

Control of the Intrinsic Pathway of Apoptosis

Presented by

Tirta (Mario) Djajawi`

0000-0003-3527-1276

Submitted in total fulfilment of the requirements of the
degree of

Doctor of Philosophy

December 2019

Department of Medical Biology

Faculty of Medicine, Dentistry and Health Sciences

The University of Melbourne

Abstract

Apoptosis is a cellular process of programmed cell death. The intrinsic pathway of apoptosis is triggered by mitochondrial outer membrane permeabilization, a point of no return that coincides with the release of cytochrome c into the cytosol where it activates the main effectors of cellular destruction: the caspases. The mitochondrial pathway that is centered on MOMP is tightly regulated by BCL2 family proteins, which includes some members that promote apoptosis and others that inhibit it. The interplay between these proteins with opposing roles determines whether a cell will die or survive. In a healthy cell, pro-survival BCL2 proteins inhibit the effector proteins BAX and BAK. BH3-only proteins are activated in response to cellular stress and promote apoptosis by neutralizing pro-survival proteins.

Targeting BCL2 proteins to provoke apoptotic cell death has proven to be a successful strategy for cancer therapy with the BCL2-selective drug venetoclax exhibiting remarkable efficacy in treating cancers that rely on BCL2 for their survival. MCL1, a protein related to BCL2, is likewise critical for the survival of many cancer cells, making it another attractive anti-cancer drug target. Selective MCL1 inhibitors have been developed and are currently being evaluated in clinical trials to establish their safety and efficacy. Safety is a particular concern for MCL1 inhibitors because MCL1 is also essential for the survival of many cells in critical organs and tissues throughout the body. It remains to be seen if a sufficient therapeutic window will exist when MCL1 is targeted systemically.

An alternative and potentially safer strategy to modulate MCL1 survival function would be to target pathways that regulate its activity in particular contexts. In **Chapter 3** and **4**, I focus on one such mechanism of MCL1 regulation: its turnover by the ubiquitin proteasome system. My work in **Chapter 3** elucidated details of how MCL1 protein turnover is regulated by BH3-only protein NOXA. Using CRISPR-Cas9 screen, I discovered that the mitochondrial E3 ligase MARCH5, the E2 conjugating enzyme UBE2K and the mitochondrial outer membrane protein MTCH2 co-operate to mark MCL1 for degradation by the proteasome. I also demonstrated that this pathway is constitutively active in cells where NOXA is abundantly expressed and showed that manipulating NOXA expression in those cells impacts on MCL1 survival function.

Having successfully demonstrated the power of CRISPR-Cas9 screen in **Chapter 3**, I undertook further screens in **Chapter 4** to identify proteins, such as deubiquinating enzymes (DUBs), that might serve to enhance MCL1 protein stability. I did not identify any strong hits from these screens, possibly because multiple DUBs act

redundantly on MCL1. Consistent with this hypothesis, only mild impacts on MCL1 protein stability were observed upon deleting DUBs previously reported to act on MCL1.

Finally, in **Chapter 5**, I investigated how BH3 mimetics mimic the activity of BH3-only proteins to induce apoptosis. I studied how selective BH3 mimetic compounds perturb interactions throughout the BCL2 protein network beyond their direct protein targets. I showed that these second order impacts are crucial for effective killing. Apoptosis induced by the BCL2 selective inhibitor venetoclax, for example, typically also involves inhibition of MCL1. The impact on MCL1 in this context occurs as a consequence of displacing BH3-only proteins normally bound to BCL2.

Declaration

In accordance with the regulations of the University of Melbourne I acknowledged that some of the data in Chapter 3 have been taken, repeated and reanalysed from my honours thesis, whereas other data in Chapter 5 were collaborative.

Specifically:

Fig. 3.1	Data were repeated and reanalysed from honours thesis
Fig. 3.2 B	Bioinformatic analysis was performed by Dr. Mark van Delft
Figs. 3.4 A-B	<i>PMAIP1</i> gene score was kindly provided by Ming-jie Luo
Sup Fig. 3.1	Data were taken from honours thesis
Sup Fig. 3.3	Data were taken from honours thesis
Sup Fig. 3.4 A	Data were kindly provided by Dr. Mark van Delft
Sup Tab.3.1	Bioinformatic analysis was performed by Dr. Mark van Delft
Fig. 4.3	Bioinformatic analysis was performed by Dr. Mark van Delft
Figs. 4.6 B-D	Bioinformatic analysis was performed by Dr. Mark van Delft
Fig. 5.2 E	Data were kindly provided by Dr. Jia-nan Gong
Figs. 5.5 B	Data were kindly provided by Dr. Jia-nan Gong
Fig. 5.6	Data were kindly provided by Dr. Jia-nan Gong

Other experiments were my original works performed between March 2016 to December 2019, under the supervision of Dr. Mark van Delft and Prof. David Huang. Overall, I assess my contribution to the work presented as greater than 90%.

This is to certify that:

1. This thesis comprises of only my original work towards the PhD degree, unless stated otherwise.
2. Due acknowledgement has been given in the text to all other materials used.
3. The thesis length is less than 100,000 words in length, exclusive of tables, charts, plots, bibliographies and appendices.

Tirta (Mario) Djajawi
Huang Laboratory, Blood Cells and Blood Cancer
Walter and Eliza Hall Institute of Medical Research
1G Royal Parade
Parkville, Victoria 3052
Australia

Publications

Parts of this thesis have been prepared or submitted for publication in the papers listed below:

Tirta Mario Djajawi, Lei Liu, Jia-nan Gong, Allan Shuai Huang, Ming-jie Luo, Zhen Xu, Toru Okamoto, Melissa J. Call, David C.S. Huang, Mark F. van Delft. MARCH5 requires MTCH2 to coordinate proteasomal turnover of the MCL1:NOXA complex. *Cell Death and Differentiation* (2020). <https://doi-org.ezp.lib.unimelb.edu.au/10.1038/s41418-020-0517-0>.

Jia-nan Gong, **Tirta Mario Djajawi**, Christoffer Flensburg, Donia M Moujalled, Yuan Yao, Tiffany Khong, Tse-Chieh Teh, Giovanna Pomilio, Mark F van Delft, Seong Lin Khaw, Mary Ann Anderson, Pasquale L Fedele, Michael S Low, Ming-jie Luo, Zhen Xu, Chris D Riffkin, Sarah E Miller, David H Segal, Christine A White, Guillaume Lessene, Marco J Herold, Andreas Strasser, Ana Leticia Maragno, Olivier Geneste, George Grigoriadis, Andrew Spencer, Andrew H Wei, Ian J Majewski, Andrew W Roberts, David CS Huang. Threshold of apoptosis induced by targeting BCL2 survival proteins in cancer is governed by secondary interactions impacting the wider BCL2 family. *Manuscript in preparation for submission to Cancer Cell*.

Acknowledgements

It has been a privilege for me to undertake a PhD program at Walter and Eliza Hall Institute and University of Melbourne. These four years have been a great enjoyment full of learning experiences, challenges, tears and rewards.

Firstly and foremost, I would like to express my special gratitude to Professor David Huang for taking me as a student from honours into the final year of PhD. Your many guidance and mentorship have irrevocably shaped me to become a resilient and critical scientist that I would always remember you for. I would also sincerely thank Dr. Mark van Delft for the support and encouragement throughout my research career. I considered myself highly fortunate for having you as my first supervisor because of your patience in guiding me when I first came into the laboratory. I am also inspired with your great knowledge and understanding in our research topics that make my PhD life less difficult and more interesting.

I would also like to express gratitude to my PhD committee, Dr. Melissa Call, Associate Professor Peter Czabotar and Dr. Marco Herold for the invaluable discussions regarding the direction of my PhD projects as well as the constructive assurance of my PhD progress. To Sue Hardy, Dr. Keely Bumsted-O'Brien, Catherine McLean, Michelle Birrell and Sabine Kelly, I am indebted to you all for providing useful and timely information regarding administrative works including PhD scholarships, confirmation and completion. Undoubtedly, I would not be able to finish my PhD without the funding from Melbourne Graduate Research Scholarship, WEHI Top-Up Scholarship and Edith Moffat Fund.

Many of my colleagues have also provided many invaluable advice, time and assistance throughout my PhD studies. I would like to particularly mention Dr. Jia-nan Gong for allowing me to collaborate in her wonderful research project. I would also like to thank Dr. Stephen Wilcox, Dr. Marco Herold, Dr. David Segal, Dr. Zhen Xu, Dr. Christine White, Dr. Kelly Rogers, Dr. Michael Mlodzianoski, Dr. Nicholas Liau, Dr. Edward Chew, Dr. Rachel Thijssen, Chris Riffkin, Angela Huang, Sandra Misfud and past as well as present members of Huang Laboratory and Blood Cells and Blood Cancer Division for scientific supports, sharing reagents and protocols. When PhD life gets tough, I am lucky to have Stephanie Hyslop, Hui San Chin, Esther Kim, Sarah Miller, Dina Stockwell and Shuai Huang to make me laugh and smile through our daily jokes and conservations.

Last but certainty not least, this thesis would not have been possible without the love, care and support from my parents Liantie and Soegiharto, and my younger brother Indra. Thank you for the extraordinary faith in me that I will get through and more

importantly, the voice of reasons to persevere and to have courage when things did not go as planned. To each of my good friends in Australia and Indonesia, Kristel, Meilinda, Priska, Stella, Charles, Winnie, Jessica, Gene, Max, Wei, Jason, Hilson, Shimun, Shuying, Swee, Katherine, Joelyn, Yuqing and Hui, I am incredibly blessed to have you all in this journey. Our meetings and conversations have brought me indispensable laughter and joy that made the hardship of PhD more bearable. Finally, to my partner Chua Shin Yiing, words cannot begin to describe how grateful I am to walk through this journey with you by my side. Thank you for the unwavering faith, countless hours of encouragement, love and hope that everything will be just fine.

Abbreviations

ADP	Adenosine diphosphate
APAF-1	Apoptotic protease activating factor 1
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATP	Adenosine triphosphate
BAK	BCL2 homologous antagonist killer
BAX	BCL2 associated X protein
BCL2	B-cell lymphoma 2
BCLxL (<i>BCL2L1</i>)	B-cell lymphoma extra large
BCLW (<i>BCL2L2</i>)	BCL2 like protein 2
BFP	Blue fluorescent protein
BH	BCL2 homology
BID	BH3 interacting domain death agonist
BIM (<i>BCL2L11</i>)	BCL2 interacting mediator of cell death/ BCL2 like protein 11
β-TrCP	Beta transducing repeat containing E3 ligase protein
Cas9	CRISPR-associated protein nuclease 9
Carb	Carbenicillin
CHX	Cycloheximide
CLL	Chronic lymphocytic leukemia
CYT C	Cytochrome c
CRISPR	Clustered regularly interspaced short palindromic repeat
DME	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxycycline
DSB	Double stranded break
DUB	Deubiquitinating enzyme
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)- N,N,N',N'-tetra acetic acid
ER	Endoplasmic reticulum
ETOP	Etoposide
FACS	Fluorescence activated cell sorting
FBS	Fetal Bovine Serum

FBW7	F-box and WD repeat domain containing 7
FCS	Fetal Calf Serum
FITC	Fluorescein-5-isothiocynate
FDR	False discovery rate
FLAG-tag	Flag-tag (DYKDDDDK)
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase 3
GZMB	Granzyme B
HA-tag	Haemagglutinin-tag (YPYDVPDYA)
HDR	Homology directed repair
HEK293T	Human embryonic kidney transformed with large T antigen
HECT	Homologous to the E6-AP carboxyl terminus
HRP	Horseradish peroxidase
HSP70	70 kDa of heat shock protein
Hygro	Hygromycin
kb	Kilobase
kDa	Kilodalton
MARCH5	Membrane associated ring finger (C3HC4) 5
MCL1	Myeloid Cell Leukemia 1
MEF	Mouse embryonic fibroblast
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MG132	<i>N</i> -Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucine Z-LLL-CHO
MID49 (<i>MIEF2</i>)	Mitochondrial dynamic protein of 49 kDa (Mitochondrial elongation factor 2)
MM	Multiple myeloma
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger ribonucleic acid
MTCH2	Mitochondrial carrier homolog 2
MULE	MCL1 ubiquitin ligase E3
MYC-tag	MYC-tag (EQKLISEEDL)
NHEJ	Non-homologous end joining
NOC	Nocodazole
nt	Nucleotides
PAM	Protospacer adjacent motif
PARKIN	Parkinson juvenile disease protein 2
PBS	Phosphate buffered saline

PCR	Polymerase Chain Reaction
PE	Phycoerythrin fluorescence
PEST	Proline, glutamic acid, serine, threonine
Pi	Propidium iodide
PINK1	PTEN-induced putative kinase 1
<i>PMAIP1</i>	Phorbol-12-myristate-13-acetate-induced protein 1
PNK	Polynucleotide kinase
PS	Phosphatidylserine
PUMA	P53 upregulated modulator of apoptosis
Puro	Puromycin
RING	Really interesting new gene
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
sd	Standard deviation
sem	Standard error of mean
sgRNA	Short guide RNA
siRNA	Small interfering RNA
TALEN	Transcription activator like effector nuclease
TPA	12-O-tetradecanoylphorbol-13-acetate
TX-100	Triton X-100
Ub	Ubiquitin
UBE2K	Ubiquitin conjugating enzyme E2K
UBE2V1	Ubiquitin conjugating enzyme E2 variant 1
UPS	Ubiquitin proteasome system
USP9X	Ubiquitin specific protease 9X
USP13	Ubiquitin specific protease 13
USP24	Ubiquitin specific protease 24
UV	Ultraviolet
VDAC	Voltage dependent anion channel (porin)
WB	Western blot
WT	Wild type

Table of Contents

Abstract.....	ii
Declaration.....	iv
Publications.....	v
Acknowledgements.....	vi
Abbreviations.....	viii
Table of Contents.....	xi
List of figures.....	xv
List of tables.....	xvii
Chapter 1: Introduction.....	1
Apoptosis: regulated cell death.....	2
The intrinsic pathway of apoptosis.....	2
BCL2 family of proteins.....	3
Pro-survival proteins.....	3
BH3-only proteins.....	4
Effector proteins: BAX, BAK and BOK.....	5
Regulation of apoptosis by members of the BCL2 family.....	6
BH3 mimetics.....	7
MCL1: an essential pro-survival protein.....	8
Multiple levels of MCL1 protein regulation.....	10
Regulation of MCL1 protein stability by the ubiquitin-proteasome pathway.....	11
Modulating pathways controlling MCL1 protein stability.....	12
Thesis outline.....	13
Chapter 2: Materials and methods.....	25
Mammalian expression constructs.....	26
Generation of sgRNA-containing vector.....	27
Cell culture of mammalian cells.....	28
Gene delivery into mammalian cells.....	30
Transient transfection.....	30
Retroviral and lentiviral infection of mammalian cells.....	30

Generation of knock out cell lines using CRISPR-Cas9 gene editing technology	31
Transient transfection of PX458 vector into adherent cell lines	31
Stable transduction of Cas9 and sgRNA into mammalian cell lines	31
Cell growth competition assay	31
DNA sequencing	32
DNA extraction from cell lysates	32
Targeted PCR of sites of CRISPR-mediated insertion-deletion mutations (indels)	32
Biochemical assays	35
Preparation of protein lysates	35
Whole cell protein extracts	35
Subcellular fractionation	35
Immunoprecipitation	36
SDS-PAGE and electroblotting	36
Protein detection: Immunoblotting	37
Biotin ID assay	38
³⁵ S-methionine labelling of BIM protein	39
³⁵ S-methionine-labelled protein detection: phosphorimaging	39
Immunofluorescence	40
Flow cytometry	40
Cell preparation	40
Gene expression assay	41
Intracellular staining of MCL1	41
Flow cytometry analysis	41
Cell death assay	41
Annexin V staining and propidium iodide assay	41
JC-1 assay	42
Cell Titer-Glo luminescent assay	42
Genome-wide CRISPR-Cas9 screen with GFP-MCL1	42
Establishment of GFP-MCL1 screening platform	42
Enrichment screen to identify novel regulators of NOXA-mediated MCL1 degradation	43
Enrichment screen to identify stabilizers of MCL1 protein	43
CRISPR-Cas9 drop out screen to identify deubiquitinating enzymes (DUBs) of MCL1	44
Bioinformatic analysis	44
Analysis of enriched sgRNAs in GFP-MCL1 screen	44
Analysis of drop out sgRNAs in an MCL1 dependent cells	44
PMAIP1 mRNA heat map	45

Chapter 3: *MARCH5* requires *MTCH2* to coordinate proteasomal turnover of the *MCL1:NOXA* complex..... 49

Background..... 50

Results..... 52

The degradation of MCL1 provoked by the BH3-only protein NOXA proceeds through a distinct pathway 52

Genome-wide loss-of-function screen to identify genes required for MCL1 degradation by NOXA..... 53

MARCH5, UBE2K and MTCH2 are required for MCL1 degradation triggered by NOXA.. 53

NOXA provokes MCL1 degradation in diverse cells and influences steady-state MCL1 levels when abundantly expressed..... 54

Context specific control of MCL1 by MARCH5 and MTCH2..... 55

Impaired turnover of the MCL1:NOXA complex does not enhance MCL1 pro-survival activity..... 55

MTCH2 cooperates with MARCH5 to degrade MCL1 but does not influence the turnover of another MARCH5 substrate: MID49 56

The C-terminal transmembrane domain of MCL1 is required for its degradation by MARCH5 and MTCH2 57

Discussion 95

Chapter 4: *Identifying novel regulators of MCL1 protein stability* 98

Background..... 99

Results..... 102

Evaluating the impact of USP9X and USP24 on MCL1 protein stability by gene knockout rather than gene knock-down 102

Genome-wide CRISPR-Cas9 screen to identify genes that stabilize MCL1 protein 102

Evaluating the impact of deleting *Prdm4* on MCL1 protein stability 103

Engineering an MCL1 dependent cell for CRISPR-Cas9 drop-out screening 104

Cell viability-based CRISPR-Cas9 drop-out screen to identify deubiquitinating enzymes of MCL1 104

Discussion 118

Chapter 5: *Direct and indirect consequences of targeting specific BCL2 proteins with BH3 mimetic compounds*..... 121

Background..... 122

Results..... 124

Establishing maximum concentrations for BH3 ligands in the BH3 profiling assay 124

Cells are less sensitive to BH3 mimetics in BH3 profiling assays than in conventional cell viability assays.....	124
Inhibiting multiple pro-survival proteins is essential for effective mitochondrial depolarization	125
Genetically deleting other pro-survival genes promotes greater sensitization to selective inhibition of MCL1	126
BIM and NOXA are essential for BCL2i killing in KMS-12-PE cells.....	127
BCL2i induces apoptosis in KMS-12-PE cells by inhibiting BCL2 and increasing the availability of free BIM to neutralize MCL1 protein	127
Discussion	145
<i>Chapter 6:Concluding remarks.....</i>	<i>147</i>
<i>Bibliography</i>	<i>150</i>

List of figures

Chapter 1: Introduction

Figure 1.1 Intrinsic pathway of apoptosis	15
Figure 1.2 Classification of the BCL2 family of proteins	17
Figure 1.3 Models of life/death determination governed by BCL2 family proteins	19
Figure 1.4 Molecular regulation of MCL1 protein stability	21
Figure 1.5 Ubiquitin-proteasome pathway	23

Chapter 2: Materials and methods

Figure 2.1 Schematic of BH3 profiling assay	47
---	----

Chapter 3: *MARCH5* requires *MTCH2* to coordinate proteasomal turnover of the *MCL1:NOXA* complex

Figure 3.1 Lysines 175 and 178 of MCL1 are critical for its degradation triggered by the BH3-only protein NOXA, but not by other stimuli	59
Figure 3.2 Genome-wide CRISPR-Cas9 screen for regulators of NOXA-induced MCL1 degradation	61
Figure 3.3 MARCH5, MTCH2 and UBE2K are critical for NOXA-induced MCL1 degradation.	63
Figure 3.4 The steady-state level of MCL1 protein is controlled by NOXA when this BH3-only protein is abundantly expressed	65
Figure 3.5 MARCH5 and MTCH2 control NOXA-driven MCL1 turnover in diverse cells	67
Figure 3.6 Distinct impacts on MCL1 pro-survival function upon deleting either <i>NOXA</i> , <i>MARCH5</i> or <i>MTCH2</i>	69
Figure 3.7 MTCH2 and MARCH5 jointly control turnover of the MCL1:NOXA complex	71
Figure 3.8 Elements of the MCL1 transmembrane domain are necessary for its turnover by MARCH5 and MTCH2	73

Supplementary Figure 3.1 The ubiquitin E3 ligases MULE, β -TRCP, FBW7 and PARKIN are not required for NOXA-mediated MCL1 degradation	75
--	----

Supplementary Figure 3.2 MCL1 lysine/arginine replacement variants retain their pro-survival function and their ability to bind NOXA	77
--	----

Supplementary Figure 3.3 Characterization of GFP-MCL1 reporter and clones utilized for CRISPR-Cas9 screen	79
---	----

Supplementary Figure 3.4 The NOXA-dependent pathway for degrading MCL1 involving MARCH5, MTCH2 and UBE2K is not required for MCL1 degradation caused by DNA damage or protein synthesis inhibition in MEFs	81
--	----

Supplementary Figure 3.5 NOXA is highly unstable in the absence of MCL1	83
---	----

Supplementary Figure 3.6 Distinct impacts of deleting <i>NOXA</i> and <i>MARCH5</i> in lymphoid cancer cell lines	85
Supplementary Figure 3.7 MTCH2 and MARCH5 do not play major roles in turnover of the BH3-only protein tBID	87
Supplementary Figure 3.8 MCL1 ^{BCL2TM} remains associated with heavy membranes, retaining its pro-survival function and ability to bind NOXA	89

Chapter 4: Identifying novel regulators of MCL1 protein stability

Figure 4.1 USP9X had a modest impact on MCL1 protein stability, while USP24 had no discernible impact	106
Figure 4.2 Genome-wide CRISPR/Cas9 screen using a GFP-MCL1 reporter to identify genes whose deletion results in reduced MCL1 protein expression	108
Figure 4.3 sgRNAs targeting <i>Prdm4</i> and <i>Mcl1</i> were enriched among the cells sorted for low GFP-MCL1 expression	110
Figure 4.4 PRDM4 does not regulate MCL1 protein post-translationally	112
Figure 4.5 Generating <i>Bcl2</i> ^{-/-} <i>Bclxl</i> ^{-/-} and the controls <i>Bcl2</i> ^{-/-} <i>Bclxl</i> ^{-/-} <i>Bax</i> ^{-/-} <i>Bak</i> ^{-/-} MEFs for the purpose of cell viability-based CRISPR/Cas9 drop-out screening	114
Figure 4.6 CRISPR/Cas9 drop-out screen identifies <i>Ubr5</i> , <i>Otud5</i> and <i>Trip12</i> as potential regulators of MCL1 protein stability	116

Chapter 5: Direct and indirect consequences of targeting specific BCL2 proteins with BH3 mimetic compounds

Figure 5.1 BH3 ligands used in BH3 profiling assay	129
Figure 5.2 BH3 profiling assay cannot accurately assess the mitochondrial potential when BH3 peptides and mimetics are used at high concentration	131
Figure 5.3 Cell lines are less sensitive to selective BH3 ligands in BH3 profiling assays compared to conventional cell viability assays	133
Figure 5.4 Combined inhibition of multiple pro-survival proteins results in greater mitochondrial depolarization in BH3 profiling assays	135
Figure 5.5 Genetic deletion of certain <i>BCL2</i> pro-survival genes enables selective BH3 ligands to depolarize mitochondria	137
Figure 5.6 Loss of <i>BIM</i> and <i>NOXA</i> prevents BCL2i induced killing in KMS-12-PE cells	139
Figure 5.7 BCL2i treatment shifts the binding of BIM from BCL2 to MCL1	141
Figure 5.8 Simplified model for the direct and indirect impacts of BH3 mimetics	143

List of tables

Chapter 2: Materials and methods

Table 2.1 List of mammalian expression constructs used	26
Table 2.2 List of 20 nt gRNA sequences	27
Table 2.3 List of cell culture media	29
Table 2.4 List of primers for sequencing indels on sgRNA-targeted DNA sites	33
Table 2.5 First PCR condition for amplification of sgRNA-targeted DNA sites	34
Table 2.6 Second PCR condition for amplification of barcoded DNA products	35
Table 2.7 Primary antibodies used for western blot	37
Table 2.8 Secondary antibodies used for western blot	38
Table 2.9 List of molecular technique kits	45
Table 2.10 List of bacterial growth media	45
Table 2.11 List of reagents and buffers	45

Chapter 3: *MARCH5* requires *MTCH2* to coordinate proteasomal turnover of the *MCL1:NOXA* complex

Supplementary Table 3.1 Quantitation of changes in sgRNA abundance after sorting for GFP ^{high} cells	91
Supplementary Table 3.2 Indels measured at sgRNA target sites by DNA sequencing.....	93

Chapter 1: Introduction

Apoptosis: regulated cell death

The ability to regulate the life and death of a cell is crucial for the survival and maintenance of multicellular organisms. There are many pathways to cellular death such as necrosis, necroptosis, pyroptosis and apoptosis. Apoptosis is a programmed form of cell death. The term apoptosis was first coined by Kerr, Wyllie and Currie in 1972 to describe a distinct type of cellular death, characterized by a series of morphological changes (Kerr, Wyllie, & Currie, 1972). These include cellular shrinkage, DNA fragmentation followed by chromatin condensation, plasma membrane blebbing and finally, fragmentation of cellular components into the formation of apoptotic bodies. When phosphatidylserine is exposed on the plasma membrane of apoptotic cells, it provides an 'eat me' signal recognized by nearby phagocytes to engulf and rapidly remove them.

The tightly controlled mechanism of apoptosis allows unwanted cells to be cleared without releasing toxic cellular contents into the surrounding environment. Clearance of cells by apoptosis is crucial for numerous physiological functions in multicellular organisms. For instance, apoptosis plays a role in shaping organs and body structure during mammalian embryogenesis (Brill, Torchinsky, Carp, & Toder, 1999). It also acts as a defensive mechanism by removing cells that have extensive DNA damage (Roos & Kaina, 2006) or are infected with viruses (Jorgensen, Rayamajhi, & Miao, 2017). Consequently, dysregulation of the apoptotic pathway can result in a variety of human pathologies including auto-immune diseases (Nagata, 2010), neurodegenerative diseases (Mattson, 2000) and cancer (Wong, 2011).

The intrinsic pathway of apoptosis

Proteins of BCL2 family govern the intrinsic pathway of apoptosis by regulating the integrity of mitochondrial outer membrane (Adams & Cory, 2018; Kale, Osterlund, & Andrews, 2018). In response to various apoptotic stimuli, the balance between pro-apoptotic and pro-survival members of BCL2 family on the mitochondrial outer membrane is altered to favour cell death. Certain pro-apoptotic members of BCL2 family cooperate to disrupt the integrity of the mitochondrial outer membrane in the event known as Mitochondrial Outer Membrane Permeabilization (MOMP). MOMP triggers the release of cytochrome c from the intermembrane space of mitochondria into the cytosol. In the cytosol, cytochrome c binds to Apoptotic Protease-Activating Factor (APAF-1) (Zou, Henzel, Liu, Lutschg, & Wang, 1997) and together, they form a heptameric oligomer that recruits pro-caspase 9 (Acehan et al., 2002). In this complex called apoptosome, pro-caspase 9 is activated (Rodriguez & Lazebnik, 1999). Once activated,

caspase 9 activates other pro-caspases to initiate a cascade of proteolytic activity (McIlwain, Berger, & Mak, 2015; Zou, Li, Liu, & Wang, 1999). Activated caspases cleave many cellular targets, leading to rapid, irreversible cell death (Fig. 1.1).

BCL2 family of proteins

The BCL2 family of proteins is evolutionarily conserved and it comprises members that are structurally related to one another through shared regions of sequence homology called BCL2 homology (BH) domains (Fig. 1.2). Members of this family are mostly localized on the mitochondrial outer membrane. They have opposing roles and are classified as either pro-survival or pro-apoptotic. Pro-survival members include BCL2, BCLxL, BCLW, MCL1 and BFL1/A1. Pro-apoptotic members are further divided into BH3-only proteins (BAD, NOXA, BIM, PUMA, BID, BIK, BMF and HRK) and effector proteins: BAX, BAK and BOK.

Pro-survival proteins

BCL2 was the first pro-survival protein identified. *BCL2* derives its name *B-cell Lymphoma-2* because its protein was found to be overexpressed in follicular lymphoma as a consequence of t(14;18) gene translocation (Akasaka et al., 1998; Cleary & Sklar, 1985). Unlike previously studied proto-oncogenes at that time, BCL2 was found to promote cell survival instead of cell proliferation (Vaux, Cory, & Adams, 1988). Subsequent pro-survival members including BCLxL, BCLW, MCL1 and A1 were found to share up to four sequences of homology (BH1-4 domains) with *BCL2* gene.

Pro-survival proteins contain short hydrophobic transmembrane spanning domains at their C-termini. They utilize the transmembrane domains to localize on the outer mitochondrial membrane (Popgeorgiev, Jabbour, & Gillet, 2018), but not exclusively. For example, BCL2 proteins are also found in the membranes of other organelles including endoplasmic reticulum and nuclear envelope (Akao, Otsuki, Kataoka, Ito, & Tsujimoto, 1994). BCL2 proteins localized in these compartments may be able to protect cells from apoptosis in some instances (Thomenius & Distelhorst, 2003).

All pro-survival proteins share a similar fold, which comprises of alpha helices bundled into a globular structure with a hydrophobic groove on its surface (Czabotar et al., 2007; Denisov et al., 2003; Herman et al., 2008; X. Liu, Dai, Zhu, Marrack, & Kappler, 2003; Petros et al., 2001). The hydrophobic groove serves as the critical interface for interacting with the BH3 domains of other proteins. In particular, the BH3 domains of BAX and BAK effectors bind into the hydrophobic groove of pro-survival proteins. These

interactions safeguard the integrity of mitochondria by holding BAX and BAK proteins in their inactive forms.

Although any of the five pro-survival proteins can protect cells against apoptosis, their role varies significantly between tissues types. This is because each pro-survival protein is expressed at different levels in various cell types and tissues (Sorenson, 2004). BCL2, for instance, is highly expressed in kidney and mature lymphocytes. When the *Bcl2* gene is deleted, mice develop polycystic kidney disease, lymphocytopenia and premature greying (Kamada et al., 1995; Nakayama et al., 1994; Veis, Sorenson, Shutter, & Korsmeyer, 1993; Yamamura et al., 1996). Depletion of other pro-survival proteins also causes abnormalities in mice at varying severity. In the case of *Bclw* deletion, it causes male sterility (Print et al., 1998; Ross et al., 1998), whereas in the case of *A1* deletion, neutrophils, dendritic cells and T-cells are reduced in number (Hamasaki et al., 1998; Schenk et al., 2017). In the more extreme cases, genetic deletion of either *Mcl1* or *Bclxl* results in embryonic lethality (Motoyama et al., 1995; Rinkenberger, Horning, Klocke, Roth, & Korsmeyer, 2000). All these examples highlight the diverse roles of pro-survival proteins in the development and maintenance of various mammalian tissues.

BH3-only proteins

BH3-only proteins share a single sequence of homology with other BCL2 proteins called the BH3 domain. Unlike pro-survival BCL2 proteins, which fold into a globular structure, most BH3-only proteins with the exception of BID are intrinsically unstructured (Hinds et al., 2007). The BH3 domain of these proteins adopts local secondary structure in the form of an amphipathic α -helix. Within the BH3-domain, there are four conserved hydrophobic residues spaced at an interval of three to four amino acids, allowing them to lie along one face of the amphipathic α -helix. A conserved aspartic acid residue in the BH3 domain forms a salt bridge to a conserved arginine on pro-survival proteins (Day et al., 2005; Hinds & Day, 2005; Kvensakul & Hinds, 2014). This interaction neutralizes the anti-apoptotic function of pro-survival proteins.

Different BH3-only proteins are activated following distinct stress signals and they serve to initiate apoptosis. For example, PUMA and NOXA are transcriptionally upregulated by P53 in response to genotoxic stress such as DNA damage (Oda et al., 2000; Yu, Zhang, Hwang, Kinzler, & Vogelstein, 2001). Other BH3-only proteins such as BIM are activated following cytokine withdrawal such as IL-3 (Dijkers, Medema, Lammers, Koenderman, & Coffey, 2000) or during endoplasmic reticulum stress (Morishima, Nakanishi, Tsuchiya, Shibata, & Seiwa, 2004). BID is activated by caspase

cleavage to generate a truncated form, tBID, which translocates to mitochondria outer membrane (Li, Zhu, Xu, & Yuan, 1998).

Effector proteins: BAX, BAK and BOK

Despite sharing sequence homology (BH1, BH2 and BH3 domains) and similar tertiary structures with pro-survival BCL2 proteins (S. Y. Hsu, Kaipia, McGee, Lomeli, & Hsueh, 1997; Moldoveanu et al., 2006; Suzuki, Youle, & Tjandra, 2000; Youle & Strasser, 2008), the canonical effector proteins BAX and BAK and also, BOK promote rather than inhibit apoptosis. Mice deficient of either BAK or BOK do not display noticeable abnormalities (F. Ke et al., 2012; Lindsten et al., 2000), whereas mice deficient of BAX have few abnormalities including lymphoid hyperplasia and defects in reproductive cells (Knudson, Tung, Tourtellotte, Brown, & Korsmeyer, 1995). In contrast, combined deletion of *Bax* and *Bak* lead to multiple abnormalities in various cell lineages (Lindsten et al., 2000; Rathmell, Lindsten, Zong, Cinalli, & Thompson, 2002) and thus, most mice do not survive to adulthood. Notably, mice deficient of BAX, BAK and BOK exhibit more severe defects and died earlier than the *Bax*^{-/-} *Bak*^{-/-} counterpart. (F. Ke et al., 2015; F. F. S. Ke et al., 2018). This suggests that BAX, BAK and BOK have overlapping functions for apoptosis regulation. Cells lacking both *BAX* and *BAK* fail to undergo apoptosis when they are treated cytotoxic agents or when BH3-only proteins are overexpressed (Cheng et al., 2001; M. C. Wei et al., 2001; Zong, Lindsten, Ross, MacGregor, & Thompson, 2001). This suggests that BAX and BAK act downstream of BH3-only proteins to induce apoptosis. In contrast, how BOK regulates intrinsic apoptosis pathway is not as clear as the upstream triggers are largely unknown and its role is limited to the endoplasmic reticulum stress signals (Carpio et al., 2015; Fernandez-Marrero et al., 2016; F. Ke et al., 2012).

In a healthy cell, BAX is predominantly located in the cytosol as an inactive soluble monomer (Y. T. Hsu, Wolter, & Youle, 1997; Wolter et al., 1997). The C-terminal transmembrane tail of BAX folds into the hydrophobic groove on its surface (Suzuki et al., 2000), preventing BAX translocation to mitochondria. In contrast to BAX, BAK is mainly located on the mitochondrial outer membrane and is kept inactive in healthy cells through interactions with the mitochondrial outer membrane protein VDAC2 (Cheng, Sheiko, Fisher, Craigen, & Korsmeyer, 2003; Lazarou et al., 2010). BOK, on the other hand, is localized in the endoplasmic reticulum and Golgi compartments (Echeverry et al., 2013).

Following stress-induced apoptotic stimuli, BAX and BAK undergo conformational rearrangement in several regions including core (hydrophobic groove

and BH3 domain), latch and transmembrane domain (Adams & Cory, 2018). The C-terminal transmembrane of BAX must first be released from its the hydrophobic pocket so it can translocate to the mitochondrial outer membrane (Antonsson, Montessuit, Sanchez, & Martinou, 2001; Nechushtan, Smith, Hsu, & Youle, 1999; Wolter et al., 1997). Similarly, BAK must be released from VDAC2 for subsequent conformational changes to occur (Lazarou et al., 2010). The most dramatic change involves separation of the BAX and BAK latch domains from their core domains (Brouwer et al., 2014). This separation exposes the hydrophobic groove and BH3 domain of BAX and BAK. The exposed domains facilitate the formation of dimers, and higher-order oligomerization later forms by involving other parts of the molecules through an unknown mechanism (Dewson et al., 2008; George, Evans, & Luo, 2007). These higher-order oligomers are predicted to form pores on the mitochondrial outer membrane, allowing the release of intermembrane space contents into the cytosol. The mechanism and nature of the pores formation by these higher oligomers remains controversial, but biochemical approaches suggest that BAX and BAK form lipid pores, instead of proteinaceous ones (Antignani & Youle, 2006; Uren, Iyer, & Kluck, 2017), to allow the release of cytochrome c (X. Liu, Kim, Yang, Jemmerson, & Wang, 1996).

Regulation of apoptosis by members of the BCL2 family

The life and death decision of a cell is determined by the activity of the canonical pro-apoptotic effectors BAX and BAK. Other members of the BCL2 family including pro-survivals and BH3-only proteins regulate BAX and BAK function. They interact with one another and also with BAX/BAK effectors to control their activation. The interactions they form will vary depending on the state of cellular well-being. Cells will live when both BAX and BAK are sufficiently inhibited, but once any of them are activated, cells will undergo apoptosis.

In a healthy cell, there are sufficient pro-survival proteins to sequester BAX and BAK. These interactions are necessary to inhibit conformational changes required for BAX and BAK activation (Cheng et al., 2001; Mikhailov et al., 2001). During cellular stress, BH3-only proteins are either upregulated or activated post-translationally. This allows more pro-survival proteins to be neutralized. When sufficient pro-survival proteins are inhibited, BAX and BAK can freely oligomerize in the mitochondrial outer membrane (Fletcher et al., 2008; O'Neill, Huang, Zhang, Chen, & Luo, 2016; Willis et al., 2005; Willis et al., 2007; Zong et al., 2001) (Fig. 1.3 A).

There is a complex network of interactions between BCL2 family proteins. BH3-only proteins selectively bind to pro-survival proteins and their binding preference

determines the potency for apoptosis induction (Chen et al., 2005)(Fig. 1.3 B). Some BH3-only proteins including BIM, PUMA and BID are potent death agonists because they promiscuously bind and neutralize all pro-survival proteins. Other members such as BAD and NOXA bind selectively to a subset of pro-survival proteins. Whereas BAD preferentially binds to BCLxL, BCL2 and BCLW, NOXA preferentially binds to MCL1 and A1. Their more restricted binding profile makes them the less potent death agonists.

The potency of BH3-only proteins to induce apoptosis, however, is determined not only by their binding selectivity to pro-survival proteins, but also through the ability to bind to BAX and BAK effectors. Some BH3-only proteins including BIM (Gavathiotis et al., 2008; Kuwana et al., 2005), BID (Desagher et al., 1999; Kuwana et al., 2002; A. Letai et al., 2002) and possibly PUMA (Cartron et al., 2004; Kim et al., 2009) have been shown to have weak affinity for BAX and BAK (Fig. 1.3 B). Those interactions have been argued as essential requirement for BAX and BAK conformational changes that lead to their oligomerization in mitochondria outer membrane (Brouwer et al., 2017; Brouwer et al., 2014). In short, for apoptosis to occur, sufficient BAX and BAK must be activated and released from their inhibition.

BH3 mimetics

Our detailed understanding of how apoptosis is regulated by BCL2 proteins paved the way to developing BH3 mimetic compounds. BH3 mimetics are small molecule inhibitors that mimic the activity of BH3-only proteins. They promote apoptosis by directly engaging and inhibiting specific BCL2 pro-survival proteins (Adams & Cory, 2018; Merino et al., 2018). Their mode of killing depends on BAX and BAK activation and they are advantageous to many standard chemotherapies because they bypass the requirement for activation of upstream stress sensor such as p53 (Delbridge & Strasser, 2015). They are also fast-acting, allowing many sensitive cells to be killed within hours.

The first 'true' BH3 mimetic compound was ABT-737, generated by AbbVie (Oltersdorf et al., 2005). It mimics the action of BAD protein and induces apoptosis in many cell lines that are dependent on BCL2, BCLxL or BCLW. An orally bioavailable version called navitoclax was later developed and tested against various lymphoid malignancies. Navitoclax is potent against some of these malignancies, and chronic lymphocytic leukemia (CLL) patients who express high levels of BCL2 show the greatest response (Mason et al., 2009; Tse et al., 2008). However, numerous on-target toxicities are observed with the most notable example being severe thrombocytopenia (Wilson et al., 2010) due to platelet dependency on BCLxL for survival (Mason et al., 2007). To

circumvent this, a structural analogue of navitoclax known as venetoclax was developed (Souers et al., 2013). Venetoclax binds more tightly to BCL2 in comparison to BCLxL, and thrombocytopenia is therefore limited. On the basis of its safety and efficacy, venetoclax has been approved by the FDA to treat BCL2-dependent CLL and acute myeloid leukemia (AML) (DiNardo et al., 2019; DiNardo et al., 2018; Majid et al., 2008; Roberts et al., 2016; Scheffold, Jebaraj, & Stilgenbauer, 2018). This success paved the way to the development of inhibitors that selectively target other pro-survival BCL2 proteins. These include A-155463 and A-1331852 that selectively bind to BCLxL (Levenson et al., 2015; Tao et al., 2014) and S63845, AMG176 and AZD5991 that selectively bind to MCL1 (Caenepeel et al., 2018; Kotschy et al., 2016; Tron et al., 2018).

Due to the complex network of interactions between BCL2 family proteins, the impact of perturbing one interaction with a BH3 mimetic causes knock-on effects on other interactions in the network. A number of studies have suggested that these knock-on effects contribute to the cell's decision whether to live or die, and if perturbed, resistance against selective BH3 mimetics may develop (Blombery et al., 2019; Del Gaizo Moore et al., 2007; Konopleva et al., 2006; Merino et al., 2012). Investigating these knock-on effects may help us to better understand how BAX and BAK are released from their inhibition by pro-survival proteins, and more importantly, how the apoptosis threshold is affected by these changes. One question that I will address in this thesis is how selective targeting of a pro-survival protein by a BH3 mimetic compound alters the interaction between proteins of the BCL2 family, and whether these changes are necessary for apoptosis to occur.

MCL1: an essential pro-survival protein

MCL1 is a pro-survival protein that plays an important role in preventing the inappropriate activation of downstream apoptotic effectors BAX and BAK (Cuconati, Mukherjee, Perez, & White, 2003; Willis et al., 2005). It was initially discovered as a protein upregulated in human myeloid leukaemia cells when these cells were induced with differentiation factor TPA (Kozopas, Yang, Buchan, Zhou, & Craig, 1993). *MCL1* was then classified as a pro-survival protein due to its sequence homology with *BCL2*.

MCL1 is widely expressed at various tissues and is important for the survival of many of cell types. The phenotypes resulting from *Mcl1* deletion are more severe and wide-ranging when compared to the deletion of other BCL2 proteins. For example, homozygous *Mcl1* deletion results in peri-implantation embryonic lethality in mice (Rinkenberger et al., 2000). Moreover, many adult cell types are heavily reliant on MCL1

expression for their survival and development. This includes a variety of haematopoietic cell types such as neutrophils (Dzhagalov, St John, & He, 2007), T-lymphocytes (Dzhagalov, Dunkle, & He, 2008) and plasma B- cells (Peperzak et al., 2013; Vikstrom et al., 2010), as well as non-blood cells such as hepatocytes (Hikita et al., 2009), cardiomyocytes (X. Wang et al., 2013) and neurons (Arbour et al., 2008).

MCL1 has a high oncogenic potential because it can contribute to cancer cell survival. *MCL1* gene is amplified in numerous solid and haematological cancers (Beroukhim et al., 2010). Cancers derived from cells in which MCL1 is an important survival factor frequently remain dependent on MCL1 for their survival. This includes myeloid leukemia (Glaser et al., 2012), lymphoma (Grabow, Delbridge, Valente, & Strasser, 2014; Kelly et al., 2014; Zhou et al., 2001), multiple myeloma (Derenne et al., 2002; Gong et al., 2016), hepatocellular carcinoma (Fleischer et al., 2006; Sieghart et al., 2006), a subset of lung (Song, Coppola, Livingston, Cress, & Haura, 2005; H. Zhang et al., 2011) and breast cancer (Goodwin, Rossanese, Olejniczak, & Fesik, 2015). These examples highlight the importance of apoptosis evasion in various cancer cells through MCL1 activity.

Elevated expression of MCL1 also frequently confers resistance to cytotoxic drugs (Song et al., 2005) and BH3 mimetic compounds (Tahir et al., 2017; van Delft et al., 2006). An important example is resistance against ABT-737, a pioneer BH3 mimetic compound that binds promiscuously to BCL2, BCLxL, BCLW, but not MCL1 (van Delft et al., 2006). Not surprisingly, increased MCL1 activity can hinder the response of tumour cells to the recently approved BCL2 selective inhibitor venetoclax (BCL2i) used to treat CLL (Bojarczuk et al., 2016; Guieze et al., 2019). It is therefore crucial that MCL1 activity be eliminated in order to achieve a successful treatment outcome.

One approach would be to inhibit MCL1 pro-survival function through selective targeting with small molecule inhibitors. Many inhibitors including S63845, AMG176 and AZD5991 can selectively target MCL1, neutralize its function and promote apoptosis of various blood cancers (Caenepeel et al., 2018; Kotschy et al., 2016; Tron et al., 2018). Whereas the safety of AZD5991 is yet to be evaluated, S63845 and AMG176 are likely to be well-tolerated because they do not kill various normal haematopoietic cells *in vitro* and *in vivo*. Notably, they do not exhibit overt systemic toxicity when they are used at their efficacious doses in humanized *MCL1* knock-in mice (Brennan et al., 2018; Caenepeel et al., 2018), although some haemopoietic cell lineages including B cells, monocytes and neutrophils are significantly reduced in numbers. All these results are in contrast with previous data showing that ubiquitous *Mcl1* knockout in mice is embryonic lethal (Rinkenberger et al., 2000). The lack of severe adverse effects from MCL1 inhibitor administration is likely due to the transient action of these agents, in contrast to the

irreversible loss-of-function caused by genetic deletion of *Mcl1*. Whether these and possibly other adverse effects can be tolerated in humans is currently under investigation, with some of these drugs recently entering Phase I clinical trials either as single agents or in combination. The safety of using these drugs in patients will need to be carefully monitored, as various normal cell types and/or their progenitors rely on MCL1 for their survival (Dzhagalov et al., 2007) (Arbour et al., 2008; Dzhagalov et al., 2008; Hikita et al., 2009; Peperzak et al., 2013; Vikstrom et al., 2010; X. Wang et al., 2013) and may be affected upon systematic inhibition of MCL1. This is why discovering an alternative way to inhibit MCL1 protein in a tissue-specific context, rather than systemically throughout the whole organism, may be advantageous. One major aim of this thesis is to identify such pathways where MCL1 protein activity may be modulated to provide a wider therapeutic window than systemically targeting MCL1 protein with BH3 mimetics.

Multiple levels of MCL1 protein regulation

There are many pathways where MCL1 protein levels and function are modulated. Given its importance for the survival of many cell types, MCL1 protein is tightly regulated at multiple levels including transcriptionally, translationally and also post-translationally.

