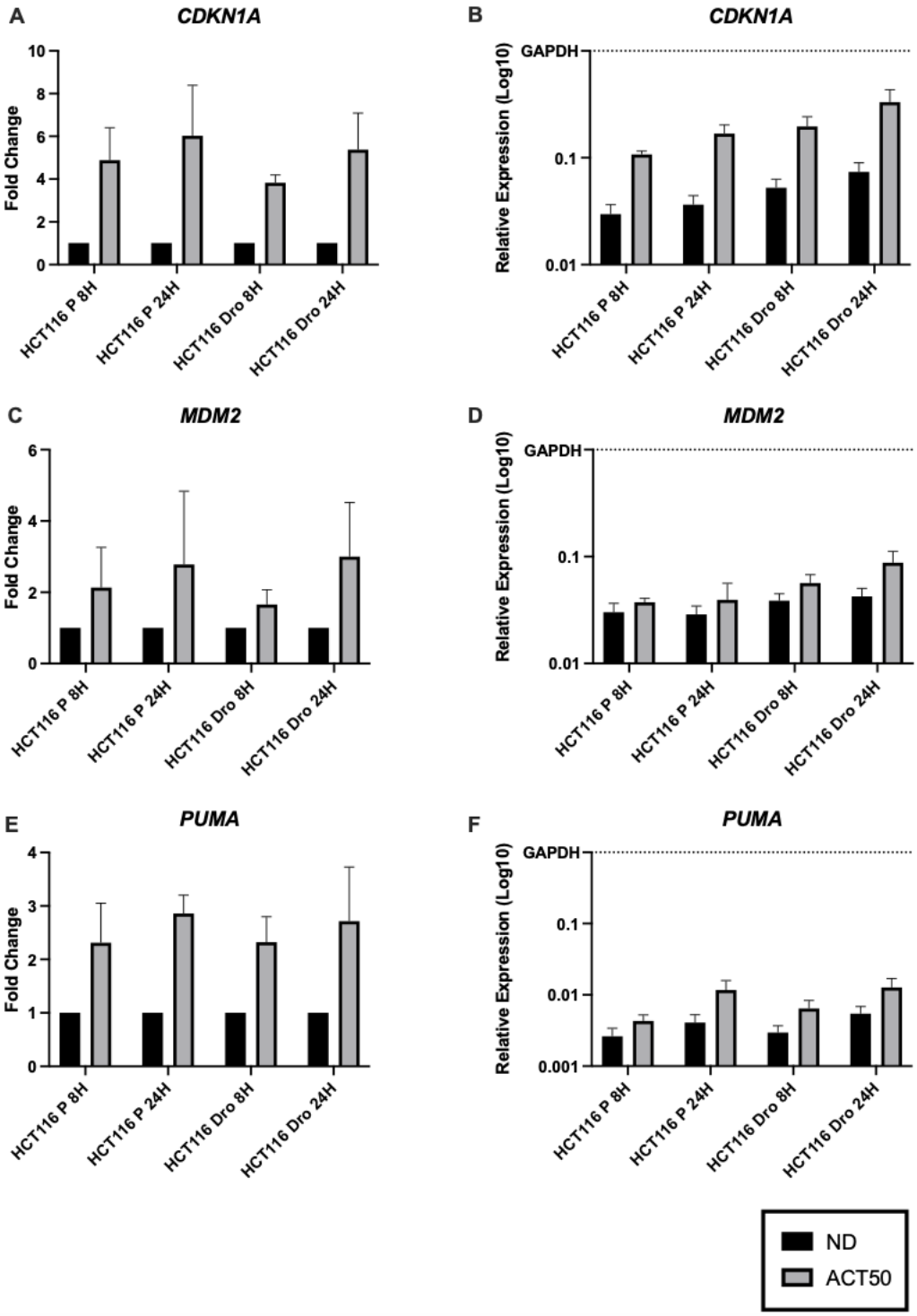

Figure 15. Gene expression analysis of p53 related genes following 50nM ACT-D (ACT50) treatments. HCT116 parental (P) and DROSHA null (Dro) cells were treated with ACT-D for 8- and 24-hours before RNA was isolated. Gene expression was measured by qRT-PCR. A, C, E) Levels of gene expression are given as fold-change relative to untreated (ND) samples. B, D, F) Levels of gene expression are shown relative to GAPDH on a Log10 scale. Error bars represent +/- SEM from five independent experiments.

3.4 Loss of DROSHA doesn't alter the effect of actinomycin-D on DNA replication, RNA synthesis, or protein synthesis

ACT-D is an intercalating agent that can block DNA and RNA polymerases, directly affecting DNA replication and transcription and indirectly affecting translation (Liu et al., 2016). A difference in the amount of intercalated DNA could affect cell viability and could manifest as a decrease in DNA or RNA synthesis and indirectly protein synthesis. Therefore, the effect of ACT-D on DNA synthesis, RNA synthesis, and translation was determined in the presence or absence of canonical miRNA processing in parental and DROSHA null HCT116 cells. For replication we used incorporation of BrdU, a thymine analog that gets incorporated into DNA during replication. The incorporation of BrdU was detected with FITC-conjugated anti-BrdU antibodies by a one-parameter flow cytometric analysis. The percentage of BrdU positive cells (percentage BrdU+) represents the fraction of cells incorporating BrdU in a 1-hour pulse treatment. There was no significant effect of ACT-D on BrdU incorporation in either cell line even at the highest concentration of ACT-D tested (Figure 16). This suggests that ACT-D treatment had no effect on DNA replication and therefore, DROSHA deficiency didn't affect DNA replication under these conditions.

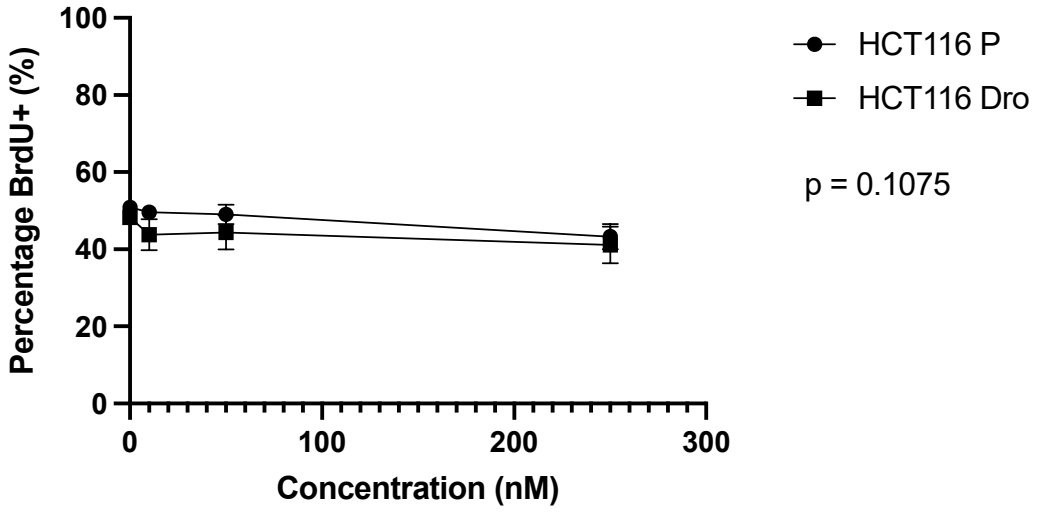

Figure 16. One-parameter flow cytometric analysis of DNA replication. Parental and DROSHA null cells were treated with ACT-D for 3 hours with a pluse BrdU treatment 1-hour before collection for BrdU incorporation. BrdU substituted DNA was detected with an FITC-conjugated anti-BrdU antibody and analyzed by flow cytometry. Each value represents the mean percentage (%) of BrdU+ cells +/- SEM determined from a minimum of 3 independent experiments. The % BrdU+ of the two cell lines was compared statistically by 2-way ANOVA using Graphpad Prism software and p-value is shown in the panel.

Nascent transcription and translation were measured using the Invitrogen Click-iT™ Alexa Fluor™ 488 kits. These kits rely on fluorescently labeled ethynyl uridine (EU) a ribonucleotide homolog, and L-homopropargylglycine (HPG) a methionine analog, incorporation into nascent transcripts and polypeptides, respectively. In both assays, green fluorescence (FL1) is proportional to nascent transcription and translation, and this can be measured using flow cytometry. Fluorescence is proportional to the amount of EU (Figure 17A) or HPG (Figure 17B) that is incorporated into transcripts and proteins during a 30-minute labelling period. Data is expressed as a ratio of FL1 in treated to untreated samples in each cell line to estimate the effect of ACT-D. Similar decreases in transcription and translation were detected in both cell lines. ACT-D decreased transcription up to ~80%, while translation decreased by about ~30% in both cell lines (Figure 17A/B). Overall, these

findings indicate that nascent RNA and protein synthesis are equally affected by ACT-D exposure in both the parental and DROSHA null subline. For this reason, the overall ability of DROSHA null cells to transcribe and translate new RNA and proteins cannot explain their increased sensitivity to apoptosis in response to ACT-D.