The expression of MCL1 protein can be controlled by modifying its protein synthesis. *MCL1* mRNA is generally short-lived and limits the rate of MCL1 protein translation (Yang, Buchan, Townsend, & Craig, 1996). The level of *MCL1* mRNA is also modulated by numerous transcriptional factors that regulate its gene transcription. Notable examples include STAT3 (H. Liu et al., 2003)), which activates *MCL1* transcription and E2F1 (Croxtan, Ma, Song, Haura, & Cress, 2002), which inhibits it. Moreover, MCL1 protein synthesis can be directly inhibited by the microRNA *MIR29B*. It binds complementarily to the 3' UTR region of *MCL1* mRNA and prevent its protein translation (Mott, Kobayashi, Bronk, & Gores, 2007).

MCL1 expression is also controlled by its protein stability. Like *MCL1* mRNA, MCL1 protein is short-lived. The short half-life of MCL1 has been attributed to its long unstructured N-terminal region that is unique among members of BCL2 protein family (Kozopas et al., 1993; Rechsteiner & Rogers, 1996). This portion of MCL1 contains a degradation motif called PEST domain. This domain is rich in prolines (P), glutamic acids (E), serines (S) and threonines (T). Some of these residues and others within the PEST domain such as aspartic acid (D) can serve as sites for a variety of post-translational modifications by distinct enzymes (Fig. 1.4 A). For example, MCL1 protein is cleaved at two aspartic acids (D127 and D157) by proteases such as caspase 3 (Clohessy, Zhuang,

& Brady, 2004; Herrant et al., 2004) and granzyme B (Han, Goldstein, Gastman, Rabinovitz, & Rabinowich, 2005). This cleavage attenuates MCL1 pro-survival function (Michels et al., 2004; Weng, Li, Xu, Shi, & Tang, 2005). Several serine and threonine residues of MCL1 including S121, S155, S159 and T163 are also associated with phosphorylation. Depending on the kinases and the phosphorylated residues, these modifications can either promote MCL1 stabilization or degradation (Figs. 1.4 B-C). In phosphorylation induced MCL1 degradation pathways, activation of certain enzymes from ubiquitin proteasome system (UPS) is usually required to mark MCL1 for the recognition by proteasome.

Regulation of MCL1 protein stability by the ubiquitin-proteasome pathway

The regulation of target proteins by the UPS utilizes a 76 amino acid polypeptide called ubiquitin (Komander, 2009; Swatek & Komander, 2016). To mark protein for proteasomal degradation, ubiquitin is covalently attached to the amine group of a lysine residue of the target protein. This process involves a series of enzymatic reactions in an ATP-dependent manner. In the first step, ubiquitin is transferred from a ubiquitin-activating enzyme (E1), of which there are two in humans (UBA1 and UBA6), to an E2 conjugating enzyme. There are more than 40 E2 conjugating enzymes in human and each of them perform the second step of ubiquitination reaction and pair with multiple E3 ligases (Markson et al., 2009; van Wijk et al., 2009). In most cases, the E3 ligase conjugates ubiquitin from an E2 enzyme onto a lysine residue of the target protein. There are currently more than 600 known E3 ligases in humans, and these enzymes are responsible for conferring substrate specificity in the ubiquitin conjugation cascade. A specific E3 ligase can form one or more types of linkages, but K48-linked poly-ubiquitin chain is the most common type recognized by the proteasome as a degradation signal. Another group of proteins, called deubiquitinating enzymes (DUBs), remove ubiquitin from proteins and can thereby protect target proteins from proteasomal degradation (Fig. 1.5)

As a labile protein, MCL1 protein level and turnover is heavily regulated by the proteasome. Although ubiquitination is commonly required to drive MCL1 to the proteasome (Mojsa, Lassot, & Desagher, 2014), a ubiquitin-independent mechanism of MCL1 targeting to proteasome has also been observed (Stewart et al., 2010). MCL1 ubiquitination is carried out by several E3 ligases and each of them controls MCL1 degradation in different contexts including the basal protein level when cells are healthy (Subramanian, Andronache, Li, & Wade, 2016; Zhong, Gao, Du, & Wang, 2005) or

during cellular stress such as DNA damage (Zhong et al., 2005) or cell cycle arrest (Wertz et al., 2011).

MULE was the first E3 ligase identified to regulate MCL1 (Zhong et al., 2005). It is unique because it has a BH3 domain that enables interaction with MCL1, but not its other pro-survival relatives. With its BH3 domain, MULE constitutively targets MCL1 for degradation and thereby controls its basal expression. MARCH5 is another E3 ligase that controls the basal level of MCL1 protein (Cherok et al., 2017; Subramanian et al., 2016; Xu et al., 2016). It is localized to the mitochondrial outer membrane but does not contain a BH3 domain. It is therefore unclear how MARCH5 recognizes MCL1 as a substrate.

Several E3 ligases recognize the phosphorylation degrons on MCL1 before they can target it for degradation. Phosphorylation sites on MCL1 are located within its PEST domain and include T92, S155, S159 and T163 (Fig. 1.4 A). These phosphorylation degrons determine which E3 ligases will recognize MCL1 as a substrate. For instance, phosphorylation on T92 of MCL1 by CDK1 promotes MCL1 ubiquitination by APC/CDC20 complex (Harley, Allan, Sanderson, & Clarke, 2010). In contrast, phosphorylation on S155, S159 and T163 of MCL1 by GSK3 β increases the binding with the E3 ligase β TRCP (Ding et al., 2007). FBW7 also recognizes these degrons, but they are phosphorylated by other kinases including JNK, p38 and CKII. How different E3 ligases are activated and compete to recognize similar phosphorylation degrons remain unclear. Finally, PARKIN is unique because its activation requires phosphorylation on itself and ubiquitin, but not MCL1. During mitochondrial depolarization, PINK1 phosphorylates S65 of PARKIN and ubiquitin (Kazlauskaitė, Kelly, et al., 2014; Kazlauskaitė, Kondapalli, et al., 2014). The binding with phosphorylated ubiquitin activates PARKIN and promotes various conformational changes that allow its translocation to the mitochondria so that it can promote MCL1 degradation (Carroll, Hollville, & Martin, 2014).

MCL1 ubiquitination can be removed by the activity of DUBs. To date, several DUBs including USP9X, USP13, USP24, KU70 and CDU1 have been reported to deubiquitinate MCL1 and promote its stability (Fischer et al., 2017; Peterson et al., 2015; Schwickart et al., 2010; B. Wang et al., 2014; S. Zhang et al., 2018). Most of these DUBs control the basal expression of MCL1 protein because MCL1 level is reduced when these DUBs are individually knocked down. As MCL1 level is reduced, these cells are more sensitive to BH3 mimetics that target other pro-survival members such as ABT-737 (Schwickart et al., 2010; S. Zhang et al., 2018).

Modulating pathways controlling MCL1 protein stability

Reducing MCL1 expression may inhibit its pro-survival activity. Drugs that reduce MCL1 protein synthesis can reduce MCL1 protein levels, but they usually do so by globally perturbing transcription (e.g. flavopiridol (Kitada, Zapata, Andreeff, & Reed, 2000)) or translation (e.g. rapamycin (G. Wei et al., 2006), silvestrol or homoharringtonine (Lindqvist et al., 2012)). These drugs are therefore likely to cause adverse side effects when used in patients.

Reducing MCL1 expression may also be achieved by controlling the rate at which it is degraded by the UPS. NOXA binding is an important mechanism through which MCL1 may be regulated by the proteasome. Although binding with other BH3-only proteins including BIM and PUMA promotes MCL1 stabilization (Mei, Du, Yang, & Wu, 2005; Ploner et al., 2008), NOXA binding promotes MCL1 proteasomal degradation (Czabotar et al., 2007; Lee et al., 2008; Willis et al., 2005). Structures of MCL1 in complex with BH3 domains of either BIM or NOXA reveal no significant changes in MCL1 conformation (Czabotar et al., 2007), suggesting that elements from both NOXA and MCL1 may serve as recognition sites for an E3 enzyme. Possible candidates for this pathway include all of MCL1's known E3 ligases. Because NOXA is expressed at a high level in some tissues but not others (Dengler et al., 2014), identifying this E3 ligase may allow us to target MCL1 in a tissue-specific context for cancer treatment. This may be achieved by enhancing the activity of this E3 ligase to reduce MCL1 protein. Alternatively, inhibiting the activity of DUBs that stabilize MCL1 protein with small molecule inhibitors (e.g. G9 (Peterson et al., 2015)) may achieve the same outcome. In this thesis, I aim to identify the E3 ligases and DUBs that regulate MCL1 protein stability so that we may target these for treatment of cancers that are heavily reliant on MCL1 for survival.

Thesis outline

In this thesis, I have investigated questions relating to the molecular control of intrinsic apoptosis. As each chapter addresses a distinct research question, I have included separate backgrounds and discussions in each chapter.

Both published and unpublished data from our laboratory suggest that NOXA could trigger ubiquitin-dependent proteasomal degradation of MCL1 through a unique pathway. I hypothesized that an E3 ligase and other UPS-associated proteins may be involved in this pathway. In Chapter 3, I investigated the mechanism by which NOXA promotes MCL1 degradation. The work in this chapter centred around identifying and characterizing the proteins that mediate this unique mechanism of MCL1 degradation.

MCL1 protein stability is regulated not only by E3 ligases but also by DUBs, although studies surrounding these MCL1-stabilizing enzymes are limited. I hypothesized that there are DUBs regulating MCL1 stability that have yet to be discovered. In Chapter 4, I performed CRISPR-Cas9 screens designed to identify novel pathways enhancing MCL1 protein stability. I focussed primarily on deubiquitinating enzymes (DUBs), with the aim to identify DUBs that could be inhibited to destabilize MCL1 and counter its pro-survival activity.

In Chapter 5, I turned my focus to better understanding how BH3 mimetic compounds induce apoptosis. Selective BH3 mimetics bind to and inhibit their direct protein targets. This prevent their targets from interacting with other BCL2 proteins, and I hypothesized that this in turn has knock-on consequences on other interactions throughout the BCL2 network. Using various assays to measure cell death and protein interactions, I studied how selective BH3 mimetic compounds alter BCL2 protein interactions, and evaluated the importance of these indirect impacts on their ability to induce apoptosis.

Figure 1.1

Intrinsic pathway of apoptosis.

Intrinsic apoptosis is activated by stress signals and is regulated by BCL2 family proteins. In a healthy cell, the pro-apoptotic effector proteins BAX and BAK are kept inactive by pro-survival proteins. Stress signals induce or activate BH3-only proteins, which antagonize pro-survival proteins and promote the activation of BAX and BAK. When activated, BAX and BAK form oligomers that initiate mitochondrial outer membrane permeabilization (MOMP). Once cytochrome c is released from the intermembrane space of mitochondria, it binds to APAF-1 to form a heptameric oligomer called apoptosome. This complex can activate caspase 9 to initiate caspases cascade to drive downstream apoptotic pathway.

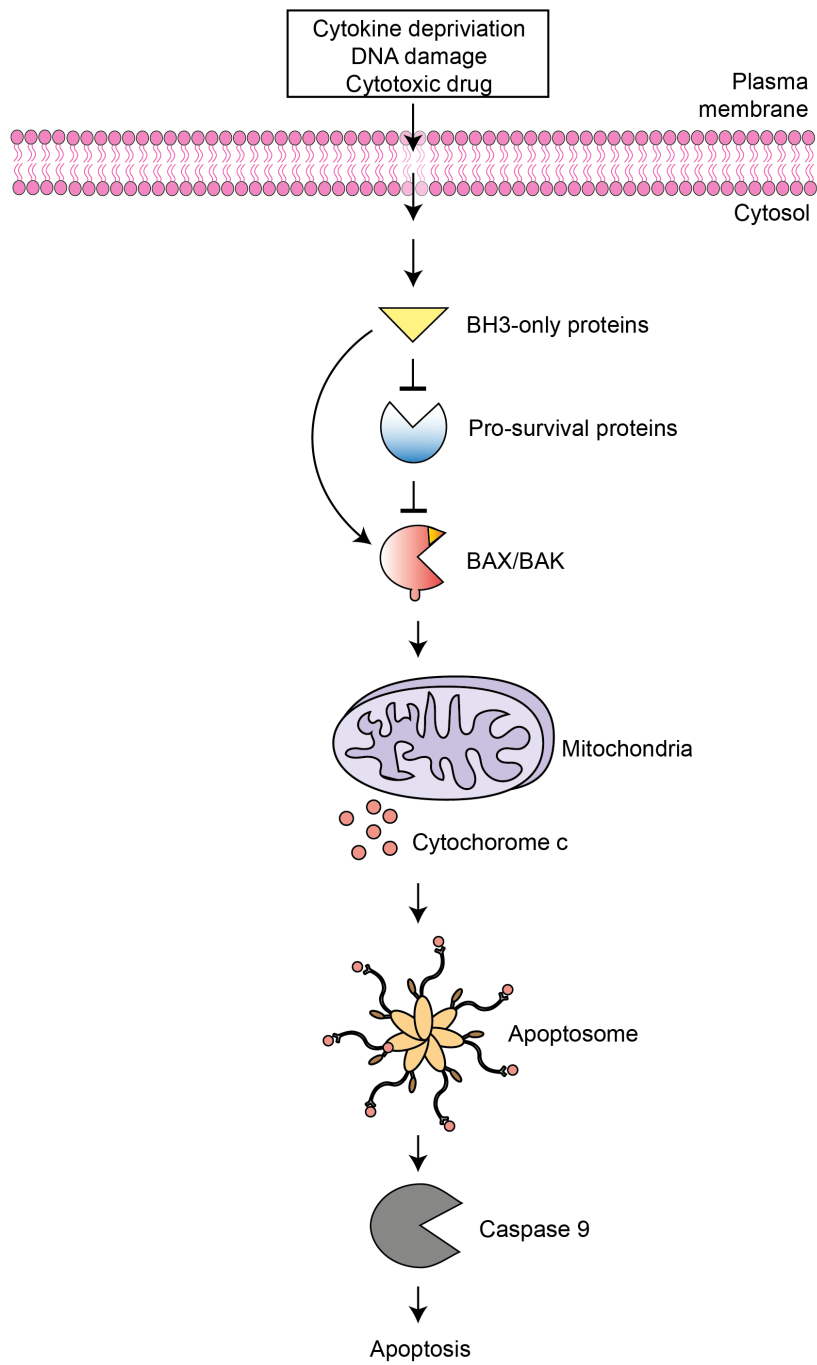


Figure 1.2

Classification of the BCL2 family of proteins.

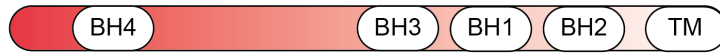
BCL2 family proteins are divided into three subgroups: pro-survivals, effectors and BH3-only proteins. All BCL2 family proteins share regions of sequence homology called BCL2 homology (BH) domains. The pro-survival and pro-apoptotic effector proteins share up to four such regions (BH1, BH2, BH3 and BH4), while BH3-only proteins share only the BH3 domain.

Pro-survival proteins



BCL2, BCLxL, BCLW,
MCL1, BFL1/A1

Pro-apoptotic effectors



BAX, BAK, BOK

BH3-only proteins



BIM, BIK, BMF
HRK, PUMA, NOXA,
BAD, BID

Figure 1.3

Models of life/death determination governed by BCL2 family proteins.

- (A) Models of BAX and BAK activation by BH3-only proteins. When cells are healthy, there are sufficient pro-survival proteins to inhibit the activity of BAX and BAK effectors. When cells are stressed, more BH3-only proteins are activated. These BH3-only proteins neutralize and overwhelm the capacity of pro-survival proteins to restrain BAX and BAK, leading to BAX/BAK activation and cell death.
- (B) Binding selectivity of BH3-only proteins to proteins of BCL2 family. PUMA, BIM and BID bind to all pro-survival proteins equally well and may directly activate BAX and BAK. BAD preferentially binds to BCLW, BCL2 and BCLxL, whilst NOXA preferentially binds to MCL1 and A1 (Chen et al., 2005; Strasser, Cory, & Adams, 2011).

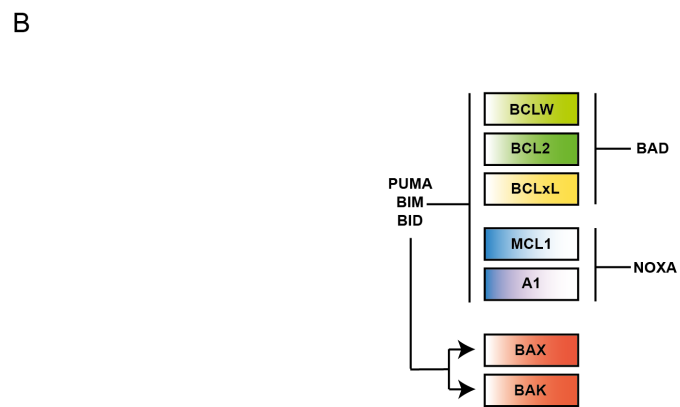
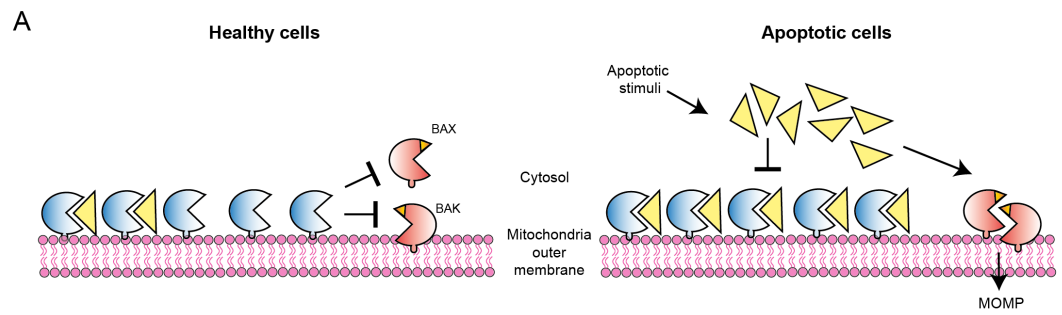
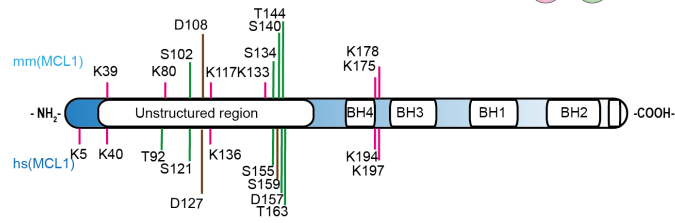


Figure 1.4

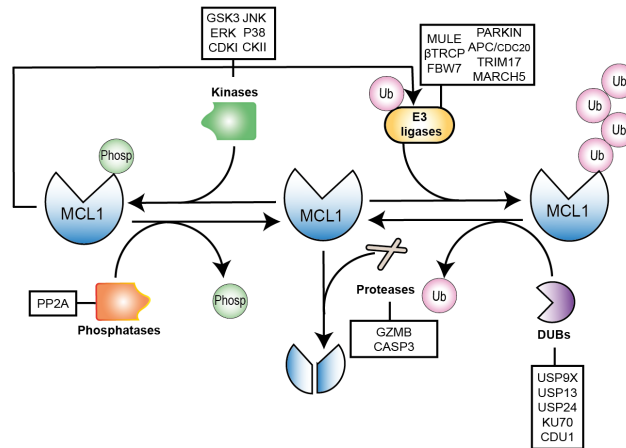
Molecular regulation of MCL1 protein stability.

- (A) Sites of post-translational modifications on MCL1. MCL1 can be ubiquitinated on several of its lysine residues. Its unstructured N-terminal PEST domain contains various sites for phosphorylation. It also contains aspartic residues that are recognized and cleaved by proteases.
- (B) Post-translational regulators of MCL1 protein stability. MCL1 is a substrate of many E3 ligases that target it for proteasomal degradation. The activity of some E3 ligases depend on the phosphorylation state of MCL1, which is in turn controlled by the action of kinases and phosphatases. MCL1 proteasomal degradation can also be reversed by deubiquitinating enzymes. Finally, MCL1 can be cleaved by proteases such as granzyme B and caspase 3.
- (C) Table showing the regulators of MCL1 protein stability and the associated post-translational modification sites.

A



B



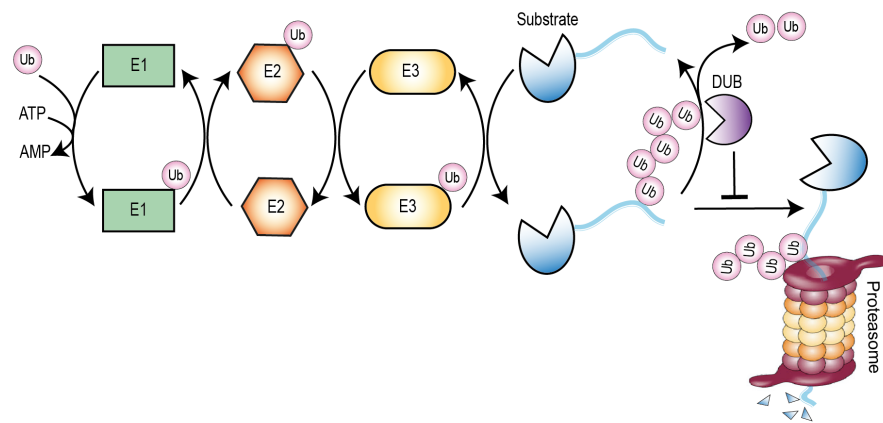
C

Regulators	Classification	Modification sites (species)	Impact on MCL1	References
GSK3β	Kinase	S155, S159, T163 (Hs)	Degradation	(Ding et al., 2007; Magiera et al., 2013)
ERK	Kinase	T92, T163 (Hs)	Stabilization	(Ding et al., 2008; Domina, Vrana, Gregory, Hann, & Craig, 2004; Nifoussi et al., 2012)
CDK1	Kinase	T92 (Hs)	Degradation	(Harley et al., 2010; Wertz et al., 2011)
JNK	Kinase	S121, T159, T163 (Hs)	Context dependent	(Inoshita et al., 2002; Kodama et al., 2009; Wertz et al., 2011)
P38	Kinase	S121 (Hs)	Context dependent	(Huelsemann et al., 2015; Inoshita et al., 2002; Wertz et al., 2011)
CKII	Kinase	S159 (Hs)	Degradation	(Wertz et al., 2011)
PP2A	Phosphatase	S159, T163 (Hs)	Stabilization	(Nifoussi et al., 2014; Wertz et al., 2011)
MULE	E3 ligase	K5, K40, K136, K194, K197 (Hs)	Degradation	(Gomez-Bougie et al., 2011; Zhong et al., 2005)
βTRCP	E3 ligase	Not determined	Degradation	(Ding et al., 2007)
FBW7	E3 ligase	Not determined	Degradation	(Inuzuka et al., 2011; Wertz et al., 2011)
PARKIN	E3 ligase	Not determined	Context dependent	(Carroll et al., 2014; Ekholm-Reed, Goldberg, Schlossmacher, & Reed, 2013)
APC/CDC20	E3 ligase	Not determined	Degradation	(Harley et al., 2010)
TRIM17	E3 ligase	K117, K175, K178 (Mm)	Degradation	(Magiera et al., 2013)
MARCH5	E3 ligase	Not determined	Degradation	(Cherok et al., 2017; Subramanian et al., 2016; Xu et al., 2016)
USP9X	DUB	Not determined	Stabilization	(Peddaboina et al., 2012; Peterson et al., 2015; Schwickart et al., 2010; Yan et al., 2014)
USP13	DUB	Not determined	Stabilization	(S. Zhang et al., 2018)
USP24	DUB	Not determined	Stabilization	(Luo et al., 2019; Peterson et al., 2015)
KU70	DUB	Not determined	Stabilization	(B. Wang et al., 2014)
CDU1	DUB	Not determined	Stabilization	(Fischer et al., 2017)
GZMB	Protease	D127, D157 (Hs)	Degradation	(Han et al., 2005)
CASP3	Protease	D127, D157 (Hs)	Degradation	(Clohessy et al., 2004; Weng et al., 2005)

Figure 1.5

Ubiquitin-proteasome pathway.

Ubiquitin is conjugated to target protein through a series of enzymatic reactions. First, ubiquitin is transferred from a ubiquitin activating E1 enzyme to a ubiquitin E2 conjugating enzyme in an ATP-dependent process. An E3 ligase then catalyses the transfer of ubiquitin from the E2 onto a lysine residue of the target protein. Proteasome recognize some forms of poly-ubiquitin chain to initiate degradation of target protein. Poly-ubiquitin chain can be removed by the action of DUB enzyme (Vucic, Dixit, & Wertz, 2011).



Chapter 2: Materials and methods

Mammalian expression constructs

Many of the constructs used in this thesis were generated and maintained by Dr. Simon Willis, Dr. Mark van Delft, Dr. Lei Liu or myself. All of the constructs used in this thesis are listed in Table 2.1.

Retroviral constructs encoding HA-tagged NOXA, NOXA3E and tBID have been described (Chen et al., 2005). 3xMYC (3xMEQKLISEEDLNE)-tagged wild-type MARCH5 or MARCH5^{C65/68S} and FLAG (MDYKDDDDKGS)-tagged MTCH2 were synthesized as gene fragments by Integrated DNA Technologies (IDT) and subcloned into pMSCV-IRES-puro (pMIP) vector. MCL1^{BCL2TM} was also synthesized as a gene fragment and subcloned in-frame to replace the C-terminus of FLAG-tagged MCL1 in pMSCV-IRES-GFP (pMIG) vector.

MCL1^{K175R}, MCL1^{K178R}, MCL1^{K175R, K178R} and MCL1^{14KR} were generated by PCR using overlap-extension site-directed mutagenesis and subcloned into pMSCV-IRES-hygro (pMIH) vector. PCR fragments for MCL1 and MCL1^{K117R, K175R, K178R} were generated in the same way and subcloned in-frame with an N-terminal GFP tag in pMIH vector to facilitate FACS-based screening. The sequences of all constructs were confirmed by Sangersequencing.

Table 2.1 List of mammalian expression constructs

Plasmid name	Construct made by
pMIH HA-(hs)NOXA	Dr. Simon Willis
pMIH HA-(hs)NOXA ^{3E}	Dr. Simon Willis
pMIH (mm)MCL1	Dr. Simon Willis
pMIH (mm)MCL1 ^{14KR}	Dr. Mark van Delft
pMIH (mm)MCL1 ^{K175R}	Dr. Mark van Delft
pMIH (mm)MCL1 ^{K178R}	Dr. Mark van Delft
pMIH (mm)MCL1 ^{K175R, K178R}	Dr. Mark van Delft
pMIH GFP-(mm)MCL1	Dr. Lei Liu
pMIH GFP-(mm)MCL1 ^{K117R, K175R, K178R}	Dr. Mark van Delft
pMIP HA-(hs)NOXA	Dr. Simon Willis
pMIP HA-(hs)NOXA ^{3E}	Dr. Simon Willis
pMIP MYC-(hs)MARCH5	Dr. Mark van Delft
pMIP MYC-(hs)MARCH5 ^{C65/68S}	Tirta Djajawi
pMIP FLAG-(hs)MTCH2	Dr. Mark van Delft
pMIG FLAG-(mm)MCL1	Dr. Mark van Delft

pMIG FLAG-(mm)MCL1 ^{BCL2TM}	Tirta Djajawi
PX458	(Ran et al., 2013)
FUCas9Cherry	(Aubrey et al., 2015)
pKLV-U6gRNA(BbsI)-PGKpuro2ABFP	(Koike-Yusa, Li, Tan, Velasco-Herrera Mdel, & Yusa, 2014)
FgH1tUTG (<i>inducible sgRNA vector</i>)	(Aubrey et al., 2015)

Generation of sgRNA-containing vector

The sgRNA sequence of a particular gene was designed using online tools (DNA 2.0 or CHOPCHOP) or taken from available whole genome libraries (Gecko or Brunello). 20 nucleotides (nt) of sgRNA sequences were cloned into PX458 (Ran et al., 2013), pKLV-U6gRNA(BbsI)-PGKpuro2ABFP (Koike-Yusa et al., 2014) or FgH1tUTG (Aubrey et al., 2015) vectors as described. The 20 nt sgRNA sequences used in this thesis are listed in Table 2.2.

Table 2.2 List of 20 nt sgRNA sequences

Gene	Species	No.	Forward sequences (5' to 3')	Vector
<i>Bak</i>	Mouse	1	CTGCCAACCCCGAGATGGAC	PX458
<i>Bax</i>	Mouse	1	GCGAATTGGAGATGAACTG	PX458
<i>Bclxl</i>	Mouse	1	GGGACCGCGTATCAGAGCT	PX458
<i>β-TrcP</i>	Mouse	1	TCCGACGTACGCGGTACCA	FgH1UTG
<i>Fbw7</i>	Mouse	1	GTATCGACAAGTCTGAGCCG	FgH1UTG
<i>MARCH5</i>	Human	1	AGGCAAGATGATTCGCTGGG	PX458
<i>MARCH5</i>	Human	2	ATTAGGCAAGATGATTCGCT	PX458, pKLV
<i>March5</i>	Mouse	1	GAGTACTCGGCGTTGCACTG	PX458
<i>March5</i>	Mouse	2	GTCTACTAAGTGGGTTCCACC	PX458
<i>MCL1</i>	Human	1	GTATCACAGACGTTCTCGTA	PX458
<i>MTCH2</i>	Human	1	TACCCCACTGTCCAGTGCAT	PX458, pKLV
<i>MTCH2</i>	Human	2	GGTCTTGTTCCCTCGCCTTCT	PX458, pKLV
<i>Mtch2</i>	Mouse	1	GACTACAAACATACCGTGGA	PX458
<i>Mtch2</i>	Mouse	2	GTTGATAACTCGGTCAAAGG	PX458
<i>Mule</i>	Mouse	1	TAAGGGGTTTCCGGTTCCAC	FgH1UTG
<i>NOXA</i>	Human	1	GTCTTGCGCGCCTTCTTCCC	PX458
<i>NOXA</i>	Human	2	GCTGGAGCCCGCGCGGGGCT	PX458
<i>NOXA</i>	Human	1	GCTACTCAACTCAGGAGATT	pKLV-U6

<i>NOXA</i>	Human	2	AATCTCCTGAGTTGAGTAGC	pKLV-U6
<i>Otud5</i>	Mouse	1	GGTGTAGGCGTGGGTGGCGG	PX458
<i>Parkin</i>	Mouse	1	TGTGGGGCTGGCGGTCATTC	PX458
<i>Parkin</i>	Mouse	2	CCCCACAGAGTCCACCGGCA	PX458
<i>Prdm4</i>	Mouse	1	GAGCACCAACTCTGTAAGCG	PX458
<i>Prdm4</i>	Mouse	2	GACATTGCAAACCGGGTCAA	PX458
<i>Trip12</i>	Mouse	1	GTGTGAGCCTTGCTTGTTGA	PX458
<i>Trip12</i>	Mouse	2	GGTCATAGTTCCACAACCAG	PX458
<i>UBE2K</i>	Human	1	GCAATGACAATAATACCGTG	PX458
<i>UBE2K</i>	Human	2	CCTAATATTAGTTCCGTCAC	PX458
<i>Ube2k</i>	Mouse	1	GAGGTTTACAATACCTTAGG	PX458
<i>Ube2k</i>	Mouse	2	GAATATTAGTTCCGTCACAG	PX458
<i>Ubr5</i>	Mouse	1	GCCCCACGCCAGAACTCCAG	PX458
<i>Ubr5</i>	Mouse	2	GTCAAACTGAACAGCAGCT	PX458
<i>USP9X</i>	Human	1	AGTATGACAGCCACGACTCG	PX458
<i>USP9X</i>	Human	2	GACCGGAGAGCCACGAGTCG	PX458
<i>USP9X</i>	Human	3	GGTTCCTCATCTTCAAGCTG	PX458
<i>Usp24</i>	Mouse	1	TTTACGAACGATAGTCTGG	PX458
<i>Usp24</i>	Mouse	2	TAGCCACCGTAGTCGAGGC	PX458

Cell culture of mammalian cells

Mouse Embryonic Fibroblasts (MEFs) were cultured in DME KELSO supplemented with 10% (v/v) Fetal Calf Serum (FCS), 100 μ M L-asparagine and 50 μ M β -Mercaptoethanol. HeLa and 293T cells were cultured in DMEM supplemented with 10% (v/v) FCS. T47D, MCF7, MDA-MB-231, NCI H522, HOP62, HOP92, IGROV1, RXF393, 786-0, TK10, HCT116, PC3, MALME3M, LOXIMVI, KMS-28-BM, RPMI8226, MOLT4, KMS-12-PE, RS4;11, GRANTA, H929, OPM2, U266B1 and SR cells were cultured in RPMI1640 supplemented with 10% (v/v) FCS. AMO1 cells were cultured in RPMI1640 supplemented with 20% (v/v) FCS. Cells cultured in DME KELSO and DMEM media were grown in humidified incubators maintained at 37°C and 10% CO₂. Cells cultured in RPMI1640 were grown in humidified incubators maintained at 37°C and 5% CO₂.

All mouse fibroblast cell lines were generated from E13.5 embryos (C57BL/6 background) and transformed with SV40 large T antigen. All human cell lines were obtained from ATCC, DSMZ, or JCRB cell line repositories.

All cell lines were cultured in the indicated media containing penicillin and streptomycin (Table 2.3). They were subjected to mycoplasma testing using MycoAlert Kit (Lonza) or PCR analysis (WEHI Bundoora). Contaminated cells were discarded or treated with BM-cyclin (Sigma) or ciprofloxacin (Sigma) using the manufacturers' guidelines.

Table 2.3 List of cell culture media

Media	Composition	Source
DMEM	Type II water Dulbecco's Modified Eagle Medium (DMEM) powder Sodium bicarbonate (NaHCO_3) – 40 mM pH = 7.10 ~ 7.15 Osmolality = 320 ~ 327 mOsm/kg	WEHI Media Lab
DME KELSO	Type II Water Dulbecco's Modified Eagle Medium (DMEM) powder Sodium bicarbonate (NaHCO_3) – 40 mM Hepes buffer – 1 mM Folic acid – 0.0135 mM L-asparagine monohydrate – 0.24 mM L-arginine monohydrochloride – 0.55 mM D-glucose – 22.2 mM pH = 7.10 ~ 7.15 Osmolality = 325 ~ 334 mOsm/kg	WEHI Media Lab
RPMI1640	Type II water RPMI1640 powder Sodium bicarbonate (NaHCO_3) – 23.8 mM Sodium pyruvate ($\text{C}_3\text{H}_3\text{NaO}_3$) – 1 mM pH = 7.14 ~ 7.16 Osmolality = 275 ~ 290 mOsm/kg	WEHI Media Lab
FCS		Sigma Aldrich
Trypsin		Sigma Aldrich

Gene delivery into mammalian cells

Transient transfection

Cells were plated at 30% confluency 24 hours prior to transfection. For each microgram of DNA, 4 µl of FuGENE6 (Promega) was added and mixed into 40 µl of serum-free Opti-Mem media (Gibco). The mixture was incubated for 10 minutes at room temperature and added drop-wise onto target cells.

Retroviral and lentiviral infection of mammalian cells

Phoenix Ecotropic and Amphotropic cells were used to generate retroviruses for infection of murine and human cells respectively. Typically, Phoenix packaging cells were plated on a 10-cm dish for a density of 3×10^6 cells at 24 hours prior to transfection. The next day, 4 µg of DNA of interest was transfected into the packaging cells using FuGENE6 (Promega).

To generate lentiviruses for infection of human cells, 293T cells were instead used as packaging cells. In this case, 1.5 µg of DNA of interest was mixed with lentiviral packaging genes (1 µg pRSV-REV, 1.5 µg pMDLg and 1 µg pVSVG) and transfected into 293T in the same way as retroviruses.

In both cases, the media of the packaging cells were replaced with fresh media the following day. At 48 hours post-transfection, viral supernatants were filtered from the packaging cells using 0.45 µm syringe filter (Millipore) to remove cell debris. Polybrene (Sigma) was subsequently added to a final concentration of 5 µg/ml. The filtered viral supernatants were now ready to use for viral transduction into mammalian cells.

Target cells were typically plated at 20 - 50% density at 24 hours prior to transduction. To enhance transduction efficiency, filtered viral supernatants were added onto target cells by spin-infection (2,500 rpm at 20 °C for 1 hour). The next day, culture media of target cells were replaced with fresh media. Three days after infection, transduced cells were distinguished from non-transduced cells either by sorting the fluorophore⁺ population (GFP, mCherry or BFP) or by passaging cells under antibiotic selection (hygromycin or puromycin).

Generation of knock out cell lines using CRISPR-Cas9 gene editing technology

Target genes were deleted either by transient transfection of Cas9 and sgRNA-containing PX458 (Ran et al., 2013) or by stable transduction of two separate plasmids: one encoding constitutive Cas9 and another encoding either constitutive or doxycycline-inducible sgRNA (Aubrey et al., 2015; Koike-Yusa et al., 2014).

Transient transfection of PX458 vector into adherent cell lines

Adherent cells were plated at a density of 2×10^5 cells at 24 hours prior to transfection. The next day, 1 μ g of PX458 vector containing a distinct sgRNA was transfected onto target cells using FuGENE6 (Promega)(Ran et al., 2013). Cells were then incubated for 24 hours in a humidified incubator before the culture medium was replaced with fresh medium. At 48 hours post-transfection, cells were harvested and sorted for single cells based on GFP expression using a BD FACS Aria machine.

Stable transduction of Cas9 and sgRNA into mammalian cell lines

Stable cell lines expressing Cas9 were generated as described (Aubrey et al., 2015). These cells were typically plated at 30 – 50% density at 24 hours prior to sgRNA transduction. They were then infected with a lentivirus encoding for constitutive- (pKLV-U6; (Koike-Yusa et al., 2014)) or doxycycline- inducible (FgH1tUTG; (Aubrey et al., 2015)) expression of the sgRNA of interest. Cells were then incubated in a humidified incubator before culture media was replaced with fresh media the following day. For constitutive sgRNA vectors, infected cells were selected at 48 hours post-transduction based on BFP expression. For inducible sgRNA vectors, infected cells were treated with 10 ng/ml doxycycline for 72 hours before selection based on GFP expression.

Cell growth competition assay

Cas9^{+ve} cells were transduced with a lentiviral vector (pKLV-U6gRNA-EF(BbsI)-PGKpuro2ABFP) encoding constitutive expression of sgRNA targeting *NOXA* or *MARCH5*. Three days after infection, these cells were subjected to flow cytometry and an equal number of infected (BFP^{+ve}) and uninfected (BFP^{-ve}) cells were sorted and placed into the same well of a 12 well-plate. The cells were cultured for 12 days and the proportion of BFP^{+ve} cells was measured every 3 days using a flow cytometer (LSRFortessa, BD Biosciences).

DNA sequencing

DNA extraction from cell lysates

Adherent cells were collected either by scraping or by trypsinisation at 37°C for 5 minutes. Suspension cells were collected directly from the tissue culture media. Cells were washed with room temperature PBS once and centrifuged at 1500 rpm, 4°C for 5 minutes. For crude DNA extracts, pellets comprising approximately 1×10^6 cells were lysed in 80 μ l of Direct PCR lysis reagent (Viagen) supplemented with 5% (v/v) Proteinase K (Sigma). The mixture was incubated with the following parameters: 55°C for 2 hours, 85°C for 40 minutes and finally, 95°C for 2 minutes. For purified DNA extracts, cells were lysed with DNeasy Blood and Tissue Kits (Qiagen) using the manufacturer's guidelines.

Targeted PCR of sites of CRISPR-mediated insertion-deletion mutations (indels)

Unique primers (Table 2.4) were designed using the online tool PrimerBlast to amplify DNA spanning the expected sites of CRISPR-mediated indels. The DNA amplicons were expected to be approximately 200 – 400 bp. Two sequencing techniques (Sanger and Miseq) were used in this thesis to confirm CRISPR-mediated indels.

To amplify DNA, 2 μ l of cell lysates were suspended in 21 μ l of GoTaq Green PCR mix supplemented with 2 μ l of primer pair (forward and reverse). The 25 μ l mixtures were then incubated in the automated thermal cycler (My Cycler) with the parameters described in Table 2.5. The DNA amplicons were then run on a 1% agarose gel to check whether the primer pair generated DNA products at the expected length. When DNA amplicons were subjected to Sanger sequencing (AGRF Melbourne), they were purified from agarose gel using an extraction kit (Qiagen). Heterozygosity in the chromatographic DNA traces from Sanger sequencing were then analysed with online tools (Mix sequence reader and CRISPR-ID).

Alternatively, CRISPR-mediated indels on the target DNA sites were analysed using next-generation sequencing (Miseq). For this method, approximately 20 base pairs of overhang sequence were added to the 5' ends of both forward and reverse primers (Table 2.4). These overhang sequences were later used for secondary PCR barcoding of DNA. CRISPR-targeted DNA sites were amplified using the parameters described in Table 2.5, but using primer pairs with overhang sequences. After the first PCR reaction, 2 μ l of DNA amplicons were re-suspended in 21 μ l of GoTaq Green PCR supplemented with 2 μ l of the unique barcoded primer pair. The 25 μ l mixtures were then incubated in the automated thermal cycler with the parameters described in Table 2.6. The size of the

barcoded DNA amplicons were expected to be approximately 50 base pairs longer than the non-barcoded amplicons from the first PCR.

To purify DNA for Miseq analysis, 5 µl of DNA amplicons from each second PCR reaction were mixed in a microcentrifuge tube and incubated with AMPure XP beads in a ratio of 1 : 1 (v/v) for 5 minutes. Beads containing the barcoded DNA amplicons were separated from non-specific PCR products by placing the microcentrifuge onto a magnetic rack. These beads were washed with 80% (v/v) ethanol twice and supernatants were carefully removed in between washes to ensure that the beads were left undisturbed. Finally, beads were left to air-dry for 15 minutes. 100 µl of nuclease-free water was then added to elute the attached DNA. The purified barcoded DNA amplicons were then sent for MiSeq analysis.

Table 2.4 List of primers for sequencing indels on sgRNA-targeted DNA sites

Gene	Species	sgRNA no.	Direction	Sequences (5' to 3')
<i>Bak</i>	Mouse	1	Forward	AACAGCAGGTTGCCAGGACACA
<i>Bak</i>	Mouse	1	Reverse	TTCCACCTAGCCCTGTCTGAA
<i>Bax</i>	Mouse	1	Forward	AGTTTCATCCAGGATCGAGCA
<i>Bax</i>	Mouse	1	Reverse	ATCTGAGTTGAGTGGAGGATACC
<i>Bclxl</i>	Mouse	1	Forward	GGAGGTGATTCCCATGGCAGCA
<i>Bclxl</i>	Mouse	1	Reverse	CTGCATCTCCTTGTCTACGCTT
<i>β-TrcP</i>	Mouse	1	Forward	GAGAAAGGGAGGCTCACCAG
<i>β-TrcP</i>	Mouse	1	Reverse	TCAGGCATGTGCTGCTCTTT
<i>Fbw7</i>	Mouse	1	Forward	GAACAGACGCAGACGCATTCT
<i>Fbw7</i>	Mouse	1	Reverse	CAAGATGCTCAGCCTGGGTAGA
<i>MARCH5</i>	Human	1&2	Forward	GAGCTTAACATGCAACACCTCA
<i>MARCH5</i>	Human	1&2	Reverse	ACAACCACAGTTCAGTGCCT
<i>March5</i>	Mouse	1&2	Forward	GTGTGCTTTGCCACCGATGA
<i>March5</i>	Mouse	1	Reverse	CAGTGAACATGTCTGTGTTGTTCTT
<i>March5</i>	Mouse	2	Reverse	GTTCTTCCATTAGTCCTTACCCAG
<i>MCL1</i>	Human	1	Forward	TTCAGCGACGGCGTAACAAA
<i>MCL1</i>	Human	1	Reverse	AGCCGTGCGTCATAAAAACC
<i>MTCH2</i>	Human	1	Forward	TGTACTGAAGAGCCTGTCATGTT
<i>MTCH2</i>	Human	1	Reverse	AGCTGCCAGATTAAAGCCAAG
<i>MTCH2</i>	Human	2	Forward	CGGGCTGGATTCTGGTGTTT
<i>MTCH2</i>	Human	2	Reverse	CGAAGGTCACAACCTACCCC
<i>Mtch2</i>	Mouse	1	Forward	TTGGAGCTAGCTTCCTGACTCT
<i>Mtch2</i>	Mouse	1	Reverse	GTCAATGCCACCCTAAGCTAT
<i>Mtch2</i>	Mouse	2	Forward	GGGGTGGCCATGAGTTTGAA

<i>Mtch2</i>	Mouse	2	Reverse	CAGGCCACGCTCTTCTTTTG
<i>Mule</i>	Mouse	1	Forward	TTGAGGTTCTCTGCGTGAGC
<i>Mule</i>	Mouse	1	Reverse	TGACTCATTGTGGAAGGGGC
<i>NOXA</i>	Human	1&2 ^(PKLV)	Forward	GACTGTTTCGTGTTTCAGCTCG
<i>NOXA</i>	Human	1&2 ^(PKLV)	Reverse	ACCCCGAAGAACTTTCTGGA
<i>NOXA</i>	Human	1&2 ^(FgH1t)	Forward	GGGCGTATTAGGTTTTGCTGG
<i>NOXA</i>	Human	1&2 ^(FgH1t)	Reverse	GGCACCCATGAATGCACCTT
<i>Otud5</i>	Mouse	1,	Forward	CGCCATGACTATTCTCCCCAAAA
<i>Otud5</i>	Mouse	1	Reverse	AGTTGTCGCACCAAGAGCGT
<i>Parkin</i>	Mouse	1&2	Forward	TGCCACTGTTTCTGGTAGCAAT
<i>Parkin</i>	Mouse	1&2	Reverse	AGTAGGTACAGGCCAACTCTCT
<i>Trip12</i>	Mouse	1	Forward	GACCAATTCTCCTAGCTCTGCG
<i>Trip12</i>	Mouse	1	Reverse	GGCACCAGTTGATTCCGGACC
<i>Trip12</i>	Mouse	2	Forward	CCACCTTTTTCCCCTAAAGGTC
<i>Trip12</i>	Mouse	2	Reverse	TGGACTAGCACTGCGTTTCA
<i>UBE2K</i>	Human	1	Forward	AGAAAATACTTTGCTTTTGCCTGC
<i>UBE2K</i>	Human	1	Reverse	GTGTTAATGTGCAAACCACTGA
<i>UBE2K</i>	Human	2	Forward	TTTCACCTAGGCCAAAATGATGTAAT
<i>UBE2K</i>	Human	2	Reverse	AGTTACTGCCCAAACAAGATAAAGT
<i>Ubr5</i>	Mouse	1	Forward	CAACAAAGGTGGAAATGATAGTGT
<i>Ubr5</i>	Mouse	1	Reverse	TGCTGGAATAACTGGCTGGG
<i>Ubr5</i>	Mouse	2	Forward	CTTGTGAGGCTGATGCCCTG
<i>Ubr5</i>	Mouse	2	Reverse	GGACCAACTTACCCCAGAGT

Table 2.5 First PCR condition for amplification of sgRNA-targeted DNA sites

Cycle	Step	Temperature (°C)	Time (seconds)	Repeats (x)
Initial Denaturation		95	300	1
Thermal cycler	Denaturation	95	20	30
	Annealing	55-60*	20-30	
	Extension	72	20-30**	
Final extension		72	480	1
Hold		12	∞	1
*annealing temperature varies based on the GC contents of primers pair				
**extension time varies based on the size of DNA products				

Table 2.6 Second PCR condition for amplification of barcoded DNA products

Cycle	Step	Temperature (°C)	Time (seconds)	Repeats (x)
Initial Denaturation		95	300	1
Thermal cycler	Denaturation	95	20	25
	Annealing	60	30	
	Extension	72	25-35*	
Final extension		72	300	1
Hold		12	∞	1
*extension time varies based on the size of DNA products				

Biochemical assays

Preparation of protein lysates

Adherent cells were collected either by scraping or by trypsinization at 37°C for 5 minutes. Suspension cells were collected directly from the tissue culture media. Following harvest, cell numbers were calculated by cell counter MOXI Z (Orflo). Cells were then washed once in ice-cold PBS and centrifuged at 1500 rpm at 4°C for 5 minutes to obtain cell pellets.

Whole cell protein extracts

Cell pellets were lysed with lysis buffer (20 mM Tris pH 7.4, 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100 and 0.5 µg/ml protease inhibitors (Roche Life Science) for 30 minutes on ice. Following lysis, they were centrifuged at 13,000 rpm at 4°C for 10 minutes to remove insoluble materials. Supernatants containing whole cell protein extracts were collected and Bradford assay was used to quantify protein concentration. For short- and long-term storage, protein lysates were stored in a -20°C or -80°C freezer respectively. To re-use, protein lysates were thawed slowly on ice.

Subcellular fractionation

Cell pellets were first permeabilized with digitonin lysis buffer (20 mM HEPES, 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 0.025% (w/v) digitonin

and 0.5 µg/ml protease inhibitors) for 30 minutes on ice. Samples were then centrifuged at 13,000 rpm at 4°C for 10 minutes to separate cytosolic proteins from membrane-associated proteins. The supernatants contained cytosolic proteins, whereas membrane-associated nuclear and mitochondrial proteins were retained in the cell pellets. The cell pellets were lysed in an equal volume of lysis buffer and centrifuged at 13,000 rpm at 4°C for 10 minutes. The supernatants from this centrifugation contained the membrane-associated proteins and were then used for electrophoresis.

Immunoprecipitation

Immunoprecipitation was performed using whole cell protein extracts prepared with lysis buffer as above. Bait proteins were captured with antibodies and Protein G Sepharose beads. Before use, beads were washed with lysis buffer three times. Beads were centrifuged at 3,000 rpm at 4°C for 1 minute in between washes to remove debris and reduce non-specific binding.

To capture bait proteins, 5-10 µg of antibodies were added to each protein lysate. The mixture was incubated by rotation for 3 hours at 4°C. The resultant antigen/antibody complex was then captured by addition of 75 µl of Protein G Sepharose beads in lysis buffer (33% (v/v)) and was incubated further by rotation for 3 hours to overnight at 4°C. The beads with the bound antigen/antibody complex were then centrifuged at 3,000 rpm, 4°C for 1 minute and were washed five times with lysis buffer to remove non-specific bound proteins. Supernatants were removed carefully in between washes so as to not disturb the beads with the bound antigen/antibody complex. After the final wash, bait proteins (antigen) and their interacting partners were eluted using 2 x SDS-PAGE sample reducing buffer by heating samples for 10 minutes at 95°C.

SDS-PAGE and electroblotting

For whole cell protein extracts, 4 x SDS-PAGE sample reducing buffer (0.25M Tris pH 5.8, 1% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.02% (v/v) bromophenol blue) was added to each protein lysate. The mixture was heated for 5 minutes at 95°C, loaded into a 4-12% pre-cast NuPAGE Bis-Tris gel (Invitrogen) and run in 2-(N-morpholino)ethanesulfonic acid (MES), sodium dodecyl sulfate (SDS) running buffer (50 mM MES pH 7.4, 50mM Tris Base, 0.1% (w/v) SDS and 1 mM EDTA) at 80V for the first 10 minutes and at 120V for the remaining time. Proteins in the gels were then transferred into nitrocellulose membrane using an iBlot Gel Transfer Device (Invitrogen) at 23V for 6 minutes.

Protein detection: Immunoblotting

Proteins on nitrocellulose membrane were detected using enhanced chemiluminescent (ECL) reagent (Millipore) or infrared fluorescent (IRF) reagent (Li-Cor). After protein transfer, membranes were incubated in blocking buffer for 1 hour in a rotating tube. Milk blocking buffer (2% (w/v) skim milk powder, 0.1% (v/v) Tween in TBS) and Odyssey blocking buffer (25% (v/v) Odyssey buffer (Li-Cor), 0.1% (v/v) Tween in TBS) were used to block membranes for ECL and IRF detection respectively.

Membranes were incubated with various primary antibodies (1 to 10 µg/ml) (Table 2.7) rotating overnight at 4°C. This was followed by incubation of secondary antibodies for 1 hour at room temperature (Table 2.8). Between antibody incubations, membranes were washed with TBS-Tween (0.01% (v/v)) with gentle rocking, 3 x 10 minutes at room temperature. Finally, proteins on the membrane were detected using a ChemiDoc Imager System (BioRad) for ECL or Odyssey Imager System (Li-Cor) for IRF detection.

Table 2.7 Primary antibodies used for western blot

Antibody	Dilution	Reactivity	Species	Type (Clonal)	Isotype	Clone/Code	Supplier
ACTIN	1:5000	Mouse/Human	Goat	Poly	IgG3	N/A	Santa Cruz Biotechnology
BAK	1:5000	Mouse	Rabbit	Poly	IgG	B5897	Sigma-Aldrich
BAK	1:1000	Human	Rat	Mono	IgG2a	7D10	WEHI Antibody Services
BAX	1:1000	Human	Rat	Mono	IgG2c	21C10	WEHI Antibody Services
BAX	1:1000	Mouse	Rat	Mono	IgG	49F9-13-3	WEHI Antibody Services
BCL2	1:1000	Mouse	Hamster	Mono	IgG	3F11	WEHI Antibody Services
BCL2	1:1000	Human	Mouse	Mono	IgG1	BCL-2-100	WEHI Antibody Services
BCLxL	1:1000	Human	Mouse	Monoc	IgG1	44	BD Pharmingen
BCLxL	1:1000	Human	Mouse	Mono	IgG2a	2H12	BD Pharmingen
BIM	1:1000	Human	Rat	Mono	IgG2a	3C5	WEHI Antibody Services
BIM	1:1000	Human	Rabbit	Poly	IgG	ADI-AAP-330	Enzo Life Science
β-TrCP	1:4000	Mouse	Rabbit	Mono	IgG	D13F10	Cell Signaling Technology
FLAG-tag	1:1000	N/A	Mouse	Mono	IgG	IC7	WEHI Antibody Services
FLAG-tag	1:1000	N/A	Mouse	Mono	IgG1	M2	Sigma-Aldrich
FLAG-tag	1:1000	N/A	Rat	Mono	IgG1k	9H1	WEHI Antibody Services
HA-tag	1:1000	N/A	Rat	Mono	IgG1	3F10	Roche Life Science
HSP70	1:4000	Mouse/Human	Mouse	Mono	IgG1	N6	WEHI Antibody Services
MARCH5	1:330	Mouse/Human	Rabbit	Poly	IgG	Ab174959	Abcam

MCL1	1:1000	Mouse/Human	Rat	Mono	IgG2a	19C4-15	WEHI Antibody Services
MCL1	1:5000	Mouse	Rabbit	Poly	IgG	600-401-394S	Rockland
MFN1	1:1000	Mouse	Mouse	Mono	IgG	11E91H12	Abcam
MFN2	1:1000	Human	Mouse	Mono	IgG	6A8	Abcam
MID49	1:500	Human	Rabbit	Poly	IgG	16413-1-AP	Proteintech
MULE	1:4000	Mouse	Mouse	Mono	IgG1	AX8D1	Cell Signaling Tech
MYC-tag	1:1000	N/A	Mouse	Mono	IgG	9E10	WEHI Antibody Services
NOXA	1:330	Human	Mouse	Mono	IgG1	114C307	Novus Biologicals
UBE2K	1:1000	Mouse/Human	Rabbit	Mono	IgG	EP1145Y	Abcam
USP9X	1:1000	Human	Mouse	Mono	IgG1k	1C4	Abnova

Table 2.8 Secondary antibodies used for western blot

Antigen	Dilution	Conjugate	Species	Method of detection	Source
Mouse IgG	1 :10000	Alexa 680	Goat	IRF: Odyssey	Molecular Probes
Mouse IgG	1 :5000	HRP	Goat	ECL: Chemidoc	Southern Biotechnology
Mouse IgG	1 :10000	IR 800	Goat	IRF: Odyssey	Rockland
Mouse IgG γ chain	1 :5000	HRP	Goat	ECL: Chemidoc	Jackson ImmunoResearch
Rabbit IgG	1 :10000	Alexa 680	Goat	IRF: Odyssey	Molecular Probes
Rabbit IgG	1 :10000	IR 800	Goat	IRF: Odyssey	Rockland
Rabbit IgG	1 :10000	HRP	Goat	ECL: Chemidoc	Southern Biotechnology
Rat IgG	1 :10000	Alexa 680	Goat	IRF: Odyssey	Molecular Probes
Rat IgG	1 :5000	HRP	Goat	ECL: Chemidoc	Southern Biotechnology
Rat IgG	1 :10000	IR 800	Goat	IRF: Odyssey	Rockland
Rat IgG Fc γ chain	1 :5000	HRP	Goat	ECL: Chemidoc	Jackson ImmunoResearch

Biotin ID assay

For biotinylated protein pulldown, cells were cultured for at least 96 hours in biotin-free DMEM media before they were treated with 100 μ M biotin for 6 hours. Cells were then harvested, washed in ice-cold PBS five times and lysed in lysis buffer. For every 1 mg of protein, 30 μ l of streptavidin beads were added to each protein lysate. The mixture was then incubated by rotation for 2 hours at 4°C. The beads with the bound biotinylated proteins were then centrifuged at 2,500 rpm at 4°C for 1 minute and washed five times with PBS in order to remove non-biotinylated proteins. The biotinylated proteins were then eluted from the beads using urea elution buffer (8M urea and 4 x SDS-PAGE sample reducing buffer (3:1) (v/v)) and were heated for 15 minutes at 95°C. The samples were later subjected to SDS-PAGE (Roux, Kim, & Burke, 2013; Roux, Kim, Raida, & Burke, 2012).

³⁵S-methionine labelling of BIM protein

³⁵S-methionine was used to label all newly made proteins including BIM protein. Along with Biotin ID assay and immunoprecipitation, the labelling of BIM with ³⁵S-methionine was used to investigate how BIM inhibits MCL1 following ABT-199 treatment (Figure 5.7).

The protocol below was used to label pre-existing BIM before ABT-199 treatment. Initially, cells were cultured in biotin-free DMEM medium for 96 hours prior to ABT-199 treatment. Forty-eight hours prior to ABT-199 treatment, cells were washed five times and starved in methionine-free medium for 1 hour. ³⁵S-methionine (500 µCi/mL) (pulse) was then added to the cells for the remaining 47 hours. Cells were then washed in PBS five times to remove excess ³⁵S-methionine (chase) before ABT-199 was added onto cells for 12 hours. Biotin (100 µM) was added in the last 6 hours of ABT-199 treatment.

The protocol below was used to label newly-synthesized BIM after ABT-199 treatment. Initially, cells were starved in the methionine- and biotin-free DMEM medium for 1 hour. Both ABT-199 and ³⁵S-methionine (500 µCi/mL) (pulse) were then added to the cells for 12 hours. This was followed by the addition of biotin (100 µM) in the last 6 hours of ABT-199 treatment. The chase step was not performed in this experiment.

In both cases, cell pellets were collected after ABT-199 treatment. They were first washed in PBS five times before they were lysed using lysis buffer. ³⁵S-methionine labelled biotinylated proteins were then pulled down from total protein lysates with streptavidin beads. To quantify BIM from ³⁵S-methionine-labelled biotinylated proteins, BIM was immunoprecipitated with rat monoclonal anti BIM (3C5; WEHI Antibody Services) and Protein G Sepharose beads (Sigma-Aldrich). ³⁵S-methionine-labelled BIM samples were then subjected to SDS-PAGE.

³⁵S-methionine-labelled protein detection: phosphorimaging

Prior to imaging, a reusable phosphor screen was exposed to bright light on an image eraser for 20 minutes. This was crucial to remove any leftover radioactive signals that might interfere with subsequent scanning.

After protein transfer, nitrocellulose membranes were left to air-dry at room temperature for 5 minutes. The membrane was then placed face-up inside an exposure cassette and the phosphor screen was placed on top. The cassette was closed, tightly sealed and left overnight. The following day, the phosphor screen was moved with a suction rod onto the phosphor stage of a Typhoon FLA 700 (GE Healthcare Life Sciences). ³⁵S-methionine labelled proteins were detected using the PhosphorImager

scanning option and the scanned image was further analysed using ImageQuant Tool (GE Healthcare Life Sciences).

Immunofluorescence

Cells grown on coverslips were incubated with 250 nM of MitoTrackerTM Deep Red FM (Cat#,M22426; Thermo Fisher Scientific) for 30 minutes at 37°C. Cells were subsequently fixed with 4% paraformaldehyde (PFA, ProSciTech) for 15 minutes at 37°C followed by three washes with PBS. Cells were permeabilized with 0.2% Triton X-100 in PBS containing 10% FCS for 20 minutes at room temperature, washed again with PBS, and then blocked with antibody dilution buffer (2.5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS) for 30 minutes. Permeabilized cells were incubated with rat monoclonal anti MCL1 antibody (19C4; WEHI Antibody Services) and mouse monoclonal anti HA antibody (16B12; Covance) in antibody dilution buffer for 2 hours with gentle shaking followed by three washes with PBS. Afterwards, cells were incubated with AlexaFluor 555-conjugated goat anti-rat secondary antibody (Cat#A-21434; Thermo Fisher Scientific) and AlexaFluor 594-conjugated goat anti-mouse secondary antibody (Cat#11005; Thermo Fisher Scientific) for 1 hour with gentle shaking, followed again by three washes with PBS. Cells were then incubated with 0.5 µg/ml DAPI in PBS for 5 minutes before a final three washes with PBS. Finally, cells were mounted onto a glass slide using SlowFade Diamond Antifade Mountant (Cat#S36963; Thermo Fisher Scientific) and a coverslip was sealed with nail polish. Images were captured using a Zeiss LSM880 confocal microscope. Images of at least 10 fields of view were analysed for each sample using Fiji software.

Flow cytometry

Cell preparation

Adherent cells were collected by trypsinization at 37°C for 5 minutes and suspension cells were collected directly from tissue culture media. Following harvest, cell numbers were calculated by cell counter MOXI Z (Orflo). Cells were then transferred into fluorescence-activated cell sorting (FACS) tubes, washed once in ice-cold PBS and centrifuged at 1500 rpm at 4°C for 5 minutes. Cells were then resuspended in FACS buffer (150 mM NaCl, 3.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 0.8 mM K₂HPO₄ 14.8 mM HEPES, pH 7.2, 2% FCS).

Gene expression assay

A reporter was used to measure the expression of the gene of interest. The reporter was either fused in-frame directly to the gene of interest (e.g. GFP-MCL1) or transcribed constitutively from a different promoter within the same construct (e.g. GFP in PX458). The reporters used in this thesis were as follows: blue fluorescent protein (BFP), green fluorescent protein (GFP), red fluorescent protein (RFP) and monomeric cherry fluorescent protein (mCherry). The intensity of fluorescence was measured using flow cytometry with the following laser excitations: 405 nm for BFP, 488 nm for GFP, and 561 nm for both RFP and mCherry.

Intracellular staining of MCL1

Cells were fixed in 1% PFA for 5 minutes and then washed with FACS buffer. Fixed cells were incubated with rat monoclonal MCL1 antibody (19C4; WEHI Antibody Services) for 30 minutes at 4°C in FACS buffer containing 0.3% saponin (Sigma-Aldrich) followed by two washes with FACS buffer containing 0.03% saponin. Cells were then incubated with PE-conjugated anti-rat secondary antibody (Cat#3030-09; Southern Biotech) for 30 minutes at 4°C in FACS buffer containing 0.3% saponin followed by two washes with FACS buffer containing 0.03% saponin. Cells were then analysed using a flow cytometer.

Flow cytometry analysis

All flow cytometry data from FACS Calibur, Fortessa or Aria were analysed using Weasel or FlowJo software.

Cell death assay

Annexin V staining and propidium iodide assay

For adherent lines, floating dead cells were collected from the culture media and the remaining viable cells were trypsinized for collection. For suspension lines, all of the cells were collected immediately from culture media. Both viable and dead cells were washed with ice-cold FACS buffer once. They were then suspended in FITC-conjugated anti Annexin V (10% (v/v); (Cat# 556547; BD Pharmingen) in FACS buffer for 30 minutes at 4°C. Afterwards, propidium iodide (Pi, Sigma-Aldrich) was added at a final concentration

of 2 µg/ml and incubated for 5 minutes at 4°C. Cell viability was calculated as the percentage of viable cells (negative for both Annexin V and PI) using flow cytometry.

JC-1 assay

Cells were collected, washed once with room-temperature PBS and later with TEB buffer (135 mM Trehalose dehydrate (Sigma), 10 mM HEPES-KOH, 50 mM KCl, 20 µM EDTA, 20 µM EGTA, 0.1% (w/v) BSA, 5mM succinate (Sigma), pH~7.5). Cells were subsequently centrifuged at 1,500 rpm at room temperature for 5 minutes and resuspended in TEB buffer at a density of 1×10^5 cells per 100 µl. Cell suspensions (100 µl per well) were plated on a round-bottomed 96-well plate. BH3 peptides or mimetic compounds were diluted to a range of concentrations in 100 µl TEB buffer supplemented with 0.002% digitonin and 20 µg/ml oligomycin. This mixture was added to each well to make a total volume of 200 µl, and the plate was incubated for 90 minutes at room temperature in the dark. Finally, 25 µl of 900 nM JC-1 dye was added to each well and the plate was incubated for another 40 minutes in the dark. Mitochondrial depolarization was identified as a reduction in the red:green JC-1 fluorescence intensity ratio when cells were excited with a 488 nM laser using flow cytometry (Fig. 2.1) (Del Gaizo Moore & Letai, 2013; Ryan, Brunelle, & Letai, 2010).

Cell Titer-Glo luminescent assay

CellTiter-Glo luminescent assay is a method to determine the number of viable cells based on the quantification of ATP present. Approximately, 3×10^3 cells were plated per well of an opaque-walled 96-well plate (Greiner) one day before drug treatment. Drugs with a range of concentrations were added to make up a volume of 100 µl of culture medium per well. The 96-well plate was then cultured for a further 24 to 96 hours at 37°C, 5 – 10% CO₂ in a humidified incubator. On the day of analysis, 40 µl of CellTiter-Glo reagent (Promega) was added into each well. The solutions were mixed for 10 minutes on an orbital shaker in an opaque container. The 96-well plate was then analysed using a luminometer.

Genome-wide CRISPR-Cas9 screen with GFP-MCL1

Establishment of GFP-MCL1 screening platform

Cells intended for screening were first infected with a lentiviral construct encoding for constitutive Cas9 expression linked to an mCherry reporter by a self-cleaving peptide

(T2A)(Aubrey et al., 2015). These cells were then infected with a retroviral construct encoding an N-terminally-tagged GFP-MCL1 fusion gene linked to a hygromycin selectable marker by an internal ribosome entry site (IRES). Transduced cells were cultured in hygromycin-containing medium (500 µg/ml) for 7 days, after which cells with high expression of GFP and mCherry were sorted as single cell clones by flow cytometry.

Enrichment screen to identify novel regulators of NOXA-mediated MCL1 degradation

To identify proteins regulating NOXA-mediated MCL1 degradation, these GFP^{high} and Cas9^{high} cells were transduced with a second retroviral construct encoding an HA-NOXA fusion gene linked to a puromycin selectable marker by IRES. Transduced cells were cultured in puromycin-containing medium (3 µg/ml) for 7 days, after which MCL1 degradation induced by NOXA was confirmed by a low level of GFP using flow cytometry. Cells with a low level of GFP-MCL1 due to NOXA overexpression were sorted by flow cytometry.

Three independent screening clones (*Bax*^{-/-} *Bak*^{-/-} MEF, GFP^{low}, Cas9^{high}, NOXA^{high}) were plated at a density of 1 x 10⁶ cells in a 10-cm dish. One day later, they were transduced with a genome-wide lentiviral murine sgRNA library (vector: pKLV-U6sgRNA-EF(BbsI)-PGKpuro2ABFP) at a multiplicity of infection (MOI) of 0.3 - 1.3. Five to six days after transduction with the sgRNA library, GFP^{high} cells were sorted by flow cytometry and expanded. Flow sorting of GFP^{high} cells was repeated three times to further enrich cells containing sgRNAs that abrogated the ability of NOXA to degrade GFP-MCL1. Genomic DNA from each sorting step was extracted using a DNeasy Blood and Tissue Kit (Qiagen). sgRNA sequences were then amplified by PCR using barcoded primers and quantitated by Miseq as previously described in this thesis. The screen was performed in duplicate using three independently-derived clones, for a total of six biological replicate samples.

Enrichment screen to identify stabilizers of MCL1 protein

To identify proteins that can stabilize MCL1 protein, two independent screening clones (*Mcl1*^{-/-} MEF, GFP^{high}, Cas9^{high}) were plated at a density of 1 x 10⁶ cells in a 10-cm dish. One day later, they were transduced with a genome wide lentiviral murine sgRNA library at a MOI of 0.6 – 1.5. Six days after transduction with the sgRNA library, GFP^{low} cells were sorted by flow cytometry and expanded. Flow sorting of GFP^{low} cells was repeated four times to further enrich cells containing sgRNAs that abrogated the ability of their

target proteins to stabilize MCL1. Genomic DNA from each sorting step was extracted and sgRNA sequences in GFP^{low} cells were quantitated as previously described.

CRISPR-Cas9 drop out screen to identify deubiquitinating enzymes (DUBs) of MCL1

Two independently-derived screening clones (*Bcl2*^{-/-} *Bclxl*^{-/-} and *Bcl2*^{-/-} *Bclxl*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs) were grown to a density of 1.2×10^7 cells per genotype. These cells were transduced with a murine sgRNA library targeting genes associated with the ubiquitin proteasome system (UPS) and BCL2 family (vector: pRSG16-U6-sgRNA-HTS6C-UbiC-TagRFP-2A-Puro) at a similar MOI between the two genotypes. Two days after transduction with the sgRNA library, these cells were selected with puromycin (3 µg/ml) for 4 days. At 6 and 16 days after transduction, 4×10^6 cells were collected and sgRNAs sequences inside viable cells were quantitated by Miseq analysis.

Bioinformatic analysis

Analysis of enriched sgRNAs in GFP-MCL1 screen

Raw counts from each sgRNA read were normalized to counts per million (CPM) and transformed into log₂ scale. An offset buffer of 10 CPM was added to each sgRNA read in order to minimize the effect of calculating log₂ fold-changes on sgRNAs with extremely low read counts. The frequency of each sgRNA read was then averaged across biological replicate samples between the post-sort and pre-sort values. The post-sort values were obtained from FACS-sorted GFP^{high} cells for 'NOXA-mediated MCL1 degradation' screen or FACS-sorted GFP^{low} cells for 'stabilizers of MCL1 screen'.

The mean log₂ fold-change for each sgRNA read was then calculated as the difference between the mean of sgRNA frequency post- and pre- cell sorting. The mean log₂ fold-changes were ranked in descending order to identify screening hits. Minimum hypergeometric p-values were then calculated from the descending ranked sgRNA list for each of the genes represented in the library using an established algorithm method (Eden, Lipson, Yogev, & Yakhini, 2007). Finally, false discovery rates (FDR) were calculated using the Benjamini-Hochberg procedure.

Analysis of drop out sgRNAs in an MCL1 dependent cells

Raw counts from each sgRNA read were normalized to CPM, transformed into log₂ scale and an offset buffer of 10 CPM was added to each sgRNA read. The frequency of each

sgRNA read was obtained from *Bcl2*^{-/-} *Bclxl*^{-/-} (DKO) and *Bcl2*^{-/-} *Bclxl*^{-/-} *Bax*^{-/-} *Bak*^{-/-} (QKO) MEFs collected at day 6 and 16 after infection, and then averaged across biological replicates. The mean log₂ fold change for each sgRNA read was then calculated as the difference of mean of sgRNA frequency between DKO and QKO MEFs at day 6 and 16 respectively. This mean log₂ fold change was normalized to the sgRNA frequency in QKO MEFs and then ranked in ascending order for identification of genes that dropped out in DKO MEFs but not in QKO MEFs.

***PMAIP1* mRNA heat map**

PMAIP1 (NOXA) mRNA expression levels in diverse cell lines were obtained from three online resources: CCLE (<http://www.broadinstitute.org/ccle>), CellMiner (<https://discover.nci.nih.gov/cellminer/>) and MMRC (<http://portals.broadinstitute.org/mmgp/>).

Z-scores of *PMAIP1* mRNA were calculated and normalized based on the *PMAIP1* mRNA levels in cell lines that appear on two or more databases.

Table 2.9 List of molecular technique kits

Kit	Function	Source
DNeasy blood and tissue kit	DNA extraction from mammalian cells	Qiagen
Gel extraction kit	DNA extraction from agarose gel	Qiagen
Maxiprep kit	DNA purification for up to 1 mg of plasmid DNA	Thermo Fisher Scientific
Midiprep kit	DNA purification for up to 200 µg plasmid DNA	Thermo Fisher Scientific
Miniprep kit	DNA purification for up to 20 µg plasmid DNA	Qiagen

Table 2.10 List of bacterial growth media

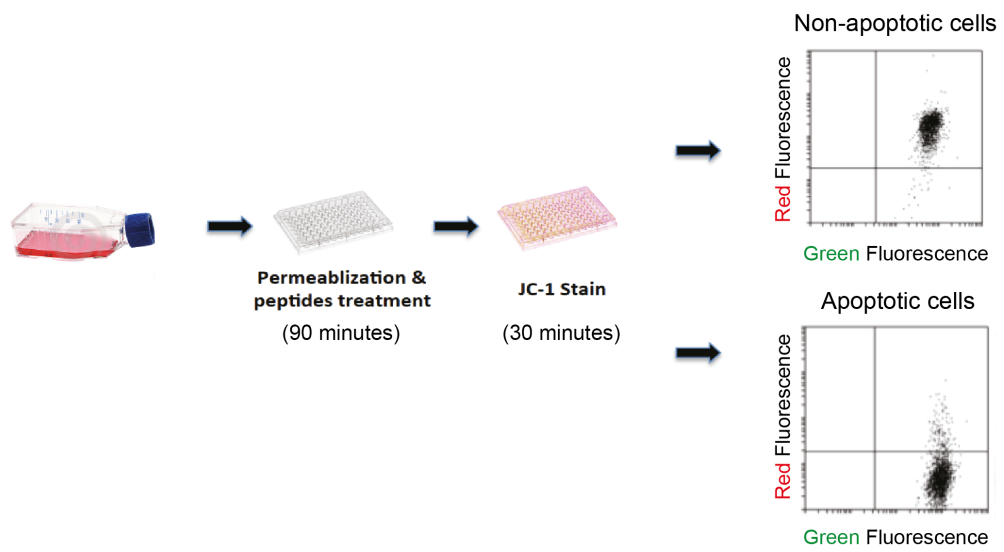
Growth media	Function	Source
LG agar	Solid growth medium for bacteria under antibiotic treatment	WEHI Media Kitchen
S broth	Liquid growth medium for bacteria following transformation	WEHI Media Kitchen
SOB	Liquid growth medium for expanding bacteria colonies	WEHI Media Kitchen

Table 2.11 List of reagents and buffers

Reagents	Purpose	Source
Agarose	Component of agarose gel electrophoresis	Bioline
AMPure XP beads	Magnetic beads for DNA sequestration	Beckman Coulter, Inc
Carbenicillin	Antibiotic for bacterial selection	Gibco

CutSmart buffer	Buffer for DNA restriction digest reaction	NEB
Cycloheximide	Protein synthesis inhibitor	Sigma
Direct PCR lysis reagent	DNA extraction reagent for mammalian cells	Viagen Biotech
DNA ladders	Molecular weight marker for agarose gel electrophoresis	Qiagen
Doxycycline	Inducer of gene expression for Tet-On/Off system	Sigma
Etoposide	DNA damage agent	Hospira
FuGENE6	Transfection reagent	Promega
Hygromycin B	Selection of transduced mammalian cells	Roche Life Science
Nocodazole	Cell cycle arrest agent	Sigma
NuPAGE Bis-Tris Gel	Precast gel for protein separation based on size	Novex
NuPAGE MES SDS	SDS-PAGE running buffer	Life Technologies
Odyssey blocking buffer	Western blot blocking buffer	Li-COR
Opti-Mem	Transfection media	Gibco
GoTaq green PCR	Components for PCR	Thermo Fisher Scientific
Polybrene	Transduction reagent	Sigma
Polynucleotide kinase (PNK)	Phosphorylation of DNA oligoes at the overhang site	Promega
Precision plus standard	Molecular weight marker for SDS-PAGE	Biorad
Propidium iodide	Fluorophore staining for dead cells	Sigma
Protease inhibitor	Components for protein lysis buffer	Roche Life Science
Proteinase K	Serine protease to digest contaminating proteins	Sigma
Puromycin	Selection of transduced mammalian cells	Roche Life Science
SDS-PAGE sample buffer	Sample loading buffer for SDS-PAGE	Biorad
T4 ligase	Enzyme to join DNA phosphodiester bond	Promega
TAE buffer	Buffer for agarose gel electrophoresis	WEHI Media Kitchen
TBS	Component of TBS-T for western blot washing	WEHI Media Kitchen
Tween (50%)	Component of TBS-T for western blot washing	Sigma
UV	DNA damage agent	Fisher Biotech

Figure 2.1
Schematic of BH3 profiling assay.



Chapter 3: MARCH5 requires MTCH2 to coordinate proteasomal turnover of the MCL1:NOXA complex

In accordance to University of Melbourne Thesis Policy, manuscript can be included as a thesis chapter.

The work in this Chapter has been accepted to Cell Death and Differentiation.

Background

As discussed in Chapter 1, intrinsic apoptosis is a fundamental process that must be carefully balanced to maintain tissue homeostasis and preserve the well-being of multicellular organisms. In vertebrates, the most critical regulators of apoptosis are members of the BCL2 protein family, which include BCL2 and its pro-survival relatives (MCL1, BCLxL, BCLW and BFL1/A1), the pro-apoptotic BH3-only proteins (NOXA, BIM, PUMA, BAD and BID) and the effector proteins BAX and BAK. The balance of interactions between these proteins dictate whether BAX and BAK become activated to permeabilize the mitochondrial outer membrane and cause apoptosis (Adams & Cory, 2018; Anthony Letai, 2017). Precise control of apoptosis is achieved by regulating BCL2 proteins in multiple ways including through post-translational modifications that impact their stability, localization, and/or propensity to interact (Kale et al., 2018; Kutuk & Letai, 2008).

MCL1 is a pro-survival relative of BCL2 and has been implicated in tumorigenesis and the resistance of cancer cells to therapy (Perciavalle & Opferman, 2013). A distinct feature of MCL1 is its rapid turnover, which has been proposed as a mechanism to drive apoptosis in various settings (Carroll et al., 2014; Cuconati et al., 2003; Ding et al., 2007; Kozopas et al., 1993; Nijhawan et al., 2003; Yang, Kozopas, & Craig, 1995). Degradation of MCL1 provoked by the ubiquitin E3 ligases MULE and FBW7, for example, has been shown to facilitate the clearance of unfit cells following DNA damage and cell cycle arrest, respectively (Inuzuka et al., 2011; Wertz et al., 2011; Zhong et al., 2005).

MCL1 is also rapidly degraded when it forms a complex with the BH3-only protein NOXA, but the mechanism responsible has remained unclear (Czabotar et al., 2007; Lee et al., 2008; Willis et al., 2005). Intriguingly, NOXA is the only BH3-only protein that targets MCL1 for degradation. Most others that interact with MCL1, including BIM, instead enhance MCL1 stability (Lee et al., 2008; Ploner et al., 2008). Structures of MCL1 in complex with the BH3 domains of either NOXA or BIM reveal no significant alterations to MCL1 conformation (Czabotar et al., 2007), suggesting that elements from both MCL1 and NOXA may combine to promote rapid degradation of the complex.

To interrogate the molecular pathway by which NOXA causes MCL1 to be degraded, I performed an unbiased loss-of-function genome wide CRISPR-Cas9 screen. Here, I identify the mitochondrial ubiquitin E3 ligase MARCH5, the ubiquitin E2 conjugating enzyme UBE2K, and the mitochondrial protein MTCH2 as regulators that cooperate to target the MCL1:NOXA complex for degradation by the proteasome. MARCH5 has previously been reported to ubiquitinate MCL1 and drive its degradation (Cherok et al., 2017; Subramanian et al., 2016), but the requirements for MTCH2 and NOXA in this process have not been appreciated. The observations here indicate that

this pathway contributes to MCL1 turnover in diverse cells and influences MCL1 steady-state levels in those that express significant amounts of NOXA.

Results

The degradation of MCL1 provoked by the BH3-only protein NOXA proceeds through a distinct pathway

To study how the BH3-only protein NOXA provokes MCL1 degradation, I first established a cellular system suitable for genetic and biochemical studies. Apoptosis relies on the essential mediators BAX and BAK and cannot proceed in their absence (Lindsten et al., 2000; M. C. Wei et al., 2001). By using *Bax*^{-/-}*Bak*^{-/-} mouse embryonic fibroblasts (MEFs), I circumvented the downstream consequences of apoptosis signalling such as the degradation of MCL1 by caspases (Herrant et al., 2004).

Initially, I confirmed previous observations that a range of stress signals (growth arrest, DNA damage or protein synthesis inhibition) (Fig. 3.1 A), and the enforced expression of NOXA (Fig. 3.1 B) could all drive proteasomal degradation of MCL1 (Nijhawan et al., 2003; Wertz et al., 2011; Willis et al., 2005). I noted that although NOXA caused MCL1 to be degraded by the proteasome, this was unperturbed in cells devoid of several E3 ligases known to ubiquitinate MCL1 in response to other signals, including MULE, β -TrCP, FBW7 and PARKIN (Sup Fig. 3.1) (Carroll et al., 2014; Ding et al., 2007; Wertz et al., 2011; Zhong et al., 2005). These observations suggest distinct regulation of MCL1 by NOXA, and prompted us to ask whether certain lysines on MCL1 were required for NOXA-driven MCL1 degradation – this residue being the most common site for protein ubiquitination (Hershko & Ciechanover, 1998; Swatek & Komander, 2016).

To this end, I re-constituted *Mcl1*^{-/-}*Bax*^{-/-}*Bak*^{-/-} MEFs with wild-type (WT) mouse MCL1 or a series of mutants in which one or more of its 14 lysines were replaced with arginine. Ultimately, I focussed my attention on lysines 175 and 178 (Sup Fig. 3.2 A). While replacing these residues did not impact upon the ability of MCL1 to block apoptosis (Sup Fig. 3.2 B) or to bind NOXA (Sup Fig. 3.2 C), the MCL1^{K175,K178R} mutant was no longer targeted for degradation by NOXA (Fig. 3.1 C). By contrast, its degradation in response to several other stress signals remained indistinguishable from WT MCL1 (Fig. 3.1 D). Thus, lysine 175 and lysine 178 on MCL1 are uniquely required for NOXA-induced degradation.

Taken together, these data strongly suggest that a distinct molecular pathway is likely to drive the degradation of MCL1 when bound by NOXA, separate from those described for other signals such as DNA damage-induced degradation of MCL1 by MULE (Zhong et al., 2005).

Genome-wide loss-of-function screen to identify genes required for MCL1 degradation by NOXA

To elucidate such a pathway, I undertook an unbiased CRISPR-Cas9 screen to identify genes required for NOXA to degrade MCL1. I introduced a N-terminally GFP-tagged form of MCL1 into *Bax*^{-/-}*Bak*^{-/-} MEFs. While abundantly expressed, the GFP-tagged reporter was barely detectable when NOXA was co-expressed to provoke its degradation (Sup Fig. 3.3). I anticipated that the level of the reporter would increase by genetically deleting factor(s) that were essential for NOXA to drive MCL1 turnover.

A genome-wide lentiviral sgRNA library targeting mouse genes was introduced into independently derived MEF clones harboring the GFP-MCL1 reporter held in check by NOXA, upon which a small but distinct fraction of the cells became GFP^{high} (Fig. 3.2 A). These GFP^{high} cells were enriched to near homogeneity after multiple rounds of flow cytometric sorting (Fig. 3.2 A). sgRNAs enriched by this selection process were identified by sequencing (Fig. 3.2 B and Sup Table. 3.1). One of the hits of the screen was *Mcl1* itself; while unexpected, I surmised that the *Mcl1* portion of the reporter gene would have been disrupted by *Mcl1* sgRNAs leaving behind the N-terminal GFP fragment linked to a truncated form of MCL1, now more stable and unable to bind NOXA.

Importantly, I also observed clear enrichment for multiple guides targeting three other genes: these encoded for the mitochondrial E3 ligase MARCH5, the E2 conjugating enzyme UBE2K and the mitochondrial outer membrane protein MTCH2 (Fig. 3.2 B and Sup Table. 3.1). Next, we sought to confirm a role for these genes in degrading MCL1.

MARCH5, UBE2K and MTCH2 are required for MCL1 degradation triggered by NOXA

To validate the candidates identified by the screen, I assessed what impact their deletion had on the degradation of endogenous MCL1. Successful gene deletion was confirmed by DNA sequencing and, where antibodies were available, by the loss of protein expression (Figs. 3.3 A-C). While NOXA promoted the degradation of MCL1 in the parental MEFs, deleting *March5* abrogated this in several independent clones (Fig. 3.3 A). Interestingly, the levels of NOXA were concomitantly higher in cells lacking MARCH5, indicating that NOXA also became more stable when bound to MCL1 and the resulting protein complex was no longer marked for degradation. When bound to MCL1, NOXA was likely protected from additional ubiquitin-independent mechanisms that otherwise drive its rapid turnover (Pang et al., 2014). Similar results were obtained with clones

lacking MTCH2 (Fig. 3.3 B) or UBE2K (Fig. 3.3 C). Of note, the impact was less pronounced upon *Ube2K* deletion, suggesting that alternate E2 enzymes may supply ubiquitin to MARCH5 in the absence of UBE2K, as has been noted for E2s in other contexts (Markson et al., 2009; van Wijk et al., 2009).

Despite the marked impact that deleting *March5*, *Mtch2* or *Ube2k* had on MCL1 degradation triggered by NOXA overexpression in MEFs, little difference was observed on steady-state MCL1 levels in the knockout clones (Figs. 3.3 A-C). Deleting *Noxa* also had little impact on MCL1 levels in MEFs (Sup Fig. 3.4 A), suggesting that at steady-state MEFs express insufficient NOXA to meaningfully engage this pathway for degrading MCL1.

I explored further whether these genes contributed to MCL1 degradation triggered by other stress signals. However, consistent with the observation that NOXA provoked MCL1 degradation through a distinct pathway in MEFs (Fig. 3.1 D), the absence of MARCH5, MTCH2 or UBE2K had little or no impact on MCL1 degradation caused by DNA damaging agents (Figs. 3.3 D-E and Sup Figs. 3.4 B-C) or on its basal turnover (Fig. 3.3 F and Sup Fig. 3.4 D). Thus, MARCH5, MTCH2 and UBE2K appear to operate in a specific pathway for degrading the MCL1:NOXA complex, which in MEFs becomes most readily apparent when NOXA expression is elevated experimentally.

NOXA provokes MCL1 degradation in diverse cells and influences steady-state MCL1 levels when abundantly expressed

I postulated that some cells would express enough NOXA at steady-state to elicit constitutive MCL1 degradation by this pathway. To identify such examples, I examined NOXA protein expression across a large panel of cancer cell lines derived from both solid tumors (Fig. 3.4 A) and hematological malignancies (Fig. 3.4 B). There was a broad range of NOXA expression across these cell lines, providing an opportunity to hone in on those with low NOXA expression (e.g. HELA, KMS-28-BM, MCF7) or those with abundant NOXA (e.g. HCT116, RS4;11 and KMS-12-PE).

I predicted that modulating NOXA expression would have different consequences in these two groups of cell lines, and this was indeed the case. In the cells that expressed minimal NOXA, MCL1 levels were largely unperturbed upon deleting NOXA whereas MCL1 was degraded when exogenous NOXA was introduced (Figs. 3.4 C-D), consistent with what I had observed in MEFs (Fig. 3.1 B). Conversely, the cells with abundant NOXA had sufficient amounts to constitutively degrade MCL1. In these cells, deleting NOXA resulted in elevated steady-state MCL1 levels while overexpressing NOXA had little impact (Figs. 3.4 C-D), presumably because the MARCH5 pathway was already fully

engaged in these cells. Thus, NOXA can provoke MCL1 degradation in diverse cells and does so constitutively when expressed at sufficient levels.

Context specific control of MCL1 by MARCH5 and MTCH2

Given these results, I proceeded to explore whether the roles I had identified for MARCH5, MTCH2 and UBE2K in enabling NOXA to degrade MCL1 in MEFs were conserved in other cell types. I first tested whether deleting these genes would alter MCL1 levels in HCT116 cells, which express NOXA abundantly. Indeed, MCL1 was markedly stabilized in these cells upon deleting either *MARCH5* or *MTCH2* (Figs. 3.5 A-D). Deleting *UBE2K* also stabilized MCL1 but only to a modest degree (Figs. 3.5 E-F), which was consistent with the intermediate impact of *Ube2k* deletion observed in MEFs (Fig. 3.3 C). The degradation of MCL1 in HELA cells upon enforced NOXA expression was also blocked by deleting either *MARCH5* or *MTCH2* (Figs. 3.5 G-H).

Thus, the involvement of both MARCH5 and MTCH2 in NOXA-driven MCL1 degradation is conserved in diverse cells and can impact steady-state MCL1 levels in those that express high levels of NOXA. UBE2K, on the other hand, appears to be less critical. While it may serve as the principal E2 enzyme supplying ubiquitin to MARCH5 in some cases, alternative E2 partners can likely fulfil this role in the absence of UBE2K.

Impaired turnover of the MCL1:NOXA complex does not enhance MCL1 pro-survival activity

My results indicated that deleting *MARCH5* or *MTCH2* caused both NOXA and MCL1 to be stabilized concurrently (Figs. 3.3 A-C and 3.5 A-H). As stated above, this is likely because NOXA is rapidly degraded in a ubiquitin-independent manner when not bound to MCL1, and protected from such rapid turnover when engaged in complex (Pang et al., 2014). Consistent with this hypothesis, NOXA was found to be extremely unstable in cells lacking MCL1 (Sup Fig. 3.5).

The parallel changes in NOXA and MCL1 upon deleting *MARCH5* or *MTCH2* prompted me to consider the resulting impact on MCL1 pro-survival function. I evaluated this in the lymphoid cancer cell lines RS4;11 and GRANTA-519, both of which express high levels of NOXA and are sensitive to the BCL2 inhibitor ABT-199. MCL1 levels were stabilized in each of these cell lines following the deletion of either *NOXA*, *MARCH5* or *MTCH2*, with NOXA levels rising in tandem with MCL1 upon removing *MARCH5* or *MTCH2* (Figs. 3.6 A-B). Importantly, while deleting *NOXA* rendered both cell lines less sensitive to ABT-199 and several other apoptosis triggers, deleting *MARCH5* or *MTCH2* did not (Figs. 3.6 C-D). Thus, the elevated levels of MCL1 that result from disabling

turnover of the MCL1:NOXA complex do not give rise to elevated MCL1 pro-survival function, which is consistent with the notion that BH3-only proteins need not cause MCL1 degradation to provoke apoptosis (Lee et al., 2008).

The findings indicate that deleting *MARCH5* has a very different outcome on the response of cells to apoptosis triggers compared to deleting *NOXA*. Notably, despite having elevated levels of MCL1 (Figs. 3.6 A-B), RS4;11 and GRANTA-519 cells lacking *MARCH5* were even more sensitive than their parental counterparts to some apoptosis triggers, particularly ABT-199 (Figs. 3.6 C-D). I sought to confirm these results in two additional lymphoid cancer cell lines. Indeed, deleting *NOXA* also rendered both KMS-12-PE and RPMI8226 cells less sensitive BH3 mimetics (Sup Figs. 3.6 A-B). However, these cell lines proved difficult to culture following transduction with lentiviruses to delete *MARCH5*. To quantitate this observation, I compared the relative outgrowth of gene-targeted cells in a competitive assay. Indeed, *MARCH5* gene-targeted cells exhibited a significant growth disadvantage in both KMS-12-PE and RPMI8226 cell lines, indicating that *MARCH5* is essential for their fitness (Sup Figs. 3.6 C-E). This observation is consistent with large-scale genetic screens that have found *MARCH5* to be an essential gene in roughly one third of all human cancer cell lines (Meyers et al., 2017).

Altogether, these data suggests that impairing *MARCH5* E3 ligase function would not enhance MCL1 pro-survival activity despite stabilizing MCL1 protein levels. Modulating *NOXA* expression, on the other hand, should have a more significant impact in many cases as it influences both MCL1 stability and the ability of MCL1 to engage other BCL2 proteins. *MARCH5* controls the turnover of multiple substrates in addition to MCL1, which may account for why I and others have found *MARCH5*^{-/-} cells to be more sensitive to certain triggers of apoptosis (Xu et al., 2016).

MTCH2 cooperates with *MARCH5* to degrade MCL1 but does not influence the turnover of another *MARCH5* substrate: MID49

The common requirement for both *MARCH5* and *MTCH2* to degrade MCL1 in diverse cells suggested that these proteins likely cooperate with one another in this process. To test this, I overexpressed FLAG-MTCH2 or MYC-MARCH5 in *MTCH2*^{-/-} and *MARCH5*^{-/-} HCT116 cells. The overexpressed proteins could compensate for their own absence to restore MCL1 degradation but *MARCH5* overexpression could not overcome the absence of *MTCH2* and likewise, *MTCH2* overexpression could not overcome the absence of *MARCH5* (Fig. 3.7 A). Thus, *MTCH2* and *MARCH5* function in a mutually dependent manner to degrade the MCL1:NOXA complex.

MARCH5 possesses ubiquitin E3 ligase activity, enabling it to transfer ubiquitin to substrate proteins and mark them for degradation by the proteasome. I confirmed that the E3 ligase function of MARCH5 was required to drive MCL1 degradation, as reintroducing WT MARCH5 into *MARCH5*^{-/-} HCT116 cells restored MCL1 turnover, but reintroducing an E3 ligase defective mutant (*MARCH5*^{C65/68S}) did not (Fig. 3.7 B).

In contrast to MARCH5, MTCH2 lacks enzymatic activity and it is not obvious how it might impact protein stability. MTCH2 has been reported to interact with another BCL2 family protein, tBID, and regulate its localization to the mitochondria (Raemy et al., 2016; Zaltsman et al., 2010). I explored whether MTCH2 might also control tBID turnover but found that it did not (Sup Fig. 3.7). I next tested whether MTCH2 controlled the subcellular localization of MARCH5, MCL1, or NOXA, but each of these proteins remained associated with mitochondria-enriched heavy membrane fractions in the absence of MTCH2 (Fig. 3.7 C). NOXA and MCL1 also continued to interact with one another in the absence of MTCH2 (Fig. 3.7 D). Thus, how MTCH2 cooperates with MARCH5 to degrade MCL1 remains unclear.