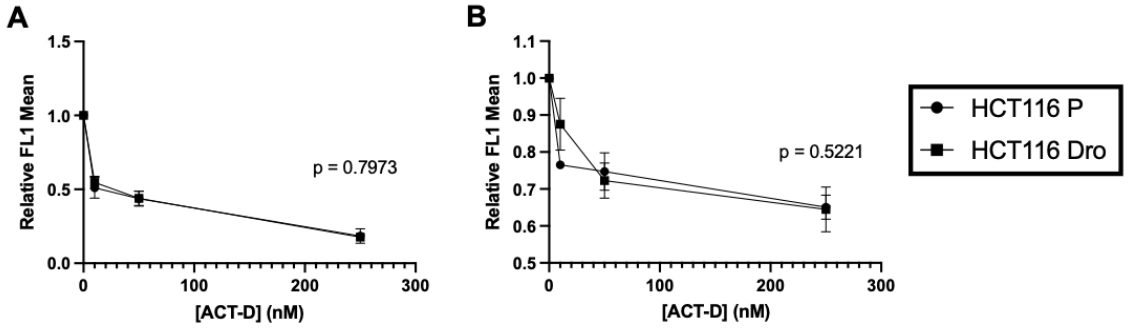

Figure 17. The effect of ACT-D on transcription and translation. (A) Nascent transcription and (B) translation were measured in parental (P) and DROSHA null (Dro) HCT116 cells treated with ACT-D for 3H with a 1H pulse of either EU (transcription) or 0.5H pulse of HPG (translation). EU and HPG substitutions were detected by green fluorescent (FL1) labels and analyzed by flow cytometry. Each value represents the mean relative FL1 +/- SEM determined from a minimum of 3 independent experiments. Mean relative FL1 of the two cell lines was compared statistically by 2-way ANOVA using Graphpad Prism software and p-values are shown in each panel.

3.5 Loss of DROSHA does not increase the sensitivity of cells to other forms of cell death

Decreased metabolic activity is associated with loss of cell viability (van Meerloo et al., 2011). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assays are colorimetric assays that rely upon metabolic activity to indicate viability (Stockert et al., 2012). The rate of reduction of MTT by mitochondrial dehydrogenases can be measured using spectrophotometry (Stockert et al., 2012). MTT reduction correlates with overall metabolic activity and is often a good indicator of cell viability (van Meerloo et al., 2011). Here, we assessed the effect of ACT-D on parental and DROSHA null HCT116 cells by MTT assay. Relative metabolic activity was assessed for both parental and

DROSHA null HCT116 cells by normalizing absorbance readings of treated cells to their vehicle control. In both cell lines, as the concentration of ACT-D increased, metabolic activity decreased in a similar manner relative to the untreated cells (Figure 18). There was no statistical difference between parental and DROSHA null cells across this range of ACT-D concentrations. This means that in the absence of canonical miRNA processing, metabolic activity appears to be equally affected by ACT-D in both cell lines. This implies that the cause of elevated levels of cell death observed in DROSHA null cells treated with ACT-D are independent of metabolic activity and rather consequences of other cellular processes.

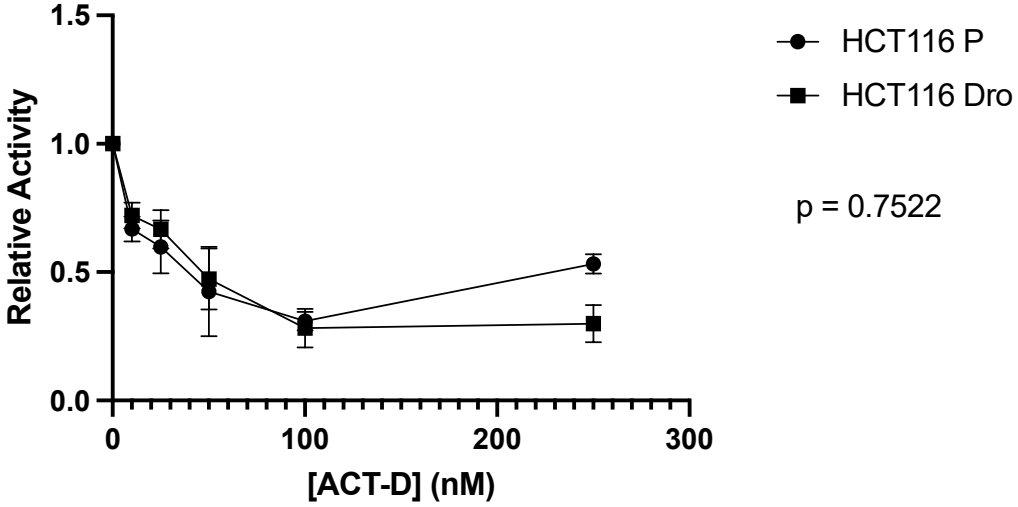

Figure 18. The effect of ACT-D on metabolic activity. An MTT assay was used as a measure of cell viability by comparison of relative metabolic activity in parental (P) and DROSHA null (Dro) HCT116 cells treated with ACT-D for 48-hours. Reduction of MTT was detected by colorimetric spectrophotometry. Each value represents the mean relative metabolic activity +/- SEM determined from a minimum of 3 independent experiments. Relative metabolic activity of the two cell lines was compared statistically by 2-way ANOVA using Graphpad Prism software and p-value is shown in the panel.

DNA damage inducing agents can induce permanent exit from the cell cycle, termed replicative senescence, in a process called stress-induced premature senescence

(SIPS) (Mijit et al., 2020). Senescence can be activated to protect the integrity of DNA following high levels of genomic stress. The induction of senescence could delay or inhibit the induction of apoptotic cell death which may contribute to the reduced levels of apoptosis observed in parental cells compared to DROSHA null HCT116 cells. The expression of senescence-associated β-galactosidase (SA-β-gal) is a change in the cellular microenvironment often associated with senescent phenotype (Itahana et al., 2001; Mijit et al., 2020). By staining SA-β-gal expressing cells, the proportion of senescent cells can be counted by microscopy to measure the induction of SIPS following drug treatment. This SA-β-gal assay was used to measure the induction of senescence in the presence and absence of DROSHA. Late passage (~25-30) GM38 primary fibroblast were used as a positive control and telomerase-overexpressing immortalized primary fibroblasts (NFhTrt) were used as a negative control for senescence. The positive control for senescence, GM38, had around ~51% SA-β-gal positive cells while the negative control, NFhTrt, had ~14% (Figure 19A). The patterns observed in these controls confirm that this is an effective technique for measuring senescence. Before treatment, about 10% of parental and DROSHA null cells were SA-β-gal positive, similar to the NFhTrt cells. In response to ACT-D, the proportion of SA-β-gal positive cells increased more at 50nM than at 250nM and there was no significant difference between cell lines (Figure 19B). These results indicate that the induction of SIPS does not contribute to differences in the level of cell death in either parental or DROSHA null HCT116 cells observed by other measures of cell sensitivity.

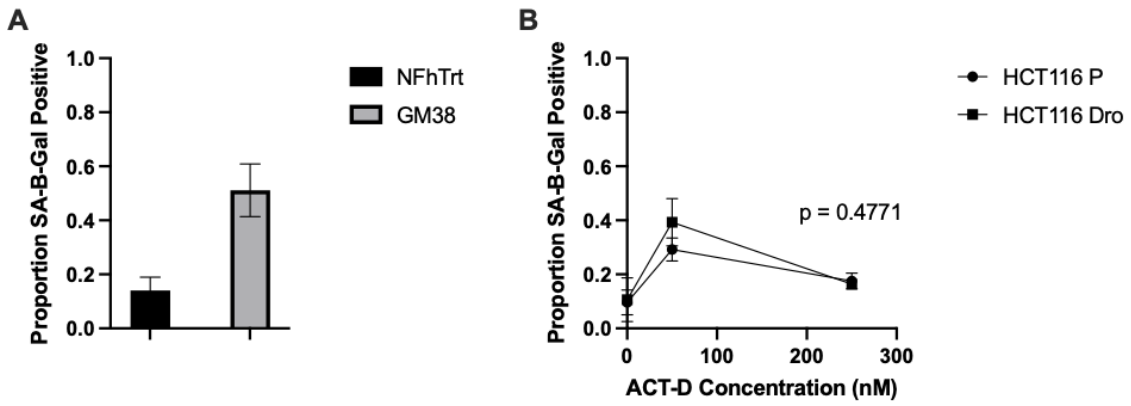

Figure 19. The effect of ACT-D on induction of senescence-associated β-galactosidase (SA-β-gal). (A) NFhTrt and GM38 cells were grown in normal conditions (B) Parental (P) and DROSHA null (Dro) HCT116 cells were treated with ACT-D for 48 hours. All cell lines were incubated for 12 hours in SA-β-gal mixture and imaged using brightfield microscopy. Senescence was measured by counting the proportion of SA-β-gal positive (blue-stained) cells over total cells. Each value represents the mean +/- SEM determined from a minimum of 3 independent experiments. The proportion of blue cells in HCT116 cells was compared statistically by 2-way ANOVA using Graphpad Prism software and the p-value is shown in panel A.