MARCH5 has been reported to target other mitochondrial proteins for degradation, of which MID49 is the best characterized (Cherok et al., 2017; Xu et al., 2016). I therefore investigated whether MTCH2 also cooperated with MARCH5 to recognize and degrade this substrate. Although MID49 was stabilized in *MARCH5*^{-/-} HCT116 cells, its stability and rate of turnover were largely unaltered in cells lacking MTCH2 (Figs. 3.7 E-F). This indicates that MTCH2 is not required for the degradation of all MARCH5 substrates and makes it unlikely that MTCH2 influences MCL1 degradation through a general impact on MARCH5 enzymatic activity. Instead, the impact of MTCH2 is more likely specific to either MCL1 or NOXA, perhaps influencing how the MCL1:NOXA complex is recognized by MARCH5.

The C-terminal transmembrane domain of MCL1 is required for its degradation by MARCH5 and MTCH2

The ability of NOXA to provoke MCL1 degradation is unique among BH3-only proteins and is driven by specific amino acids within its BH3 domain (Czabotar et al., 2007). Given the requirement for MARCH5 and MTCH2 to degrade the MCL1:NOXA complex, I next explored whether there were additional molecular requirements for MCL1 degradation to proceed. In particular, we noted that MARCH family E3 ligases often recognize their substrates through interactions between transmembrane domains (Corcoran et al., 2011; Jahnke, Trowsdale, & Kelly, 2012; Tan, Byrne, Ah-Cann, Call, & Call, 2019; Tze

et al., 2011). I therefore postulated that the C-terminal transmembrane domain of MCL1 might contribute to its recognition and degradation by MARCH5.

To test this idea directly, I replaced the membrane targeting region of MCL1 with equivalent residues from a related pro-survival protein, BCL2 (MCL1^{BCL2TM}; Fig. 3.8 A). These substitutions did not affect the ability of MCL1 to block apoptosis (Sup Fig. 3.8 A) or to bind NOXA (Sup Fig. 3.8 B), but did prevent NOXA from causing its degradation (Fig. 3.8 B). Subcellular fractionation and confocal imaging confirmed that MCL1^{BCL2TM} was properly localized at heavy membranes including mitochondria, nuclear envelope and endoplasmic reticulum (Fig. 3.8 C and Sup Fig. 3.8 C), consistent with the intracellular distributions of both BCL2 and MCL1 (Akao et al., 1994; Schinzel, Kaufmann, & Borner, 2004). Despite being distributed slightly more broadly than MCL1 itself, the MCL1^{BCL2TM} localized at mitochondria was clearly not degraded when co-expressed with NOXA (Fig. 3.8 C). Hence, I conclude that in addition to motifs contributed by the BH3 domain of NOXA within the MCL1:NOXA complex (Czabotar et al., 2007), elements of the MCL1 transmembrane domain are also necessary for recognition by MARCH5. Along with the requirement for MTCH2 in this process and the identification of specifically required lysine residues on MCL1, I can begin to formulate a model for how these observations fit together (Fig. 3.8 D). I postulate that MARCH5 and MCL1 may interact directly, potentially through their transmembrane domains, and possibly aided by a second interface involving NOXA. MTCH2 may also bridge interactions that take place within or adjacent to the mitochondrial outer membrane. I propose that these proteins come together in a specific orientation that allows MARCH5 to discharge ubiquitin onto K175/K178 of MCL1, thereby marking it for degradation.

Figure 3.1

Lysines 175 and 178 of MCL1 are critical for its degradation triggered by the BH3-only protein NOXA, but not by other stimuli.

- (A) A variety of stress stimuli provoke MCL1 degradation by the proteasome. *Bax*^{-/-} *Bak*^{-/-} MEFs were exposed to nocodazole (NOC; 400 ng/mL for 16 h), etoposide (ETOP; 50 μ M for 16 h), cycloheximide (CHX; 50 μ g/mL for 16 h) or UV irradiation (200 J/m²; then cultured for 4 h). Where indicated, the cells were also cultured with the proteasome inhibitor MG132 (10 μ M).
- (B) NOXA promotes MCL1 degradation by the proteasome. *Bax*^{-/-} *Bak*^{-/-} MEFs were engineered to express HA-NOXA (WT) or HA-NOXA^{3E} (3E), a mutant that does not bind MCL1 (Chen et al., 2005). Where indicated, the cells were also cultured with the proteasome inhibitor MG132 (10 μ M) for 16h.
- (C) Lysines 175 and 178 of mouse MCL1 are critical for NOXA to trigger its degradation. *Mcl1*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs were engineered to express WT MCL1 or the indicated MCL1 mutants together with HA-NOXA or HA-NOXA^{3E}.
- (D) Unlike NOXA-induced degradation, other stress stimuli degrade MCL1 independently of K175 and K178. *Mcl1*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs were engineered to express WT MCL1 or MCL1^{K175R,K178R} together with HA-NOXA or HA-NOXA^{3E}, or exposed to nocodazole (NOC; 400 ng/mL for 16 h), etoposide (ETOP; 50 μ M for 16 h), cycloheximide (CHX; 50 μ g/mL for 16 h) or UV irradiation (200 J/m²; then cultured for 4 h).

* Asterisks denote MCL1^{Matrix}, a form of the protein that has undergone N-terminal proteolysis associated with import into the mitochondrial matrix (Huang & Yang-Yen, 2010; Perciavalle et al., 2012). The absence of MCL1^{Matrix} from cells expressing wild-type HA-NOXA suggests that the binding of NOXA to MCL1 may prevent such import from taking place.

Representative images in A-D from two to three independent experiments.

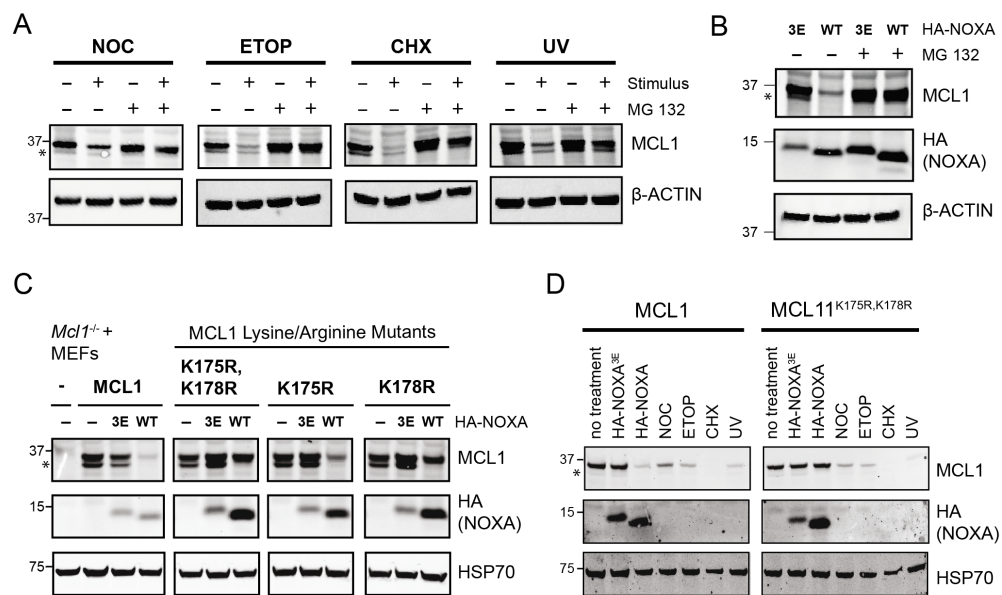


Figure 3.2

Genome-wide CRISPR-Cas9 screen for regulators of NOXA-induced MCL1 degradation.

- (A) Selection strategy to enrich cells in which the ability of NOXA to provoke MCL1 degradation has been disabled. *Bax*^{-/-}*Bak*^{-/-} MEFs engineered to express GFP-MCL1 along with HA-NOXA and Cas9 (i) were transduced with a genome-wide sgRNA lentivirus library (Koike-Yusa et al., 2014). Following transduction, a small proportion of GFP^{high} cells were detected (ii) and enriched through three rounds of flow cytometric sorting (iii – v).
- (B) Quantitation of sgRNA abundance in the transduced cells before and after sorting. Multiple independent sgRNAs targeting *Mcl1*, *March5*, *Mtch2* and *Ube2k* were enriched within the sorted GFP^{high} cell population. These data are also summarized in Supplementary Table 3.3.

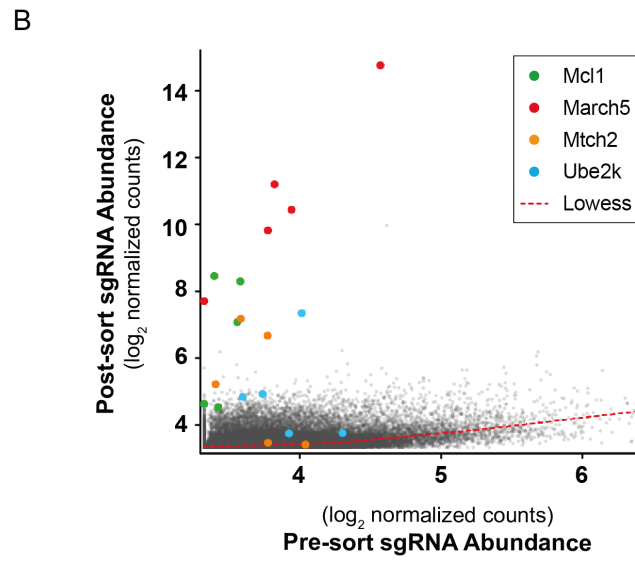
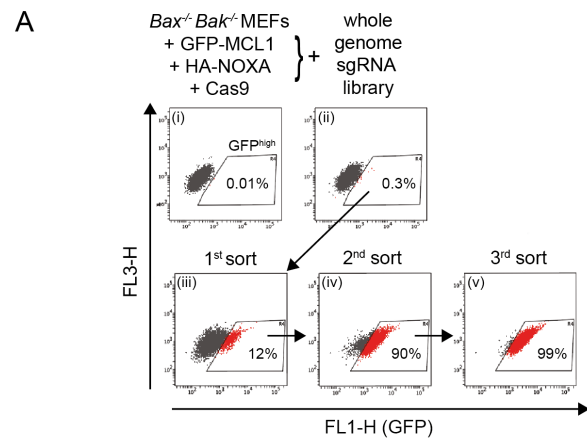


Figure 3.3

MARCH5, MTCH2 and UBE2K are critical for NOXA-induced MCL1 degradation.

(A-C) *March5* (A), *Mtch2* (B), or *Ube2k* (C) were deleted from *Bax*^{-/-}*Bak*^{-/-} MEFs by CRISPR-Cas9 gene targeting. DNA sequencing was performed to confirm indel generation at sgRNA target sites (Sup Table. 3.2). Confirmed knockout clones were then engineered to express HA-NOXA. The ability of NOXA to trigger MCL1 degradation was abrogated in the knockout cells. Representative images in A-C from three independent experiments.

(D-F) MARCH5 is not required for MCL1 degradation in MEFs subjected to DNA damage or protein synthesis inhibition. *Bax*^{-/-}*Bak*^{-/-} and *Bax*^{-/-}*Bak*^{-/-}*March5*^{-/-} MEFs were exposed to (D) UV radiation (200 J/m²; then cultured for 4 h), (E) etoposide (50 μM for 16 h; ETOP), or (F) the protein synthesis inhibitor cycloheximide (50 μg/mL for up to 6 h; CHX).

* Asterisks denote MCL1^{Matrix}, a truncated form of MCL1 that appears to be absent from cells expressing wild-type HA-NOXA for the reasons outlined in the legend to Figure

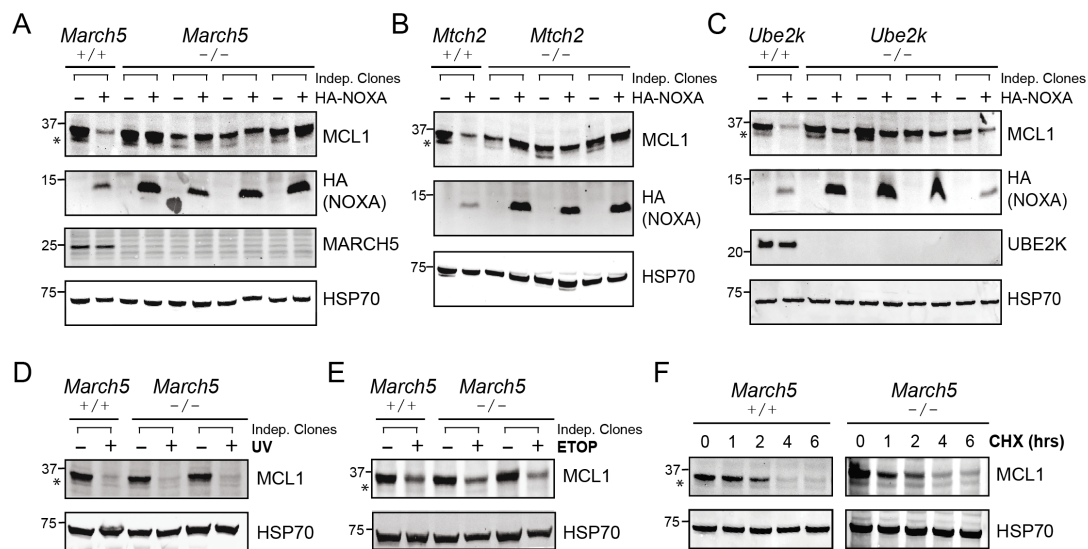


Figure 3.4

The steady-state level of MCL1 protein is controlled by NOXA when this BH3-only protein is abundantly expressed.

- (A-B) Expression of select BCL2 family proteins in (A) solid and (B) haematological cancer cell lines.
- (C) In cells that express high levels of NOXA, this BH3 only protein controls steady-state MCL1 protein levels. NOXA was deleted using CRISPR-Cas9 gene targeting from a selection of cell lines that normally express either low (HELA, KMS-28-BM) or high (HCT116, RS4;11, KMS-12-PE) amounts of NOXA.
- (D) HA-NOXA overexpression promoted MCL1 degradation in cell lines that normally express low amounts of NOXA (e.g., HELA, MCF7), but had minimal impact in those with high NOXA expression (e.g., HCT116, RS4;11).

Representative images in A-D from two to three independent experiments.

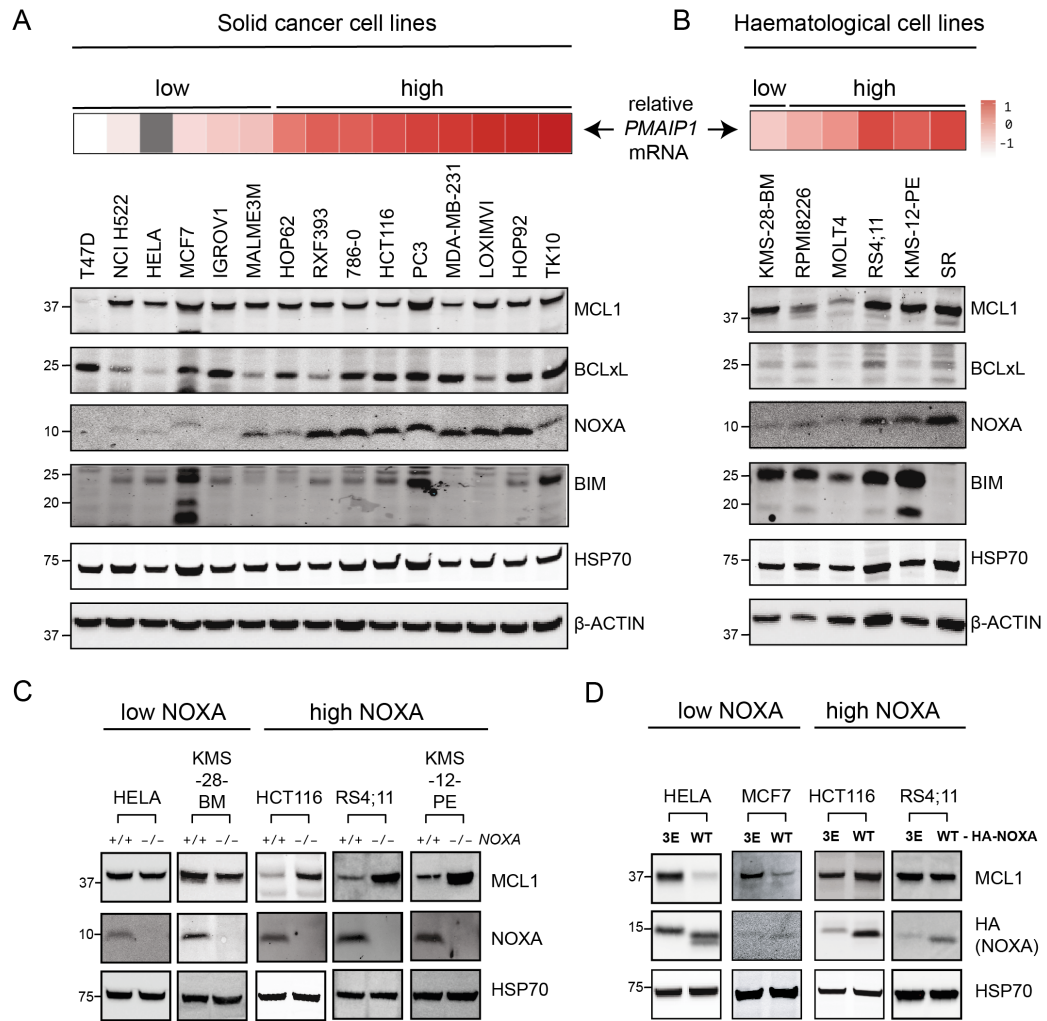


Figure 3.5

MARCH5 and MTCH2 control NOXA-driven MCL1 turnover in diverse cells.

- (A-F) *MARCH5* (A,B), *MTCH2* (C,D) or *UBE2K* (E,F) were deleted from HCT116 cells by CRISPR-Cas9 gene targeting. Indel generation at sgRNA target sites was confirmed by DNA sequencing (Sup Table. 3.2). Steady-state MCL1 expression (A,C,E) and the rate of MCL1 turnover (B,D,F) were examined in confirmed knockout clones. Cells were cultured with the protein synthesis inhibitor cycloheximide (50 µg/mL; CHX) for up to 4 h where indicated. MCL1 was markedly stabilised in clones lacking either *MARCH5* or *MTCH2* (A-D) and to a lesser degree in clones lacking *UBE2K* (E-F).
- (G-H) *MARCH5* (G) or *MTCH2* (H) were deleted from HELA cells by CRISPR-Cas9 gene targeting. Indel generation at sgRNA target sites was again confirmed by DNA sequencing (Sup Table. 3.2). MCL1 degradation caused by HA-NOXA overexpression was abolished in the knockout clones.

Representative images in A-H from two to three independent experiments.

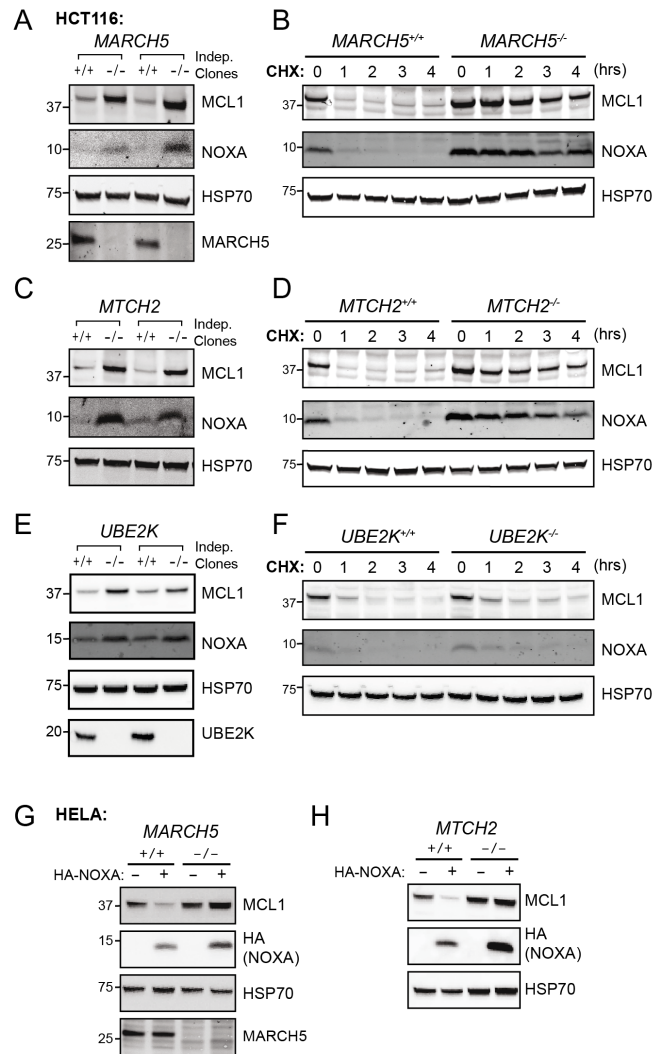


Figure 3.6

Distinct impacts on MCL1 pro-survival function upon deleting either *NOXA*, *MARCH5* or *MTCH2*.

(A-B) Steady-state MCL1 protein levels were elevated in both RS4;11 (A) and GRANTA-519 (B) cells transduced with lentiviruses to express Cas9 and sgRNA targeting either *NOXA*, *MARCH5* or *MTCH2*. The proportion of cells in the transduced populations bearing indels at the sgRNA target sites (as measured by DNA sequencing and reported in Sup Table. 3.2) is indicated below the gels. Representative images in A-B from two independent experiments.

(C-D) Deleting *NOXA* rendered RS4;11 (C) and GRANTA-519 (D) cells less sensitive to several triggers of apoptosis, but deleting *MARCH5* and *MTCH2* did not despite MCL1 being stabilized to comparable levels in all cases. Transduced cell populations from (A) and (B) were exposed to the indicated concentrations of ABT-199, RG7388, cisplatin or etoposide for 24-48 h. Cell viability was measured by PI exclusion. Data represent mean $\pm$ standard deviation of three independent experiments using two independent sgRNAs per gene. Statistical testing was not performed.

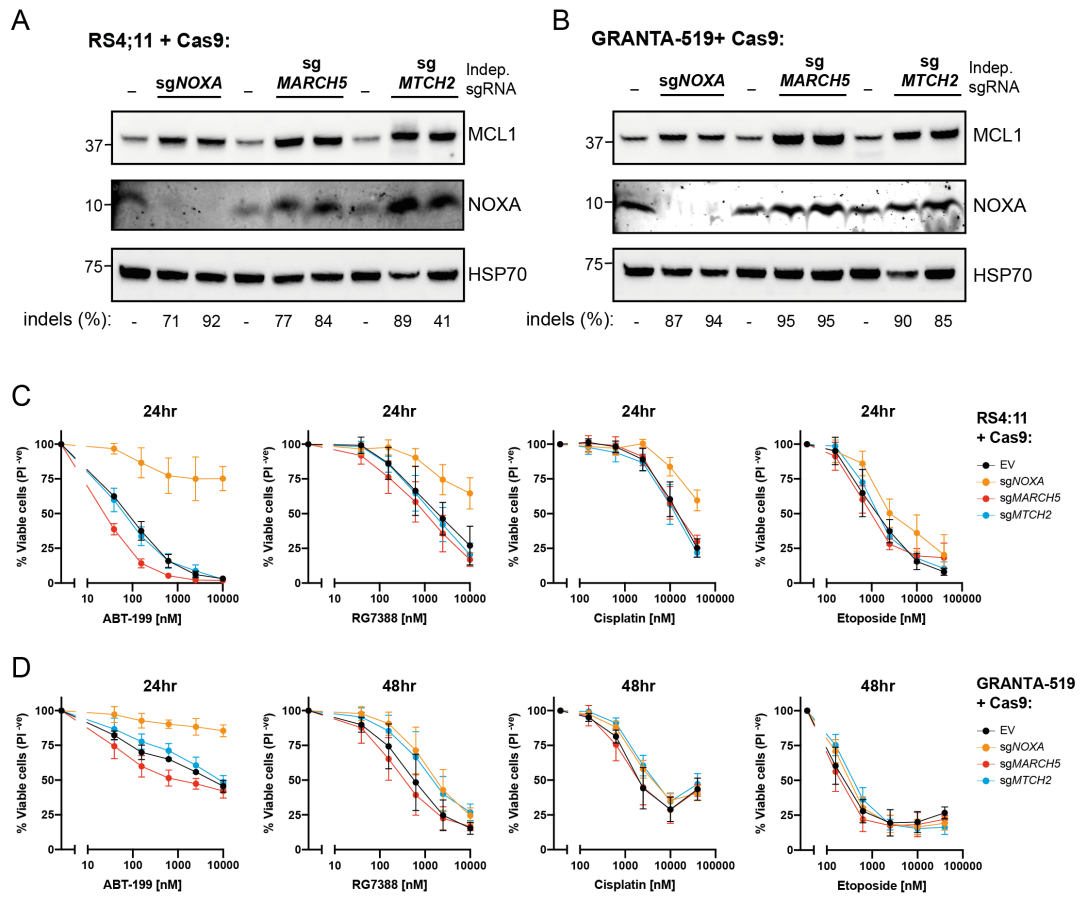


Figure 3.7

MTCH2 and MARCH5 jointly control turnover of the MCL1:NOXA complex.

- (A) MARCH5 and MTCH2 require one another to regulate MCL1 degradation by NOXA. *WT*, *MARCH5*^{-/-} or *MTCH2*^{-/-} HCT116 cells were engineered to stably express either MYC-MARCH5 (MYC-M5) or FLAG-MTCH2 (FLAG-M2). MYC-MARCH5 restored NOXA-driven MCL1 turnover in *MARCH5*^{-/-} but not *MTCH2*^{-/-} cells, while FLAG-MTCH2 did so in *MTCH2*^{-/-} but not *MARCH5*^{-/-} cells.
- (B) The E3 ubiquitin ligase function of MARCH5 is critical for regulating MCL1 degradation. *MARCH5*^{-/-} HCT116 cells were engineered to express either WT MARCH5 or a ligase-defective mutant (*MARCH5*^{C65/68S}). While WT MARCH5 restored NOXA-driven MCL1 turnover, *MARCH5*^{C65/68S} did not.
- (C) MTCH2 is not required for the heavy membrane association of key proteins required for NOXA-driven MCL1 turnover. Protein lysates were prepared from cytosol (C) and heavy membrane (HM) fractions derived from *WT* and *MTCH2*^{-/-} HCT116 cells.
- (D) MCL1 was immunoprecipitated from HCT116 cells (*WT*, *MCL1*^{-/-}, *MARCH5*^{-/-} and *MTCH2*^{-/-}) cultured with proteasome inhibitor MG132 (10 μ M for 8 h). NOXA continued to interact with MCL1 even when MTCH2 was absent.
- (E) The steady-state expression level of MID49 was elevated in *MARCH5*^{-/-} but not *MTCH2*^{-/-} HCT116 cells.
- (F) The rate MID49 protein turnover was reduced in *MARCH5*^{-/-} but not *MTCH2*^{-/-} HCT116 cells. Cells were cultured with the protein synthesis inhibitor cycloheximide (50 μ g/mL; CHX) for up to 8 h.

Representative images in A-F from two independent experiments.

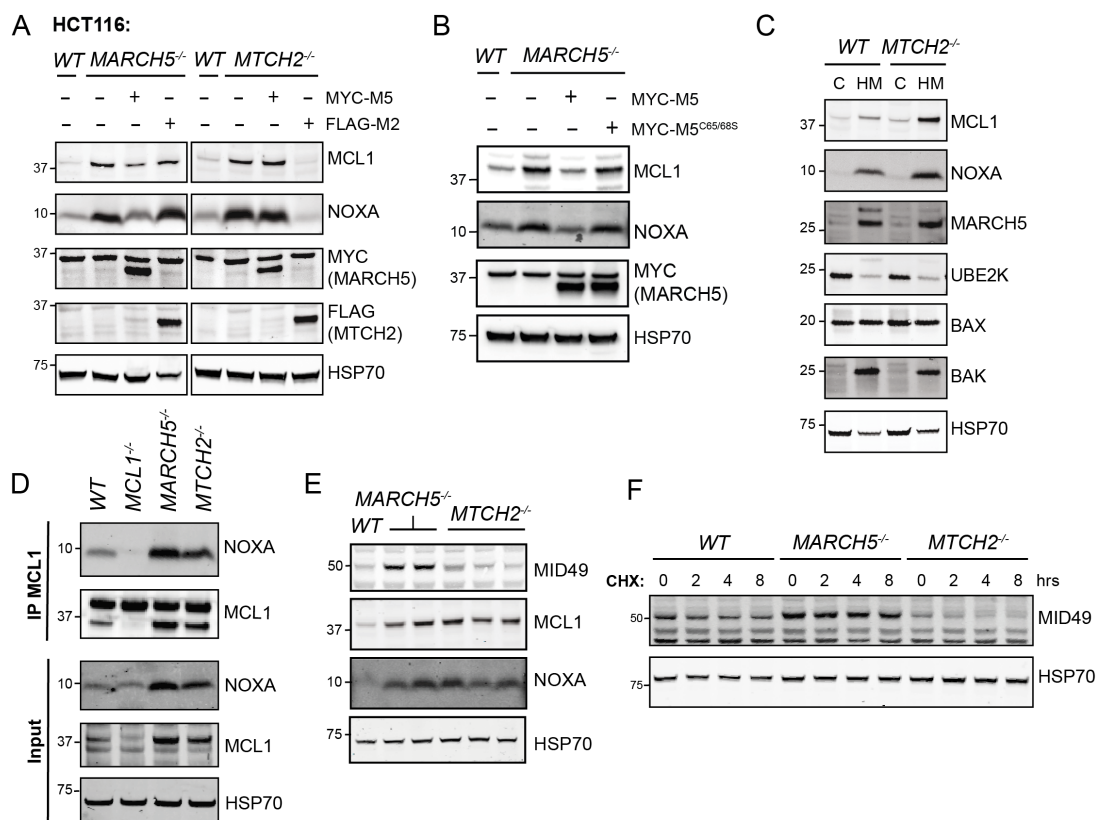
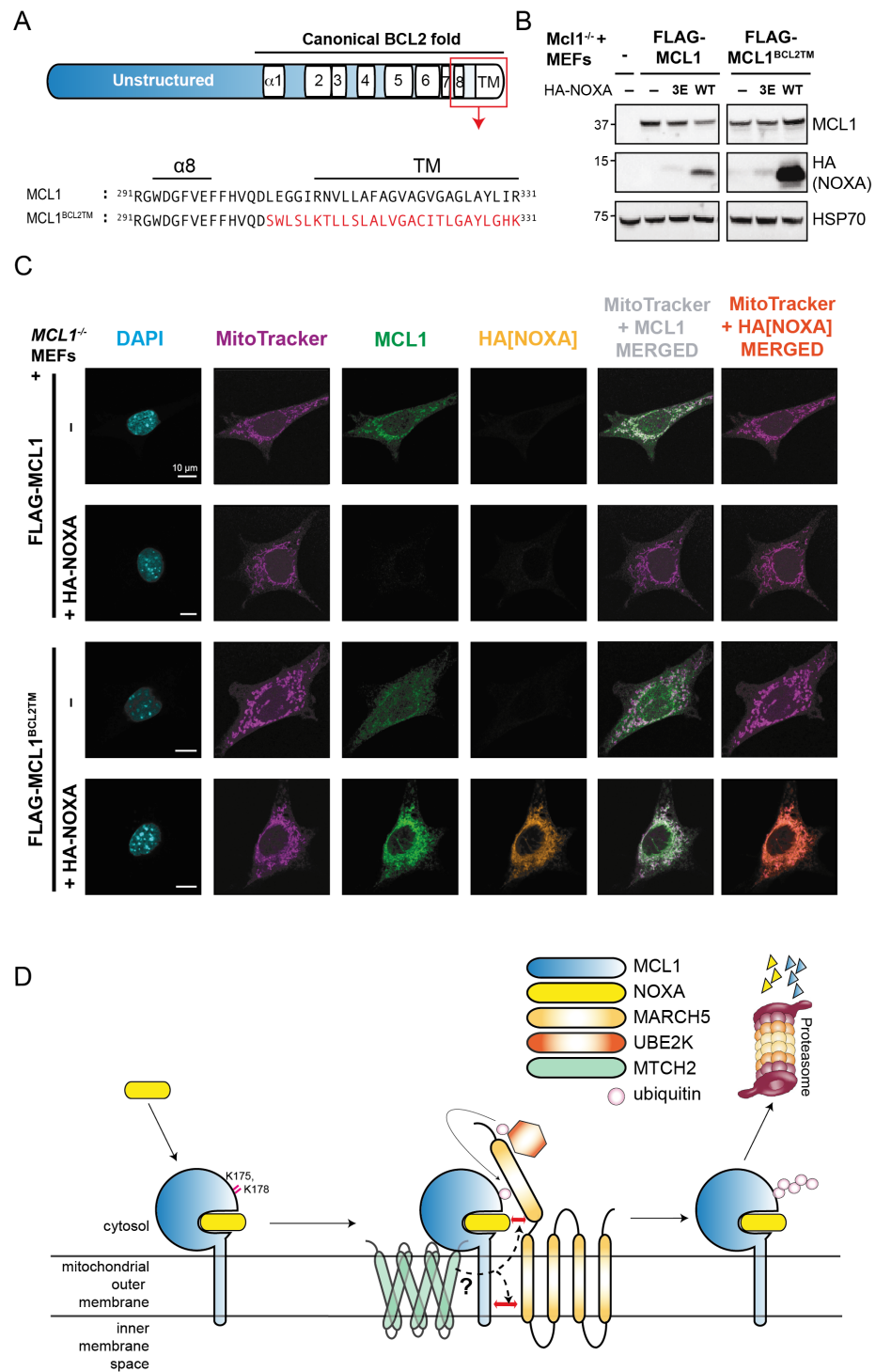


Figure 3.8

Elements of the MCL1 transmembrane domain are necessary for its turnover by MARCH5 and MTCH2.

- (A) Schematic highlighting the MCL1 transmembrane domain and a mutant in which this region of MCL1 was replaced by corresponding sequence from BCL2.
- (B) The MCL1 transmembrane domain is critical for NOXA to trigger MCL1 degradation. *Mcl1*^{-/-} MEFs were engineered to express FLAG-MCL1 or FLAG-MCL1^{BCL2TM} along with HA-NOXA or HA-NOXA^{3E}. Representative image from two independent experiments.
- (C) *Mcl1*^{-/-} MEFs engineered to express FLAG-MCL1 or FLAG-MCL1^{BCL2TM} with or without HA-NOXA were imaged by confocal immunofluorescence microscopy. MCL1 (green) and NOXA (orange) were revealed by MCL1 and HA immunofluorescence, whilst DAPI (blue) and MitoTracker (purple) provided counterstain for the nucleus and mitochondrial network respectively. When co-expressed, FLAG-MCL1^{BCL2TM} and HA-NOXA were both present at high levels on the mitochondrial network (merged channels) indicating that degradation of the MCL1:NOXA complex by MARCH5/MTCH2 requires the MCL1 transmembrane domain. Scale bars: 10 µm. Images are representative of at least 10 fields of view captured for each condition. Equal laser intensities and thresholds were used in capturing and representing the MCL1 and HA immunofluorescence signals to faithfully represent their relative levels between samples.
- (D) Model highlighting key aspects of MARCH5-mediated turnover of MCL1. MARCH5 degrades MCL1 specifically when it is engaged by NOXA, suggesting that NOXA may contribute to the motif through which MARCH5 recognizes MCL1 as a substrate. These findings also indicate that the integral mitochondrial outer membrane protein MTCH2, the transmembrane domain of MCL1, and key lysine residues on MCL1 are all critical elements of this mechanism.

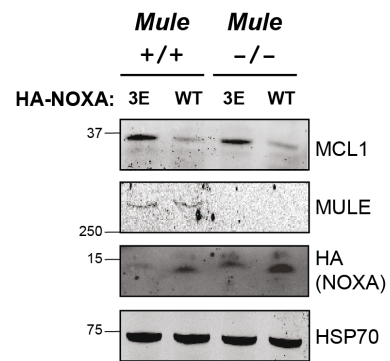


Supplementary Figure 3.1

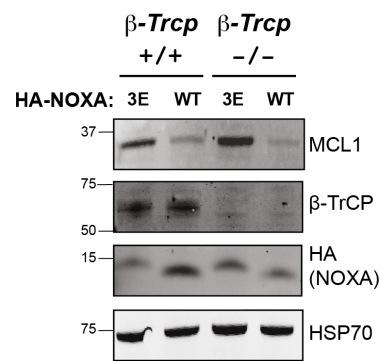
The ubiquitin E3 ligases MULE, β -TRCP, FBW7 and PARKIN are not required for NOXA-mediated MCL1 degradation.

(A) *Mule*, (B) *β -Trcp*, (C) *Fbw7* and (D) *Parkin* were deleted from wild-type (A) or *Bax*^{-/-} *Bak*^{-/-} MEFs (B-D) by CRISPR-Cas9 gene targeting. Indel generation at sgRNA target sites was confirmed by DNA sequencing (Sup Table. 3.2). Confirmed knockout clones were then engineered to express HA-NOXA or HA-NOXA^{3E}. Blots are representative of at least two independent knockout clones examined for each gene.

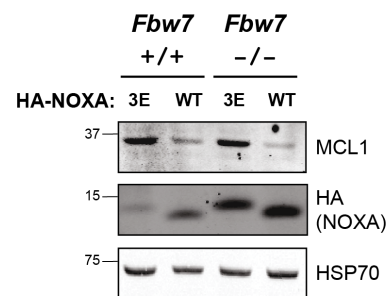
A



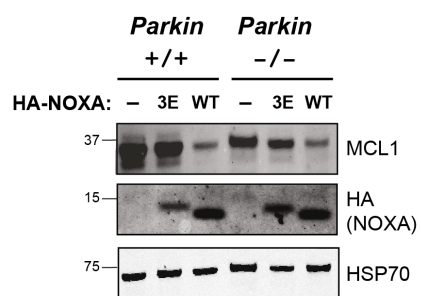
B



C



D

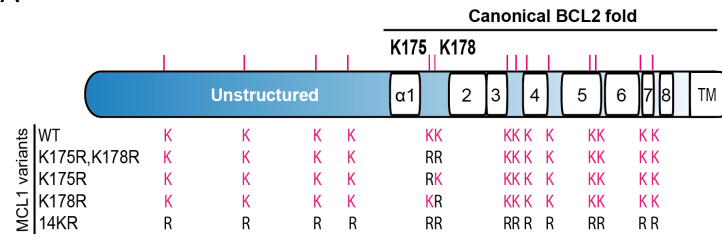


Supplementary Figure 3.2

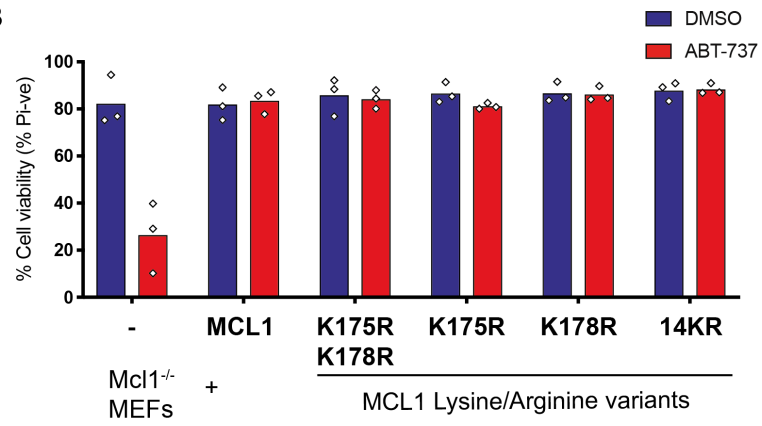
MCL1 lysine/arginine replacement variants retain their pro-survival function and their ability to bind NOXA.

- (A) Schematic indicating lysine/arginine replacement mutations made to MCL1.
- (B) All MCL1 lysine/arginine replacement mutants retained their ability to inhibit apoptosis. *Mcl1*^{-/-} MEFs were engineered to express WT MCL1, MCL1^{K175R}, MCL1^{K178R}, MCL1^{K175R,K178R} and MCL1^{14KR} then exposed to ABT-737 (2 μM) for 24 h. Cell viability was measured by propidium iodide exclusion. n = 3 independent experiments.
- (C) All MCL1 lysine/arginine replacement mutants retained their ability to bind NOXA. *Mcl1*^{-/-} MEFs were engineered to express WT MCL1, MCL1^{K175R,K178R} or MCL1^{14KR} together with HA-NOXA or HA-NOXA^{3E}, a mutant that does not bind MCL1. HA immunoprecipitations were performed on lysates prepared from cells cultured with the proteasome inhibitor MG132 (10 μM) for 8 h. Representative image from two independent experiments.

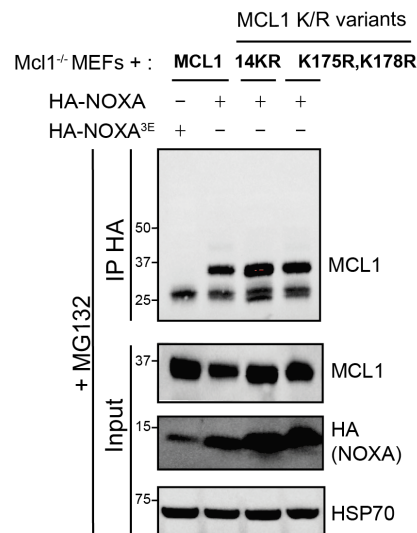
A



B



C

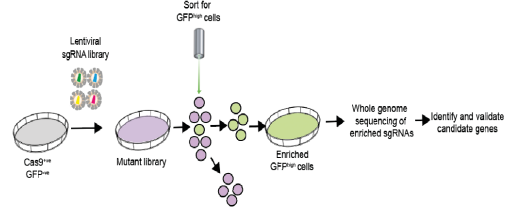


Supplementary Figure 3.3

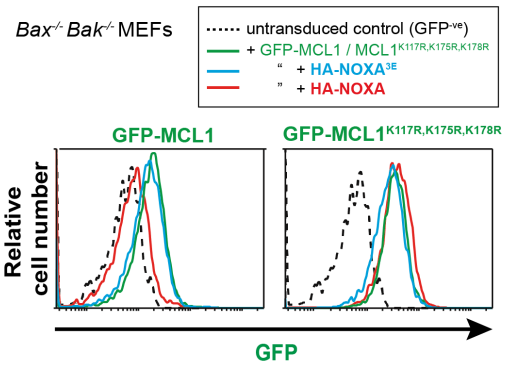
Characterization of GFP-MCL1 reporter and clones utilized for CRISPR-Cas9 screen.

- (A) Outline of the genome-wide CRISPR-Cas9 enrichment screen to identify key players of NOXA-mediated MCL1 degradation.
- (B) GFP-tagged MCL1 is targeted for degradation upon co-expressing NOXA. *Bax*^{-/-} *Bak*^{-/-} MEFs were engineered to express GFP-MCL1 or GFP-MCL1^{K117R,K175R,K178R} and also HA-NOXA or HA-NOXA^{3E} where indicated.
- (C) Selection of clones for CRISPR-Cas9 screen. *Bax*^{-/-} *Bak*^{-/-} MEFs were engineered to express GFP-MCL1 together with Cas9-T2A-mCherry and HA-NOXA. Single cells with low GFP-MCL1 expression (resulting from HA-NOXA co-expression) were sorted and expanded from the population.
- (D) Characterization of clones utilized for screen. Individual clones were sorted and expanded (as described in C) and then exposed to proteasome inhibitor MG132 (10 μ M) for 6h. Each of the selected clones exhibited robust recovery of GFP-MCL1 expression following MG132 treatment.

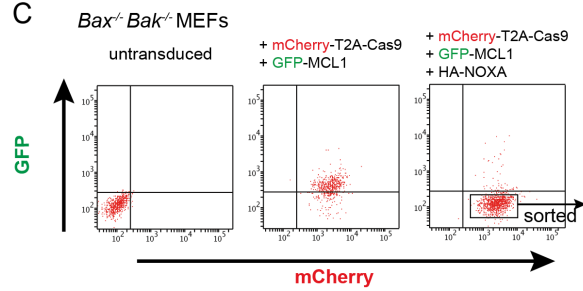
A



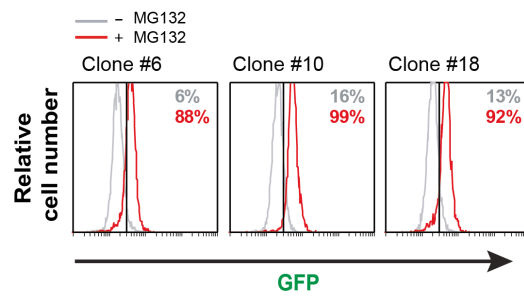
B



C



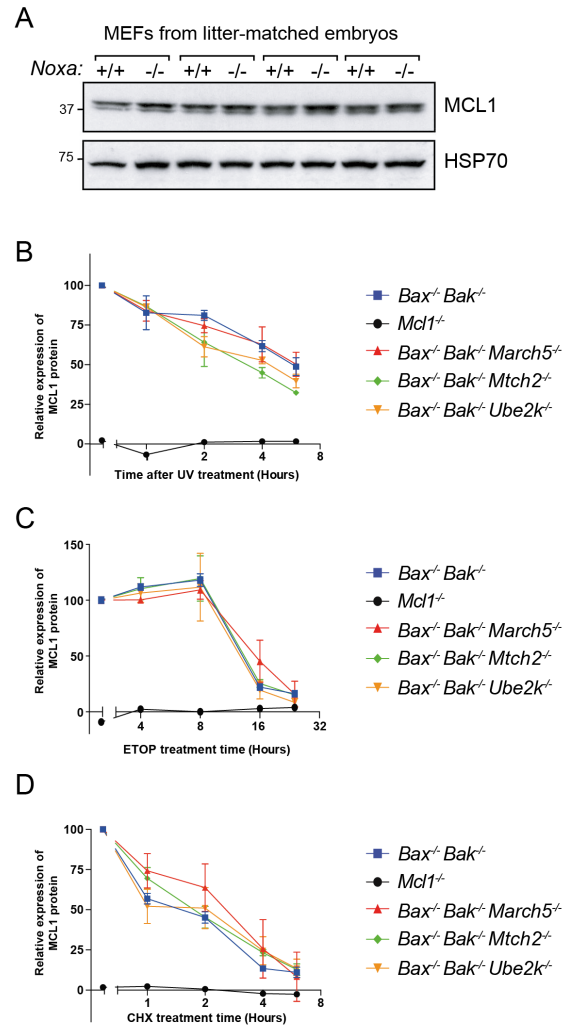
D



Supplementary Figure 3.4

The NOXA-dependent pathway for degrading MCL1 involving MARCH5, MTCH2 and UBE2K is not required for MCL1 degradation caused by DNA damage or protein synthesis inhibition in MEFs.