4. Discussion

Micro RNAs regulate numerous cellular processes and are essential for maintaining the healthy function of multicellular life (Chong et al., 2008; Rukov & Shomron, 2011). Due to their ubiquity, it is not surprising that miRNAs play important roles in the regulation of gene expression during cellular responses to stressors like exogenous chemical agents (Rukov & Shomron, 2011). Recent research describing the roles of miRNAs in the context of cellular drug responses tends to focus on specific miRNAs, the genes that they regulate, and how they affect pharmacokinetics and biological responses (Rukov & Shomron, 2011). We know that individual miRNAs can target numerous mRNAs through redundancy in 5' seed sequence and 3' UTR interactions. In addition to this, multiple miRNAs can affect the expression of a single gene (Bracken et al., 2016). Considering this overlap in function, the up- and down-regulation of miRNAs could have offsetting effects on gene expression during cellular stress responses to pharmacological agents.

The regulation of miRNA-induced gene silencing itself is regulated at both transcriptional and post-transcriptional levels. The transcription of miRNAs can be modulated by other regulatory biomolecules like transcription factors (Gulyaeva & Kushlinskiy, 2016). Post-transcriptional regulation of miRNA function can also be affected by RNA binding proteins (RBP) which, like miRNAs, can target the 3'UTR of mRNAs. These RBP-mRNA interactions at the 3'UTR can both positively and negatively regulate the expression of the mRNA target (Szostak & Gebauer, 2013). Genes like *TP53* exemplify how a single transcription factor can transactivate a complex network of many miRNAs that can simultaneously promote and inhibit critical cellular processes affecting cell survival and cell death pathways (Bracken et al., 2016; Hernández Borrero & El-Deiry,

2021; Liu et al., 2017). This fine-tuning of gene expression through cooperative miRNA activity presents a more complex yet realistic view of how miRNAs affect cell fate in response to stress. For this reason, we proposed that using an experimental model devoid of canonical miRNA biogenesis could provide valuable insights into the overall importance of miRNA regulatory activity in cellular responses to pharmacological agents. To do this we used a genetically modified HCT116 colorectal carcinoma cell line with aberrant expression of the miRNA processing ribonuclease III enzyme, DROSHA. This model provided a means by which we could assess cellular responses to drugs in the presence and absence of canonical miRNA processing.

4.1 The absence of DROSHA appears to sensitize cells to apoptosis induced by some pharmacological agents

The sensitivity of parental and DROSHA null HCT116 cells to apoptosis was initially assessed following exposure to 10 different chemical agents with several distinct modes of action. Sub-G_1 DNA content is often used as a measure of DNA fragmentation, a common characteristic of apoptotic cells (Kajstura et al., 2007; Riccardi & Nicoletti, 2006; Zhang & Xu, 2000). We found that 5/10 drugs tested (actinomycin-D, vincristine, doxorubicin, DRB, and mimosine) induced significantly more apoptosis in DROSHA null cells compared to their parental cells. Conversely, parental and DROSHA null cells were equally sensitive to the other 5 drugs (cisplatin, cytochalasin-D, paclitaxel, cycloheximide, and hydroxyurea).

Our screening results suggest that the disruption of DROSHA sensitizes cells to apoptosis induced by ACT-D and four other drugs. From this initial drug screening, actinomycin-D (ACT-D) was chosen as the candidate drug for further study because it

exhibited the greatest difference in sensitivity to apoptosis between cell lines. Furthermore, this difference was highly reproducible. In programmed cell death like apoptosis, the activation of intracellular proteases and nucleases leads to degradation of major cellular structures including the fragmentation of DNA (Riccardi & Nicoletti, 2006). Fragments of DNA are released from the nucleus leading to a reduction nuclear DNA content. The sub-G_1 assay detects the fragmentation of DNA by staining cells with propidium iodide (PI) which binds to DNA so that DNA content can be measured (Riccardi & Nicoletti, 2006). Reduced levels of PI staining indicate a decrease in nuclear DNA content and thereby apoptosis. However, DNA fragmentation may also be present in other forms of cell death, like necrosis (Riccardi & Nicoletti, 2006). Several additional measures of cell sensitivity were also assessed in response to ACT-D.

4.2 Sensitivity of DROSHA null cells is characterized by increased caspase activation and loss of plasma membrane integrity

Our data from the sub-G_1 assay suggested that DROSHA null cells were more sensitive than parental HCT116 cells to ACT-D induced apoptosis. ACT-D is known to induce apoptosis through activation of a p53 response however, surprisingly differences in the p53-response did not account for this increase in DROSHA null cell death. To reinforce the observations made in the sub-G_1 assay, we employed additional techniques to measure cell sensitivity to ACT-D treatments. Caspase activity is a major component of both extrinsic and intrinsic pathways of apoptotic cell death. Caspases 3, 6, and 7 are known as executioner caspases because they conduct and promote much of the proteolytic and nucleolytic activity that occurs during apoptosis (D'Arcy, 2019; Hotchkiss et al., 2009). These caspases are effectors of apoptosis that are activated downstream of the p53 response

but upstream of DNA fragmentation, which is what is measured in sub-G_1 assays. We used a caspase 3/7 assay accordingly to determine if the activation of caspases contributes to cell death in the absence of canonical miRNA processing. We found that ACT-D increased caspase activity more in DROSHA null cells than the parental controls (Figure 10). This finding indicates that ACT-D induced cell death in DROSHA null cells observed in screening experiments correlates with increased caspase 3/7 activity. p53 related mediators of the intrinsic pathway of apoptosis were measured in this work however no differences were observed between cell lines. Caspases 3/7 are activated by both the intrinsic and extrinsic pathways of apoptosis (Hotchkiss et al., 2009). It would be beneficial in future work to measure whether the extrinsic pathway of apoptosis is activated. This work could measure the expression of p53-inducible cell surface death-receptors like FAS and TRAIL (Helton & Chen, 2007; Hernández Borrero & El-Deiry, 2021). Exogenous siRNAs could also be used to knockdown the expression of p53 in both parental and DROSHA null cells to assess whether p53 plays a role in DROSHA null sensitivity to apoptosis through the extrinsic pathway.

In addition to increased relative caspase activity, DROSHA null HCT116 cells treated with ACT-D were more permeable to propidium iodide in PI dye exclusion assays (Figure 11). Dye exclusion assays are one of the most common techniques used to measure cell viability (Stoddart, 2011). Live cells have intact plasma membranes that are impermeable to certain molecules like propidium iodide (Adan et al., 2016; Stoddart, 2011). During necrotic and late apoptotic cell death, changes in plasma membrane integrity can result in permeability to these molecules (Elmore, 2007). We detected an increase in the proportion of PI positive cells following 24- and 48-hour ACT-D treatments. In both

treatment periods, the DROSHA null cells had a significantly higher relative proportion of PI positive cells than the parental cell line. The loss of membrane integrity in DROSHA null cells 24-hours after treatment was interesting as changes in membrane integrity usually are associated with late-stage apoptosis (Elmore, 2007; Hotchkiss et al., 2009). The loss of membrane integrity can be also associated with necrosis however apoptosis appears to be the major contributor to cell death here as indicated by increased caspase 3/7 activity in the absence of DROSHA.

4.3 Sensitivity of DROSHA null cells cannot be attributed to differences in the p53 response

ACT-D has been reported to induce p53 and both G_1-phase arrest and apoptosis in several cancer cell lines (Kastan et al., 1991; Liu et al., 2016). There is also early evidence to show that p53 contributes to drug-induced apoptosis in HCT116 cells specifically (Blagosklonny & El-Deiry, 1998). Many miRNAs are induced by p53 and reportedly promote both survival and death through repression of pro- and anti-apoptotic mRNAs and proteins. MDM2 is also reported to be negatively regulated by p53-induced miRNAs (Hernández Borrero & El-Deiry, 2021; Liu et al., 2017). The net effect of miRNA-induced gene silencing of pro- and anti-apoptotic proteins is not clear. We therefore assessed the role of p53 in ACT-D induced cell death in parental and DROSHA null HCT116 cells. By measuring the expression of genes and proteins like p53 itself and its downstream targets we aimed to determine if the p53 response differed in the presence or absence of DROSHA.

When assessing levels of apoptosis following treatment with the highest concentration of ACT-D (250nM) we observed large and statistically significant increases in apoptotic cell death in the absence of DROSHA. At this dose, p53 protein levels were

increased but p53-responsive genes (*CDKN1A*, *MDM2*, and *BBC3*) were decreased or unchanged following up to 24-hour ACT-D treatments in both cell lines. The protein levels corresponding to these genes (p21, MDM2, and PUMA) were similarly decreased or unchanged in both cell lines (Figure 12/13). This indicates that this high dose of ACT-D induced p53 but failed to activate its downstream targets in the presence/absence of DROSHA. ACT-D is a known inhibitor of transcription, so this high concentration likely interferes with execution of the p53-transcription program (Liu et al., 2016). It is also possible that high levels of cell death also contributed to decreases in p21, MDM2, and PUMA observed exclusively at this high concentration of ACT-D.