- (A) Deleting *Noxa* has no discernible impact on MCL1 stability in MEFs. Representative image from three independent experiments.
- (B-D) MARCH5, MTCH2 and UBE2K are dispensable for MCL1 degradation induced by exposure to UV radiation, etoposide, or cycloheximide. *Bax*^{-/-} *Bak*^{-/-} MEFs and clones in which *March5*, *Mtch2*, or *Ube2k* were deleted by CRISPR-Cas9 gene targeting were exposed to (B) 200 J/m² UV radiation, (C) 50 μM etoposide (ETOP), or (D) 50 μM cycloheximide (CHX). Cells were harvested at the indicated times and MCL1 protein levels were determined by intracellular FACS staining. *Mcl1*^{-/-} MEFs served as a negative control. Data presented as mean ± standard error of mean of biological replicates using independently derived knockout clones (n=3 for *March5*^{-/-} *Bax*^{-/-} *Bak*^{-/-}, *Ube2k*^{-/-} *Bax*^{-/-} *Bak*^{-/-} or *Mtch2*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs) and (n=2 for *Bax*^{-/-} *Bak*^{-/-} (control) and UV-treated *Mtch2*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs).

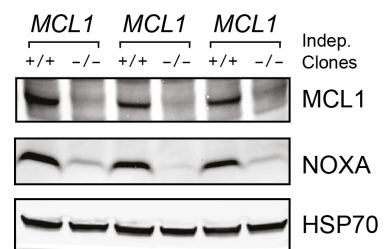


Supplementary Figure 3.5

NOXA is highly unstable in the absence of MCL1.

MCL1^{-/-} HCT116 clones were generated by CRISPR-Cas9 gene editing. *MCL1* gene disruption was verified in the knockout clones by DNA sequencing (Sup Table. 3.2). NOXA protein levels were dramatically reduced in the absence of MCL1.

HCT116:

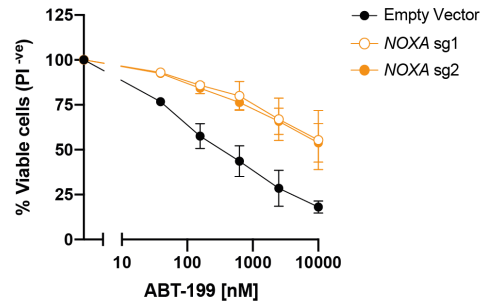


Supplementary Figure 3.6

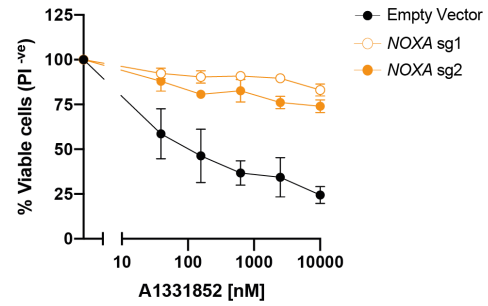
Distinct impacts of deleting *NOXA* and *MARCH5* in lymphoid cancer cell lines.

- (A-B) Deleting *NOXA* rendered KMS-12-PE and RPMI8226 cells resistant to selective BH3 mimetics. KMS-12-PE (A) and RPMI8226 (B) cells transduced with lentiviruses to express Cas9 and independent sgRNA targeting *NOXA*. Indel generation at the *NOXA* locus was confirmed to be $\geq 90\%$ in all cases (Sup Table. 3.2). Gene-edited cell populations were then exposed to titrated concentrations of the BCL-2 inhibitor ABT-199 (A) or the BCL-XL inhibitor A1331852 (B) for 24 h. Cell viability was measured by PI exclusion. Data represent mean $\pm$ standard deviation of three independent experiments.
- (C-E) Cell growth competition assay. KMS-12-PE (C), RPMI8226 (D), and GRANTA-519 (E) cells expressing Cas9 were transduced with a control lentivirus (Empty Vector) encoding only for the fluorescent marker protein BFP or with lentiviruses encoding for both BFP and independent sgRNA targeting either *NOXA* or *MARCH5*. Equal numbers of transduced cells (BFP⁺) and untransduced cells (BFP⁻) were sorted from the cultures 72 h after infection. Sorted cells were co-cultured for 12 days, with the proportion of BFP⁺ cells measured every three days. Whilst GRANTA-519 cells could tolerate *MARCH5* deletion (E), both KMS-12-PE and RPMI8226 cells exhibited a marked loss of fitness upon *MARCH5* deletion (C-D). Data represent mean $\pm$ standard deviation of three independent experiments.

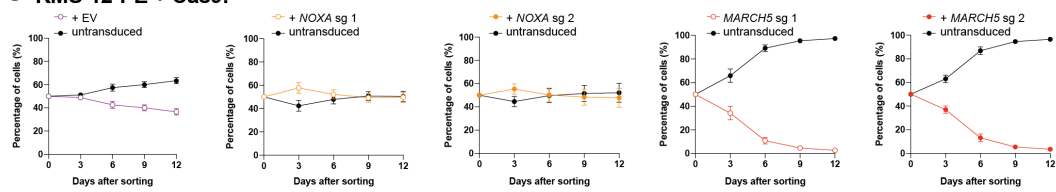
A KMS-12-PE + Cas9:



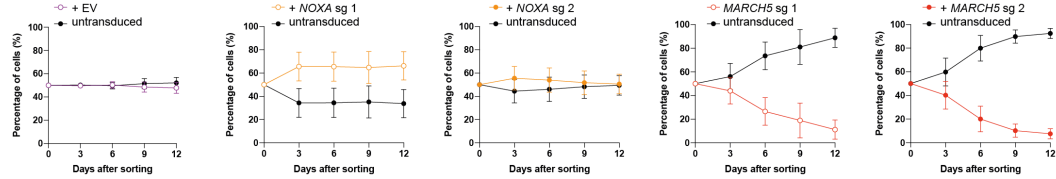
B RPMI8226 + Cas9:



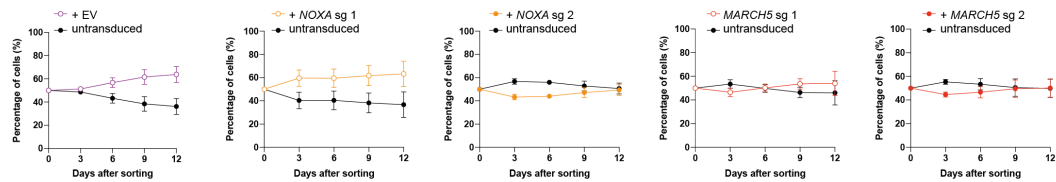
C KMS-12-PE + Cas9:



D RPMI8226 + Cas9:



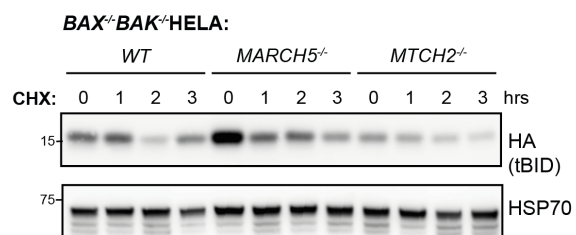
E GRANTA-519 + Cas9:



Supplementary Figure 3.7

MTCH2 and MARCH5 do not play major roles in turnover of the BH3-only protein tBID.

BAX^{-/-}*BAK*^{-/-} HELA cells and clones lacking either MARCH5 or MTCH2 were engineered to express HA-tBID. Whole cell protein extracts were prepared from cells cultured with the protein synthesis inhibitor cycloheximide (50 µg/ml; CHX) for up to 3 h. Representative image from two independent experiments.



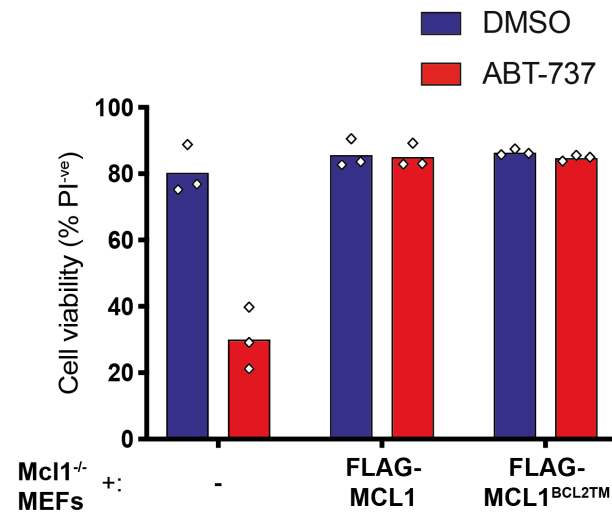
Supplementary Figure 3.8

MCL1^{BCL2TM} remains associated with heavy membranes, retaining its pro-survival function and ability to bind NOXA.

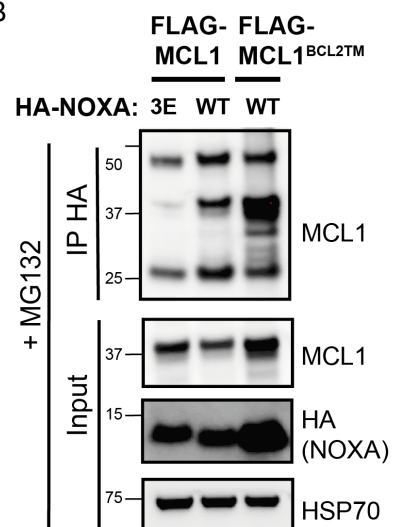
- (A) MCL1^{BCL2TM} retains the ability to inhibit apoptosis. *Mcl1*^{-/-} MEFs were engineered to express FLAG-MCL1 or FLAG-MCL1^{BCL2TM} then exposed to ABT-737 (2 µM) for 24 h. Cell viability was measured by propidium iodide exclusion. n = 3 independent experiments.
- (B) MCL1^{BCL2TM} retains the ability to bind NOXA. *Mcl1*^{-/-} MEFs stably expressing HA-NOXA or HA-NOXA^{3E} (the mutant that does not bind MCL1) were engineered to also express FLAG-MCL1 or FLAG-MCL1^{BCL2TM}. Lysates were prepared from cells cultured with proteasome inhibitor MG132 (10 µM for 8 h) and subjected to HA immunoprecipitation.
- (C) MCL1^{BCL2TM} associates predominantly with the heavy membrane fraction. Protein lysates were prepared from cytosolic (C) and heavy membrane (HM) fractions derived from *Mcl1*^{-/-} MEFs expressing either FLAG-MCL1 or FLAG-MCL1^{BCL2TM}.

Representative images in B-C from two independent experiments.

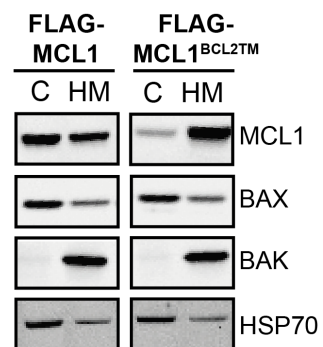
A



B



C



Supplementary Table 3.1 Quantitation of changes in sgRNA abundance after sorting for GFP^{high} cells.

The table comprises the following elements for top 50 enriched genes represented in the library: the total number of sgRNA targeting that gene in the library, the Log2 fold change for each of those sgRNA (calculated as residuals to the lowess curve fitted to the data in Fig. 3.2 B), and the ranked order for the Log2 fold change of each sgRNA. (N, B, n, b) correspond to the parameters that yielded the minimum hypergeometric p-value (Eden et al., 2007), where N is the population size (the total size of the library), B is the number of successes present in the library (sgRNA targeting a particular gene), n is the sample size, and b is the number of successes in the sample. p-values were corrected to false discovery rates (FDR) using the Benjamini-Hochberg method.

gene	Total sg	sg1.Log2FC	sg2.Log2FC	sg3.Log2FC	sg4.Log2FC	sg5.Log2FC	sg1.rank	sg2.rank	sg3.rank	sg4.rank	sg5.rank	(N,B,nb)	mHG.pValue	FDR
	Mar05	5	11.41	7.77	7.06	6.41	4.35	1	2	3	5	8 (67435, 5, 8, 5)	1.32E-21	2.52E-17
Mcl1		5	5.06	4.93	3.69	1.26	1.16	6	7	11	338	463 (67435, 5, 463, 5)	4.07E-12	3.90E-08
Mth2		5	3.84	3.2	1.85	0.12	0.05	10	12	56	18155	29407 (67435, 5, 56, 3)	2.49E-09	1.59E-05
Ube2k		5	4	1.56	1.5	0.33	0.32	9	122	155	6244	6467 (67435, 5, 155, 3)	5.45E-08	0.000260903
Krtap24-1		5	1.03	0.5	0.36	0.34	0.24	736	3563	5561	6007	9157 (67435, 5, 9157, 5)	1.26E-05	0.048207387
Ccdc150		5	1.16	0.6	0.39	0.29	0.23	463	2650	4989	7277	9606 (67435, 5, 9606, 5)	1.60E-05	0.04896702
Olftr1107		4	1.23	0.92	0.82	0.01 NA		368	1056	1448	39138 NA	(67435, 4, 1448, 3)	1.79E-05	0.04896702
Itm2a		5	0.74	0.62	0.62	0.45	-0.15	1830	2532	2532	4122	83386 (67435, 5, 4122, 4)	2.37E-05	0.056814143
Ccdc152		3	1.34	1.26	-0.12 NA	NA		256	338	81709 NA	NA	(67435, 3, 338, 2)	4.46E-05	0.09486683
Olftr482		4	0.56	0.39	0.32	0.27 NA		2985	4989	6467	7974 NA	(67435, 4, 7974, 4)	6.91E-05	0.132383975
Alpk2		5	1.02	0.46	0.27	0.21	0.17	761	4000	7974	10704	13325 (67435, 5, 13325, 5)	8.22E-05	0.14065027
Zbt25		5	1.31	0.76	0.45	0.35	-0.08	285	1728	4122	5787	75636 (67435, 5, 5787, 4)	9.08E-05	0.14085027
Vmn249		2	1.38	0.97 NA	NA	NA		225	894 NA	NA	NA	(67435, 2, 894, 2)	0.00010443	0.14085027
Nck1		5	0.47	0.38	0.35	0.21	0.16	3875	5182	5787	10704	14165 (67435, 5, 14165, 5)	0.000111532	0.14085027
Nlip9b		5	1.4	0.8	0.71	0.05	0	208	1539	1991	29407	41727 (67435, 5, 1991, 3)	0.000113917	0.14085027
Oog2		2	1.5	0.95 NA	NA	NA		155	949 NA	NA	NA	(67435, 2, 949, 2)	0.000117682	0.14085027
5530400C23Rik		4	1.86	0.41	0.29	0.23 NA		86	4648	7277	9606 NA	(67435, 4, 9606, 4)	0.000145609	0.153309719
4930428D18Rik		5	1.25	0.97	0.22	0.15	0.15	346	894	10154	15044	15044 (67435, 5, 15044, 5)	0.000150713	0.153309719
Olftr245		4	0.68	0.59	0.55	-0.09 NA		2167	2731	3080	77653 NA	(67435, 4, 3080, 3)	0.000170071	0.153309719
Gm20594		2	1.2	0.89 NA	NA	NA		401	1165 NA	NA	NA	(67435, 2, 1165, 2)	0.000177384	0.153309719
Plcl3		4	0.74	0.52	0.26	0.22 NA		1830	3368	8344	10154 NA	(67435, 4, 10154, 4)	0.000181794	0.153309719
Tex15		5	0.62	0.48	0.44	0.3	0.09	2532	3765	4237	7001	22193 (67435, 5, 7001, 4)	0.000192217	0.153309719
Vmn171		5	1.18	0.4	0.4	0.3	0.03	430	4814	4814	7001	33753 (67435, 5, 7001, 4)	0.000192217	0.153309719
Olftr505		4	0.63	0.58	0.53	-0.03 NA		2483	2826	3257	55691 NA	(67435, 4, 3257, 3)	0.000200806	0.153309719
Itnar1		5	2.18	0.85	0.52	0.29	0.07	29	1323	3368	7277	25510 (67435, 5, 7277, 4)	0.00022377	0.153309719
Tuba3b		5	0.77	0.39	0.35	0.29	-0.06	1676	4989	5787	7277	68998 (67435, 5, 7277, 4)	0.00022377	0.153309719
Fat2		5	6.63	0.37	0.02	-0.1	-0.17	4	5368	36413	79344	83713 (67435, 5, 4, 1)	0.000228726	0.153309719
Prr13		5	1.36	0.78	0.62	-0.06	-0.47	235	1634	2532	68998	86734 (67435, 5, 2532, 3)	0.000232166	0.153309719
Trim31		5	1.34	0.85	0.62	0.14	-0.06	256	1323	2532	16026	68998 (67435, 5, 2532, 3)	0.000232166	0.153309719
Acad7		3	0.63	0.58	0.36 NA	NA		2483	2826	5561 NA	NA	(67435, 3, 5561, 3)	0.000257148	0.157194031
Aida		4	0.58	0.53	0.48	0.2 NA		2826	3257	3765	11282 NA	(67435, 4, 11282, 4)	0.000277078	0.157194031
Olftr668		5	1.23	1.14	0.39	0.14	0	368	495	4989	16026	41727 (67435, 5, 495, 2)	0.000316273	0.157194031
Npm2		5	0.45	0.38	0.29	0.27	0	4122	5182	7277	7974	41727 (67435, 5, 7974, 4)	0.000320443	0.157194031
Stc2		5	0.83	0.68	0.46	0.27	-0.13	1413	2167	4000	7974	82448 (67435, 5, 7974, 4)	0.000320443	0.157194031
Olftr24		3	1.28	0.53	0.34 NA	NA		316	3257	6007 NA	NA	(67435, 3, 6007, 3)	0.000324127	0.157194031
Gm7714		4	1.83	1.06	0.34	0 NA		60	665	6007	41727 NA	(67435, 4, 665, 2)	0.000343064	0.157194031
Sh2t1b2		4	0.49	0.27	0.22	0.19 NA		3671	7974	10154	11902 NA	(67435, 4, 11902, 4)	0.000343202	0.157194031
Rad51ap2		5	1.41	1.13	0.08	0.03	-0.02	203	522	23825	33753	51012 (67435, 5, 522, 2)	0.000351535	0.157194031
Olftr250		3	1.83	0.34	0.33 NA	NA		60	6007	6244 NA	NA	(67435, 3, 6244, 3)	0.000364031	0.157194031
Pax6		4	0.99	0.91	0.46	0 NA		838	1086	4000	41727 NA	(67435, 4, 4000, 3)	0.000369591	0.157194031
Ace		5	0.87	0.81	0.37	0.26	-0.02	1246	1490	5368	8344	51012 (67435, 5, 8344, 4)	0.000382797	0.157194031
Il17b		5	1.42	0.48	0.41	0.26	-0.04	199	3765	4648	8344	60764 (67435, 5, 8344, 4)	0.000382797	0.157194031
Mic1		5	0.93	0.65	0.36	0.26	-0.11	1007	2336	5561	8344	80579 (67435, 5, 8344, 4)	0.000382797	0.157194031
Migpd		5	0.74	0.45	0.3	0.26	-0.04	1830	4122	7001	8344	60764 (67435, 5, 8344, 4)	0.000382797	0.157194031
Olftr497		5	0.79	0.3	0.27	0.26	-0.06	1595	7001	7974	8344	68998 (67435, 5, 8344, 4)	0.000382797	0.157194031
Mga		5	0.28	0.27	0.25	0.14	0.12	7596	7974	8760	16026	18155 (67435, 5, 18155, 5)	0.000385803	0.157194031
Slc38a11		5	0.37	0.29	0.23	0.13	0.12	5368	7277	9606	17006	18155 (67435, 5, 18155, 5)	0.000385803	0.157194031
Pramel5		4	0.52	0.41	0.29	0.18 NA		3368	4648	7277	12548 NA	(67435, 4, 12548, 4)	0.000424013	0.1657111
Stim1		4	0.75	0.57	0.23	0.18 NA		1778	2908	9606	12548 NA	(67435, 4, 12548, 4)	0.000424013	0.1657111

Supplementary Table 3.2 Indels measured at sgRNA target sites by DNA sequencing.

Indels were measured by sequencing PCR amplicons encompassing sgRNA target sites and aligning reads to the wild-type locus. In clones where more than two indel sizes were observed the parental cells likely possessed an additional copy (or copies) of the targeted gene. In clones where only one allele size was observed the parental cells may have had only one copy of the gene, or both alleles may have been repaired similarly to generate indels of the same size. No clones for which wild-type sequence remained were used in the study.

Cell Type	Target Gene	Clone/Pool Reference	sgRNA number	Indel sizes observed (for single cell-derived clones)	% of reads with indels (for uncloned populations)
WT MEF	<i>Mule</i>	2	1	Deletion of 3, Insertion of 9	N/A
WT MEF	<i>Mule</i>	5	1	Deletion of 15 and 9	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>β-TrCP</i>	2	1	Deletion of 7	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>β-TrCP</i>	4	1	Deletion of 7	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>Fbw7</i>	5	1	Deletion of 26 and 9	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>Fbw7</i>	7	1	Deletion of 10 and 5, Insertion of 7	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>Parkin</i>	1.3	1	Deletion of 2, Insertion of 1	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>Parkin</i>	2.3	2	Deletion of 5	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>March5</i>	1a	1	Deletion of 16, 10 and 1	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>March5</i>	1d	1	Deletion of 2, Insertion of 1	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>March5</i>	2a	2	Deletion of 13 and 10	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>March5</i>	2e	2	Deletion of 1 and 2	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>Mtch2</i>	1a	1	Deletion of 2 and 3, Insertion of 1	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>Mtch2</i>	1c	1	Deletion of 14 and 6	N/A
Bax ^{-/-} Bak ^{-/-} MEFs	<i>Mtch2</i>	2a	2	Insertion of 1	N/A
HCT116	<i>MARCH5</i>	1f	1	Insertion of 1	N/A
HCT116	<i>MARCH5</i>	2e	2	Deletion of 23 and 13	N/A
HCT116	<i>MTCH2</i>	1i	1	Deletion of 7 and 4	N/A
HCT116	<i>MTCH2</i>	1l	1	Deletion of 4	N/A
HCT116	<i>MTCH2</i>	1q	1	Deletion of 4, Insertion of 4	N/A
HCT116	<i>UBE2K</i>	1a	1	Deletion of 1	N/A
HCT116	<i>UBE2K</i>	1b	1	Deletion of 1, Insertion of 1	N/A
HCT116	<i>UBE2K</i>	2d	2	Deletion of 14, Insertion of 1	N/A
HCT116	<i>NOXA</i>	1d	1	Deletion of 5	N/A
HCT116	<i>NOXA</i>	2a	2	Insertion of 1	N/A
HCT116	<i>NOXA</i>	2c	2	Deletion of 1, Insertion of 2	N/A
HCT116	<i>MCL1</i>	1a	1	Deletion of 37	N/A
HCT116	<i>MCL1</i>	1c	1	Deletion of 17	N/A
HCT116	<i>MCL1</i>	1d	1	Deletion of 1 and 25	N/A
BAX ^{-/-} BAK ^{-/-} HELA	<i>MARCH5</i>	1h	1	Deletion of 5, Insertion of 1	N/A
BAX ^{-/-} BAK ^{-/-} HELA	<i>MARCH5</i>	2b	2	Deletion of 4 and 2, Insertion of 1	N/A
BAX ^{-/-} BAK ^{-/-} HELA	<i>MTCH2</i>	1a	1	Deletion of 13, 4 and 2	N/A
BAX ^{-/-} BAK ^{-/-} HELA	<i>MTCH2</i>	2f	2	Deletion of 10, 2 and 1	N/A
BAX ^{-/-} BAK ^{-/-} HELA	<i>NOXA</i>	1b	1	Deletion of 2, Insertion of 1	N/A
BAX ^{-/-} BAK ^{-/-} HELA	<i>NOXA</i>	2d	2	Deletion of 8 and 1	N/A
RS4;11	<i>MARCH5</i>	1	1	N/A	77
RS4;11	<i>MARCH5</i>	2	2	N/A	84
RS4;11	<i>MTCH2</i>	1	1	N/A	89
RS4;11	<i>MTCH2</i>	2	2	N/A	41
RS4;11	<i>NOXA</i>	1a	1	Deletion of 22, Insertion of 2	N/A
RS4;11	<i>NOXA</i>	1b	1	Deletion of 5, Insertion of 2	N/A
RS4;11	<i>NOXA</i>	1	1	N/A	71
RS4;11	<i>NOXA</i>	2	2	N/A	92
KMS-12-PE	<i>NOXA</i>	1c	1	Insertion of 1	N/A
KMS-12-PE	<i>NOXA</i>	2b	2	Deletion of 25	N/A
KMS-12-PE	<i>NOXA</i>	1	1	N/A	96
KMS-12-PE	<i>NOXA</i>	2	2	N/A	99
KMS-28-BM	<i>NOXA</i>	1a	1	Deletion of 5, Insertion of 1	N/A
KMS-28-BM	<i>NOXA</i>	2a	2	Deletion of 8	N/A
RPMI8226	<i>NOXA</i>	1	1	N/A	88
RPMI8226	<i>NOXA</i>	2	2	N/A	99
GRANTA	<i>NOXA</i>	1	1	N/A	87
GRANTA	<i>NOXA</i>	2	2	N/A	94
GRANTA	<i>MARCH5</i>	1	1	N/A	95
GRANTA	<i>MARCH5</i>	2	2	N/A	95
GRANTA	<i>MTCH2</i>	1	1	N/A	90
GRANTA	<i>MTCH2</i>	2	2	N/A	85

Discussion

It has been recognized for some time that the MCL1:NOXA complex is rapidly degraded by the proteasome, but the mechanism responsible has remained unclear (Czabotar et al., 2007; Willis et al., 2005). Using a genome-wide CRISPR-Cas9 screen I have now identified that MARCH5 and MTCH2 are both required for this process and elucidated certain aspects of how they work together.

It had been previously identified a motif (FRQKLL) within the NOXA BH3-domain that is important for causing MCL1 degradation (Czabotar et al., 2007). MARCH5 may recognize or bind to MCL1:NOXA complex in part through this motif (Fig. 3.8 D). I have now identified that the transmembrane domain of MCL1 also appears to be important, which is consistent with the notion that E3 ligases of the MARCH family commonly recognize their integral membrane protein substrates through interactions within the lipid bilayer (Corcoran et al., 2011; Jahnke et al., 2012; Tan et al., 2019; Tze et al., 2011).

My data indicates that two MCL1 lysine residues (K175 and K178) are necessary for degradation of the MCL1:NOXA complex. MARCH5 likely transfers ubiquitin to one or both MCL1 lysines located on the close proximity of FRQKLL motif upon NOXA binding (Czabotar et al., 2007). This ubiquitination marks MCL1 for degradation by the proteasome. The nature of the ubiquitin chain and whether it is formed on one or both of these lysine residues remains to be determined. These data do however indicate that the degradation of MCL1 is partly impaired when either of K175 or K178 is removed, and completely abrogated when both are absent (Fig. 3.1 C). The strict requirement for specific lysine residues is notable. In most cases E3 ligases can discharge ubiquitin onto a wide range of nearby accessible lysine residues. The MCL1 E3 ligase MULE, for example, could drive MCL1 degradation by ubiquitinating any of several lysine residues within its unstructured N-terminus (Zhong et al., 2005). That MARCH5 specifically requires K175 and K178 may imply that MCL1, NOXA, and MARCH5 must come together in a precise orientation to produce the correct environment for ubiquitin discharge to occur.

I show that MARCH5 and MTCH2 cooperate to degrade the MCL1:NOXA complex in a variety of cell types, indicating that this mechanism is well-conserved. Whilst this study does not specifically address where this degradation pathway has the greatest biological influence, I postulate that it may be most important in contexts where the ability of NOXA to counteract MCL1 is critical. Possibilities include B- and T-cell development and their responses to antigen (Wensveen et al., 2012; Wensveen et al., 2010) as well as during apoptotic responses to viral infection (Rosebeck, Sudini, Chen, & Leaman, 2011; Sun & Leaman, 2005). Notably, the induction of NOXA during viral infection is controlled in part by MAVS (Eitz Ferrer et al., 2011) – a key regulator of

antiviral signalling and another substrate of MARCH5 (Yoo et al., 2015). It is also becoming increasingly clear that NOXA can significantly influence the response of cancer cells to drug therapies, particularly BH3 mimetics such as venetoclax, as NOXA loss has been found to underpin resistance to these agents in lymphoid malignancies (Guieze et al., 2019; Tahir et al., 2017; Zhao et al., 2019). These findings confirm that NOXA can indeed be critical for the response of cancer cells to BH3 mimetics (Figs. 6 C-D and Sup Figs. 3.6 A-B).

The influence of MTCH2, an integral mitochondrial outer membrane protein, on NOXA-driven MCL1 degradation is also particularly intriguing. MTCH2 has not previously been linked to protein degradation, but has been reported to influence BCL2 family proteins through facilitating recruitment of the BH3-only protein tBID to mitochondria and thereby promoting apoptosis induction (Grinberg et al., 2005; Raemy et al., 2016; Zaltsman et al., 2010). However, in relation to MCL1 degradation by MARCH5, MTCH2 deletion did not appear to significantly impact the localization of MCL1, NOXA, or MARCH5 (Fig. 3.7 C). It is possible that MTCH2 serves as an adaptor protein that bridges interactions between MARCH5 and the MCL1:NOXA complex within the mitochondrial outer membrane (Fig. 3.8 D).

Although the precise function of MTCH2 at mitochondria remains uncertain, prior reports have primarily linked MTCH2 to two biological roles: cellular metabolism and apoptosis. The link to metabolism originates from the identification of MTCH2 as a genetic susceptibility locus for obesity (Renstrom et al., 2009; Willer et al., 2009), with subsequent studies in conditional knockout mice demonstrating altered metabolic profiles in cells lacking MTCH2 (Buzaglo-Azriel et al., 2016; Maryanovich et al., 2015). MTCH2 has also been associated with regulating mitochondrial fission and fusion dynamics (Bahat et al., 2018). The association with apoptosis, on the other hand, has until now centred on the ability of MTCH2 to recruit tBid to mitochondria (Grinberg et al., 2005; Zaltsman et al., 2010). These findings now associate MTCH2 with regulating additional BCL2 family proteins: MCL1 and NOXA. These findings strengthen the connection between MTCH2 and BCL2-regulated apoptosis and raise the possibility that MTCH2 may have a broader role in linking metabolism to apoptosis regulation.

The mechanism through which NOXA, MTCH2, and MARCH5 cooperate to drive MCL1 turnover may help to inform novel strategies for promoting MCL1 degradation as an alternative to inhibiting MCL1 with selective BH3 mimetics. MCL1 itself remains a highly compelling drug target. Although MCL1-selective inhibitors have been developed (Caenepeel et al., 2018; Kotschy et al., 2016; Tron et al., 2018), the critical importance of MCL1 in diverse healthy tissues suggests that the therapeutic window associated with inhibiting MCL1 systemically may be narrow. I anticipate that this will be especially true

in contexts where there would be advantages to combining MCL1 inhibitors with complimentary BCL2 protein inhibitors such as venetoclax. In such scenarios, the ability to inhibit MCL1 in a tissue or disease specific manner rather than systemically would be highly valuable. Harnessing protein degradation pathways, such as with PROTAC small molecules, is one way to achieve such contextual inhibition (Lai & Crews, 2017), and this is indeed a strategy being pursued for MCL1 (Papatzimas et al., 2019; Z. Wang et al., 2019). Distinct from PROTACs, compounds that act as “molecular glues” to enhance the activity of a cognate E3 ligase on its substrate have also recently been described (Simonetta et al., 2019). The novel details that I have elucidated for NOXA-driven MCL1 degradation could be highly informative for strategies such as these.

Chapter 4: Identifying novel regulators of MCL1 protein stability

Background

MCL1 is an attractive cancer therapeutic target because it is known to be overactive in a number of cancers and can promote resistance to many conventional cytotoxic drugs. Several inhibitors including S63845, AMG176 and AZD5991 directly target MCL1 and specifically induce apoptosis via BAX and BAK (Caenepeel et al., 2018; Kotschy et al., 2016; Tron et al., 2018). Both S63845 and AMG176 are considered safe to use as a single agent in mice, as they do not adversely affect normal tissues that depend on MCL1 for survival (Caenepeel et al., 2018; Kotschy et al., 2016). These mouse models, however, may not reveal potential on-target toxicities because both drugs bind less tightly to mouse MCL1 than the human counterpart. When humanized *MCL1* knock-in mice were treated with S63845, they were significantly more sensitive than wild-type controls (Brennan et al., 2018), highlighting the potentially harmful effects of inhibiting human MCL1 protein systemically. Although humanized *MCL1* knock-in mice can tolerate S63845 and AMG176 at the doses required for tumour growth inhibition, numerous adverse effects including reduction of multiple haematopoietic cell lineages still occur (Brennan et al., 2018; Caenepeel et al., 2018). Whether those adverse effects can be tolerated in humans remains to be seen.

As MCL1 plays major roles in development and maintenance of many tissues (Mojša et al., 2014; L. W. Thomas, Lam, & Edwards, 2010), systemic inhibition of MCL1 protein function may cause severe on-target toxicities. The anticipated toxicities include cardiac failure (R. L. Thomas et al., 2013; X. Wang et al., 2013) and liver damage (Vick et al., 2009) as both organs are essential for normal health. In fact, toxicities to these tissues commonly prevent compounds from progressing through development to become clinically-approved drugs. The toxicities may be even more severe when MCL1 inhibitor is used in combination with venetoclax, as multiple lymphoid cell lineages are likely to be significantly affected when both pro-survival proteins are inhibited (Dzhagalov et al., 2008; Kamada et al., 1995; Nakayama et al., 1994; Peperzak et al., 2013; Veis et al., 1993). One way to ameliorate these on-target toxicities is by directing MCL1 inhibition away from healthy tissues. Targeting MCL1 protein selectively in a subset of tissues rather than systemically may be more tolerable, and therefore safer for patients.

Reducing MCL1 protein expression represents one potential strategy to diminish its pro-survival activity. Reducing the rate of MCL1 protein synthesis has been attempted, but severe side effects may occur because those drugs target MCL1 by globally perturbing transcription or translation (Kitada et al., 2000; Lindqvist et al., 2012; G. Wei et al., 2006). Increasing the rate of MCL1 protein degradation is an alternative way to reduce its expression. MCL1 has been shown to be a substrate of several proteins of the ubiquitin proteasome system (UPS), which includes enzymes that act in opposition

to one another in respect to regulating MCL1 protein stability. For instance, several E3 ligases have been reported to ubiquitinate MCL1 and promote its degradation (Chapter 3)(Carroll et al., 2014; Ding et al., 2007; Subramanian et al., 2016; Wertz et al., 2011; Zhong et al., 2005). By enhancing the activity of these E3 ligases with a small molecule activator, MCL1 can be degraded and its pro-survival function can be inhibited. Alternatively, proteolysis targeting chimeras (PROTACs) (Ottis & Crews, 2017) could hijack unrelated E3 ligases to degrade MCL1 since many of its lysines are common sites for ubiquitin conjugation (Mojca et al., 2014).

Besides E3s, many deubiquitinating enzymes (DUBs) also regulate MCL1 protein levels. These include USP9X, USP13, USP24, KU70 and CDU1 that have been shown to remove ubiquitin from MCL1 and promote its stability (Fischer et al., 2017; Peterson et al., 2015; Schwickart et al., 2010; B. Wang et al., 2014; S. Zhang et al., 2018). Some of these DUBs such as USP9X and USP24 may be targeted by a small molecular inhibitor (e.g. G9 (Peterson et al., 2015)) to reduce the basal MCL1 protein level to promote apoptosis. This shows inhibiting a DUB with small molecule as an alternative viable option. Arresting catalytic reaction of DUB with an inhibitor that binds competitively in the enzyme active site or allosterically may successfully disrupt its activated form. However, because all DUBs bind to an identical substrate, ubiquitin, targeting a DUB selectively is still a challenge.

Most E3s and DUBs are attractive therapeutic targets because they control MCL1 protein levels only in certain contexts. Some of these enzymes are activated following distinct cellular stimuli, for example FBW7 is induced by cell cycle arrest (Wertz et al., 2011). Some are only expressed in a subset of tissues, for example USP13 is upregulated in lung and ovarian cancer cells, but not in healthy tissues (S. Zhang et al., 2018). Targeting these restrictively expressed regulators of MCL1 protein stability via small molecule inhibitors may provide a wider therapeutic window than systemic targeting of MCL1 by BH3 mimetics, as they are unlikely to affect MCL1 function in other normal tissues.

Most of the DUBs that have been described to date have only relatively modest impacts on MCL1 protein stability (Peterson et al., 2015; Schwickart et al., 2010; S. Zhang et al., 2018). In this thesis, I investigated and validated the impact of some of these DUBs on MCL1 protein stability. I hypothesized that additional DUBs may regulate MCL1 and utilized two complementary CRISPR-Cas9 screen approaches in an attempt to identify these DUBs.

The overall aims of this chapter were:

- Aim 1: Evaluate specific DUBs that have previously been reported to impact MCL1 protein stability.
- Aim 2: Perform unbiased genetic screens to identify novel DUBs impacting MCL1 protein stability.

Results

Evaluating the impact of USP9X and USP24 on MCL1 protein stability by gene knockout rather than gene knock-down

Several DUBs have been reported to act on MCL1 in different contexts, but only some of them (USP9X, USP13, USP24 and KU70) are known to control its basal protein level (Peterson et al., 2015; Schwickart et al., 2010; B. Wang et al., 2014; S. Zhang et al., 2018). Individual knock-down of these DUBs reduces the MCL1 protein level, albeit rather modestly (Peterson et al., 2015; Schwickart et al., 2010; S. Zhang et al., 2018). The redundancy of DUBs in recognizing their substrates may explain the relatively mild impact on MCL1 when these DUBs were individually knocked down (Komander, 2009), as could the incomplete inhibition of target mRNA that is sometimes observed when using RNA interference (Jackson & Linsley, 2010; Ui-Tei, 2013). The first aim of this chapter was to investigate the impact of some of these DUBs on MCL1 protein stability using gene knockout rather than the less efficient gene knock-down.

I specifically investigated the role of two DUBs (USP9X and USP24) using CRISPR-Cas9 gene targeting. When *USP9X* was deleted in HEK293T cells, there was little impact on the basal MCL1 protein level, consistent with a previous study (Schwickart et al., 2010) (Fig. 4.1 A). (HCT116), the MCL1 level was also not reduced following *USP9X* deletion, although this may be due to the increase of overall protein loading in those cells (Fig. 4.1 B). Similarly, the half-life of MCL1 protein was unaltered in *USP9X*^{-/-} HEK293T cells (Fig. 4.1 C). When *Usp24* was deleted in MEFs, there was little impact on MCL1 protein stability (Fig. 4.1 D).

These data suggest that USP9X and USP24 are not by themselves major regulators of MCL1 protein stability, although they may cooperate or act redundantly with other enzymes.

Genome-wide CRISPR-Cas9 screen to identify genes that stabilize MCL1 protein

I next undertook an unbiased CRISPR-Cas9 screen to identify genes whose deletion reduced the expression of MCL1 protein. I utilized a GFP-MCL1 reporter as described in Chapter 3, and performed the screen with similar methodology. However, I adjusted the gating and sorting strategy to instead enrich for sgRNAs that caused a reduction of GFP-MCL1 from its baseline levels (Fig. 4.2 A). These sgRNAs were expected to include target genes that may be important for stable expression of MCL1, such as DUBs.

To perform the screen, two independent *Mcl1*^{-/-} MEF single cell clones were engineered to express MCL1 tagged at its N-terminus with GFP and Cas9 (Fig. 4.2 B).

Each single clone was infected with a genome-wide lentiviral sgRNA library targeting mouse genes (Koike-Yusa et al., 2014) at MOI around 1 in 2 independent replicates. The expected coverage of the library was approximately 25 cells infected for each sgRNA. Following infection, a small fraction of cells lost GFP-MCL1 expression (Figs. 4.2 C-D). These GFP^{low} cells were enriched through multiple rounds of sorting (Figs. 4.2 C-D) and their sgRNAs were quantitated by next generation sequencing (Fig. 4.3). Details of the analysis are included in Chapter 2: Materials and Methods.

Mcl1 and *Prdm4* were the two genes for which sgRNAs were most significantly enriched. Three of the five sgRNAs targeting *Mcl1* were enriched in the screen (Figs. 4.3 B-D). Each of these three sgRNAs targets the DNA region encoding for a portion of the MCL1 protein directly downstream of its PEST domain. I hypothesized that the truncated GFP-PEST fusion proteins resulting from indels introduced by these *Mcl1* sgRNAs were likely to be more labile than the full length GFP-MCL1 and be degraded more rapidly by the proteasome. This is in contrast to the evidence presented in Chapter 3 that truncated GFP-MCL1 resulting from five independent sgRNAs was significantly more stable than full-length GFP-MCL1. This may be explained by the overexpression of NOXA in the previous screen. Although truncated GFP-PEST fusion proteins are labile, they are more stable than the full-length counterparts that are degraded following NOXA binding. Of interest, all sgRNAs targeting *Prdm4* were significantly enriched in the screen (Figs. 4.3 A-D).

No DUBs were significantly enriched in the screen. Among genes related to the UPS pathway, *Ubap1* and *Ubqln1* (highlighted in Figs. 4.3 A-D) were the only ones enriched. As the sgRNAs targeting these genes only exhibited modest enrichment, I did not evaluate these targets further.

Evaluating the impact of deleting *Prdm4* on MCL1 protein stability

To determine whether PRDM4 regulates MCL1 protein stability, I assessed the impact of deleting *Prdm4* from *Bax*^{-/-} *Bak*^{-/-} MEFs expressing the GFP-MCL1 reporter alongside endogenous MCL1. While deletion of *Prdm4* significantly reduced the expression of exogenous GFP-MCL1, it had no impact on endogenous MCL1 (Fig. 4.4 A), suggesting that PRDM4 may not specifically regulate MCL1 protein stability.

PRDM4 belongs to a family of transcription factors. It has been reported to modify histones and interact with other transcriptional activators to promote gene transcription (Bogani et al., 2013; Chittka, Nitarska, Grazini, & Richardson, 2012; H. Liu et al., 2018). I hypothesized that *Prdm4* may have been identified in the screen because it controls the expression of genes transcribed from the MSCV retroviral vector used to drive

expression of GFP-MCL1. To test this idea, *Bax*^{-/-} *Bak*^{-/-} MEFs were infected with MSCV retroviruses encoding either GFP or GFP-MCL1. The impact of *Prdm4* deletion on the expression of these genes was assessed by flow cytometry. Deleting *Prdm4* reduced the level of GFP or GFP-MCL1 (Fig. 4.4 B). Thus, it seems that PRDM4 plays a role in promoting or maintaining the expression of genes transcribed from the integrated MSCV retroviral promoter.

Engineering an MCL1 dependent cell for CRISPR-Cas9 drop-out screening

Given that no DUBs influencing MCL1 protein stability were identified in the first screen, I decided to pursue an alternative strategy. The strategy was based on selecting for cells that are highly dependent on MCL1 for their survival (Fig. 4.5 A). With intact MCL1 protein function, these cells should survive (Fig. 4.5 A (i)). However, they may be sensitive to perturbations that reduce MCL1 expression including deletion of *Mcl1* or DUBs that promote MCL1 stabilization (Fig. 4.5 A (ii-iii)). Theoretically, because of the disturbance in cellular fitness, these cells may be lost after multiple rounds of cell culture passages.

Co-inhibition of both BCLxL and MCL1 are needed for MEFs to undergo apoptosis. To generate MCL1 dependent MEFs, *Bclxl* was therefore deleted in *Bcl2*^{-/-} MEFs. *Bclxl* gene deletion was confirmed by sequencing and the lack of both BCL2 and BCLxL expression was confirmed by western blotting (Fig. 4.5 B). *Bcl2*^{-/-} *Bclxl*^{-/-} MEFs were highly sensitive to MCL1 inhibition (Fig. 4.5 C), confirming that they are highly dependent on MCL1 for survival. In parallel, *Bcl2*^{-/-} *Bclxl*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs were also generated (Fig. 4.5 D) as controls because they should be insensitive to *Mcl1* depletion or inhibition, as confirmed in the subsequent validation assay (Fig. 4.5 E).

Cell viability-based CRISPR-Cas9 drop-out screen to identify deubiquitinating enzymes of MCL1

The CRISPR-Cas9 drop-out screen was then performed using these cells. The basis of the screen was that sgRNAs that target genes that promote MCL1 stability should be depleted in *Bcl2*^{-/-} *Bclxl*^{-/-}, but not *Bcl2*^{-/-} *Bclxl*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs (Fig. 4.6 A). To conduct the screen, two independent single cell clones from both genotypes were transduced with a retrovirus encoding for Cas9. They were then transduced with lentivirus encoding a sgRNA library targeting genes associated with the UPS pathway and control genes belonging to the BCL2 protein family at a similar MOI between the two genotypes (Fig. 4.6 A). Two days after transduction, these cells were selected with puromycin for 4 days. At 6 and 16 days after infection, sgRNAs were then quantitated and ranked by the degree

to which they were depleted specifically in *Bcl2*^{-/-} *Bclxl*^{-/-}, but not *Bcl2*^{-/-} *Bclxl*^{-/-} *Bax*^{-/-} *Bak*^{-/-} clones (Figs. 4.6 B-D). Details of the analysis are included in Chapter 2: Materials and Methods.

The top three hits from the screen were *Mcl1*, *Bcl2l2* and *March5*. Multiple sgRNAs targeting *Mcl1* were depleted, which was expected because *Bcl2*^{-/-} *Bclxl*^{-/-} MEFs depend on MCL1 for their survival. sgRNAs targeting *Bcl2l2*, the gene for BCLW, were also depleted, indicating that in the absence of BCL2 and BCLXL, BCLW also contributes to the survival of these cells. *March5* sgRNAs depletion was also not surprising because *March5* deletion has been shown to impact on mitochondrial morphology and cause cellular stress (Cherok et al., 2017; Subramanian et al., 2016; Xu et al., 2016).

The next three most significantly depleted genes were HECT-type E3 ligase *Ubr5* and *Trip12*, and the DUB *Otud5* (Figs. 5.6 B-D), but their impacts were relatively mild. To validate these genes, *Ubr5*, *Otud5* or *Trip12* were individually deleted from MEFs and their deletion was confirmed by sequencing (Fig. 5.6 E). Individually deleting these genes did not have a large impact on basal MCL1 protein expression (Fig. 5.6 E). This suggests that they do not play major roles on their own in regulating MCL1 basal levels in MEFs.

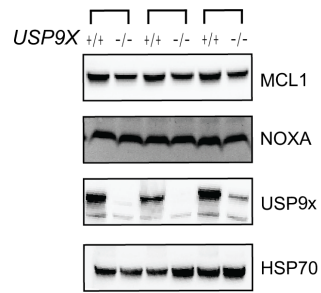
Figure 4.1

USP9X had a modest impact on MCL1 protein stability, while USP24 had no discernible impact.

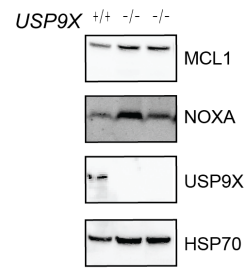
- (A-B) Deleting *USP9X* had little impact on the basal MCL1 protein level in HEK293T (A) or HCT116 (B). The levels of MCL1 protein in WT or USP9X-deficient cells were determined by immunoblotting. *USP9X*^{-/-} single clones in A and B were generated using two independent sgRNAs.
- (C) The turnover of MCL1 protein was unaltered in *USP9X*^{-/-} HEK293T cells. WT or USP9X-deficient HEK293T cells were cultured with the protein synthesis inhibitor cycloheximide (CHX; 50 µg/mL) for up to 4 h.
- (D) Deleting *Usp24* from MEFs had no discernable impact on the basal expression of MCL1. *Usp24*^{-/-} single clones were kindly provided by Shuai Huang and were generated using two independent sgRNAs.