Previous studies have reported that very high concentrations of ACT-D (>200nM) can be too high to induce a conventional p53 response due to severe transcriptional inhibition (Ljungman et al., 1999). There was no significant difference between the percentage of sub-G_1 parental and DROSHA null cells treated with 50nM ACT-D (Figure 9A). Under these conditions, p53 protein increased and the expression of p53-regulated genes (*CDKN1A, MDM2,* and *BBC3*) appeared to increase at the mRNA and protein level (Figure 14/15). Although a few minor differences may exist between the two cell lines, it appears the expression of p53-regulated genes was similar in the presence/absence of DROSHA.

This analysis of gene expression and protein abundance showed that p53, MDM2, p21, and PUMA respond similarly to ACT-D treatment in parental and DROSHA null HCT116 cells. These findings suggest that in the absence of canonical miRNA processing, the p53 response appears unchanged in response to ACT-D. Overall, this implies that increased sensitivity in DROSHA null cells to ACT-D induced apoptosis by cannot be

explained by gross differences in the p53 response. The previous suggestion to use siRNAs to knockdown p53 expression in parental and DROSHA null cells would also be informative here by helping to determine if p53 plays an unrecognized role in the cellular response to ACT-D.

4.4 The absence of DROSHA did not change the direct effects of ACT-D on biochemical processes

ACT-D intercalates between DNA base pairs which interferes with DNA and RNA polymerases thereby directly inhibiting DNA replication and transcriptional activity (Liu et al., 2016). One possible explanation for the elevated sensitivity to apoptosis observed in DROSHA null cells would be changes in the ability for ACT-D to access DNA. Efflux of ACT-D from the cell has been reported to require multidrug-resistance (MDR) transporters (Hill et al., 2013). These transporters like ABCB1, ABCC1, and ABCC2 actively export ACT-D out of the cell such that it cannot bind DNA and induce DNA damage.

The dysregulation of transporter expression could impact cellular responses to ACT-D. There is evidence that miRNAs can affect MDR transporter expression. For example, the activity of miR-873 has been reported to increase sensitivity to other drugs like paclitaxel through the downregulation of MDR transporters (Wu et al., 2016). One might predict that the absence of canonical miRNA processing could surprisingly contribute to a resistant phenotype to ACT-D-induced apoptosis. In this case, the absence of miRNAs downregulating MDR transporters would lead to their overexpression, decreasing intracellular drug levels and conferring resistance to some drugs, including ACT-D in DROSHA null cells. However, our data suggests that this is likely not the case. This is not surprising because, as previously stated, the net effect of multiple miRNAs is

often unclear in specific cellular responses. It is entirely possible that additional miRNA-induced gene silencing activity has a greater effect on positive regulators of MDR transporters. Inhibition of positive regulators could lead to a net decrease in the number of MDR transporters and foster increased sensitivity to ACT-D. The net effect of miRNAs regulating these membrane transporters, like many other cellular processes, is not well-described. For this reason, we assessed the effect of ACT-D on major biochemical processes including DNA replication, nascent transcription, and nascent translation. This was useful because differences in these processes could suggest a variable ability for ACT-D to access DNA in the presence/absence of DROSHA.

We found that ACT-D treatment did not reduce the proportion of replicating cells in either cell line (Figure 16). Furthermore, nascent transcription and nascent translation were equally inhibited by ACT-D treatment across the dose range tested (Figure 17). These results indicate that ACT-D has the same direct effects on major biochemical processes regardless of miRNA processing activity. This finding was important as it implied that the elevated levels of apoptosis observed in DROSHA null cells in sub-G_1 assays were likely not caused by altered ability for ACT-D to enter cells and interact with DNA. An alternative consequence of the absence of canonical miRNA processing must therefore be responsible for elevated sensitivity to apoptosis in DROSHA null cells.

4.5 The loss of DROSHA did not increase cell sensitivity to other forms of cell death

Metabolic activity correlates well with the number of viable cells so it is often used to measure the sensitivity of cells to various forms of cell death (Stockert et al., 2012; van Meerloo et al., 2011). Surprisingly, we found no difference in the relative metabolic activity of parental and DROSHA null cells treated with ACT-D (Figure 18). Both cell

lines equally decreased relative metabolic activity at low concentrations of ACT-D. At the highest concentration of ACT-D (250nM), the relative metabolic activity of parental HCT116 cells appeared slightly higher than that of DROSHA null cells, however, this difference was not significant ($p=0.0646$) by unpaired t-test. Our data was generated using three independent experiments, but increasing the number of replicates could reveal a statistical difference between relative metabolic activity of these two cell lines in response to 250nM ACT-D. If this were the case it is possible that parental HCT116 cells decrease proliferative activity at this dose which may delay the cytotoxic effects of ACT-D. Future work could further address this idea by measuring proliferative capacity of parental and DROSHA null HCT116 cells treated with ACT-D in a different way. Clonogenic survival assays are the gold standard for assessing proliferative capacity and would be useful for this purpose (Franken et al., 2006).

The possibility that parental HCT116 cells exclusively are transiently protected from ACT-D induced apoptosis by decreasing their rate of replication is an interesting idea that could explain their lack of sensitivity to apoptosis in comparison to DROSHA null cells. We proposed that this could occur through the induction of stress-induced premature senescence (SIPS) because senescent cells are resistant to apoptotic cell death (Yosef et al., 2017). We found that both parental and DROSHA null HCT116 cells equally increased the proportion of senescent cells in response to ACT-D treatment (Figure 19). These findings suggest that SIPS likely does not contribute to the differential levels of sensitivity to apoptosis in the presence/absence of DROSHA observed in this work. Although replicative senescence does not explain how DROSHA affects cellular responses to ACT-D, it does not rule out the possibility that alternative forms of cell death like autophagy-associated

cell death contribute to the differences observed in this work. Future work could address this idea by measuring autophagosome formation in the presence and absence of DROSHA.

4.6 Alternative explanations for elevated sensitivity in the absence of DROSHA

Recent work by an undergraduate student that I supervised aimed to compliment these findings by assessing cellular sensitivity to ACT-D in a DICER null HCT116 subline. The disruption of DROSHA and DICER ribonuclease enzymes produces similar miRNA phenotypes with a marked reduction in the production of canonical miRNAs (Kim et al., 2016). DROSHA null cells exhibit a complete loss of canonical miRNA processing while the DICER null subline still produces some canonical miRNAs from the 5' side of hairpins in a DICER-independent process (Kim et al., 2016). Non-canonical miRNAs produced in DROSHA- and DICER-independent manners also exist, however there are relatively few of these non-canonical miRNAs. If the elevated sensitivity to apoptosis in DROSHA null cells observed in this work is caused solely by the absence of canonical miRNA processing it would be reasonable to expect a similar pattern of sensitivity in DICER null cells. Surprisingly, the results of this work suggested that parental and DICER null HCT116 cells are equally sensitive to ACT-D induced cell death. These results indicate that the absence of canonical miRNAs is not the reason for heightened apoptosis in ACT-D treated DROSHA null cells. Instead, the disruption of DROSHA itself rather than loss of canonical miRNAs may explain these findings.

As previously stated, some different non-canonical miRNAs persist in DROSHA and DICER null cells. One possible explanation for DROSHA null sensitivity is that DICER-independent miRNAs (miR-451 and simtrons), which are both DROSHA-

dependent, play an important role in promoting cell survival while DROSHA-independent non-canonical miRNAs (mirtrons, snoRNAs, shRNAs, and tRNA-derived miRNAs), which are all DICER-dependent, do not. Although gene-silencing activity by miR-451 and simtrons has been reported, little is known about these miRNAs (Abdelfattah et al., 2014). More research into the functions of DICER-independent miRNAs is needed to determine the plausibility of this hypothesis.

A second hypothesis is that DROSHA provides additional functions beyond miRNA processing that are important to cell survival. Previous studies have reported that miRNA-independent activity of DROSHA may positively regulate nascent transcription (Gromak et al., 2014). In our assessment of nascent transcriptional activity, the absence of DROSHA did not affect nascent RNA synthesis as it had no effect on ACT-D dependent decreases in transcription. This evidence doesn't support the idea that a role for DROSHA in transcription is the root cause of this differential sensitivity. However, we cannot rule out the possibility that unrecognized miRNA-independent functions of DROSHA exist, therefore the likelihood of this hypothesis remains to be seen.