Representative images in A, B and C from two independent experiments.

A HEK 293T



B HCT116



C HEK 293T



D MEFs

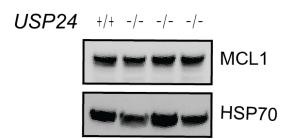


Figure 4.2

Genome-wide CRISPR/Cas9 screen using a GFP-MCL1 reporter to identify genes whose deletion results in reduced MCL1 protein expression.

- (A) Outline of the genome-wide CRISPR-Cas9 enrichment screen to identify genes whose deletion results in reduced MCL1 protein expression.
- (B) Engineering two independent *Mcl1*^{-/-} MEF single cell clones that express both GFP-MCL1 and Cas9-T2A-mCherry.
- (C) Selection strategy to enrich for cells with reduced GFP-MCL1 expression. Cells expressing GFP-MCL1 and Cas9-T2A-mCherry were transduced with a genome-wide lentiviral sgRNA library. GFP^{low} cells became more abundant following transduction (ii) and were enriched through multiple rounds of flow cytometry sorting (iii – v).
- (D) Proportion of cells expressing low GFP-MCL1 fusion protein levels after each round of sorting.

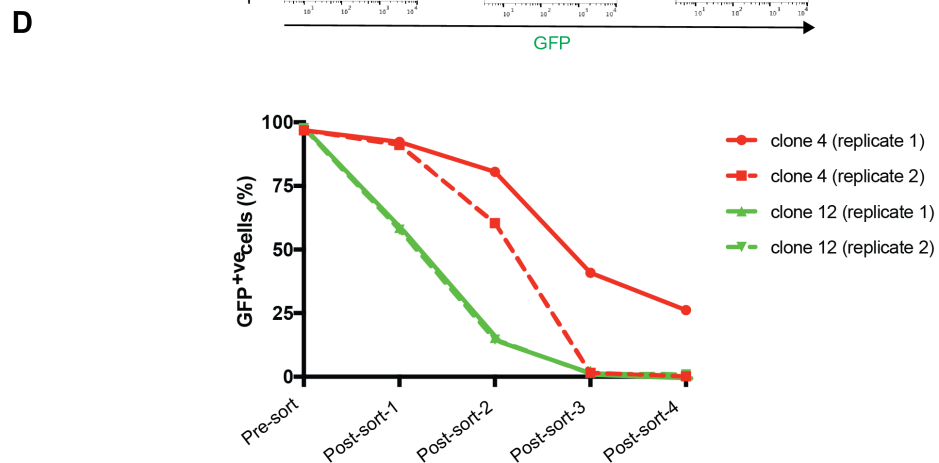
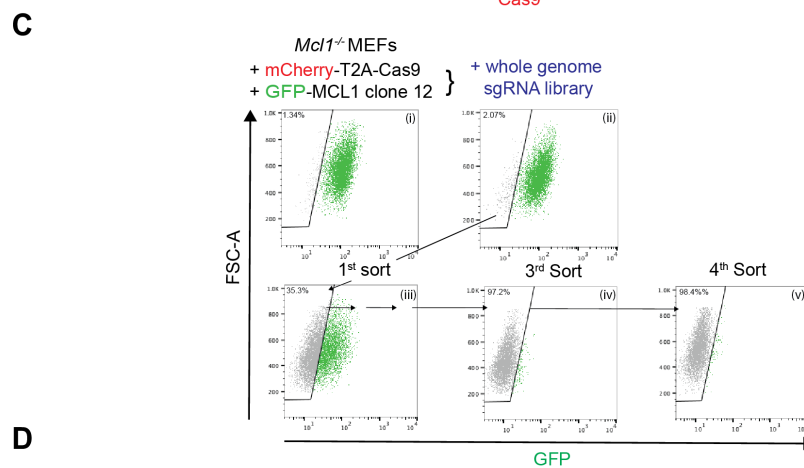
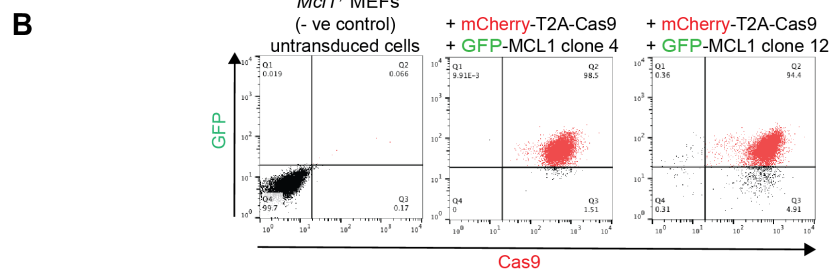
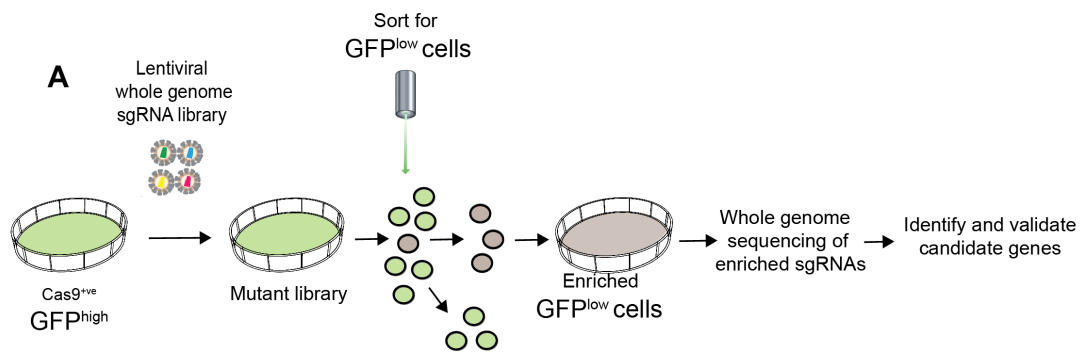


Figure 4.3

sgRNAs targeting *Prdm4* and *Mcl1* were enriched among the cells sorted for low GFP-MCL1 expression.

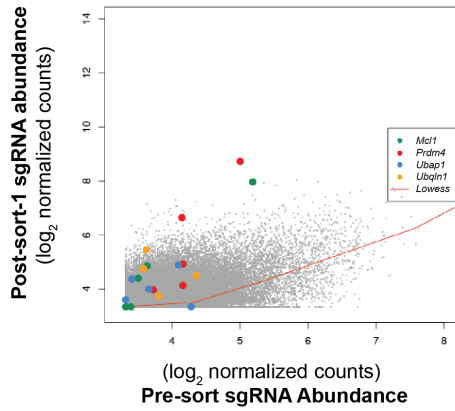
Plots show quantitation of sgRNA abundance in the transduced cells before and after each round of sorting for GFP^{low} cells (from 1st round (A) to 4th round (D) respectively). The red line represent a Lowess curve fitted to the median of data. Individual dots represent independent sgRNAs.

Tables below each plot show the ranked order of individual sgRNAs targeting the ten most enriched genes after each round of sorting (from 1st round (A) to 4th round (D) respectively). Data compiled from four independent experiments (see Fig. 4.2 D). Details of the analysis are included in the Materials and Methods.

sgRNAs targeting *Prdm4* and *Mcl1* were significantly enriched within the sorted GFP^{low} cell population (FDR < 0.001 followings sorts 2, 3, and 4). *Ubqln1* and *Ubap1* are highlighted as the most significantly enriched genes related to the UPS pathway, although both exhibited only very modest enrichment and did not reach an FDR value of less than 0.1 at any stage.

A

Post-Sort-1

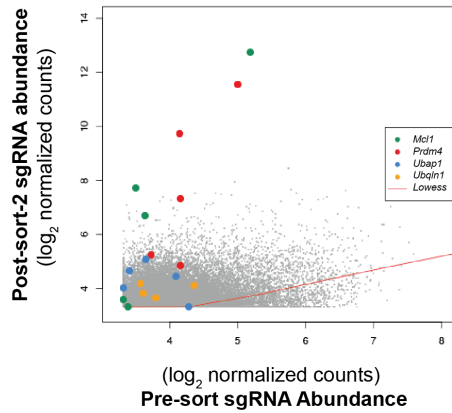


Gene	sg1.rank	sg2.rank	sg3.rank	sg4.rank	sg5.rank	FDR.qValue
Mefta13	688	2658	4628	6207	8088	0.06439838
Prdm4	2	87	2487	11650	15436	0.06439838
Olfr307	1352	1917	2973	6917	8923	0.06439838
Adcy3	3004	5039	5408	6717	9413	0.06439838
Olfr707	315	1074	1418	11112	NA	0.06439838
Vmn1r8	271	1266	5582	6109	NA	0.067068571
Ram2	645	7248	7822	8677	10464	0.067068571
Pion	1401	2912	4323	4411	60437	0.071790142
Mib1	2712	7486	9320	10890	11154	0.071790142
Cohn3	1881	2552	3121	NA	NA	0.086646989
Cyp2t12	692	1069	2051	26874	NA	0.086646989
Mbr4	1	18002	20043	22113	69118	0.086646989

sgRNA Rank
1 - 10
11 - 100
101 - 1000
1001 - 10000
> 10000

B

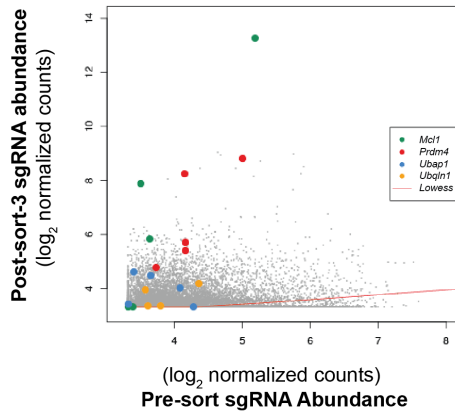
Post-Sort-2



Gene	sg1.rank	sg2.rank	sg3.rank	sg4.rank	sg5.rank	FDR.qValue
Prdm4	3	4	18	607	1334	1.40E-06
Mcl1	1	8	31	15972	57329	1.79E-05
Cideb	45	109	32465	66031	79148	0.066798626
Smadca4	77	2036	2715	4116	43706	0.066798626
Smadca4	657	1040	1185	31648	79097	0.066798626
Ankrd7	825	1957	3232	4188	34768	0.066798626
Ical	393	1613	3354	4238	61832	0.066798626
Olfr707	3873	4850	4893	6357	NA	0.066798626
Adcy2	6250	9488	11033	11238	11807	0.08672145
Olfr1412	1980	8811	9730	12077	12347	0.08672145
Pdp1	210	3098	3639	5204	26340	0.08672145
Fam48a	46	278	60328	72203	NA	0.08672145

C

Post-Sort-3

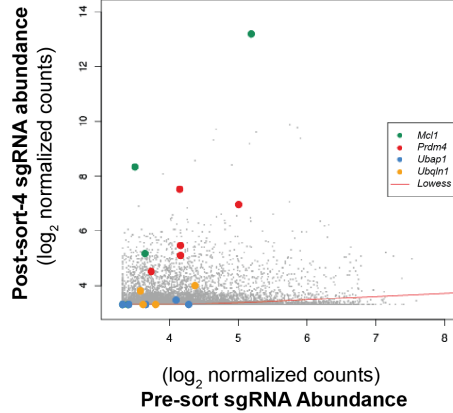


Gene	sg1.rank	sg2.rank	sg3.rank	sg4.rank	sg5.rank	FDR.qValue
Prdm4	6	11	104	155	506	1.22E-07
Mcl1	1	15	82	74730	74730	7.60E-05
Olfr6	794	1583	1844	NA	NA	0.059763133
Ical	788	965	1044	74730	74730	0.077418232
Aoad8	68	125	74730	74730	74730	0.077418232
Ubqln1	1442	2664	6709	6732	NA	0.112020289
Pcdha12	2131	3959	4455	4831	74730	0.121653157
Bcl2l2	183	845	2774	5186	74730	0.138752881
Olfr707	490	2014	3159	8200	NA	0.147977688
Rdh9	144	3763	7010	8200	NA	0.147977688
Ubap1	709	872	1853	6393	74730	0.153235845
Smadca4	542	1380	2662	5879	74730	0.153235845

sgRNA Rank
1 - 10
11 - 100
101 - 1000
1001 - 10000
> 10000

D

Post-Sort-4

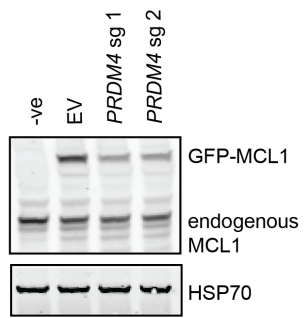


Gene	sg1.rank	sg2.rank	sg3.rank	sg4.rank	sg5.rank	FDR.qValue
Prdm4	14	16	118	198	538	1.66E-07
Mcl1	1	12	183	74734	74734	0.000860613
Cmb1	298	1095	3290	3522	74734	0.068911515
Aoad8	55	104	74734	74734	74734	0.068911515
Fam48a	25	193	74734	74734	NA	0.111039609
Olfr6	835	2359	3196	NA	NA	0.155682582
Foxp1	312	854	1642	5942	80555	0.175072328
Tipcl	322	1129	1750	74734	74734	0.175072328
Olfr68	51	275	74734	74734	74734	0.175072328
Mefta13	91	1042	5942	5942	74734	0.175072328
Sic10a2	1483	2140	2405	6007	74734	0.175072328
Mbr4	2	4118	74734	74734	74734	0.175072328

Figure 4.4

PRDM4 does not regulate MCL1 protein post-translationally.

- (A) Deleting *Prdm4* reduces the expression of exogenous GFP-MCL1, but not endogenous MCL1. *Bax*^{-/-} *Bak*^{-/-} MEFs expressing GFP-MCL1 and Cas9 were transduced with a lentivirus encoding two independent sgRNAs targeting *Prdm4* or an empty vector control. The cells were analyzed as a polyclonal population. Representative image from two independent experiments.
- (B) Deleting *Prdm4* reduces the expression of genes transcribed from the MSCV retroviral promoter. *Bax*^{-/-} *Bak*^{-/-} MEFs expressing Cas9 were infected with an MSCV retrovirus encoding for either GFP or GFP-MCL1. Where indicated, the cells were subsequently transduced with a lentivirus encoding two independent sgRNAs targeting *Prdm4* or an empty vector control. The expression of GFP (or GFP-MCL1) in these polyclonal populations was then measured by flow cytometry.

A*Bax*^{-/-} *Bak*^{-/-} MEFs+ GFP-MCL1
+ Cas9**B***Bax*^{-/-} *Bak*^{-/-} MEFs

neg control
 + Cas9 + GFP-MCL1 + EV
 + Cas9 + GFP-MCL1 + *Prdm4* sg1
 + Cas9 + GFP-MCL1 + *Prdm4* sg2

neg control
 + Cas9 + acGFP + EV
 + Cas9 + acGFP + *Prdm4* sg1
 + Cas9 + acGFP + *Prdm4* sg2

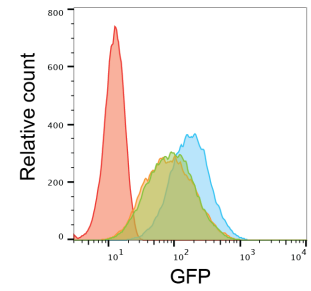
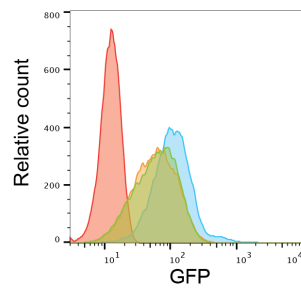


Figure 4.5

Generating *Bcl2*^{-/-} *Bclxl*^{-/-} and the controls *Bcl2*^{-/-} *Bclxl*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs for the purpose of cell viability-based CRISPR/Cas9 drop-out screening.

- (A) Schematic detailing the cellular fitness of an MCL1-dependent cell. Cells with intact MCL1 will survive (i), but cells in which *Mcl1* is deleted will not (ii). Alternatively, deleting DUBs that stabilize MCL1 (iii) may lead to loss of these targeted cells.
- (B) MCL1-dependent MEFs were generated by deleting *Bclxl* from *Bcl2*^{-/-} MEFs using CRISPR-Cas9 gene targeting. The lack of expression of BCLxL and BCL2 was confirmed by immunoblotting.
- (C) *Bcl2*^{-/-} *Bclxl*^{-/-} MEFs are sensitive to selective inhibition of MCL1 protein. WT, *Bcl2*^{-/-} or *Bcl2*^{-/-} *Bclxl*^{-/-} MEFs were treated with MCL1 inhibitor S63845 (MCLi) for 24h. Cell viability was determined by PI exclusion.
- (D) *Bcl2*^{-/-} *Bclxl*^{-/-} MEFs lacking BAX and BAK were generated as control cell lines for contrast because reducing MCL1 expression in these cells should not affect cellular fitness. The lack of expression of both BAX and BAK was confirmed by immunoblotting.
- (E) *Bcl2*^{-/-} *Bclxl*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs are resistant to selective inhibition of MCL1 protein. *Bcl2*^{-/-} *Bclxl*^{-/-} MEFs and clones derived from them lacking both BAX and BAK were treated with MCL1i for 24h. Cell viability was determined by PI exclusion.

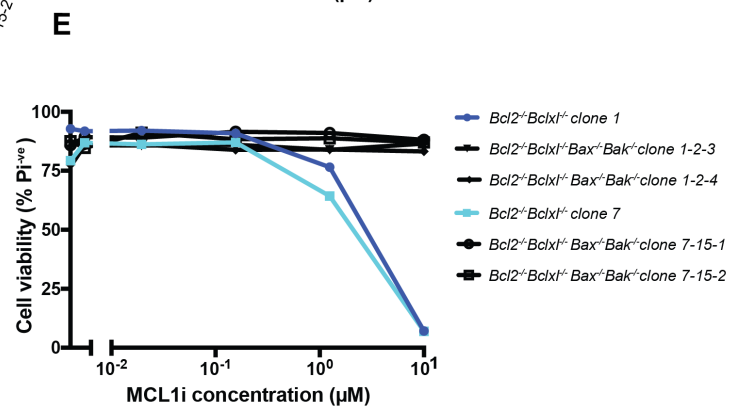
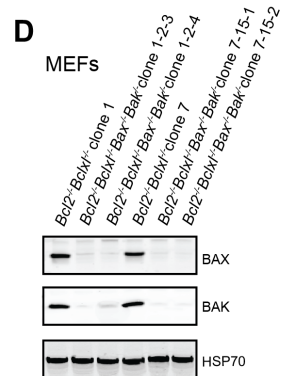
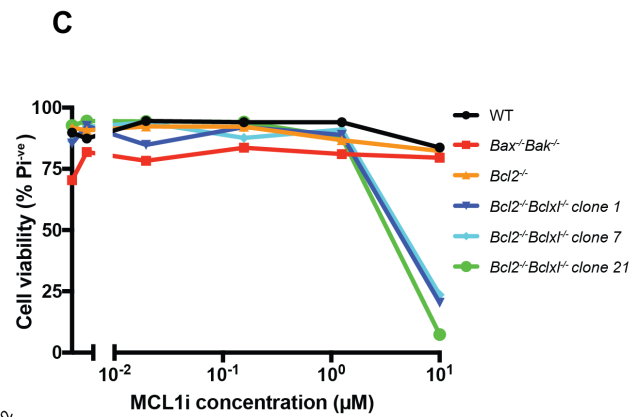
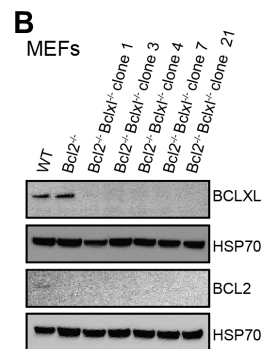
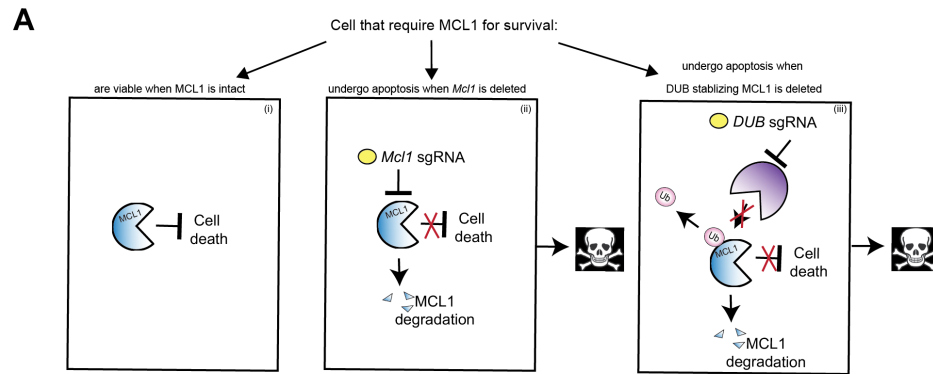
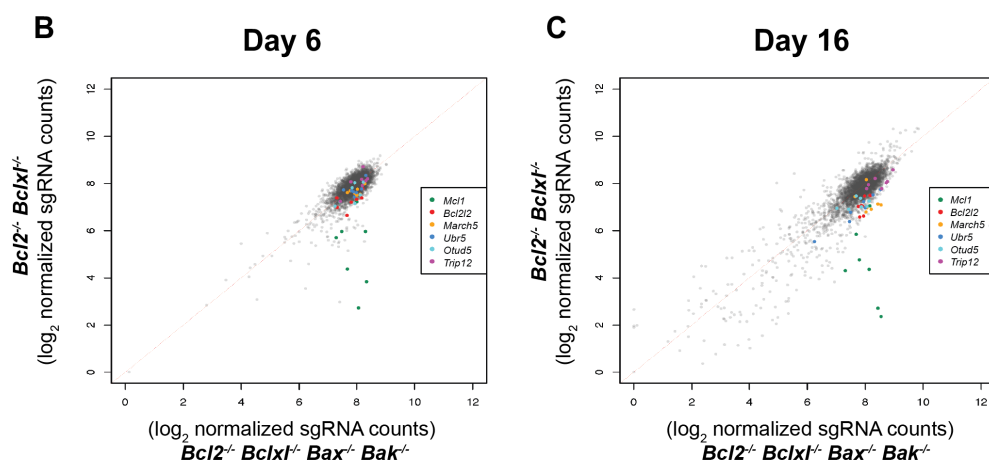
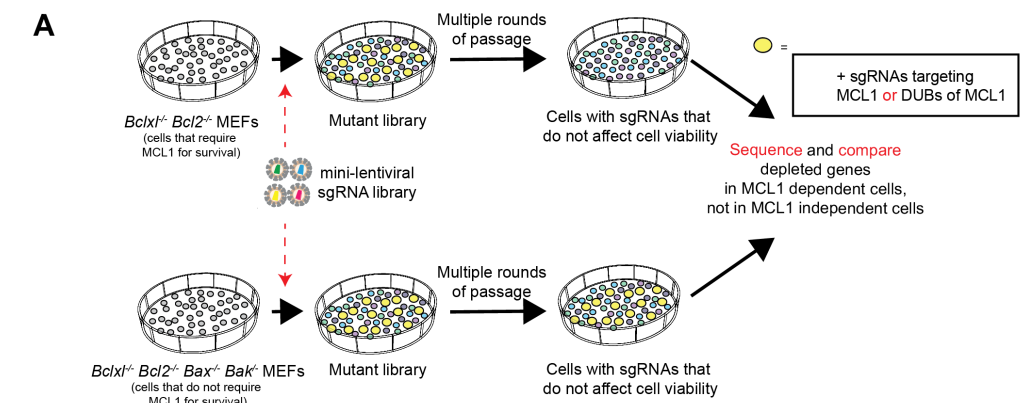


Figure 4.6

CRISPR/Cas9 drop-out screen identifies *Ubr5*, *Otud5* and *Trip12* as potential regulators of MCL1 protein stability.

- (A) Outline of the CRISPR-Cas9 drop-out screen using a sgRNA library targeting genes of the UPS (including E3 ligases, DUBs and UPS-associated proteins) and members of the BCL2 protein family.
- (B-C) Quantitation of sgRNA abundance in *Bcl2*^{-/-} *Bclxl*^{-/-} (DKO) compared to *Bcl2*^{-/-} *Bclxl*^{-/-} *Bax*^{-/-} *Bak*^{-/-} (QKO) MEFs after 6 days (B) or 16 days (C) of culture following transduction with the sgRNA library. The red line represents a Lowess curve fitted to the data median. Individual dots represent independent sgRNAs. Individual sgRNAs targeting *Mcl1*, *Bcl2l2*, *March5*, *Ubr5*, *Otud5* and *Trip12* are highlighted as genes for which sgRNAs were consistently depleted to a greater extent in *Bcl2*^{-/-} *Bclxl*^{-/-} MEFs compared to *Bcl2*^{-/-} *Bclxl*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs.
- (D) Table showing the rank order of individual sgRNAs by their degree of depletion in *Bcl2*^{-/-} *Bclxl*^{-/-} compared to *Bcl2*^{-/-} *Bclxl*^{-/-} *Bax*^{-/-} *Bak*^{-/-} MEFs for the ten most significantly depleted genes. Data compiled from two independent experiments. Details of the analysis are included in the Materials and Methods.
- (E) Deleting *Ubr5*, *Otud5* or *Trip12* had little impact on MCL1 basal expression in MEFs. The levels of MCL1 protein were determined in WT MEFs or clones in which *Ubr5*, *Otud5* or *Trip12* had been deleted with specific sgRNAs. *Ubr5*^{-/-} and *Trip12*^{-/-} MEF single cell clones were generated using two independent sgRNAs, whereas *Otud5*^{-/-} MEF single cell clones were generated using one independent sgRNA. Gene indels was confirmed by sequencing in each of the knockout clones.

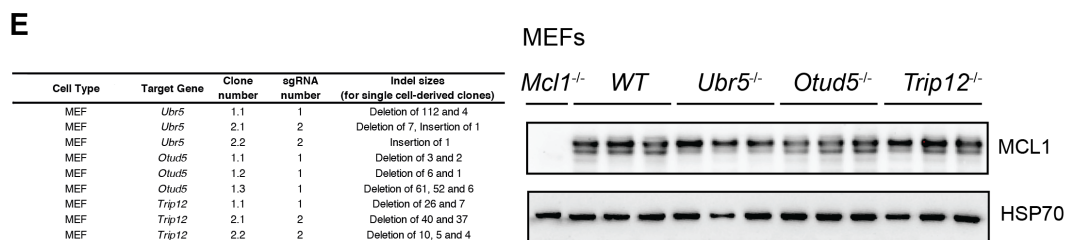


D

Gene	sg1.rank	sg2.rank	sg3.rank	sg4.rank	sg5.rank	sg6.rank	sg7.rank	FDR.qValue
<i>Mcl1</i>	1	2	3	4	5	6	7	1.58E-19
<i>Bcl2l2</i>	9	13	17	21	23	150	450	2.16E-08
<i>Mar-05</i>	8	12	15	16	26	854	2487	2.82E-08
<i>Ubr5</i>	49	50	73	159	492	639	3835	0.01019657
<i>Otud5</i>	66	92	109	200	387	1113	2001	0.01550155
<i>Trip12</i>	113	225	407	493	711	818	1884	0.03610722
<i>Cbl</i>	324	586	680	997	1213	1231	1369	0.03804993
<i>Usp2</i>	256	365	425	445	514	2590	2908	0.03804993
<i>Usp22</i>	39	391	404	462	712	899	1623	0.03881227
<i>Mib1</i>	54	81	117	369	2649	3372	3473	0.03881227
<i>Trim58</i>	115	722	752	1057	1139	1440	1461	0.03881227

sgRNA Rank

- 1 - 15
- 16 - 60
- 61 - 250
- 251 - 1000
- > 1000



Discussion

MCL1 is a labile protein that is heavily regulated by E3 ligases and DUBs. Altering the activity of these enzymes can alter MCL1 protein expression, and potentially its pro-survival function (Mojša et al., 2014). Deleting *USP9X* or *USP13*, for example, renders cells more sensitive to apoptotic stimuli, albeit mildly (Schwickart et al., 2010; S. Zhang et al., 2018). Identifying a DUB that has a much larger impact may provide an indirect and effective way to target MCL1 for cancer treatment. This is important because targeting MCL1 directly with small molecule inhibitors may not be well tolerated in human patients given the diverse roles of MCL1 in the survival of many normal tissues (Arbour et al., 2008; Dzhagalov et al., 2008; Dzhagalov et al., 2007; Hikita et al., 2009; Rinkenberger et al., 2000; Vikstrom et al., 2010; X. Wang et al., 2013). Identifying a deubiquitinating enzyme that promotes MCL1 stabilization in a tissue-specific context (examples include *USP9X* and *USP24* in B cell tumours (Peterson et al., 2015)) may allow a larger therapeutic window to modulate MCL1 activity. A small molecule inhibitor that targets such a DUB could be safer to use in humans than a systemic MCL1 inhibitor.

From the examples I investigated, individually deleting known DUBs of MCL1 has little impact on MCL1 stability (Fig. 4.1). I therefore performed two complementary screens to identify novel candidates, but neither of these uncovered genes that had a major impact on MCL1 levels (Figs. 4.3, 4.4 and 4.6).

In the screen where GFP-MCL1^{low} cells were enriched, the most significant hit was not a DUB (Fig 4.3), but instead was PRDM4. This protein was a false positive hit that impacted the expression of any proteins whose genes were transcribed from the MSCV vector used in this screen (Fig. 4.4) (Bogani et al., 2013; Chittka et al., 2012; H. Liu et al., 2018). Since this chapter focussed on the identification of novel UPS proteins controlling MCL1 protein stability, some ubiquitin-associated genes such as *Ubap1* and *Ubqln1* were intentionally highlighted despite them being enriched very mildly. These genes may impact on MCL1 protein indirectly since they have been reported to be critical for proteasomal degradation of various ubiquitinated substrates (Ko, Uehara, Tsuruma, & Nomura, 2004; Lu et al., 2009; Stefani et al., 2011). However, they were not investigated further since targeting them is unlikely to have significant impact on MCL1 protein levels.

In the MCL1-dependent cell viability screen, novel candidates such *Ubr5*, *Otud5* and *Trip12* were identified (Figs. 4.6 B-D). Although individually deleting these genes did not have a large impact on basal MCL1 protein expression in MEFs (Fig. 4.6 E), these candidates may play a more prominent role in regulating MCL1 expression in other contexts. For example, UBR5 has been reported to promote *Mcl1* transcription and its deletion sensitizes cells to DNA damage (Bradley et al., 2014). UBR5 may also act as a

complex with other candidates, OTUD5 and TRIP12. While OTUD5 can stabilize UBR5 (de Vivo et al., 2019), TRIP12 has been reported to act with UBR5 to suppress the silencing of various genes at damaged chromatin (Gudjonsson et al., 2012). This highlights the possibility that UBR5, OTUD5 and TRIP12 may act in the same pathway to control *Mcl1* transcription following DNA damage or in other contexts.

It was disappointing that neither screen identified DUB candidates with strong enough evidence to justify follow-up. However, both screens identified expected genes (for example *Mcl1*, *Bcl2l2* and *March5* (Figs. 4.3 and 4.6 B-D)), indicating that the screens worked properly in a technical sense. Other factors, therefore, may be associated with how DUBs stabilize their substrates and may have prevented discovery of a novel DUB of MCL1. The following paragraphs offer potential explanations for the lack of promising hits, and possible improvements to the design of the screens to circumvent these limitations.

DUBs may be acting redundantly on MCL1, and therefore knockout of a single DUB may be insufficient to lower MCL1 protein levels. In hindsight, many DUBs of MCL1 (e.g. USP9X, USP13 and USP24) are members of the Ubiquitin-Specific Protease (USP) family. Members of this family have been shown to co-regulate a single substrate, such as P53 (Graner et al., 2004; Potu et al., 2014; Stevenson et al., 2007). MCL1 is also co-regulated by USP24 and USP9X in multiple myeloma and deletion of *USP9X* has been shown to be compensated by USP24 upregulation (Peterson et al., 2015). In MEFs, where both screens were conducted, there could be potential redundancy of DUBs regulating basal MCL1 protein expression. If this is the case, the impact of individually deleting a DUB may be compensated by the activity of another DUB. This precludes identification of such DUBs because MCL1 levels could remain high following a single DUB deletion.

The redundant compensatory mechanism of MCL1's DUBs may be triggered when cells recognize complete loss of function of the primary DUB. If a DUB is inhibited only transiently, rather than completely, the redundancy with other DUBs may not be initiated. This is useful in a context where transient inhibition of a DUB is rapid and disruptive enough to reduce MCL1 levels to induce apoptosis without activating the compensation mechanism. To test this, a drop out screen using siRNA or CRISPRi targeting DUBs should be used.

Moreover, DUBs often have many other substrates, including those known to stabilize MCL1 such as USP9X (Kodani et al., 2019; Y. Liu et al., 2019; Schwickart et al., 2010; Skowyra, Allan, Saurin, & Clarke, 2018). In some cases, these substrates have crucial roles in cell cycle and proliferation (Skowyra et al., 2018; Song & Rape, 2008). Consequently, deleting these Dubs with specific sgRNAs may disrupt cell cycle and

proliferation. This would likely reduce the fitness of cells bearing those sgRNAs. Such genes would not be read out in the GFP-MCL1 reporter (enrichment) screen because they are out-competed by others. They would also not be read out in the MCL1-dependent cell viability (drop-out) screen because such genes would be depleted in both MCL1- dependent and the corresponding *BAX*- and *BAK*-deleted cells. One method to circumvent this would be to use an alternative approach such as a CRISPR-Cas9 arrayed screen in a 96-well plate (Agrotis & Ketteler, 2015; Costa et al., 2004). This approach allows multiple parameters to be simultaneously read out. Since it is only one gene that is targeted by multiple sgRNAs in each well, it is possible to track cells that not only lose GFP-MCL1 expression, but also grow slowly as a consequence of sgRNAs targeting. This allows identification of DUBs that regulate MCL1 protein stability when their deletion negatively impacts other cellular functions independent of apoptosis.

DUBs may be activated only under specific contexts. Since certain E3 ligases degrade MCL1 following distinct apoptotic stimuli (Mojca et al., 2014), DUBs may also be working in this context-specific manner. One notable example is CDU1 that can deubiquitinate MCL1 following bacterial infection (Fischer et al., 2017). Since both screens in this chapter were conducted under basal conditions, some DUBs acting in this manner may be missed. Future screens could focus on other cellular stimuli that may result in MCL1 protein stabilization.

The extent to which a DUB stabilizes MCL1 may also be a contributing factor in the lack of promising hits. In the MCL1-dependent viability screen, it is unclear by how much the MCL1 protein level must be reduced in order to render cells vulnerable to apoptosis. When establishing an MCL1-dependent cell line (Fig. 4.5), an additional control could be included to determine how much MCL1 protein must be reduced for apoptosis to occur. This would allow us to optimise the screening platform using cells that are most sensitive to the reduction of MCL1 protein levels.

Despite being unable to identify a novel DUB of MCL1, the strategies outlined above may improve the design of the screens. This should increase the chances of finding novel DUBs for MCL1. Ideally, the above strategies should be implemented with the screens that mimic therapeutic situations, such as when cancer cells were treated with chemotherapeutic agents or BH3 mimetics. This allow us to identify DUB that can be potentially co-targeted to improve treatment outcome of BH3 mimetics.

**Chapter 5: Direct and indirect consequences of targeting
specific BCL2 proteins with BH3 mimetic compounds**

Background

In order for cancer cells to survive, they must evade apoptosis (Hanahan & Weinberg, 2011). Deregulated apoptosis is frequently observed in cancers with examples including inactivation of the upstream P53 signalling pathway (Aubrey, Kelly, Janic, Herold, & Strasser, 2018; Aubrey, Strasser, & Kelly, 2016) as well as perturbation of the balance between BCL2 proteins governing mitochondrial apoptosis (Adams & Cory, 2018). Downregulating pro-apoptotic BH3-only proteins such as BIM or PUMA (Garrison et al., 2008; Karst, Dai, Martinka, & Li, 2005; Tagawa et al., 2005) or upregulating pro-survival proteins such as BCL2 (Majid et al., 2008; Tsujimoto, Cossman, Jaffe, & Croce, 1985), tips the cellular balance in favour of survival and thus, inhibits apoptosis.

Impaired apoptosis signalling in cancer cells can be circumvented in some cases using BH3 mimetics: small molecule inhibitors that mimic the activity of BH3-only proteins. BH3 mimetics induce apoptosis by selectively targeting one or more pro-survival BCL2 proteins. (Adams & Cory, 2018; Merino et al., 2018) (Figs. 5.1 A-B). The most successful BH3 mimetic to date has been venetoclax (ABT-199/BCL2i): a BCL2 selective inhibitor that is approved by FDA for treating chronic lymphocytic leukemia (CLL) as a single agent and acute myeloid leukemia (AML) as combination therapy with azacitidine or decitabine (DiNardo et al., 2019; DiNardo et al., 2018; Roberts et al., 2016; Scheffold et al., 2018). In both cases, although the majority of patients respond well when they were first introduced to venetoclax, there are some who are unresponsive, while some others acquire resistance whilst on therapy.

There is a complex network of interactions between members of BCL2 family of proteins. The balance of these interactions determine whether cells live or die. BH3 mimetic compounds alter this balance of interactions both directly and indirectly. Their direct impacts derive from binding to and directly inhibiting their pro-survival target proteins. Their indirect impacts arise because proteins that would otherwise interact with their direct targets proceed to form new interactions with other members of the BCL2 family. Understanding how these changes throughout the network of interactions between BCL2 proteins cause BAX and BAK to become activated and promote cell death may provide insight to combat primary and acquired resistance to BH3 mimetic compounds.

In this chapter, I investigated two selective BH3 mimetics: BCL2i and MCL1 selective inhibitor S63845 (MCL1i) and evaluated how they impact the network of interactions between BCL2 proteins. Previous studies have suggested that cancer cells with high BCL2 expression are generally more sensitive to BCL2i (Merino et al., 2012; Souers et al., 2013). It has been proposed that BCL2 acts as a reservoir for BIM and accordingly, treatment with BCL2i displaces BIM from BCL2 (Merino et al., 2012). The

displaced BIM is thought to have secondary actions, such as inactivating other pro-survival proteins (e.g. MCL1). Whether MCL1 inhibition by BIM following BCL2i treatment is necessary for cell death to occur remain unclear. It is also unclear whether other BH3-only proteins play a role in killing by BCL2i or other BH3 mimetics.

In this chapter, I explored how BH3 mimetic compounds perturb BCL2 protein interactions to induce apoptosis. I utilized a variety of approaches including cell viability and BH3 profiling assays to assess the sensitivity of cells to BH3 peptides and mimetic compounds. I also performed Biotin ID and immunoprecipitation studies to investigate changes to protein:protein interactions between BCL2 family proteins following treatment with BH3 mimetics.

In this chapter, I will aim to address the following questions:

1. How does the direct inhibition of BCL2 pro-survival proteins with BH3 mimetic compounds indirectly alter interactions between other BCL2 proteins?
2. Are the indirect impacts of BH3 mimetics on other BCL2 protein interactions necessary for efficient killing by these drugs?

Results

Establishing maximum concentrations for BH3 ligands in the BH3 profiling assay

Mitochondrial outer membrane permeabilization (MOMP) is considered a tipping point where cells irreversibly commit to cell death. BH3 profiling is a method that measures the impact of BH3 ligands on mitochondrial membrane potential. When MOMP occurs, the mitochondria becomes depolarized. This depolarization is conveniently measured with the potential sensitive JC-1 dye (Fig. 5.1 C). After MOMP, JC-1 ceases to form red fluorescence aggregates that result from retention of JC-1 within the mitochondria of healthy cells. This assay has been widely used and proposed to predict patient responses to BH3 mimetic therapy (Del Gaizo Moore & Letai, 2013; Ryan et al., 2010). However, BH3 ligands are often used at high concentrations and appropriate controls to indicate that BH3 ligands drive BAX and BAK dependent MOMP are often lacking. Thus, I decided to first establish the maximum concentration to which BH3 ligands (BH3 peptides and mimetics; Figs. 5.1 A-B) can be titrated in FACS-based BH3 profiling.

Two cell lines lacking BAX and BAK were generated to serve as controls in which BH3 ligands should not cause MOMP (OPM2; Figs. 5.2 A-B and AMO1; Figs. 5.2 C-E). When used at high concentrations, however, BH3 ligands caused loss of red JC-1 fluorescence aggregates (Figs. 5.2 A-D), suggesting that the mitochondria had become depolarized even when BAX and BAK were absent.

To test whether the loss of red JC-1 fluorescence signal in cells lacking BAX and BAK resulted from MOMP, I utilized an alternative assay that measures cytochrome c (CYT C) release. High concentration of BH3 ligands did not induce cytochrome c release from the mitochondria of *BAX^{-/-} BAK^{-/-}* AMO1 (Fig. 5.2 E), indicating that the response in JC-1 assay was unlikely to be MOMP-related since the effectors BAX and BAK were absent. To ensure that JC-1 assay reflects BAX- and BAK- driven MOMP, lower concentrations of BH3 ligands were used in the future experiments. BH3 peptides were used at a maximum concentration of 10 μ M (dashed line; Figs. 5.2 A and C) and BH3 mimetics were used at a maximum concentration of 1.25 μ M (dashed line; Figs. 5.2 B and D).

Cells are less sensitive to BH3 mimetics in BH3 profiling assays than in conventional cell viability assays

Having established maximum concentrations for BH3 ligands in the BH3 profiling assay, I next sought to compare the response of cells to selective inhibition of BCL2 proteins in BH3 profiling and conventional cell viability assays (PI/Annexin V staining). Seven blood

cancer cell lines were selected. Using conventional cell viability assays, three of these cell lines were sensitive to BCL2 inhibition (KMS-12-PE, RS4;11 and GRANTA; Figs. 5.3 A-C; top panel), three were sensitive to MCL1 inhibition (AMO1, OPM2 and H929; Figs. 5.3 D-F; top panel) and one was sensitive to combined inhibition of BCLxL and MCL1 (U266B1; Fig. 5.3 G; top panel). Surprisingly, minimal responses were observed when selective BH3 ligands were used in BH3 profiling assay (Figs. 5.3 A-G; bottom panel).

One major difference between the two assays is that in the BH3 profiling assay the plasma membrane has been permeabilized to allow direct access of BH3 ligands to the mitochondria. A potential consequence of this is that cytosolic proteins get diluted out from cells, presumably along with BCL2 proteins that are being displaced from pro-survivals by BH3 ligands. I hypothesized that in the permeabilized cells, the indirect impacts of selectively targeting pro-survival proteins with BH3 ligands would occur less efficiently. This is because BH3-only proteins that are displaced from pro-survival proteins by BH3 ligands may not efficiently inhibit other pro-survival proteins as they are diluted out from cells. Thus, MOMP may not occur. This hypothesis would account for why BIM peptide promoted mitochondrial depolarization at a relatively low concentration in all cell lines tested (Fig. 5.3; red line). One possible reason is that BIM peptide inhibits all pro-survival proteins with high affinity (Fig. 5.1 A) (Chen et al., 2005). The other possibility is that BIM peptide may activate BAX and BAK directly (Kuwana et al., 2005; A. Letai et al., 2002). The next parts of this chapter aim to investigate the discrepancy between the two assays and whether the indirect impacts of selectively inhibiting BCL2 proteins are necessary for MOMP to occur.

Inhibiting multiple pro-survival proteins is essential for effective mitochondrial depolarization

I next sought to investigate whether the lack of response observed in BH3 profiling assay when using selective inhibitors are resulted from the inability of these drugs to promote inhibition of other pro-survival proteins they do not directly engage. If this hypothesis is true, I predict that combining multiple selective inhibitors would cause MOMP more efficiently.

KMS-12-PE cells were investigated first. They were sensitive to BCL2i in the cell viability assay, but not in the BH3 profiling assay (Fig. 5.3 A). MOMP was readily induced in these cells in BH3 profiling assay when BCL2i and MCL1i were combined (Fig. 5.4 A). MOMP depended on BAX- and BAK- because it was abrogated by deleting both effectors (Fig. 5.4 A). This suggests that BCL2 and MCL1 must both be inhibited for MOMP to

occur in KMS-12-PE in BH3 profiling assay. Inhibiting BCLxL did not appear to be important to drive MOMP in these cells.

A similar phenomenon was observed in AMO1 and H929 cells, which were both sensitive to MCL1i in the cell viability assay but not the BH3 profiling assay. Whereas MOMP occurred most efficiently when both MCL1 and BCL2 were directly inhibited in AMO1 cells (Fig. 5.4 B), direct inhibition of both MCL1 and BCLxL were required for the most efficient MOMP in H929 cells (Fig. 5.4 C). In U266B1 cells, MOMP occurred most efficiently only when MCL1, BCL2 and BCLxL were all inhibited (Fig. 5.4 D). All these suggest that inhibition of multiple pro-survival proteins is a necessary requirement for mitochondrial depolarization to occur.

Genetically deleting other pro-survival genes promotes greater sensitization to selective inhibition of MCL1

In the BH3 profiling assay, the indirect impacts from selectively targeting pro-survivals with selective inhibitors on other BCL2 proteins in the network may not occur efficiently. This was supported by the fact that MOMP could only be triggered by combining selective BH3 mimetics that directly inhibit multiple pro-survivals or by treatment with BIM peptide that binds to all pro-survivals (Chen et al., 2005). Here, I utilized genetic deletion of pro-survival proteins as a complementary approach to address why BH3 mimetics differ in their ability to promote MOMP in intact vs permeabilized cells.

In AMO1, deleting *BCL2* promoted greater MOMP induced by MCL1i, but deleting other pro-survival genes did not (Fig. 5.5 A). The impact of deleting *BCL2* on MOMP was comparable to deleting *BCL2*, *BCLxL*, *BCLW* and *A1* together. This observation was also confirmed in the orthogonal cytochrome c release assay (Fig. 5.5 B). These data suggest that *BCL2* deletion alone is sufficient for MCLi to cause MOMP and that other pro-survival proteins are not major contributors for AMO1 survival. *BCL2*^{-/-}*BAX*^{-/-}*BAK*^{-/-} cells did not respond to MCL1i (Fig. 5.5 A), confirming that MOMP occurred through a BAX- and BAK-dependent mechanism.

In H929 cells, it was deletion of *BCLxL*, not other pro-survival genes, that promoted greater MOMP induced by MCL1i (Fig. 5.5 C) Because of the high variability in mitochondrial depolarization for *BCLxL*^{-/-} H929 cells, individual clones were investigated more thoroughly. Some *BCLxL*^{-/-} clones were highly sensitive to MCL1i (e.g. clone 5; Fig. 5.5 D), whereas others were less sensitive (e.g. clone 7; Fig. 5.5 D). This suggests that although BCLxL and MCL1 play major role for H929 survival, other pro-survival proteins may also play a more active role in subclonal population. Overall, all

these data show that the indirect impacts of BH3 mimetics treatment on other BCL2 protein interactions as essential for BAX- and BAK- induced MOMP.

BIM and NOXA are essential for BCL2i killing in KMS-12-PE cells

The experiments performed thus far have supported the hypothesis that the indirect impacts of selectively targeting pro-survival proteins with BH3 ligands are essential for MOMP induction. I hypothesized this process occurs less efficiently in permeabilized cells because BH3-only proteins are diluted out from cytosol and thus, can not inhibit other critical pro-survival proteins. It has been suggested that BH3-only proteins such as BIM contributes to killing induced by BH3 mimetics (Merino et al., 2012). A genetic screen was therefore conducted to investigate whether BIM and also other BH3-only proteins are required for apoptosis induced by BCL2i.

To identify which BH3-only proteins are required for the killing by BCL2i, KMS-12-PE cells were infected with a small lentiviral sgRNA library targeting BH3-only proteins. Afterwards, the cells were cultured with either DMSO (vehicle) or BCL2i for 14 days. Surviving cells were collected and sgRNAs were quantified by next generation sequencing. SgRNAs targeting *NOXA* and *BIM* were enriched in cells treated with BCL2i (Fig. 5.6 A). To validate these findings, *NOXA* or *BIM* or both of them were deleted from KMS-12-PE cells and their sensitivity to BCL2i was evaluated. Deleting either *NOXA* or *BIM* caused modest resistance against BCL2i. Combined loss of both *BIM* and *NOXA* conferred much greater resistance (Fig. 5.6 B), confirming that both are important for BCL2i-induced apoptosis in KMS-12-PE cells. This suggest that multiple BH3-only proteins may play significant roles in the killing induced by BCL2i.

BCL2i induces apoptosis in KMS-12-PE cells by inhibiting BCL2 and increasing the availability of free BIM to neutralize MCL1 protein

Having shown that both *BIM* and *NOXA* are important for the response to BCL2i, I next sought to investigate how BCL2i alters the interactions of *BIM* and *NOXA* with other BCL2 proteins. To do this, I employed a method called Biotin ID. In this method, proteins of interest are fused to a promiscuous biotin ligase: BirA. BirA can catalyse the covalent attachment of biotin to lysine residues on nearby proteins. This method has advantages over other methods of measuring protein:protein interactions, especially in the context of membrane-embedded proteins where their extraction from membranes with detergents can lead to artefacts (Lindsay, Esposti, & Gilmore, 2011). Biotin ID can also detect weakly or transiently interacting proteins because of BirA labels any proteins within the radius of 10 nM , even without a direct contact (Roux et al., 2013; Roux et al., 2012).

To investigate how BCL2i perturbs interactions between BCL2 family proteins, a series of biotin ID binding experiments was performed. KMS-12-PE cells were selected and engineered to express BirA-FLAG-MCL1 (Fig. 5.7 A) or BirA-FLAG-BCL2 (Fig. 5.7 B). When cells expressing BirA-FLAG-MCL1 were exposed to BCL2i, increasing amounts of BIM and BAX were observed (Fig. 5.7 A). Likewise, there was a corresponding decrease in the amount of BIM and BAX associated with BirA-FLAG-BCL2 upon BCL2i treatment (Fig. 5.7 B). Whereas the significance of BAX binding to MCL1 remains unclear, BIM binding to MCL1 may be important for the pro-survival neutralization. Although NOXA plays a role in the KMS-12-PE killing by BCL2i, NOXA association to BirA-FLAG-MCL1 remained unaltered (Fig. 5.7 A).

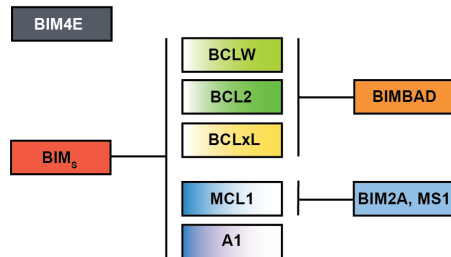
It has been previously suggested that BH3 mimetics targeting BCL2 displace BIM from the BCL2 hydrophobic binding groove, creating free BIM that can neutralize other pro-survival proteins such as MCL1 (Merino et al., 2012; Niu et al., 2016). Another possible source of free BIM is newly synthesized protein that cannot engage with the BCL2 hydrophobic groove when occupied by BH3 mimetics (Fig. 5.7 C). To test whether the BIM interacting with MCL1 protein following BCL2i treatment was derived from displaced or newly synthesized protein, I performed ³⁵S methionine labelling experiments. When ³⁵S methionine was added before BCL2i treatment, it should have labelled pre-existing BIM including the one that binds to BCL2. When ³⁵S methionine was added after BCL2i treatment, it should have labelled only newly synthesized BIM. In both cases (Figs. 5.7 D-E), the amount ³⁵S-labelled BIM bound to MCL1 increased when KMS-12-PE cells were exposed to BCL2i. This suggests that both displaced and newly synthesized BIM contributed to the indirect inhibition of MCL1 by BCL2i treatment.

Figure 5.1

BH3 ligands used in BH3 profiling assay.

- (A) Selectivity of BH3 peptides used in this thesis chapter. BIM4E is an inert BIM peptide in which 4 hydrophobic residues (h1-h4) important for binding to pro-survival proteins are replaced by glutamate (Chen et al., 2005). Some BH3 peptides (BIM_s and BIMBAD) bind to several pro-survival proteins, while others (BIM2A and MS1) are more selective (Lee et al., 2008; Merino et al., 2009) (Foight, Ryan, Gulla, Letai, & Keating, 2014). The sequence and binding affinity of these BH3 peptides were collated from previous studies (Chen et al., 2005; Foight et al., 2014; Lee et al., 2008; Merino et al., 2009).
- (B) Selectivity of BH3 mimetic compounds used in this thesis chapter. Some BH3 mimetics (ABT-737) bind to several pro-survival proteins, while others (ABT-199 (BCL2i), A1331852 (BCLxLi) and S63845 (MCL1i)) are more selective (Kotschy et al., 2016; Levenson et al., 2015; Oltersdorf et al., 2005; Souers et al., 2013).
- (C) BH3 profiling assay utilizes JC-1 dye to measure the polarization state of mitochondria. JC-1 is a potential-sensitive dye. It accumulates in polarized (healthy) mitochondria, forming JC-1 aggregates that emit red fluorescence. The properties of JC-1 aggregates differ from the unaggregated dyes that emit green fluorescence. MOMP promotes the loss of mitochondria potential and causes the loss of red JC-1 fluorescence aggregates (Del Gaizo Moore & Letai, 2013; Ryan et al., 2010).

A BH3 peptides

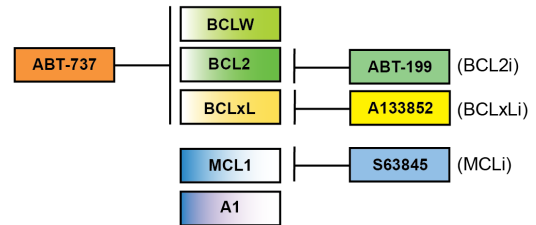


Peptide sequence	h1	h2	h3	h4
BIM ^{83D}	L	R	P	E
BIM4E	L	R	P	E
BIMBAD	L	W	A	A
BIM2A	L	W	A	A
MS1	R	P	E	I

Peptide binding affinity

BH3 peptide	Binding affinity (Kd)				
	BCL2 (nM)	BCLxL (nM)	BCLW (nM)	MCL1 (nM)	A1 (nM)
BIMs	4.5	0.8	1.6	0.2	0.5
BIM4E	>10,000	>10,000	>10,000	>10,000	>10,000
BIMBAD	16	5.3	30	>100,000	15,000
BIM2A	>10,000	>10,000	>10,000	19	520
MS1	2,300	1,600	1,300	1.9	5,000

B BH3 mimetics



C BH3 profiling assay

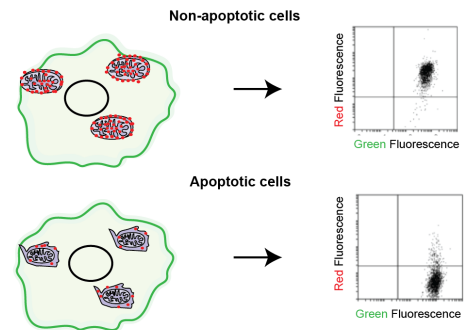


Figure 5.2

BH3 profiling assay cannot accurately assess the mitochondrial potential when BH3 peptides and mimetics are used at high concentration.

- (A-D) BH3 profiling assays were performed using titrated doses of BH3 peptides and BH3 mimetics in *BAX^{-/-}BAK^{-/-}* OPM2 (A,B) and *BAX^{-/-}BAK^{-/-}* AMO1 cells (C,D). At BH3 peptide concentrations above 10 μ M and BH3 mimetic concentrations above 1.25 μ M, these ligands appeared to cause mitochondrial depolarization independently of BAX and BAK as measured by the loss of red JC-1 aggregate fluorescent signal.
- (E) High concentration of BH3 peptides and BH3 mimetics do not trigger the release of cytochrome c (CYT C) from the mitochondria of *BAX^{-/-}BAK^{-/-}* AMO1 cells. The cells were exposed to titrated doses of BH3 peptides and BH3 mimetics for 30 minutes and then separated into cytosolic and membrane fractions. CYT C and ACTIN levels in the corresponding fractions were determined by immunoblotting.

Data shown in A-D are mean $\pm$ standard deviation of independent replicates (n = 2-3).

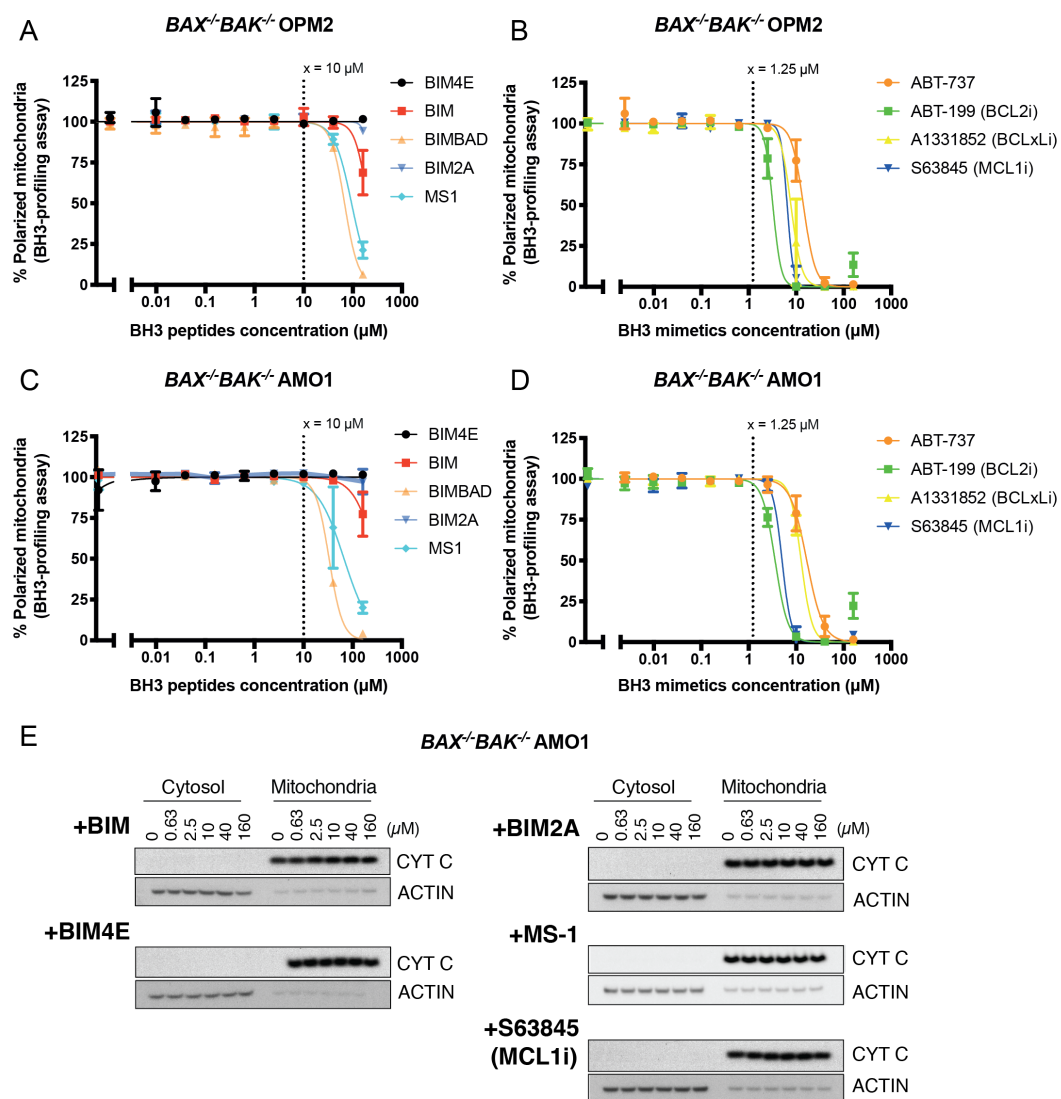


Figure 5.3

Cell lines are less sensitive to selective BH3 ligands in BH3 profiling assays compared to conventional cell viability assays.

(A-G) Cell viability assays (upper panels) were performed by exposing cultured cells to titrated doses of BH3 mimetics for 24 h and then measuring cell viability by PI and Annexin V staining. BH3 profiling assays (lower panels) were performed by exposing permeabilized cells to titrated doses of BH3 peptides and BH3 mimetics for 2 h and then measuring mitochondrial polarization by JC-1 fluorescence. Seven cell lines were examined. In the cell viability assays (top panels), three cell lines were sensitive to a selective BCL2 inhibitor (KMS-12-PE (A), RS4;11 (B) and GRANTA (C)), three were sensitive to a selective MCL1 inhibitor (AMO1 (D), OPM2 (E) and H929 (F)) and one was sensitive to combined inhibition of BCLxL and MCL1 (U266B1 (G)). In the BH3 profiling assays (bottom panels), cells were generally less responsive to selective BH3 ligands. BIM, which binds promiscuously to BCL2 proteins, was the only BH3 ligand that caused significant mitochondrial depolarization in the BH3 profiling assays.

Data shown in A-G are mean $\pm$ standard deviation of independent replicates (n = 2-4).

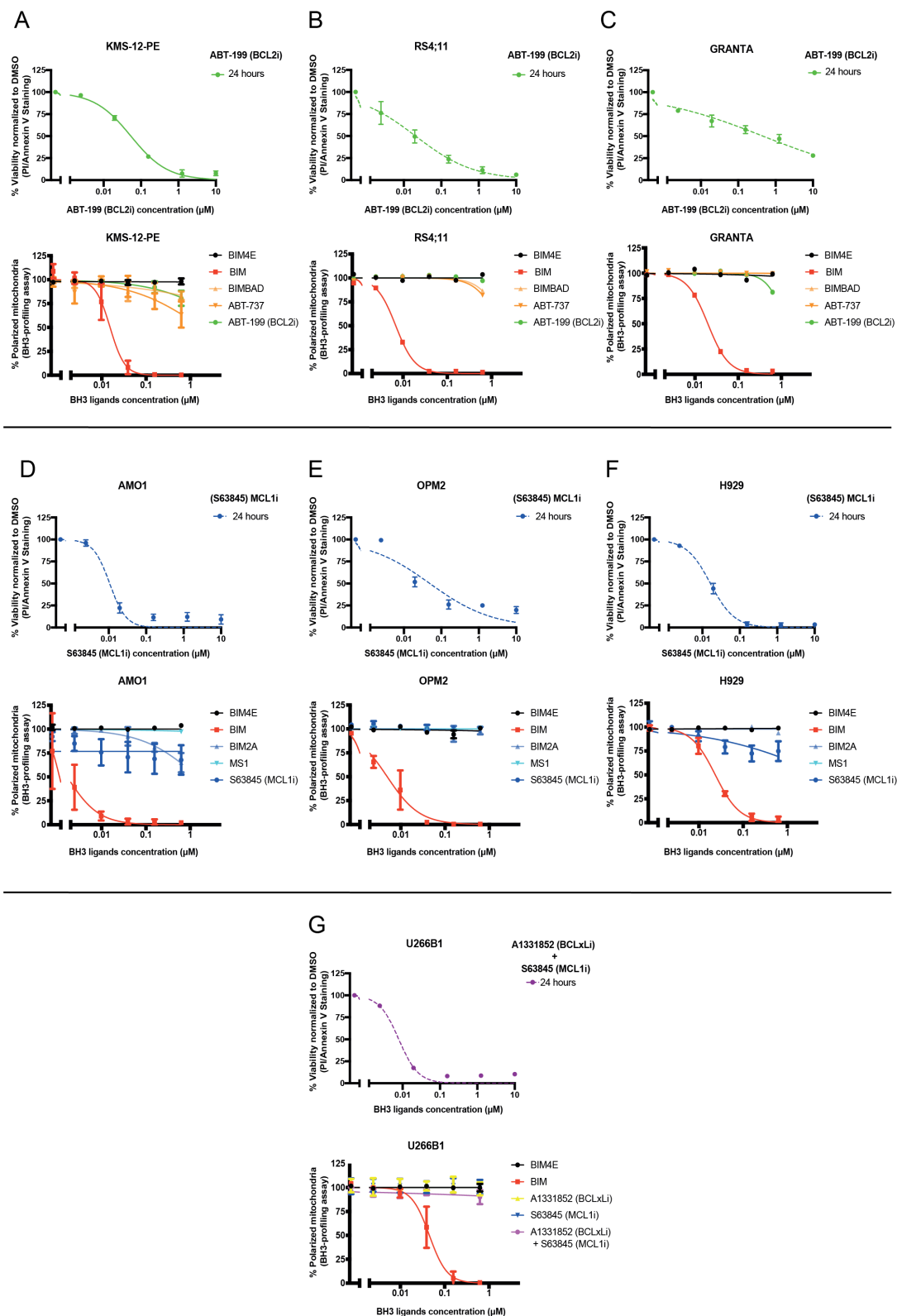


Figure 5.4

Combined inhibition of multiple pro-survival proteins results in greater mitochondrial depolarization in BH3 profiling assays.

(A-D) Four cell lines were examined using the BH3 profiling assay: KMS-12-PE (A), AMO1 (B), H929 (C) and U266B1 (D). These cells were permeabilized and then exposed to BH3 mimetics either alone or in combination (titrated in equimolar amounts) for 2 h. The mitochondrial polarization was then measured by the loss of red JC-1 aggregates. In two cases (A-B), *BAX*^{-/-} *BAK*^{-/-} deficient cells were included as controls.

Data shown in A-D are mean $\pm$ standard deviation of independent replicates (n = 3-5).

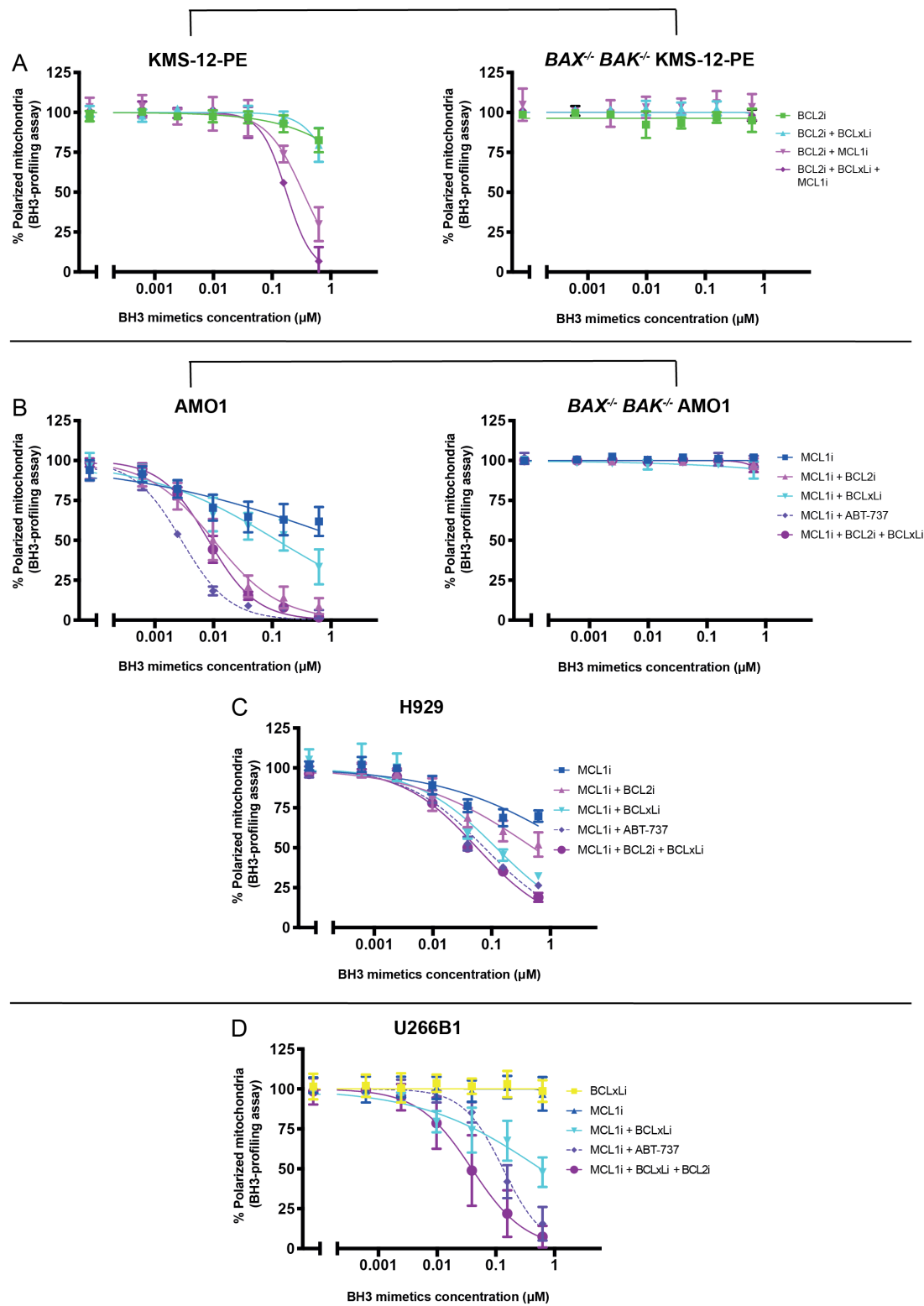


Figure 5.5

Genetic deletion of certain *BCL2* pro-survival genes enables selective BH3 ligands to depolarize mitochondria.

(A-D) AMO1 (A-B) and H929 (C-D) clones, of which the indicated *BCL2* genes were deleted, were treated with titrated doses of MCL1i for 2 h. Where indicated, parental cells were infected with empty vector (sgEV) as control. MOMP was determined by either the loss of red JC-1 aggregates (BH3 profiling assay; A, C-D) or the release of CYT C into cytosol (western blotting; B).

Data shown in A, C and D are mean $\pm$ standard deviation of the independent replicates (n = 3-5).

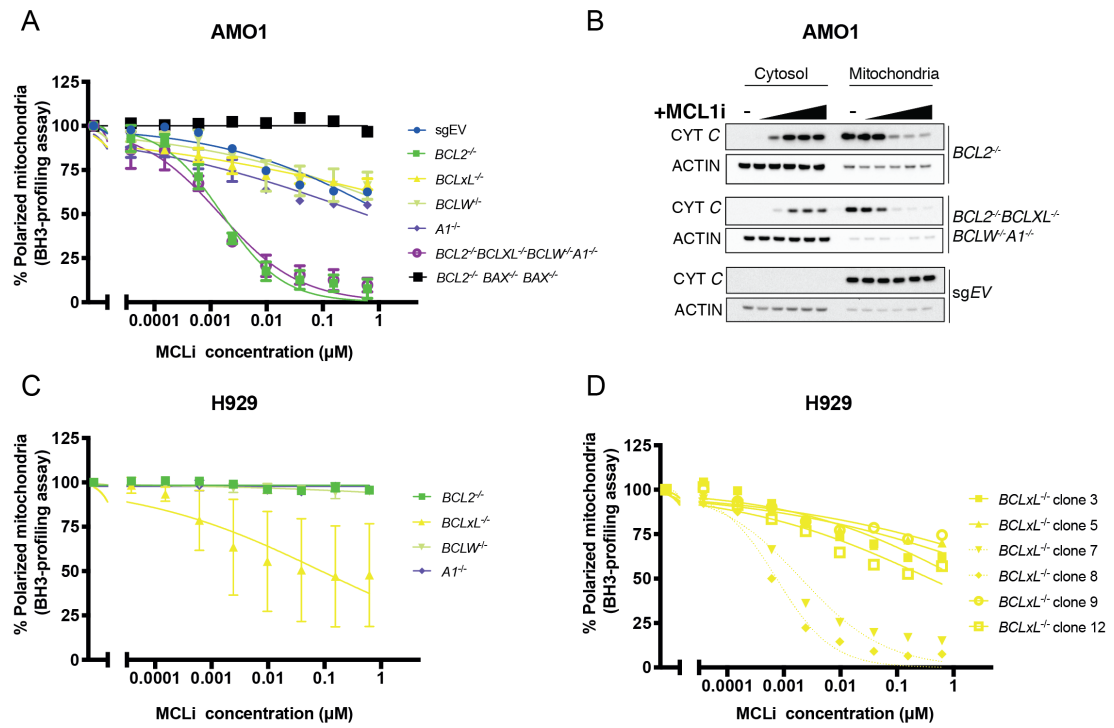


Figure 5.6

Loss of *BIM* and *NOXA* prevents BCL2i induced killing in KMS-12-PE cells.

- (A) Genetic screen with a focused sgRNA library targeting BH3-only proteins identified NOXA and BIM as essential for BCL2i-induced killing in KMS-12-PE cells. Briefly, Cas9 expressing KMS-12-PE were transduced lentivirus encoding the sgRNA library. These cells were cultured for 14 days under selection with 10 μ M of BCL2i or DMSO. sgRNA abundance in surviving cells was determined by sequencing. Plot shows fold change in counts for sgRNA targeting the indicated BH3-only proteins in BCL2i treated KMS-12-PE relative to DMSO treated cells. Each data point represents an independent sgRNA.
- (B) Whereas individually deleting either *BIM* or *NOXA* confers a degree of resistance against BCL2i, deleting both genes together confers an even greater resistance in KMS-12-PE cells. Cell viability was determined using CellTiter-Glo assay.

Data shown in B are mean $\pm$ standard deviation of the independent replicates (n = 3).

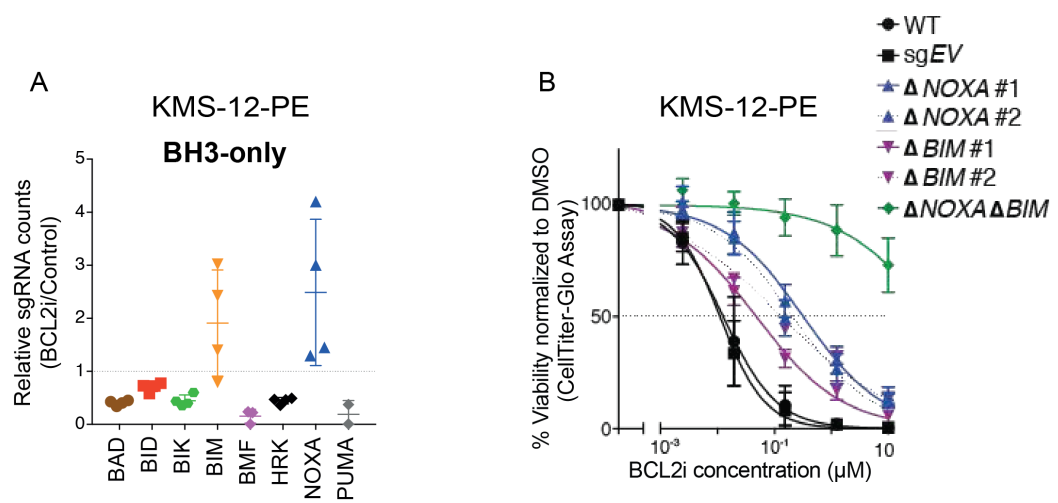


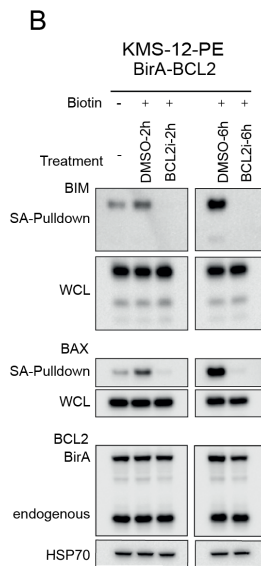
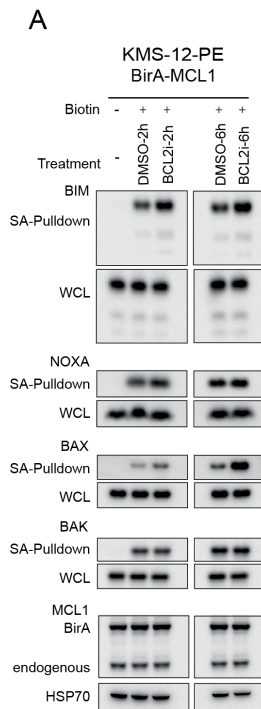
Figure 5.7

BCL2i treatment shifts the binding of BIM from BCL2 to MCL1.

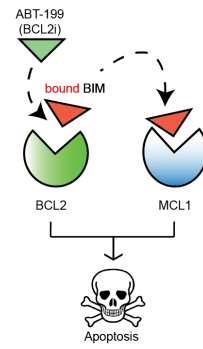
- (A) BCL2i treatment increases the binding of BIM and BAX to MCL1. KMS-12-PE cells were engineered to express BirA-FLAG-MCL1. These cells were cultured in biotin-free media for at least 96 h before they were treated with BCL2i or DMSO for the indicated length of time. Biotin was added simultaneously to biotinylate proteins in close proximity to BirA-FLAG-MCL1 following drug treatment. Biotinylated proteins were pulled down using streptavidin. Select BCL2 family proteins (BIM, NOXA, BAX, BAK, MCL1) were then analysed by western blotting.
- (B) BCL2i treatment reduces the binding of BIM and BAX from BCL2. This experiment was performed in the same manner as (A), except that KMS-12-PE cells were engineered to express BirA-FLAG-BCL2. The amounts of BIM, BAX and BAK pulled down with streptavidin were determined by western blotting.
- (C) The sources of BIM with the potential to inhibit MCL1 following BCL2i treatment. Occupation of BCL2 by BCL2i may displace previously bound BIM (i) or prevent interaction with newly synthesized BIM (ii), allowing BIM to engage MCL1 instead.
- (D) BCL2i promotes the binding of pre-existing BIM to MCL1. KMS-12-PE cells overexpressing BirA-FLAG-MCL1 were treated with ³⁵S-methionine to label pre-existing proteins including BIM. The pre-existing BIM labelled here could be the unbound proteins, or ones that **bound to BCL2 (C-i)** or other BCL2 family proteins. The labelled cells were then co-treated biotin and either BCL2i or DMSO control for 6 h. Biotinylated proteins were first pulled down with streptavidin and eluted. BIM was then specifically immunoprecipitated from the eluted protein fraction. The amount of ³⁵S-methionine radiolabelled BIM associated with BirA-FLAG-MCL1 was then determined by phosphorimaging and western blotting.
- (E) BCL2i occupies BCL2 and promotes the binding of newly synthesized BIM to MCL1. BirA-FLAG-MCL1 overexpressing KMS-12-PE cells were co-treated with BCL2i or DMSO control and ³⁵S-methionine for 12 h to label all **newly synthesized proteins** including BIM (**C-ii**) after drug treatment. Biotin was later added in the last 6 h of drug treatment. The amount of ³⁵S-methionine radiolabelled BIM associated with BirA-FLAG-MCL1 was then determined in the same manner as above (D).

An IgG isotype antibody (D-E) in the 1st lane served as the control for BIM pulldown for subsequent lanes. For more details on D-E, see '³⁵S-methionine labelling of BIM protein' in *Materials and Methods* section.

Representative images in A-B and D-E from two independent experiments.



C (i) DISPLACEMENT OF BIM MODEL



(ii) NEWLY SYNTHESIZED BIM MODEL

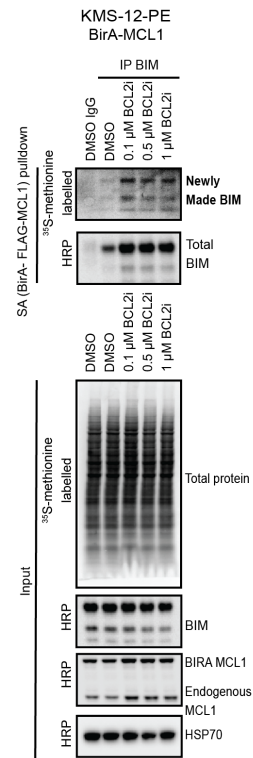
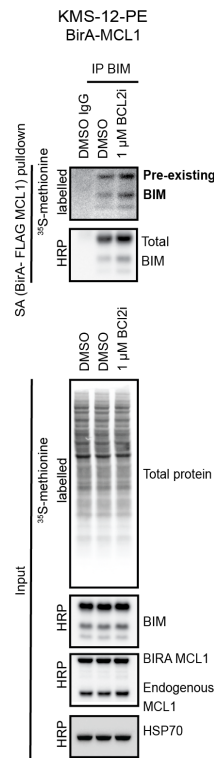
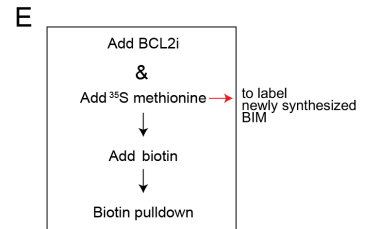
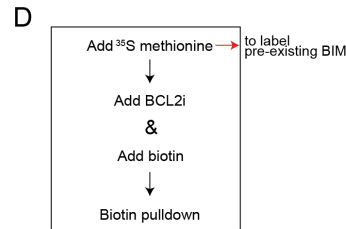
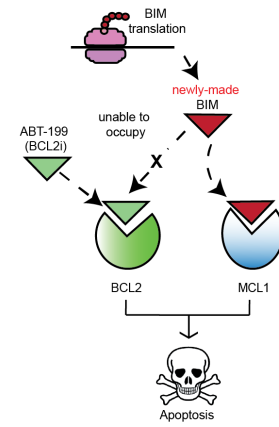
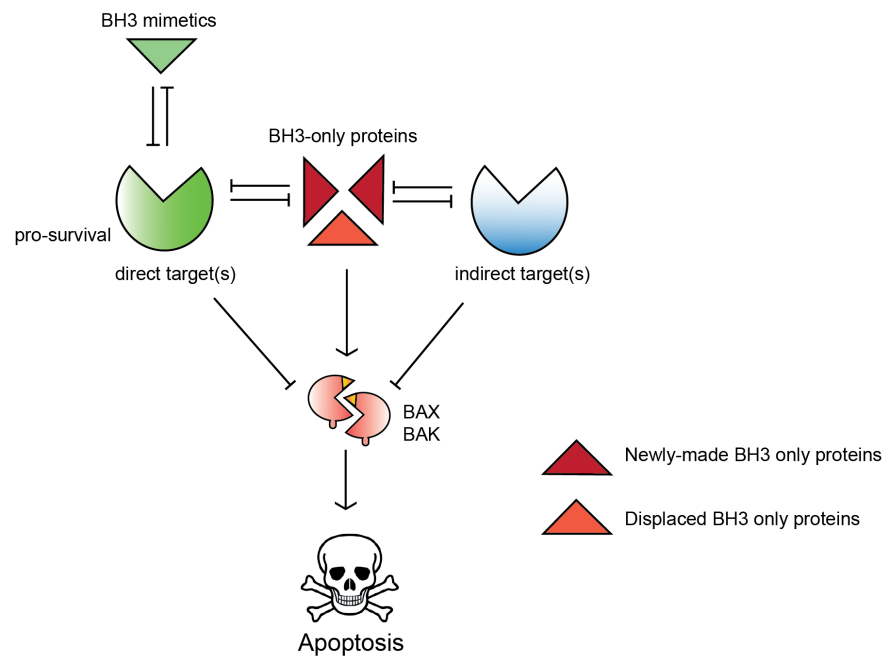


Figure 5.8**Simplified model for the direct and indirect impacts of BH3 mimetics.**

BH3 mimetics directly engage specific pro-survival proteins of BCL2 family. This interaction displaces the previously bound BH3-only proteins. At the same time, this interaction prevents the occupied pro-survivals for binding with the newly synthesised BH3-only proteins (Fig. 5.7 C). These free BH3-only proteins can then engage with other BCL2 proteins to promote apoptosis.



Discussion

A complex network of interactions occurs between proteins of the BCL2 family. In this chapter, I have explored the hypothesis that perturbing one interaction with a selective BH3 mimetic compound is likely to impact interactions between other BCL2 proteins. Beyond the consequence of inhibiting their direct targets, BH3 mimetics also have indirect impacts on other proteins that influence their ability to promote apoptosis. The indirect impacts are mediated by BH3-only proteins that are either newly synthesized or displaced from interactions with their BCL2 pro-survival partners. When other non-targeted pro-survival proteins are sufficiently inhibited by these free BH3-only proteins, MOMP can proceed (Fig. 5.8).

My data suggest that the results from BH3 profiling assays need to be interpreted with caution. Unlike in conventional cell viability assays, BH3 profiling is performed in cells that have their plasma membrane permeabilized. In this state, the indirect impacts of selectively targeting pro-survival proteins by BH3 mimetics, which would normally lead to other pro-survivals being inhibited, appear not to occur very efficiently. My data suggest that this lack of secondary consequence prevents MOMP because the pro-survival proteins that are not directly targeted remain sufficiently active to keep BAX and BAK in check (Figs. 5.3-5.5). This lack of secondary consequence may occur because there are less available BH3-only proteins for apoptosis induction. BH3-only proteins may not be synthesized when cells undergo stress due to permeabilization. Moreover, pre-existing BH3-only proteins may be diluted out from the cytosol into extracellular spaces through the holes in plasma membrane. All these are likely to reduce the ability to neutralize other pro-survival proteins. Although BH3 profiling assay does not fully recapitulate the secondary consequence of targeting pro-survival proteins by selective BH3 mimetics, it may be useful tool to identify pro-survival proteins that must be inhibited for cancer cells to undergo apoptosis.

Resistance to BH3 mimetics can occur because of the changes in the expression of BCL2 proteins. One common way is through overexpression of non-targeted pro-survival BCL2 proteins. In the case of small molecule inhibitors that directly target BCL2, MCL1 often limits their action (Figs. 5.3 A and 5.4 A) (Guieze et al., 2019; Tahir et al., 2017; van Delft et al., 2006) and less commonly BCLxL (Agarwal et al., 2019). Combining BCL2i with inhibitors that directly target MCL1 or BCLxL is an obvious choice (Fig. 5.4), provided they are tolerable in patients. In fact, combination of BCL2i with other BH3 mimetics is currently under clinical trials. It is likely, however, that they are not well tolerated. Combination treatment with MCL1i is likely to cause severe on-target toxicities given its prominent role in the maintenance of many critical organs (Hikita et al., 2009; Rinkenberger et al., 2000; R. L. Thomas et al., 2013; X. Wang et al., 2013), whereas

combination treatment with BCLxLi is likely to have significant impact on platelet production and cause thrombocytopenia (Mason et al., 2007). Finding alternative ways where these pro-survival proteins are targeted only in cancer cells, but not in other tissues may provide a wider therapeutical window. This is why identifying pathways that regulate MCL1 protein stability (Chapter 3 and Chapter 4) in context specific manners may provide us a solution, and the same can be applied by studying BCLxL protein regulation.

Reduced expression of certain BH3-only proteins can also cause resistance against BCL2i because they enhance MCL1 pro-survival function (Fig. 5.6). For example, NOXA downregulation can lead to MCL1 upregulation because MCL1 is no longer degraded (Chapter 3)(Czabotar et al., 2007; Willis et al., 2005). Notably, NOXA loss in this manner has been shown to compromise killing by BCL2 inhibitors in many cell lines studied (Nakajima, Hicks, Tanaka, Krystal, & Harada, 2014; Tahir et al., 2017; Tromp et al., 2012). This makes strategies that activate NOXA transcription or alleviate its repression (Yamashita et al., 2008) as attractive, but such strategies may only be useful in a limited number of settings where NOXA expression can be controlled in a tissue specific manner. Obviously, these strategies can also be applied to BIM that has been shown by many studies as essential for BCL2i killing (Merino et al., 2012; Souers et al., 2013).

Taken together, my studies suggest that both direct and indirect consequences of selectively targeting BCL2 pro-survival proteins with BH3 mimetics are essential for apoptosis to occur. When non-targeted pro-survival proteins are not sufficiently inhibited, cells may become resistant to selective BH3 mimetics treatment. My work in this chapter has provided rationale to target multiple pro-survival proteins for cancer treatment, but we must first develop strategies where they are targeted specifically in cancers but not other tissues to reduce toxicity.

Chapter 6: Concluding remarks

It is well established that many cancer cells evade apoptosis to survive. Using BH3 mimetics to target members of the BCL2 family that govern the mitochondrial pathway has been shown to circumvent this by directly engaging these pro-survival proteins. The consequences of selective BH3 mimetic treatment are thought to extend beyond the specifically targeted pro-survival protein to other BCL2 proteins in the network (Konopleva et al., 2006; Merino et al., 2012). The work I have presented in this thesis expands our understanding of how BH3 mimetics work, and ultimately how BAX and BAK are activated by these induced changes in the BCL2 protein family interaction network.

In Chapter 5, I investigated the direct and indirect consequences of BH3 mimetic treatment. I found that BH3 mimetics induce apoptosis by inhibiting not only the pro-survival proteins that they directly engage, but also others to which they do not bind. Intact BH3-only proteins are required to neutralize these non-targeted pro-survival proteins, because when some of them are downregulated, resistance to BH3 mimetics arises. In the case of venetoclax, MCL1 is often a resistance factor that may be well overactive because of NOXA or BIM downregulation (Chapter 5)(Guieze et al., 2019; Tahir et al., 2017; van Delft et al., 2006). Combining venetoclax with MCL1-selective inhibitors may overcome this resistance, but this combination is likely to be toxic due to multiple tissues being significantly affected (Arbour et al., 2008; Dzhagalov et al., 2008; Dzhagalov et al., 2007; Hikita et al., 2009; Kamada et al., 1995; Nakayama et al., 1994; Veis et al., 1993; Vikstrom et al., 2010; X. Wang et al., 2013; Yamamura et al., 1996). Therefore, in Chapters 3 and 4, I investigated the novel pathways controlling MCL1 protein stability as an alternative strategy to indirectly target MCL1 in a context-specific manner.

In Chapter 3, I investigated the mechanism by which NOXA causes MCL1 degradation (Czabotar et al., 2007; Willis et al., 2005). I demonstrated that MCL1 expression is regulated by MARCH5 (Cherok et al., 2017; Subramanian et al., 2016; Xu et al., 2016) and MTCH2 when NOXA is abundantly expressed. Individual elements from both NOXA and MCL1 appear to be critical for MARCH5 to recognize them as substrates. Because both MCL1 and NOXA are co-regulated by MARCH5 as a complex, enhancing MARCH5 activity may not translate well to increased apoptosis, if NOXA is equally degraded. The activity of MARCH5 on MCL1 depends on NOXA binding, therefore modulating NOXA transcription or using a small molecule activator that mimics NOXA protein may be potential strategies to control MCL1 function.

Alternatively, harnessing protein degradation pathway, such as with PROTAC is one strategy to inhibit MCL1 protein. Few PROTAC based approaches for MCL1 inhibition has been pursued including dMCL1-2 and C3 (Papatzimas et al., 2019; Z.

Wang et al., 2019). Both PROTACs enhance the proximity between unrelated E3 ligase CRBN and the target protein: MCL1. These PROTACs act as 'molecular glues' by having one part recognizing MCL1 and the other part recognizing CRBN. How to design a PROTAC that enhances the proximity of MCL1 and MARCH5, however, may require different approaches. This is because unlike CRBN, MARCH5 is the natural E3 ligase for MCL1. To enhance MARCH5 binding on MCL1, a logical approach will be to design a PROTAC that mimics NOXA. Although potential interaction sites in NOXA:MCL1 complex have been identified in this study, it is still unclear where is the portion of MARCH5 that recognizes this complex. To successfully design a PROTAC that mimics NOXA, this portion of MARCH5 needs to be identified. I speculate that some critical binding portions will be located in the MARCH5 transmembrane domain, since MCL1 transmembrane is indispensable for its degradation. Developing a PROTAC that binds to deeply embedded MARCH5 transmembrane domain may be a challenge. Whether it is necessary is also unclear since it is unknown if NOXA transmembrane is required for MARCH5 recognition. At this stage, no PROTAC has been reported to hijack MARCH E3 ligases to ubiquitinate unrelated substrates.

In Chapter 4, I identified novel deubiquitinating enzymes (DUBs) that are responsible for enhancing MCL1 protein stability. Top candidates from both CRISPR-Cas9 screens were the anticipated control or genes whose deletion does not directly impact on MCL1 post-translational control. Improving the screen design could focus on identifying a DUB that deubiquitinates MCL1 only in a specific subset of tissues. Identifying a DUB that works in this manner may provide a way to modulate MCL1 expression, and ultimately its pro-survival function.

In conclusion, this thesis provides insights into how MCL1 protein stability is regulated by UPS and how manipulating this pathway may present an opportunity to control MCL1 levels in cancer therapy. Harnessing this pathway to induce MCL1 protein degradation using either a PROTAC or a DUB inhibitor is one strategy to achieve inhibition of MCL1 pro-survival activity. Targeting the E3s or DUBs in tissue-specific contexts may allow a wider therapeutic window than systemic targeting of MCL1 protein with direct inhibitors and is likely to be better tolerated in patients.