One final explanation points to the fact that in the absence of functional DROSHA, canonical pri-miRNAs are not processed by the microprocessor complex into pre-miRNAs. This could lead to accumulation of unprocessed pri-miRNAs in the nucleus. It may be possible that the buildup of RNA material in the nucleus could lead to increases in cell sensitivity. We speculate that this could occur through activation of innate immune responses which normally respond to RNA viruses, however to our knowledge this has never been examined in DROSHA null cells (Koyama et al., 2008). PKR is a double stranded RNA (dsRNA) -dependent kinase that is activated in response to ER stress to

inhibit cellular and viral protein synthesis. PKR has been reported to play a role in the induction of ER-stress mediated apoptosis through eIF2α/ATF4/CHOP pathway (Lee et al., 2007). We speculate that dsRNA hairpins of pri-miRNAs could activate PKR and contribute to elevated sensitivity to apoptosis in DROSHA null cells. One way to begin assessing this hypothesis in future work would be to measure the sensitivity of parental and DROSHA null cells to ACT-D-induced apoptosis following knockdown of PKR using siRNAs. A second way to approach this hypothesis could be to assess the sensitivity of nuclear export protein (NEP) -deficient cells to apoptosis by the same ACT-D treatments. The disruption of multiple NEPs may be necessary as recent work suggests that some miRNAs are exported from the nucleus by not only XPO5, but other NEPs like XPO1 (Kim et al., 2016). In the absence of NEPs, many canonical pri-miRNAs would presumably accumulate in the nucleus. If NEP deficient cells exhibit similar patterns of sensitivity as DROSHA null cells, this may support the idea that the accumulation of pri-miRNAs in the nucleus sensitises cells to ACT-D induced cell death.

5. Conclusion

We hypothesized that the presence/absence of DROSHA affects cellular responses to pharmacological agents. The data collected in this research supports this hypothesis as we observed increases in sensitivity to apoptosis by multiple measures in a DROSHA null subline in comparison to wild-type (parental) cells. The measures of sensitivity employed suggest that DROSHA null HCT116 cell sensitivity is characterized by increased DNA fragmentation, increased caspase 3/7 activity and loss of plasma membrane integrity. Although it has been reported that ACT-D induces cell death by induction of apoptosis through p53, no differences were observed between the two cell lines in the expression of

p53 or its downstream targets at the protein and gene levels. Furthermore, no differences were found between the two cell lines in experiments measuring DNA replication, nascent transcription, nascent translation, metabolic activity, and induction of replicative senescence. This indicates that although the DROSHA null cells are more sensitive to apoptosis than the parental cells, the two cell lines are equally susceptible to ACT-D treatment as assessed by the direct effects of the intercalating agent on replication, transcription, and translation. We speculate that the parental cells do not survive exposure to ACT-D but rather die through an alternative form of cell death in comparison to DROSHA null cells. It remains to be seen whether differences in the type of cell death occurring are a function of a lack of canonical miRNA activity or the absence of DROSHA itself. Continuing this work using the proposed future directions will help determine whether a shift in the type of cell death occurring in DROSHA null HCT116 cells explains the elevated levels of death observed in cell sensitivity assays. Understanding how the modulation of miRNAs and miRNA processing activity/machinery affects cell death pathways could be important in therapeutic responses.

References

Abdelfattah, A. M., Park, C., & Choi, M. Y. (2014). Update on non-canonical microRNAs. *Biomol Concepts*, *5*(4), 275-287.

Adams, J. M., & Cory, S. (2007). Bcl-2-regulated apoptosis: mechanism and therapeutic potential. *Curr Opin Immunol*, *19*(5), 488-496.

Adan, A., Kiraz, Y., & Baran, Y. (2016). Cell proliferation and cytotoxicity assays. *Curr Pharm Biotechnol*, *17*(14), 1213-1221.

Agrawal, R. K., Patel, R. K., Shah, V., Nainiwal, L., & Trivedi, B. (2014). Hydroxyurea in sickle cell disease: drug review. *Indian J Hematol Blood Transfus*, *30*(2), 91-96.

Auyeung, V. C., Ulitsky, I., McGeary, S. E., & Bartel, D. P. (2013). Beyond secondary structure: primary-sequence determinants license pri-miRNA hairpins for processing. *Cell*, *152*(4), 844-858.

Babiarz, J. E., Ruby, J. G., Wang, Y., Bartel, D. P., & Blelloch, R. (2008). Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. *Genes Dev*, *22*(20), 2773-2785.

Bano, D., Young, K. W., Guerin, C. J., Lefeuvre, R., Rothwell, N. J., Naldini, L., . . . Nicotera, P. (2005). Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity. *Cell*, *120*(2), 275-285.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, *116*(2), 281-297.

Bentley, D. L. (2014). Coupling mRNA processing with transcription in time and space. *Nat Rev Genet*, *15*(3), 163-175.

Berezikov, E., Chung, W. J., Willis, J., Cuppen, E., & Lai, E. C. (2007). Mammalian mirtron genes. *Mol Cell*, *28*(2), 328-336.

Blagosklonny, M. V., & El-Deiry, W. S. (1998). Acute overexpression of wt p53 facilitates anticancer drug-induced death of cancer and normal cells. *Int J Cancer*, *75*(6), 933-940.

Bouillet, P., Metcalf, D., Huang, D. C., Tarlinton, D. M., Kay, T. W., Köntgen, F., . . . Strasser, A. (1999). Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science*, *286*(5445), 1735-1738.

Bracken, C. P., Scott, H. S., & Goodall, G. J. (2016). A network-biology perspective of microRNA function and dysfunction in cancer. *Nat Rev Genet*, *17*(12), 719-732.

Brady, C. A., Jiang, D., Mello, S. S., Johnson, T. M., Jarvis, L. A., Kozak, M. M., . . . Attardi, L. D. (2011). Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell*, *145*(4), 571-583.

Bringold, F., & Serrano, M. (2000). Tumor suppressors and oncogenes in cellular senescence. *Exp Gerontol*, *35*(3), 317-329.

Buccitelli, C., & Selbach, M. (2020). mRNAs, proteins and the emerging principles of gene expression control. *Nat Rev Genet*, *21*(10), 630-644.

Böhlig, L., Friedrich, M., & Engeland, K. (2011). p53 activates the PANK1/miRNA-107 gene leading to downregulation of CDK6 and p130 cell cycle proteins. *Nucleic Acids Res*, *39*(2), 440-453.

Cabrita, M. A., Bose, R., Vanzyl, E. J., Pastic, A., Marcellus, K. A., Pan, E., . . . McKay, B. C. (2017). The p53 protein induces stable miRNAs that have the potential to modify subsequent p53 responses. *Gene*, *608*, 86-94.

Cabrita, M. A., Vanzyl, E. J., Hamill, J. D., Pan, E., Marcellus, K. A., Tolls, V. J., . . . McKay, B. C. (2016). A temperature sensitive variant of p53 drives p53-dependent microRNA expression without evidence of widespread post-transcriptional gene silencing. *PLoS One*, *11*(2), e0148529.

Carrasco, R. A., Stamm, N. B., & Patel, B. K. (2003). One-step cellular caspase-3/7 assay. *Biotechniques*, *34*(5), 1064-1067.

Cheloufi, S., Dos Santos, C. O., Chong, M. M., & Hannon, G. J. (2010). A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature*, *465*(7298), 584-589.

Chen, K., & Rajewsky, N. (2007). The evolution of gene regulation by transcription factors and microRNAs. *Nat Rev Genet*, *8*(2), 93-103.

Chen, S., Jiao, J. W., Sun, K. X., Zong, Z. H., & Zhao, Y. (2015). MicroRNA-133b targets glutathione S-transferase π expression to increase ovarian cancer cell sensitivity to chemotherapy drugs. *Drug Des Devel Ther*, *9*, 5225-5235.

Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., & Korsmeyer, S. J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell*, *8*(3), 705-711.

Cheng, Y., & Zhang, C. (2010). MicroRNA-21 in cardiovascular disease. *J Cardiovasc Transl Res*, *3*(3), 251-255.

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., & Green, D. R. (2004). Direct activation of Bax by p53 mediates

mitochondrial membrane permeabilization and apoptosis. *Science*, *303*(5660), 1010-1014.

Chong, M. M., Rasmussen, J. P., Rudensky, A. Y., Rundensky, A. Y., & Littman, D. R. (2008). The RNAseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease. *J Exp Med*, *205*(9), 2005-2017.

Conradt, B. (2009). Genetic control of programmed cell death during animal development. *Annu Rev Genet*, *43*, 493-523.

D'Arcy, M. S. (2019). Cell death: a review of the major forms of apoptosis, necrosis and autophagy. *Cell Biol Int*, *43*(6), 582-592.

Dasari, S., & Tchounwou, P. B. (2014). Cisplatin in cancer therapy: molecular mechanisms of action. *Eur J Pharmacol*, *740*, 364-378.

Dash, B. C., & El-Deiry, W. S. (2005). Phosphorylation of p21 in G2/M promotes cyclin B-Cdc2 kinase activity. *Mol Cell Biol*, *25*(8), 3364-3387.

Dice, J. F. (2007). Chaperone-mediated autophagy. *Autophagy*, *3*(4), 295-299.

Djuranovic, S., Nahvi, A., & Green, R. (2011). A parsimonious model for gene regulation by miRNAs. *Science*, *331*(6017), 550-553.

Doonan, F., & Cotter, T. G. (2008). Morphological assessment of apoptosis. *Methods*, *44*(3), 200-204.

Eichhorn, S. W., Guo, H., McGeary, S. E., Rodriguez-Mias, R. A., Shin, C., Baek, D., . . . Bartel, D. P. (2014). mRNA destabilization is the dominant effect of mammalian microRNAs by the time substantial repression ensues. *Mol Cell*, *56*(1), 104-115.

Elbashir, S. M., Lendeckel, W., & Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev*, *15*(2), 188-200.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol*, *35*(4), 495-516.

Feng, S., Cong, S., Zhang, X., Bao, X., Wang, W., Li, H., . . . Zhang, B. (2011). MicroRNA-192 targeting retinoblastoma 1 inhibits cell proliferation and induces cell apoptosis in lung cancer cells. *Nucleic Acids Res*, *39*(15), 6669-6678.

Franken, N. A., Rodermond, H. M., Stap, J., Haveman, J., & van Bree, C. (2006). Clonogenic assay of cells *in vitro*. *Nat Protoc*, *1*(5), 2315-2319.

Friedman, R. C., Farh, K. K., Burge, C. B., & Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*, *19*(1), 92-105.

Fukunaga, R., Han, B. W., Hung, J. H., Xu, J., Weng, Z., & Zamore, P. D. (2012). Dicer partner proteins tune the length of mature miRNAs in flies and mammals. *Cell*, *151*(4), 912.

Furuta, T., Ueda, T., Aune, G., Sarasin, A., Kraemer, K. H., & Pommier, Y. (2002). Transcription-coupled nucleotide excision repair as a determinant of cisplatin sensitivity of human cells. *Cancer Res*, *62*(17), 4899-4902.

Galluzzi, L., Maiuri, M. C., Vitale, I., Zischka, H., Castedo, M., Zitvogel, L., & Kroemer, G. (2007). Cell death modalities: classification and pathophysiological implications. *Cell Death Differ*, *14*(7), 1237-1243.

Gebert, L. F. R., & MacRae, I. J. (2019). Regulation of microRNA function in animals. *Nat Rev Mol Cell Biol*, *20*(1), 21-37.

Georges, S. A., Biery, M. C., Kim, S. Y., Schelter, J. M., Guo, J., Chang, A. N., . . . Chau, B. N. (2008). Coordinated regulation of cell cycle transcripts by p53-inducible microRNAs, miR-192 and miR-215. *Cancer Res*, *68*(24), 10105-10112.

Goldstein, J. C., Muñoz-Pinedo, C., Ricci, J. E., Adams, S. R., Kelekar, A., Schuler, M., . . . Green, D. R. (2005). Cytochrome c is released in a single step during apoptosis. *Cell Death Differ*, *12*(5), 453-462.

Gonzalez, V. M., Fuertes, M. A., Alonso, C., & Perez, J. M. (2001). Is cisplatin-induced cell death always produced by apoptosis? *Mol Pharmacol*, *59*(4), 657-663.

Gregory, R. I., Chendrimada, T. P., Cooch, N., & Shiekhattar, R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell*, *123*(4), 631-640.

Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., & Shiekhattar, R. (2004). The microprocessor complex mediates the genesis of microRNAs. *Nature*, *432*(7014), 235-240.

Gromak, N., Dienstbier, M., Macias, S., Plass, M., Eyras, E., Cáceres, J. F., & Proudfoot, N. J. (2014). Drosha regulates gene expression independently of RNA cleavage function. *Cell Rep*, *7*(5), 1753-1754.

Grosswendt, S., Filipchyk, A., Manzano, M., Klironomos, F., Schilling, M., Herzog, M., . . . Rajewsky, N. (2014). Unambiguous identification of miRNA:target site interactions by different types of ligation reactions. *Mol Cell*, *54*(6), 1042-1054.

Gulyaeva, L. F., & Kushlinskiy, N. E. (2016). Regulatory mechanisms of microRNA expression. *J Transl Med*, *14*(1), 143.

Guo, Y., Wu, Y., Li, N., & Wang, Z. (2021). Up-regulation of miRNA-151-3p enhanced the neuroprotective effect of dexmedetomidine against β-amyloid by targeting DAPK-1 and TP53. *Exp Mol Pathol*, *118*, 104587.

Halazonetis, T. D., & Kandil, A. N. (1993). Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. *EMBO J*, *12*(13), 5057-5064.

Hansen, T. B., Wiklund, E. D., Bramsen, J. B., Villadsen, S. B., Statham, A. L., Clark, S. J., & Kjems, J. (2011). miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA. *EMBO J*, *30*(21), 4414-4422.

Harris, C. C. (1996). Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. *J Natl Cancer Inst*, *88*(20), 1442-1455.

Harrison, R. A., & Vickers, S. E. (1990). Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J Reprod Fertil*, *88*(1), 343-352.

Havens, M. A., Reich, A. A., Duelli, D. M., & Hastings, M. L. (2012). Biogenesis of mammalian microRNAs by a non-canonical processing pathway. *Nucleic Acids Res*, *40*(10), 4626-4640.

Helton, E. S., & Chen, X. (2007). p53 modulation of the DNA damage response. *J Cell Biochem*, *100*(4), 883-896.

Hentze, M. W., Castello, A., Schwarzl, T., & Preiss, T. (2018). A brave new world of RNA-binding proteins. *Nat Rev Mol Cell Biol*, *19*(5), 327-341.

Hermeking, H. (2012). MicroRNAs in the p53 network: micromanagement of tumour suppression. *Nat Rev Cancer*, *12*(9), 613-626.

Hernández Borrero, L. J., & El-Deiry, W. S. (2021). Tumor suppressor p53: Biology, signaling pathways, and therapeutic targeting. *Biochim Biophys Acta Rev Cancer*, *1876*(1), 188556.

Hill, C. R., Jamieson, D., Thomas, H. D., Brown, C. D., Boddy, A. V., & Veal, G. J. (2013). Characterisation of the roles of ABCB1, ABCC1, ABCC2 and ABCG2 in the transport and pharmacokinetics of actinomycin D *in vitro* and *in vivo*. *Biochem Pharmacol*, *85*(1), 29-37.

Hotchkiss, R. S., Strasser, A., McDunn, J. E., & Swanson, P. E. (2009). Cell death. *N Engl J Med*, *361*(16), 1570-1583.

Hu, W., Feng, Z., & Levine, A. J. (2012). The regulation of multiple p53 stress responses is mediated through MDM2. *Genes Cancer*, *3*(3-4), 199-208.

Hughes, P. D., Belz, G. T., Fortner, K. A., Budd, R. C., Strasser, A., & Bouillet, P. (2008). Apoptosis regulators Fas and Bim cooperate in shutdown of chronic immune responses and prevention of autoimmunity. *Immunity*, *28*(2), 197-205.

Humphreys, D. T., Westman, B. J., Martin, D. I., & Preiss, T. (2005). MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *Proc Natl Acad Sci U S A*, *102*(47), 16961-16966.

Hupp, T. R., Meek, D. W., Midgley, C. A., & Lane, D. P. (1992). Regulation of the specific DNA binding function of p53. *Cell*, *71*(5), 875-886.

Iorio, M. V., & Croce, C. M. (2012). MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med*, *4*(3), 143-159.

Itahana, K., Dimri, G., & Campisi, J. (2001). Regulation of cellular senescence by p53. *Eur J Biochem*, *268*(10), 2784-2791.

Iwasaki, S., Kobayashi, M., Yoda, M., Sakaguchi, Y., Katsuma, S., Suzuki, T., & Tomari, Y. (2010). Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol Cell*, *39*(2), 292-299.

Jeffers, J. R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., . . . Zambetti, G. P. (2003). Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell*, *4*(4), 321-328.

Jung, S., Jeong, H., & Yu, S. W. (2020). Autophagy as a decisive process for cell death. *Exp Mol Med*, *52*(6), 921-930.

Jung, S. H., Hwang, H. J., Kang, D., Park, H. A., Lee, H. C., Jeong, D., . . . Lee, J. S. (2019). mTOR kinase leads to PTEN-loss-induced cellular senescence by phosphorylating p53. *Oncogene*, *38*(10), 1639-1650.

Kaikkonen, M. U., Lam, M. T., & Glass, C. K. (2011). Non-coding RNAs as regulators of gene expression and epigenetics. *Cardiovasc Res*, *90*(3), 430-440.

Kajstura, M., Halicka, H. D., Pryjma, J., & Darzynkiewicz, Z. (2007). Discontinuous fragmentation of nuclear DNA during apoptosis revealed by discrete "sub-G1" peaks on DNA content histograms. *Cytometry A*, *71*(3), 125-131.

Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., & Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res*, *51*(23 Pt 1), 6304-6311.

Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., . . . Fornace, A. J. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, *71*(4), 587-597.

Kastenhuber, E. R., & Lowe, S. W. (2017). Putting p53 in context. *Cell*, *170*(6), 1062-1078.

Kehl, T., Backes, C., Kern, F., Fehlmann, T., Ludwig, N., Meese, E., . . . Keller, A. (2017). About miRNAs, miRNA seeds, target genes and target pathways. *Oncotarget*, *8*(63), 107167-107175.

Kim, H., Rafiuddin-Shah, M., Tu, H. C., Jeffers, J. R., Zambetti, G. P., Hsieh, J. J., & Cheng, E. H. (2006). Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol*, *8*(12), 1348-1358.

Kim, Y. K., Kim, B., & Kim, V. N. (2016). Re-evaluation of the roles of DROSHA, Export in 5, and DICER in microRNA biogenesis. *Proc Natl Acad Sci U S A*, *113*(13), E1881-1889.

Knudsen, B. S., & Vasioukhin, V. (2010). Mechanisms of prostate cancer initiation and progression. *Adv Cancer Res*, *109*, 1-50.

Kobayashi, H., & Tomari, Y. (2016). RISC assembly: Coordination between small RNAs and Argonaute proteins. *Biochim Biophys Acta*, *1859*(1), 71-81.

Koyama, S., Ishii, K. J., Coban, C., & Akira, S. (2008). Innate immune response to viral infection. *Cytokine*, *43*(3), 336-341.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., . . . 2009, N. C. o. C. D. (2009). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ*, *16*(1), 3-11.

Kroemer, G., & Levine, B. (2008). Autophagic cell death: the story of a misnomer. *Nat Rev Mol Cell Biol*, *9*(12), 1004-1010.

Lain, S., & Lane, D. (2003). Improving cancer therapy by non-genotoxic activation of p53. *Eur J Cancer*, *39*(8), 1053-1060.

Laster, S. M., Wood, J. G., & Gooding, L. R. (1988). Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. *J Immunol*, *141*(8), 2629-2634.

Lee, E. S., Yoon, C. H., Kim, Y. S., & Bae, Y. S. (2007). The double-strand RNA-dependent protein kinase PKR plays a significant role in a sustained ER stress-induced apoptosis. *FEBS Lett*, *581*(22), 4325-4332.

Lee, Y., Han, J., Yeom, K. H., Jin, H., & Kim, V. N. (2006). Drosha in primary microRNA processing. *Cold Spring Harb Symp Quant Biol*, *71*, 51-57.

Lee, Y., Hur, I., Park, S. Y., Kim, Y. K., Suh, M. R., & Kim, V. N. (2006). The role of PACT in the RNA silencing pathway. *EMBO J*, *25*(3), 522-532.

Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H., & Kim, V. N. (2004). MicroRNA genes are transcribed by RNA polymerase II. *EMBO J*, *23*(20), 4051-4060.

Levine, B., & Deretic, V. (2007). Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol*, *7*(10), 767-777.

Li, W. W., Li, J., & Bao, J. K. (2012). Microautophagy: lesser-known self-eating. *Cell Mol Life Sci*, *69*(7), 1125-1136.

Liao, J. M., Cao, B., Zhou, X., & Lu, H. (2014). New insights into p53 functions through its target microRNAs. *J Mol Cell Biol*, *6*(3), 206-213.

Lin, J., Chen, J., Elenbaas, B., & Levine, A. J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev*, *8*(10), 1235-1246.

Ling, H. Y., Ou, H. S., Feng, S. D., Zhang, X. Y., Tuo, Q. H., Chen, L. X., . . . Liao, D. F. (2009). Changes in microRNA (miR) profile and effects of miR-320 in insulin-resistant 3T3-L1 adipocytes. *Clin Exp Pharmacol Physiol*, *36*(9), e32-39.

Liu, J., Zhang, C., Zhao, Y., & Feng, Z. (2017). MicroRNA control of p53. *J Cell Biochem*, *118*(1), 7-14.

Liu, X. F., Xiang, L., Zhou, Q., Carralot, J. P., Prunotto, M., Niederfellner, G., & Pastan, I. (2016). Actinomycin D enhances killing of cancer cells by immunotoxin RG7787 through activation of the extrinsic pathway of apoptosis. *Proc Natl Acad Sci U S A*, *113*(38), 10666-10671.

Liu, Y., Lagowski, J. P., Vanderbeek, G. E., & Kulesz-Martin, M. F. (2004). Facilitated search for specific genomic targets by p53 C-terminal basic DNA binding domain. *Cancer Biol Ther*, *3*(11), 1102-1108.

Ljungman, M., Zhang, F., Chen, F., Rainbow, A. J., & McKay, B. C. (1999). Inhibition of RNA polymerase II as a trigger for the p53 response. *Oncogene*, *18*(3), 583-592.

Lohrum, M. A., Woods, D. B., Ludwig, R. L., Bálint, E., & Vousden, K. H. (2001). C-terminal ubiquitination of p53 contributes to nuclear export. *Mol Cell Biol*, *21*(24), 8521-8532.

Loreto, C., La Rocca, G., Anzalone, R., Caltabiano, R., Vespasiani, G., Castorina, S., . . . Sansalone, S. (2014). The role of intrinsic pathway in apoptosis activation and progression in Peyronie's disease. *Biomed Res Int*, *2014*, 616149.

Los, M., Mozoluk, M., Ferrari, D., Stepczynska, A., Stroh, C., Renz, A., . . . Schulze-Osthoff, K. (2002). Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. *Mol Biol Cell*, *13*(3), 978-988.

Maelfait, J., & Beyaert, R. (2008). Non-apoptotic functions of caspase-8. *Biochem Pharmacol*, *76*(11), 1365-1373.

Maki, C. G. (1999). Oligomerization is required for p53 to be efficiently ubiquitinated by MDM2. *J Biol Chem*, *274*(23), 16531-16535.

Mandke, P., Wyatt, N., Fraser, J., Bates, B., Berberich, S. J., & Markey, M. P. (2012). MicroRNA-34a modulates MDM4 expression via a target site in the open reading frame. *PLoS One*, *7*(8), e42034.

Medley, J. C., Panzade, G., & Zinovyeva, A. Y. (2021). microRNA strand selection: Unwinding the rules. *Wiley Interdiscip Rev RNA*, *12*(3), e1627.

Michlewski, G., & Cáceres, J. F. (2019). Post-transcriptional control of miRNA biogenesis. *RNA*, *25*(1), 1-16.

Mijit, M., Caracciolo, V., Melillo, A., Amicarelli, F., & Giordano, A. (2020). Role of p53 in the Regulation of Cellular Senescence. *Biomolecules*, *10*(3).

Morris, P. G., & Fornier, M. N. (2008). Microtubule active agents: beyond the taxane frontier. *Clin Cancer Res*, *14*(22), 7167-7172.

Mosca, P. J., Lin, H. B., & Hamlin, J. L. (1995). Mimosine, a novel inhibitor of DNA replication, binds to a 50 kDa protein in Chinese hamster cells. *Nucleic Acids Res*, *23*(2), 261-268.

Naeli, P., Winter, T., Hackett, A. P., Alboushi, L., & Jafarnejad, S. M. (2022). The intricate balance between microRNA-induced mRNA decay and translational repression. *FEBS J*.

Newton, K., Harris, A. W., Bath, M. L., Smith, K. G., & Strasser, A. (1998). A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *EMBO J*, *17*(3), 706-718.

Nikolaou, M., Pavlopoulou, A., Georgakilas, A. G., & Kyrodimos, E. (2018). The challenge of drug resistance in cancer treatment: a current overview. *Clin Exp Metastasis*, *35*(4), 309-318.

Nishimura, Y., & Lemasters, J. J. (2001). Glycine blocks opening of a death channel in cultured hepatic sinusoidal endothelial cells during chemical hypoxia. *Cell Death Differ*, *8*(8), 850-858.

O'Hagan, H. M., & Ljungman, M. (2004). Efficient NES-dependent protein nuclear export requires ongoing synthesis and export of mRNAs. *Exp Cell Res*, *297*(2), 548-559.

Ohler, U., Yekta, S., Lim, L. P., Bartel, D. P., & Burge, C. B. (2004). Patterns of flanking sequence conservation and a characteristic upstream motif for microRNA gene identification. *RNA*, *10*(9), 1309-1322.

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., & Bao, J. K. (2012). Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Prolif*, *45*(6), 487-498.

Pandey, V. K., Mathur, A., & Kakkar, P. (2019). Emerging role of Unfolded Protein Response (UPR) mediated proteotoxic apoptosis in diabetes. *Life Sci*, *216*, 246-258.

Park, C. Y., Choi, Y. S., & McManus, M. T. (2010). Analysis of microRNA knockouts in mice. *Hum Mol Genet*, *19*(R2), R169-175.

Park, S. Y., Im, J. S., Park, S. R., Kim, S. E., Wang, H. J., & Lee, J. K. (2012). Mimosine arrests the cell cycle prior to the onset of DNA replication by preventing the binding of human Ctf4/And-1 to chromatin via HIF-1α activation in HeLa cells. *Cell Cycle*, *11*(4), 761-766.

Peter, M. E., & Krammer, P. H. (2003). The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ*, *10*(1), 26-35.

Pichiorri, F., Suh, S. S., Rocci, A., De Luca, L., Taccioli, C., Santhanam, R., . . . Croce, C. M. (2010). Downregulation of p53-inducible microRNAs 192, 194, and 215 impairs the p53/MDM2 autoregulatory loop in multiple myeloma development. *Cancer Cell*, *18*(4), 367-381.

Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., . . . Filipowicz, W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science*, *309*(5740), 1573-1576.

Piovan, C., Palmieri, D., Di Leva, G., Braccioli, L., Casalini, P., Nuovo, G., . . . Croce, C. M. (2012). Oncosuppressive role of p53-induced miR-205 in triple negative breast cancer. *Mol Oncol*, *6*(4), 458-472.

Pogribny, I. P., Filkowski, J. N., Tryndyak, V. P., Golubov, A., Shpyleva, S. I., & Kovalchuk, O. (2010). Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin. *Int J Cancer*, *127*(8), 1785-1794.

Proskuryakov, S. Y., Konoplyannikov, A. G., & Gabai, V. L. (2003). Necrosis: a specific form of programmed cell death? *Exp Cell Res*, *283*(1), 1-16.

Provost, P. (2010). MicroRNAs as a molecular basis for mental retardation, Alzheimer's and prion diseases. *Brain Res*, *1338*, 58-66.

Riccardi, C., & Nicoletti, I. (2006). Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc*, *1*(3), 1458-1461.

Rossi, J. J. (2005). Mammalian Dicer finds a partner. *EMBO Rep*, *6*(10), 927-929.

Rukov, J. L., & Shomron, N. (2011). MicroRNA pharmacogenomics: post-transcriptional regulation of drug response. *Trends Mol Med*, *17*(8), 412-423.

Saini, H. K., Griffiths-Jones, S., & Enright, A. J. (2007). Genomic analysis of human microRNA transcripts. *Proc Natl Acad Sci U S A*, *104*(45), 17719-17724.

Sayers, T. J. (2011). Targeting the extrinsic apoptosis signaling pathway for cancer therapy. *Cancer Immunol Immunother*, *60*(8), 1173-1180.

Schmittgen, T. D. (2008). Regulation of microRNA processing in development, differentiation and cancer. *J Cell Mol Med*, *12*(5B), 1811-1819.

Schneider-Poetsch, T., Ju, J., Eyler, D. E., Dang, Y., Bhat, S., Merrick, W. C., . . . Liu, J. O. (2010). Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nat Chem Biol*, *6*(3), 209-217.

Shadfan, M., Lopez-Pajares, V., & Yuan, Z. M. (2012). MDM2 and MDMX: Alone and together in regulation of p53. *Transl Cancer Res*, *1*(2), 88-89.

Singh, R., Letai, A., & Sarosiek, K. (2019). Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. *Nat Rev Mol Cell Biol*, *20*(3), 175-193.

Smith, N. D., Rubenstein, J. N., Eggener, S. E., & Kozlowski, J. M. (2003). The p53 tumor suppressor gene and nuclear protein: basic science review and relevance in the management of bladder cancer. *J Urol*, *169*(4), 1219-1228.

Stavast, C. J., & Erkeland, S. J. (2019). The Non-Canonical Aspects of MicroRNAs: Many Roads to Gene Regulation. *Cells*, *8*(11).

Stewart, Z. A., Leach, S. D., & Pietenpol, J. A. (1999). p21(Waf1/Cip1) inhibition of cyclin E/Cdk2 activity prevents endoreduplication after mitotic spindle disruption. *Mol Cell Biol*, *19*(1), 205-215.

Stockert, J. C., Blázquez-Castro, A., Cañete, M., Horobin, R. W., & Villanueva, A. (2012). MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta Histochem*, *114*(8), 785-796.

Stoddart, M. J. (2011). Mammalian cell viability : methods and protocols. Humana Press/Springer.

Strasser, A. (2005). The role of BH3-only proteins in the immune system. *Nat Rev Immunol*, *5*(3), 189-200.

Strober, W. (2015). Trypan blue exclusion test of cell viability. *Curr Protoc Immunol*, *111*, A3.B.1-A3.B.3.

Sui, X., Chen, R., Wang, Z., Huang, Z., Kong, N., Zhang, M., . . . Pan, H. (2013). Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death Dis*, *4*, e838.

Szostak, E., & Gebauer, F. (2013). Translational control by 3'-UTR-binding proteins. *Brief Funct Genomics*, *12*(1), 58-65.

Tacar, O., Sriamornsak, P., & Dass, C. R. (2013). Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. *J Pharm Pharmacol*, *65*(2), 157-170.

Tahmasebi, S., Khoutorsky, A., Mathews, M. B., & Sonenberg, N. (2018). Translation deregulation in human disease. *Nat Rev Mol Cell Biol*, *19*(12), 791-807.

Teufel, D. P., Bycroft, M., & Fersht, A. R. (2009). Regulation by phosphorylation of the relative affinities of the N-terminal transactivation domains of p53 for p300 domains and Mdm2. *Oncogene*, *28*(20), 2112-2118.

Trendowski, M. (2015). Using cytochalasins to improve current chemotherapeutic approaches. *Anticancer Agents Med Chem*, *15*(3), 327-335.

van Deursen, J. M. (2014). The role of senescent cells in ageing. *Nature*, *509*(7501), 439-446.

van Meerloo, J., Kaspers, G. J., & Cloos, J. (2011). Cell sensitivity assays: the MTT assay. *Methods Mol Biol*, *731*, 237-245.

Vanzyl, E. J., Rick, K. R. C., Blackmore, A. B., MacFarlane, E. M., & McKay, B. C. (2018). Flow cytometric analysis identifies changes in S and M phases as novel cell cycle alterations induced by the splicing inhibitor isoginkgetin. *PLoS One*, *13*(1), e0191178.

Vermeulen, K., Berneman, Z. N., & Van Bockstaele, D. R. (2003). Cell cycle and apoptosis. *Cell Prolif*, *36*(3), 165-175.

Walker, K. K., & Levine, A. J. (1996). Identification of a novel p53 functional domain that is necessary for efficient growth suppression. *Proc Natl Acad Sci U S A*, *93*(26), 15335-15340.

Wang, P. Y., Li, Y. J., Zhang, S., Li, Z. L., Yue, Z., Xie, N., & Xie, S. Y. (2010). Regulating A549 cells growth by ASO inhibiting miRNA expression. *Mol Cell Biochem*, *339*(1-2), 163-171.

Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., . . . Harris, C. C. (1999). GADD45 induction of a G2/M cell cycle checkpoint. *Proc Natl Acad Sci U S A*, *96*(7), 3706-3711.

Weaver, B. A. (2014). How Taxol/paclitaxel kills cancer cells. *Mol Biol Cell*, *25*(18), 2677-2681.

Wu, D. D., Li, X. S., Meng, X. N., Yan, J., & Zong, Z. H. (2016). MicroRNA-873 mediates multidrug resistance in ovarian cancer cells by targeting ABCB1. *Tumour Biol*, *37*(8), 10499-10506.

Wu, K., He, J., Pu, W., & Peng, Y. (2018). The role of exportin-5 in microRNA biogenesis and cancer. *Genom Proteom Bioinform*, *16*(2), 120-126.

Xu, J., & Morris, G. F. (1999). p53-mediated regulation of proliferating cell nuclear antigen expression in cells exposed to ionizing radiation. *Mol Cell Biol*, *19*(1), 12-20.

Yosef, R., Pilpel, N., Papismadov, N., Gal, H., Ovadya, Y., Vadai, E., . . . Krizhanovsky, V. (2017). p21 maintains senescent cell viability under persistent DNA damage response by restraining JNK and caspase signaling. *EMBO J*, *36*(15), 2280-2295.

Yuan, J., Najafov, A., & Py, B. F. (2016). Roles of caspases in necrotic cell death. *Cell*, *167*(7), 1693-1704.

Zhang, J., Sun, Q., Zhang, Z., Ge, S., Han, Z. G., & Chen, W. T. (2013). Loss of microRNA-143/145 disturbs cellular growth and apoptosis of human epithelial cancers by impairing the MDM2-p53 feedback loop. *Oncogene*, *32*(1), 61-69.

Zhang, J. H., & Xu, M. (2000). DNA fragmentation in apoptosis. *Cell Res*, *10*(3), 205-211.

Zong, W. X., & Thompson, C. B. (2006). Necrotic death as a cell fate. *Genes Dev*, *20*(1), 1-15.

Zorio, D. A., & Bentley, D. L. (2001). Transcription elongation: the 'Foggy' is liftingellipsis. *Curr Biol*, *11*(4), R144-146.