Bibliography

- Acehan, D., Jiang, X., Morgan, D. G., Heuser, J. E., Wang, X., & Akey, C. W. (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell*, 9(2), 423-432.
- Adams, J. M., & Cory, S. (2018). The BCL-2 arbiters of apoptosis and their growing role as cancer targets. *Cell Death Differ*, 25(1), 27-36. doi:10.1038/cdd.2017.161
- Agarwal, R., Chan, Y. C., Tam, C. S., Hunter, T., Vassiliadis, D., Teh, C. E., . . . Dawson, S. J. (2019). Dynamic molecular monitoring reveals that SWI-SNF mutations mediate resistance to ibrutinib plus venetoclax in mantle cell lymphoma. *Nat Med*, 25(1), 119-129. doi:10.1038/s41591-018-0243-z
- Agrotis, A., & Ketteler, R. (2015). A new age in functional genomics using CRISPR/Cas9 in arrayed library screening. *Front Genet*, 6, 300. doi:10.3389/fgene.2015.00300
- Akao, Y., Otsuki, Y., Kataoka, S., Ito, Y., & Tsujimoto, Y. (1994). Multiple subcellular localization of bcl-2: detection in nuclear outer membrane, endoplasmic reticulum membrane, and mitochondrial membranes. *Cancer Res*, 54(9), 2468-2471.
- Akasaka, T., Akasaka, H., Yonetani, N., Ohno, H., Yamabe, H., Fukuhara, S., & Okuma, M. (1998). Refinement of the BCL2/immunoglobulin heavy chain fusion gene in t(14;18)(q32;q21) by polymerase chain reaction amplification for long targets. *Genes Chromosomes Cancer*, 21(1), 17-29.
- Antignani, A., & Youle, R. J. (2006). How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane? *Curr Opin Cell Biol*, 18(6), 685-689. doi:10.1016/j.ceb.2006.10.004
- Antonsson, B., Montessuit, S., Sanchez, B., & Martinou, J. C. (2001). Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. *J Biol Chem*, 276(15), 11615-11623. doi:10.1074/jbc.M010810200
- Arbour, N., Vanderluit, J. L., Le Grand, J. N., Jahani-Asl, A., Ruzhynsky, V. A., Cheung, E. C., . . . Slack, R. S. (2008). Mcl-1 is a key regulator of apoptosis during CNS development and after DNA damage. *J Neurosci*, 28(24), 6068-6078. doi:10.1523/jneurosci.4940-07.2008
- Aubrey, B. J., Kelly, G. L., Janic, A., Herold, M. J., & Strasser, A. (2018). How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? *Cell Death Differ*, 25(1), 104-113. doi:10.1038/cdd.2017.169
- Aubrey, B. J., Kelly, G. L., Kueh, A. J., Brennan, M. S., O'Connor, L., Milla, L., . . . Herold, M. J. (2015). An inducible lentiviral guide RNA platform enables the identification of tumor-essential genes and tumor-promoting mutations in vivo. *Cell Rep*, 10(8), 1422-1432. doi:10.1016/j.celrep.2015.02.002
- Aubrey, B. J., Strasser, A., & Kelly, G. L. (2016). Tumor-Suppressor Functions of the TP53 Pathway. *Cold Spring Harb Perspect Med*, 6(5). doi:10.1101/cshperspect.a026062
- Bahat, A., Goldman, A., Zaltsman, Y., Khan, D. H., Halperin, C., Amzallag, E., . . . Gross, A. (2018). MTCH2-mediated mitochondrial fusion drives exit from naive pluripotency in embryonic stem cells. *Nat Commun*, 9(1), 5132. doi:10.1038/s41467-018-07519-w

- Beroukhi, R., Mermel, C. H., Porter, D., Wei, G., Raychaudhuri, S., Donovan, J., . . . Meyerson, M. (2010). The landscape of somatic copy-number alteration across human cancers. *Nature*, 463(7283), 899-905. doi:10.1038/nature08822
- Blombery, P., Anderson, M. A., Gong, J. N., Thijssen, R., Birkinshaw, R. W., Thompson, E. R., . . . Roberts, A. W. (2019). Acquisition of the Recurrent Gly101Val Mutation in BCL2 Confers Resistance to Venetoclax in Patients with Progressive Chronic Lymphocytic Leukemia. *Cancer Discov*, 9(3), 342-353. doi:10.1158/2159-8290.Cd-18-1119
- Bogani, D., Morgan, M. A., Nelson, A. C., Costello, I., McGouran, J. F., Kessler, B. M., . . . Bikoff, E. K. (2013). The PR/SET domain zinc finger protein Prdm4 regulates gene expression in embryonic stem cells but plays a nonessential role in the developing mouse embryo. *Mol Cell Biol*, 33(19), 3936-3950. doi:10.1128/mcb.00498-13
- Bojarczuk, K., Sasi, B. K., Gobessi, S., Innocenti, I., Pozzato, G., Laurenti, L., & Efremov, D. G. (2016). BCR signaling inhibitors differ in their ability to overcome Mcl-1-mediated resistance of CLL B cells to ABT-199. *Blood*, 127(25), 3192-3201. doi:10.1182/blood-2015-10-675009
- Bradley, A., Zheng, H., Ziebarth, A., Sakati, W., Branham-O'Connor, M., Blumer, J. B., . . . Eblen, S. T. (2014). EDD enhances cell survival and cisplatin resistance and is a therapeutic target for epithelial ovarian cancer. *Carcinogenesis*, 35(5), 1100-1109. doi:10.1093/carcin/bgt489
- Brennan, M. S., Chang, C., Tai, L., Lessene, G., Strasser, A., Dewson, G., . . . Herold, M. J. (2018). Humanized Mcl-1 mice enable accurate preclinical evaluation of MCL-1 inhibitors destined for clinical use. *Blood*, 132(15), 1573-1583. doi:10.1182/blood-2018-06-859405
- Brill, A., Torchinsky, A., Carp, H., & Toder, V. (1999). The role of apoptosis in normal and abnormal embryonic development. *J Assist Reprod Genet*, 16(10), 512-519. doi:10.1023/a:1020541019347
- Brouwer, J. M., Lan, P., Cowan, A. D., Bernardini, J. P., Birkinshaw, R. W., van Delft, M. F., . . . Czabotar, P. E. (2017). Conversion of Bim-BH3 from Activator to Inhibitor of Bak through Structure-Based Design. *Mol Cell*, 68(4), 659-672.e659. doi:10.1016/j.molcel.2017.11.001
- Brouwer, J. M., Westphal, D., Dewson, G., Robin, A. Y., Uren, R. T., Bartolo, R., . . . Czabotar, P. E. (2014). Bak core and latch domains separate during activation, and freed core domains form symmetric homodimers. *Mol Cell*, 55(6), 938-946. doi:10.1016/j.molcel.2014.07.016
- Buzaglo-Azriel, L., Kuperman, Y., Tsoory, M., Zaltsman, Y., Shachnai, L., Zaidman, S. L., . . . Gross, A. (2016). Loss of Muscle MTCH2 Increases Whole-Body Energy Utilization and Protects from Diet-Induced Obesity. *Cell Rep*, 14(7), 1602-1610. doi:10.1016/j.celrep.2016.01.046
- Caenepeel, S., Brown, S. P., Belmontes, B., Moody, G., Keegan, K. S., Chui, D., . . . Hughes, P. E. (2018). AMG 176, a Selective MCL1 Inhibitor, Is Effective in Hematologic Cancer Models Alone and in Combination with Established Therapies. *Cancer Discov*, 8(12), 1582-1597. doi:10.1158/2159-8290.Cd-18-0387
- Carpio, M. A., Michaud, M., Zhou, W., Fisher, J. K., Walensky, L. D., & Katz, S. G. (2015). BCL-2 family member BOK promotes apoptosis in response to endoplasmic reticulum stress. *Proc Natl Acad Sci U S A*, 112(23), 7201-7206. doi:10.1073/pnas.1421063112

- Carroll, R. G., Hollville, E., & Martin, S. J. (2014). Parkin sensitizes toward apoptosis induced by mitochondrial depolarization through promoting degradation of Mcl-1. *Cell Rep*, 9(4), 1538-1553. doi:10.1016/j.celrep.2014.10.046
- Cartron, P. F., Gallenne, T., Bougras, G., Gautier, F., Manero, F., Vusio, P., . . . Juin, P. (2004). The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. *Mol Cell*, 16(5), 807-818. doi:10.1016/j.molcel.2004.10.028
- Chen, L., Willis, S. N., Wei, A., Smith, B. J., Fletcher, J. I., Hinds, M. G., . . . Huang, D. C. (2005). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell*, 17(3), 393-403. doi:10.1016/j.molcel.2004.12.030
- Cheng, E. H., Sheiko, T. V., Fisher, J. K., Craigen, W. J., & Korsmeyer, S. J. (2003). VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science*, 301(5632), 513-517. doi:10.1126/science.1083995
- 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.
- Cherok, E., Xu, S., Li, S., Das, S., Meltzer, W. A., Zalzman, M., . . . Karbowski, M. (2017). Novel regulatory roles of Mff and Drp1 in E3 ubiquitin ligase MARCH5-dependent degradation of MiD49 and Mcl1 and control of mitochondrial dynamics. *Mol Biol Cell*, 28(3), 396-410. doi:10.1091/mbc.E16-04-0208
- Chittka, A., Nitarska, J., Grazini, U., & Richardson, W. D. (2012). Transcription factor positive regulatory domain 4 (PRDM4) recruits protein arginine methyltransferase 5 (PRMT5) to mediate histone arginine methylation and control neural stem cell proliferation and differentiation. *J Biol Chem*, 287(51), 42995-43006. doi:10.1074/jbc.M112.392746
- Cleary, M. L., & Sklar, J. (1985). Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. *Proc Natl Acad Sci U S A*, 82(21), 7439-7443. doi:10.1073/pnas.82.21.7439
- Clohessy, J. G., Zhuang, J., & Brady, H. J. (2004). Characterisation of Mcl-1 cleavage during apoptosis of haematopoietic cells. *Br J Haematol*, 125(5), 655-665. doi:10.1111/j.1365-2141.2004.04949.x
- Corcoran, K., Jabbour, M., Bhagwandin, C., Deymier, M. J., Theisen, D. L., & Lybarger, L. (2011). Ubiquitin-mediated regulation of CD86 protein expression by the ubiquitin ligase membrane-associated RING-CH-1 (MARCH1). *J Biol Chem*, 286(43), 37168-37180. doi:10.1074/jbc.M110.204040
- Costa, J. R., Bejcek, B. E., McGee, J. E., Fogel, A. I., Brimacombe, K. R., & Ketteler, R. (2004). Genome Editing Using Engineered Nucleases and Their Use in Genomic Screening. In G. S. Sittampalam, A. Grossman, K. Brimacombe, M. Arkin, D. Auld, C. Austin, J. Baell, B. Bejcek, J. M. M. Caaveiro, T. D. Y. Chung, N. P. Coussens, J. L. Dahlin, V. Devanaryan, T. L. Foley, M. Glicksman, M. D. Hall, J. V. Haas, S. R. J. Hoare, J. Inglese, P. W. Iversen, S. D. Kahl, S. C. Kales, S. Kirshner, M. Lal-Nag, Z. Li, J. McGee, O. McManus, T. Riss, O. J. Trask, Jr., J. R. Weidner, M. J. Wildey, M. Xia, & X. Xu (Eds.), *Assay Guidance Manual*. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences.

- Croxton, R., Ma, Y., Song, L., Haura, E. B., & Cress, W. D. (2002). Direct repression of the Mcl-1 promoter by E2F1. *Oncogene*, *21*(9), 1359-1369. doi:10.1038/sj.onc.1205157
- Cuconati, A., Mukherjee, C., Perez, D., & White, E. (2003). DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. *Genes Dev*, *17*(23), 2922-2932. doi:10.1101/gad.1156903
- Czabotar, P. E., Lee, E. F., van Delft, M. F., Day, C. L., Smith, B. J., Huang, D. C., . . . Colman, P. M. (2007). Structural insights into the degradation of Mcl-1 induced by BH3 domains. *Proc Natl Acad Sci U S A*, *104*(15), 6217-6222. doi:10.1073/pnas.0701297104
- Day, C. L., Chen, L., Richardson, S. J., Harrison, P. J., Huang, D. C., & Hinds, M. G. (2005). Solution structure of prosurvival Mcl-1 and characterization of its binding by proapoptotic BH3-only ligands. *J Biol Chem*, *280*(6), 4738-4744. doi:10.1074/jbc.M411434200
- de Vivo, A., Sanchez, A., Yegres, J., Kim, J., Emly, S., & Kee, Y. (2019). The OTUD5-UBR5 complex regulates FACT-mediated transcription at damaged chromatin. *Nucleic Acids Res*, *47*(2), 729-746. doi:10.1093/nar/gky1219
- Del Gaizo Moore, V., Brown, J. R., Certo, M., Love, T. M., Novina, C. D., & Letai, A. (2007). Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *J Clin Invest*, *117*(1), 112-121. doi:10.1172/jci28281
- Del Gaizo Moore, V., & Letai, A. (2013). BH3 profiling--measuring integrated function of the mitochondrial apoptotic pathway to predict cell fate decisions. *Cancer Lett*, *332*(2), 202-205. doi:10.1016/j.canlet.2011.12.021
- Delbridge, A. R., & Strasser, A. (2015). The BCL-2 protein family, BH3-mimetics and cancer therapy. *Cell Death Differ*, *22*(7), 1071-1080. doi:10.1038/cdd.2015.50
- Dengler, M. A., Weilbacher, A., Gutekunst, M., Staiger, A. M., Vohringer, M. C., Horn, H., . . . van der Kuip, H. (2014). Discrepant NOXA (PMAIP1) transcript and NOXA protein levels: a potential Achilles' heel in mantle cell lymphoma. *Cell Death Dis*, *5*, e1013. doi:10.1038/cddis.2013.552
- Denisov, A. Y., Madiraju, M. S., Chen, G., Khadir, A., Beauparlant, P., Attardo, G., . . . Gehring, K. (2003). Solution structure of human BCL-w: modulation of ligand binding by the C-terminal helix. *J Biol Chem*, *278*(23), 21124-21128. doi:10.1074/jbc.M301798200
- Derenne, S., Monia, B., Dean, N. M., Taylor, J. K., Rapp, M. J., Harousseau, J. L., . . . Amiot, M. (2002). Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. *Blood*, *100*(1), 194-199. doi:10.1182/blood.v100.1.194
- Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., . . . Martinou, J. C. (1999). Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol*, *144*(5), 891-901. doi:10.1083/jcb.144.5.891
- Dewson, G., Kratina, T., Sim, H. W., Puthalakath, H., Adams, J. M., Colman, P. M., & Kluck, R. M. (2008). To trigger apoptosis, Bak exposes its BH3 domain and homodimerizes via BH3:groove interactions. *Mol Cell*, *30*(3), 369-380. doi:10.1016/j.molcel.2008.04.005
- Dijkers, P. F., Medema, R. H., Lammers, J. W., Koenderman, L., & Coffey, P. J. (2000). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol*, *10*(19), 1201-1204. doi:10.1016/s0960-9822(00)00728-4

- DiNardo, C. D., Pratz, K., Pullarkat, V., Jonas, B. A., Arellano, M., Becker, P. S., . . . Letai, A. (2019). Venetoclax combined with decitabine or azacitidine in treatment-naïve, elderly patients with acute myeloid leukemia. *Blood*, *133*(1), 7-17. doi:10.1182/blood-2018-08-868752
- DiNardo, C. D., Pratz, K. W., Letai, A., Jonas, B. A., Wei, A. H., Thirman, M., . . . Pollyea, D. A. (2018). Safety and preliminary efficacy of venetoclax with decitabine or azacitidine in elderly patients with previously untreated acute myeloid leukaemia: a non-randomised, open-label, phase 1b study. *Lancet Oncol*, *19*(2), 216-228. doi:10.1016/s1470-2045(18)30010-x
- Ding, Q., He, X., Hsu, J. M., Xia, W., Chen, C. T., Li, L. Y., . . . Hung, M. C. (2007). Degradation of Mcl-1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. *Mol Cell Biol*, *27*(11), 4006-4017. doi:10.1128/mcb.00620-06
- Ding, Q., Huo, L., Yang, J. Y., Xia, W., Wei, Y., Liao, Y., . . . Hung, M. C. (2008). Down-regulation of myeloid cell leukemia-1 through inhibiting Erk/Pin 1 pathway by sorafenib facilitates chemosensitization in breast cancer. *Cancer Res*, *68*(15), 6109-6117. doi:10.1158/0008-5472.Can-08-0579
- Domina, A. M., Vrana, J. A., Gregory, M. A., Hann, S. R., & Craig, R. W. (2004). MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. *Oncogene*, *23*(31), 5301-5315. doi:10.1038/sj.onc.1207692
- Dzhagalov, I., Dunkle, A., & He, Y. W. (2008). The anti-apoptotic Bcl-2 family member Mcl-1 promotes T lymphocyte survival at multiple stages. *J Immunol*, *181*(1), 521-528. doi:10.4049/jimmunol.181.1.521
- Dzhagalov, I., St John, A., & He, Y. W. (2007). The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages. *Blood*, *109*(4), 1620-1626. doi:10.1182/blood-2006-03-013771
- Echeverry, N., Bachmann, D., Ke, F., Strasser, A., Simon, H. U., & Kaufmann, T. (2013). Intracellular localization of the BCL-2 family member BOK and functional implications. *Cell Death Differ*, *20*(6), 785-799. doi:10.1038/cdd.2013.10
- Eden, E., Lipson, D., Yogev, S., & Yakhini, Z. (2007). Discovering motifs in ranked lists of DNA sequences. *PLoS Comput Biol*, *3*(3), e39. doi:10.1371/journal.pcbi.0030039
- Eitz Ferrer, P., Potthoff, S., Kirschnek, S., Gasteiger, G., Kastenmuller, W., Ludwig, H., . . . Hacker, G. (2011). Induction of Noxa-mediated apoptosis by modified vaccinia virus Ankara depends on viral recognition by cytosolic helicases, leading to IRF-3/IFN-beta-dependent induction of pro-apoptotic Noxa. *PLoS Pathog*, *7*(6), e1002083. doi:10.1371/journal.ppat.1002083
- Ekholm-Reed, S., Goldberg, M. S., Schlossmacher, M. G., & Reed, S. I. (2013). Parkin-dependent degradation of the F-box protein Fbw7beta promotes neuronal survival in response to oxidative stress by stabilizing Mcl-1. *Mol Cell Biol*, *33*(18), 3627-3643. doi:10.1128/mcb.00535-13
- Fernandez-Marrero, Y., Ke, F., Echeverry, N., Bouillet, P., Bachmann, D., Strasser, A., & Kaufmann, T. (2016). Is BOK required for apoptosis induced by endoplasmic reticulum stress? *Proc Natl Acad Sci U S A*, *113*(5), E492-493. doi:10.1073/pnas.1516347113
- Fischer, A., Harrison, K. S., Ramirez, Y., Auer, D., Chowdhury, S. R., Prusty, B. K., . . . Rudel, T. (2017). Chlamydia trachomatis-containing vacuole serves as

- deubiquitination platform to stabilize Mcl-1 and to interfere with host defense. *Elife*, 6. doi:10.7554/eLife.21465
- Fleischer, B., Schulze-Bergkamen, H., Schuchmann, M., Weber, A., Biesterfeld, S., Muller, M., . . . Galle, P. R. (2006). Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. *Int J Oncol*, 28(1), 25-32.
- Fletcher, J. I., Meusburger, S., Hawkins, C. J., Riglar, D. T., Lee, E. F., Fairlie, W. D., . . . Adams, J. M. (2008). Apoptosis is triggered when prosurvival Bcl-2 proteins cannot restrain Bax. *Proc Natl Acad Sci U S A*, 105(47), 18081-18087. doi:10.1073/pnas.0808691105
- Foight, G. W., Ryan, J. A., Gulla, S. V., Letai, A., & Keating, A. E. (2014). Designed BH3 peptides with high affinity and specificity for targeting Mcl-1 in cells. *ACS Chem Biol*, 9(9), 1962-1968. doi:10.1021/cb500340w
- Garrison, S. P., Jeffers, J. R., Yang, C., Nilsson, J. A., Hall, M. A., Rehg, J. E., . . . Zambetti, G. P. (2008). Selection against PUMA gene expression in Myc-driven B-cell lymphomagenesis. *Mol Cell Biol*, 28(17), 5391-5402. doi:10.1128/mcb.00907-07
- Gavathiotis, E., Suzuki, M., Davis, M. L., Pitter, K., Bird, G. H., Katz, S. G., . . . Walensky, L. D. (2008). BAX activation is initiated at a novel interaction site. *Nature*, 455(7216), 1076-1081. doi:10.1038/nature07396
- George, N. M., Evans, J. J., & Luo, X. (2007). A three-helix homo-oligomerization domain containing BH3 and BH1 is responsible for the apoptotic activity of Bax. *Genes Dev*, 21(15), 1937-1948. doi:10.1101/gad.1553607
- Glaser, S. P., Lee, E. F., Trounson, E., Bouillet, P., Wei, A., Fairlie, W. D., . . . Strasser, A. (2012). Anti-apoptotic Mcl-1 is essential for the development and sustained growth of acute myeloid leukemia. *Genes Dev*, 26(2), 120-125. doi:10.1101/gad.182980.111
- Gomez-Bougie, P., Menoret, E., Juin, P., Dousset, C., Pellat-Deceunynck, C., & Amiot, M. (2011). Noxa controls Mule-dependent Mcl-1 ubiquitination through the regulation of the Mcl-1/USP9X interaction. *Biochem Biophys Res Commun*, 413(3), 460-464. doi:10.1016/j.bbrc.2011.08.118
- Gong, J. N., Khong, T., Segal, D., Yao, Y., Riffkin, C. D., Garnier, J. M., . . . Huang, D. C. S. (2016). Hierarchy for targeting prosurvival BCL2 family proteins in multiple myeloma: pivotal role of MCL1. *Blood*, 128(14), 1834-1844. doi:10.1182/blood-2016-03-704908
- Goodwin, C. M., Rossanese, O. W., Olejniczak, E. T., & Fesik, S. W. (2015). Myeloid cell leukemia-1 is an important apoptotic survival factor in triple-negative breast cancer. *Cell Death Differ*, 22(12), 2098-2106. doi:10.1038/cdd.2015.73
- Grabow, S., Delbridge, A. R., Valente, L. J., & Strasser, A. (2014). MCL-1 but not BCL-XL is critical for the development and sustained expansion of thymic lymphoma in p53-deficient mice. *Blood*, 124(26), 3939-3946. doi:10.1182/blood-2014-09-601567
- Graner, E., Tang, D., Rossi, S., Baron, A., Migita, T., Weinstein, L. J., . . . Loda, M. (2004). The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. *Cancer Cell*, 5(3), 253-261.
- Grinberg, M., Schwarz, M., Zaltsman, Y., Eini, T., Niv, H., Pietrokovski, S., & Gross, A. (2005). Mitochondrial carrier homolog 2 is a target of tBID in cells signaled to die by tumor necrosis factor alpha. *Mol Cell Biol*, 25(11), 4579-4590. doi:10.1128/mcb.25.11.4579-4590.2005

- Gudjonsson, T., Altmeyer, M., Savic, V., Toledo, L., Dinant, C., Grofte, M., . . . Lukas, C. (2012). TRIP12 and UBR5 suppress spreading of chromatin ubiquitylation at damaged chromosomes. *Cell*, 150(4), 697-709. doi:10.1016/j.cell.2012.06.039
- Guieze, R., Liu, V. M., Rosebrock, D., Jourdain, A. A., Hernandez-Sanchez, M., Martinez Zurita, A., . . . Wu, C. J. (2019). Mitochondrial Reprogramming Underlies Resistance to BCL-2 Inhibition in Lymphoid Malignancies. *Cancer Cell*, 36(4), 369-384.e313. doi:10.1016/j.ccell.2019.08.005
- Hamasaki, A., Sendo, F., Nakayama, K., Ishida, N., Negishi, I., Nakayama, K., & Hatakeyama, S. (1998). Accelerated neutrophil apoptosis in mice lacking A1-a, a subtype of the bcl-2-related A1 gene. *J Exp Med*, 188(11), 1985-1992. doi:10.1084/jem.188.11.1985
- Han, J., Goldstein, L. A., Gastman, B. R., Rabinovitz, A., & Rabinowich, H. (2005). Disruption of Mcl-1.Bim complex in granzyme B-mediated mitochondrial apoptosis. *J Biol Chem*, 280(16), 16383-16392. doi:10.1074/jbc.M411377200
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, 144(5), 646-674. doi:10.1016/j.cell.2011.02.013
- Harley, M. E., Allan, L. A., Sanderson, H. S., & Clarke, P. R. (2010). Phosphorylation of Mcl-1 by CDK1-cyclin B1 initiates its Cdc20-dependent destruction during mitotic arrest. *Embo j*, 29(14), 2407-2420. doi:10.1038/emboj.2010.112
- Herman, M. D., Nyman, T., Welin, M., Lehtio, L., Flodin, S., Tresaugues, L., . . . Nordlund, P. (2008). Completing the family portrait of the anti-apoptotic Bcl-2 proteins: crystal structure of human Bfl-1 in complex with Bim. *FEBS Lett*, 582(25-26), 3590-3594. doi:10.1016/j.febslet.2008.09.028
- Herrant, M., Jacquet, A., Marchetti, S., Belhacene, N., Colosetti, P., Luciano, F., & Auberger, P. (2004). Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. *Oncogene*, 23(47), 7863-7873. doi:10.1038/sj.onc.1208069
- Hershko, A., & Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem*, 67, 425-479. doi:10.1146/annurev.biochem.67.1.425
- Hikita, H., Takehara, T., Shimizu, S., Kodama, T., Li, W., Miyagi, T., . . . Hayashi, N. (2009). Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. *Hepatology*, 50(4), 1217-1226. doi:10.1002/hep.23126
- Hinds, M. G., & Day, C. L. (2005). Regulation of apoptosis: uncovering the binding determinants. *Curr Opin Struct Biol*, 15(6), 690-699. doi:10.1016/j.sbi.2005.10.003
- Hinds, M. G., Smits, C., Fredericks-Short, R., Risk, J. M., Bailey, M., Huang, D. C., & Day, C. L. (2007). Bim, Bad and Bmf: intrinsically unstructured BH3-only proteins that undergo a localized conformational change upon binding to prosurvival Bcl-2 targets. *Cell Death Differ*, 14(1), 128-136. doi:10.1038/sj.cdd.4401934
- Hsu, S. Y., Kaipia, A., McGee, E., Lomeli, M., & Hsueh, A. J. (1997). Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. *Proc Natl Acad Sci U S A*, 94(23), 12401-12406. doi:10.1073/pnas.94.23.12401
- Hsu, Y. T., Wolter, K. G., & Youle, R. J. (1997). Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proc Natl Acad Sci U S A*, 94(8), 3668-3672. doi:10.1073/pnas.94.8.3668

- Huang, C. R., & Yang-Yen, H. F. (2010). The fast-mobility isoform of mouse Mcl-1 is a mitochondrial matrix-localized protein with attenuated anti-apoptotic activity. *FEBS Lett*, 584(15), 3323-3330. doi:10.1016/j.febslet.2010.07.013
- Huelsemann, M. F., Patz, M., Beckmann, L., Brinkmann, K., Otto, T., Fandrey, J., . . . Frenzel, L. P. (2015). Hypoxia-induced p38 MAPK activation reduces Mcl-1 expression and facilitates sensitivity towards BH3 mimetics in chronic lymphocytic leukemia. *Leukemia*, 29(4), 981-984. doi:10.1038/leu.2014.320
- Inoshita, S., Takeda, K., Hatai, T., Terada, Y., Sano, M., Hata, J., . . . Ichijo, H. (2002). Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress. *J Biol Chem*, 277(46), 43730-43734. doi:10.1074/jbc.M207951200
- Inuzuka, H., Shaik, S., Onoyama, I., Gao, D., Tseng, A., Maser, R. S., . . . Wei, W. (2011). SCF(FBW7) regulates cellular apoptosis by targeting MCL1 for ubiquitylation and destruction. *Nature*, 471(7336), 104-109. doi:10.1038/nature09732
- Jackson, A. L., & Linsley, P. S. (2010). Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat Rev Drug Discov*, 9(1), 57-67. doi:10.1038/nrd3010
- Jahnke, M., Trowsdale, J., & Kelly, A. P. (2012). Ubiquitination of human leukocyte antigen (HLA)-DM by different membrane-associated RING-CH (MARCH) protein family E3 ligases targets different endocytic pathways. *J Biol Chem*, 287(10), 7256-7264. doi:10.1074/jbc.M111.305961
- Jorgensen, I., Rayamajhi, M., & Miao, E. A. (2017). Programmed cell death as a defence against infection. *Nat Rev Immunol*, 17(3), 151-164. doi:10.1038/nri.2016.147
- Kale, J., Osterlund, E. J., & Andrews, D. W. (2018). BCL-2 family proteins: changing partners in the dance towards death. *Cell Death Differ*, 25(1), 65-80. doi:10.1038/cdd.2017.186
- Kamada, S., Shimono, A., Shinto, Y., Tsujimura, T., Takahashi, T., Noda, T., . . . Tsujimoto, Y. (1995). bcl-2 deficiency in mice leads to pleiotropic abnormalities: accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. *Cancer Res*, 55(2), 354-359.
- Karst, A. M., Dai, D. L., Martinka, M., & Li, G. (2005). PUMA expression is significantly reduced in human cutaneous melanomas. *Oncogene*, 24(6), 1111-1116. doi:10.1038/sj.onc.1208374
- Kazlauskaitė, A., Kelly, V., Johnson, C., Baillie, C., Hastie, C. J., Pegg, M., . . . Muqit, M. M. (2014). Phosphorylation of Parkin at Serine65 is essential for activation: elaboration of a Miro1 substrate-based assay of Parkin E3 ligase activity. *Open Biol*, 4, 130213. doi:10.1098/rsob.130213
- Kazlauskaitė, A., Kondapalli, C., Gourlay, R., Campbell, D. G., Ritorto, M. S., Hofmann, K., . . . Muqit, M. M. (2014). Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser65. *Biochem J*, 460(1), 127-139. doi:10.1042/bj20140334
- Ke, F., Grabow, S., Kelly, G. L., Lin, A., O'Reilly, L. A., & Strasser, A. (2015). Impact of the combined loss of BOK, BAX and BAK on the hematopoietic system is slightly more severe than compound loss of BAX and BAK. *Cell Death Dis*, 6, e1938. doi:10.1038/cddis.2015.304
- Ke, F., Voss, A., Kerr, J. B., O'Reilly, L. A., Tai, L., Echeverry, N., . . . Kaufmann, T. (2012). BCL-2 family member BOK is widely expressed but its loss has only

- minimal impact in mice. *Cell Death Differ*, 19(6), 915-925.
doi:10.1038/cdd.2011.210
- Ke, F. F. S., Vanyai, H. K., Cowan, A. D., Delbridge, A. R. D., Whitehead, L., Grabow, S., . . . Strasser, A. (2018). Embryogenesis and Adult Life in the Absence of Intrinsic Apoptosis Effectors BAX, BAK, and BOK. *Cell*, 173(5), 1217-1230.e1217. doi:10.1016/j.cell.2018.04.036
- Kelly, G. L., Grabow, S., Glaser, S. P., Fitzsimmons, L., Aubrey, B. J., Okamoto, T., . . . Strasser, A. (2014). Targeting of MCL-1 kills MYC-driven mouse and human lymphomas even when they bear mutations in p53. *Genes Dev*, 28(1), 58-70. doi:10.1101/gad.232009.113
- Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, 26(4), 239-257. doi:10.1038/bjc.1972.33
- Kim, H., Tu, H. C., Ren, D., Takeuchi, O., Jeffers, J. R., Zambetti, G. P., . . . Cheng, E. H. (2009). Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis. *Mol Cell*, 36(3), 487-499. doi:10.1016/j.molcel.2009.09.030
- Kitada, S., Zapata, J. M., Andreeff, M., & Reed, J. C. (2000). Protein kinase inhibitors flavopiridol and 7-hydroxy-staurosporine down-regulate antiapoptosis proteins in B-cell chronic lymphocytic leukemia. *Blood*, 96(2), 393-397.
- Knudson, C. M., Tung, K. S., Tourtellotte, W. G., Brown, G. A., & Korsmeyer, S. J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*, 270(5233), 96-99. doi:10.1126/science.270.5233.96
- Ko, H. S., Uehara, T., Tsuruma, K., & Nomura, Y. (2004). Ubiquitin interacts with ubiquitylated proteins and proteasome through its ubiquitin-associated and ubiquitin-like domains. *FEBS Lett*, 566(1-3), 110-114. doi:10.1016/j.febslet.2004.04.031
- Kodama, Y., Taura, K., Miura, K., Schnabl, B., Osawa, Y., & Brenner, D. A. (2009). Antiapoptotic effect of c-Jun N-terminal Kinase-1 through Mcl-1 stabilization in TNF-induced hepatocyte apoptosis. *Gastroenterology*, 136(4), 1423-1434. doi:10.1053/j.gastro.2008.12.064
- Kodani, A., Moyer, T., Chen, A., Holland, A., Walsh, C. A., & Reiter, J. F. (2019). SFI1 promotes centriole duplication by recruiting USP9X to stabilize the microcephaly protein STIL. *J Cell Biol*, 218(7), 2185-2197. doi:10.1083/jcb.201803041
- Koike-Yusa, H., Li, Y., Tan, E. P., Velasco-Herrera Mdel, C., & Yusa, K. (2014). Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol*, 32(3), 267-273. doi:10.1038/nbt.2800
- Komander, D. (2009). The emerging complexity of protein ubiquitination. *Biochem Soc Trans*, 37(Pt 5), 937-953. doi:10.1042/bst0370937
- Konopleva, M., Contractor, R., Tsao, T., Samudio, I., Ruvolo, P. P., Kitada, S., . . . Andreeff, M. (2006). Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell*, 10(5), 375-388. doi:10.1016/j.ccr.2006.10.006
- Kotschy, A., Szlavik, Z., Murray, J., Davidson, J., Maragno, A. L., Le Toumelin-Braizat, G., . . . Geneste, O. (2016). The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. *Nature*, 538(7626), 477-482. doi:10.1038/nature19830

- Kozopas, K. M., Yang, T., Buchan, H. L., Zhou, P., & Craig, R. W. (1993). MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc Natl Acad Sci U S A*, 90(8), 3516-3520.
- Kutuk, O., & Letai, A. (2008). Regulation of Bcl-2 family proteins by posttranslational modifications. *Curr Mol Med*, 8(2), 102-118.
- Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., & Newmeyer, D. D. (2005). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell*, 17(4), 525-535. doi:10.1016/j.molcel.2005.02.003
- Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneider, R., . . . Newmeyer, D. D. (2002). Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell*, 111(3), 331-342. doi:10.1016/s0092-8674(02)01036-x
- Kvansakul, M., & Hinds, M. G. (2014). The structural biology of BH3-only proteins. *Methods Enzymol*, 544, 49-74. doi:10.1016/b978-0-12-417158-9.00003-0
- Lai, A. C., & Crews, C. M. (2017). Induced protein degradation: an emerging drug discovery paradigm. *Nat Rev Drug Discov*, 16(2), 101-114. doi:10.1038/nrd.2016.211
- Lazarou, M., Stojanovski, D., Frazier, A. E., Kotevski, A., Dewson, G., Craigen, W. J., . . . Ryan, M. T. (2010). Inhibition of Bak activation by VDAC2 is dependent on the Bak transmembrane anchor. *J Biol Chem*, 285(47), 36876-36883. doi:10.1074/jbc.M110.159301
- Lee, E. F., Czabotar, P. E., van Delft, M. F., Michalak, E. M., Boyle, M. J., Willis, S. N., . . . Fairlie, W. D. (2008). A novel BH3 ligand that selectively targets Mcl-1 reveals that apoptosis can proceed without Mcl-1 degradation. *J Cell Biol*, 180(2), 341-355. doi:10.1083/jcb.200708096
- Letai, A. (2017). Apoptosis and Cancer. *Annual Review of Cancer Biology*, 1(1), 275-294. doi:10.1146/annurev-cancerbio-050216-121933
- Letai, A., Bassik, M. C., Walensky, L. D., Sorcinelli, M. D., Weiler, S., & Korsmeyer, S. J. (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell*, 2(3), 183-192.
- Levenson, J. D., Phillips, D. C., Mitten, M. J., Boghaert, E. R., Diaz, D., Tahir, S. K., . . . Souers, A. J. (2015). Exploiting selective BCL-2 family inhibitors to dissect cell survival dependencies and define improved strategies for cancer therapy. *Sci Transl Med*, 7(279), 279ra240. doi:10.1126/scitranslmed.aaa4642
- Li, H., Zhu, H., Xu, C. J., & Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*, 94(4), 491-501. doi:10.1016/s0092-8674(00)81590-1
- Lindqvist, L. M., Vikstrom, I., Chambers, J. M., McArthur, K., Ann Anderson, M., Henley, K. J., . . . Huang, D. C. (2012). Translation inhibitors induce cell death by multiple mechanisms and Mcl-1 reduction is only a minor contributor. *Cell Death Dis*, 3, e409. doi:10.1038/cddis.2012.149
- Lindsay, J., Esposti, M. D., & Gilmore, A. P. (2011). Bcl-2 proteins and mitochondria--specificity in membrane targeting for death. *Biochim Biophys Acta*, 1813(4), 532-539. doi:10.1016/j.bbamcr.2010.10.017
- Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., . . . Thompson, C. B. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell*, 6(6), 1389-1399.

- Liu, H., Dai, X., Cao, X., Yan, H., Ji, X., Zhang, H., . . . Zhao, B. (2018). PRDM4 mediates YAP-induced cell invasion by activating leukocyte-specific integrin beta2 expression. *EMBO Rep*, 19(6). doi:10.15252/embr.201745180
- Liu, H., Ma, Y., Cole, S. M., Zander, C., Chen, K. H., Karras, J., & Pope, R. M. (2003). Serine phosphorylation of STAT3 is essential for Mcl-1 expression and macrophage survival. *Blood*, 102(1), 344-352. doi:10.1182/blood-2002-11-3396
- Liu, X., Dai, S., Zhu, Y., Marrack, P., & Kappler, J. W. (2003). The structure of a Bcl-xL/Bim fragment complex: implications for Bim function. *Immunity*, 19(3), 341-352.
- Liu, X., Kim, C. N., Yang, J., Jemmerson, R., & Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*, 86(1), 147-157. doi:10.1016/s0092-8674(00)80085-9
- Liu, Y., Xu, X., Lin, P., He, Y., Zhang, Y., Cao, B., . . . Mao, X. (2019). Inhibition of the deubiquitinase USP9x induces pre-B cell homeobox 1 (PBX1) degradation and thereby stimulates prostate cancer cell apoptosis. *J Biol Chem*, 294(12), 4572-4582. doi:10.1074/jbc.RA118.006057
- Lu, A., Hiltunen, M., Romano, D. M., Soininen, H., Hyman, B. T., Bertram, L., & Tanzi, R. E. (2009). Effects of ubiquilin 1 on the unfolded protein response. *J Mol Neurosci*, 38(1), 19-30. doi:10.1007/s12031-008-9155-6
- Luo, H., Jing, B., Xia, Y., Zhang, Y., Hu, M., Cai, H., . . . Wu, Y. (2019). WP1130 reveals USP24 as a novel target in T-cell acute lymphoblastic leukemia. *Cancer Cell Int*, 19, 56. doi:10.1186/s12935-019-0773-6
- Magiera, M. M., Mora, S., Mojsa, B., Robbins, I., Lassot, I., & Desagher, S. (2013). Trim17-mediated ubiquitination and degradation of Mcl-1 initiate apoptosis in neurons. *Cell Death Differ*, 20(2), 281-292. doi:10.1038/cdd.2012.124
- Majid, A., Tsoulakis, O., Walewska, R., Gesk, S., Siebert, R., Kennedy, D. B., & Dyer, M. J. (2008). BCL2 expression in chronic lymphocytic leukemia: lack of association with the BCL2 938A>C promoter single nucleotide polymorphism. *Blood*, 111(2), 874-877. doi:10.1182/blood-2007-07-098681
- Markson, G., Kiel, C., Hyde, R., Brown, S., Charalabous, P., Bremm, A., . . . Sanderson, C. M. (2009). Analysis of the human E2 ubiquitin conjugating enzyme protein interaction network. *Genome Res*, 19(10), 1905-1911. doi:10.1101/gr.093963.109
- Maryanovich, M., Zaltsman, Y., Ruggiero, A., Goldman, A., Shachnai, L., Zaidman, S. L., . . . Gross, A. (2015). An MTCH2 pathway repressing mitochondria metabolism regulates haematopoietic stem cell fate. *Nat Commun*, 6, 7901. doi:10.1038/ncomms8901
- Mason, K. D., Carpinelli, M. R., Fletcher, J. I., Collinge, J. E., Hilton, A. A., Ellis, S., . . . Kile, B. T. (2007). Programmed anuclear cell death delimits platelet life span. *Cell*, 128(6), 1173-1186. doi:10.1016/j.cell.2007.01.037
- Mason, K. D., Khaw, S. L., Rayeroux, K. C., Chew, E., Lee, E. F., Fairlie, W. D., . . . Roberts, A. W. (2009). The BH3 mimetic compound, ABT-737, synergizes with a range of cytotoxic chemotherapy agents in chronic lymphocytic leukemia. *Leukemia*, 23(11), 2034-2041. doi:10.1038/leu.2009.151
- Mattson, M. P. (2000). Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol*, 1(2), 120-129. doi:10.1038/35040009
- McIlwain, D. R., Berger, T., & Mak, T. W. (2015). Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol*, 7(4). doi:10.1101/cshperspect.a026716

- Mei, Y., Du, W., Yang, Y., & Wu, M. (2005). Puma(*)Mcl-1 interaction is not sufficient to prevent rapid degradation of Mcl-1. *Oncogene*, 24(48), 7224-7237. doi:10.1038/sj.onc.1208873
- Merino, D., Giam, M., Hughes, P. D., Siggs, O. M., Heger, K., O'Reilly, L. A., . . . Bouillet, P. (2009). The role of BH3-only protein Bim extends beyond inhibiting Bcl-2-like prosurvival proteins. *J Cell Biol*, 186(3), 355-362. doi:10.1083/jcb.200905153
- Merino, D., Kelly, G. L., Lessene, G., Wei, A. H., Roberts, A. W., & Strasser, A. (2018). BH3-Mimetic Drugs: Blazing the Trail for New Cancer Medicines. *Cancer Cell*, 34(6), 879-891. doi:10.1016/j.ccell.2018.11.004
- Merino, D., Khaw, S. L., Glaser, S. P., Anderson, D. J., Belmont, L. D., Wong, C., . . . Bouillet, P. (2012). Bcl-2, Bcl-x(L), and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. *Blood*, 119(24), 5807-5816. doi:10.1182/blood-2011-12-400929
- Meyers, R. M., Bryan, J. G., McFarland, J. M., Weir, B. A., Sizemore, A. E., Xu, H., . . . Tsherniak, A. (2017). Computational correction of copy number effect improves specificity of CRISPR-Cas9 essentiality screens in cancer cells. *Nat Genet*, 49(12), 1779-1784. doi:10.1038/ng.3984
- Michels, J., O'Neill, J. W., Dallman, C. L., Mouzakiti, A., Habens, F., Brimmell, M., . . . Packham, G. (2004). Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage. *Oncogene*, 23(28), 4818-4827. doi:10.1038/sj.onc.1207648
- Mikhailov, V., Mikhailova, M., Pulkrabek, D. J., Dong, Z., Venkatachalam, M. A., & Saikumar, P. (2001). Bcl-2 prevents Bax oligomerization in the mitochondrial outer membrane. *J Biol Chem*, 276(21), 18361-18374. doi:10.1074/jbc.M100655200
- Mojsa, B., Lassot, I., & Desagher, S. (2014). Mcl-1 ubiquitination: unique regulation of an essential survival protein. *Cells*, 3(2), 418-437. doi:10.3390/cells3020418
- Moldoveanu, T., Liu, Q., Tocilj, A., Watson, M., Shore, G., & Gehring, K. (2006). The X-ray structure of a BAK homodimer reveals an inhibitory zinc binding site. *Mol Cell*, 24(5), 677-688. doi:10.1016/j.molcel.2006.10.014
- Morishima, N., Nakanishi, K., Tsuchiya, K., Shibata, T., & Seiwa, E. (2004). Translocation of Bim to the endoplasmic reticulum (ER) mediates ER stress signaling for activation of caspase-12 during ER stress-induced apoptosis. *J Biol Chem*, 279(48), 50375-50381. doi:10.1074/jbc.M408493200
- Motoyama, N., Wang, F., Roth, K. A., Sawa, H., Nakayama, K., Nakayama, K., . . . et al. (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science*, 267(5203), 1506-1510. doi:10.1126/science.7878471
- Mott, J. L., Kobayashi, S., Bronk, S. F., & Gores, G. J. (2007). mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene*, 26(42), 6133-6140. doi:10.1038/sj.onc.1210436
- Nagata, S. (2010). Apoptosis and autoimmune diseases. *Ann N Y Acad Sci*, 1209, 10-16. doi:10.1111/j.1749-6632.2010.05749.x
- Nakajima, W., Hicks, M. A., Tanaka, N., Krystal, G. W., & Harada, H. (2014). Noxa determines localization and stability of MCL-1 and consequently ABT-737 sensitivity in small cell lung cancer. *Cell Death Dis*, 5, e1052. doi:10.1038/cddis.2014.6
- Nakayama, K., Nakayama, K., Negishi, I., Kuida, K., Sawa, H., & Loh, D. Y. (1994). Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair,

- polycystic kidney disease, and lymphocytopenia. *Proc Natl Acad Sci U S A*, 91(9), 3700-3704. doi:10.1073/pnas.91.9.3700
- Nechushtan, A., Smith, C. L., Hsu, Y. T., & Youle, R. J. (1999). Conformation of the Bax C-terminus regulates subcellular location and cell death. *Embo j*, 18(9), 2330-2341. doi:10.1093/emboj/18.9.2330
- Nifoussi, S. K., Ratcliffe, N. R., Ornstein, D. L., Kasof, G., Strack, S., & Craig, R. W. (2014). Inhibition of protein phosphatase 2A (PP2A) prevents Mcl-1 protein dephosphorylation at the Thr-163/Ser-159 phosphodegron, dramatically reducing expression in Mcl-1-amplified lymphoma cells. *J Biol Chem*, 289(32), 21950-21959. doi:10.1074/jbc.M114.587873
- Nifoussi, S. K., Vrana, J. A., Domina, A. M., De Biasio, A., Gui, J., Gregory, M. A., . . . Craig, R. W. (2012). Thr 163 phosphorylation causes Mcl-1 stabilization when degradation is independent of the adjacent GSK3-targeted phosphodegron, promoting drug resistance in cancer. *PLoS One*, 7(10), e47060. doi:10.1371/journal.pone.0047060
- Nijhawan, D., Fang, M., Traer, E., Zhong, Q., Gao, W., Du, F., & Wang, X. (2003). Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev*, 17(12), 1475-1486. doi:10.1101/gad.1093903
- Niu, X., Zhao, J., Ma, J., Xie, C., Edwards, H., Wang, G., . . . Ge, Y. (2016). Binding of Released Bim to Mcl-1 is a Mechanism of Intrinsic Resistance to ABT-199 which can be Overcome by Combination with Daunorubicin or Cytarabine in AML Cells. *Clin Cancer Res*, 22(17), 4440-4451. doi:10.1158/1078-0432.Ccr-15-3057
- O'Neill, K. L., Huang, K., Zhang, J., Chen, Y., & Luo, X. (2016). Inactivation of prosurvival Bcl-2 proteins activates Bax/Bak through the outer mitochondrial membrane. *Genes Dev*, 30(8), 973-988. doi:10.1101/gad.276725.115
- Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., . . . Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*, 288(5468), 1053-1058. doi:10.1126/science.288.5468.1053
- Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., . . . Rosenberg, S. H. (2005). An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature*, 435(7042), 677-681. doi:10.1038/nature03579
- Ottis, P., & Crews, C. M. (2017). Proteolysis-Targeting Chimeras: Induced Protein Degradation as a Therapeutic Strategy. *ACS Chem Biol*, 12(4), 892-898. doi:10.1021/acscchembio.6b01068
- Pang, X., Zhang, J., Lopez, H., Wang, Y., Li, W., O'Neill, K. L., . . . Luo, X. (2014). The carboxyl-terminal tail of Noxa protein regulates the stability of Noxa and Mcl-1. *J Biol Chem*, 289(25), 17802-17811. doi:10.1074/jbc.M114.548172
- Papatzimas, J. W., Gorobets, E., Maity, R., Muniyat, M. I., MacCallum, J. L., Neri, P., . . . Derksen, D. J. (2019). From Inhibition to Degradation: Targeting the Antiapoptotic Protein Myeloid Cell Leukemia 1 (MCL1). *J Med Chem*, 62(11), 5522-5540. doi:10.1021/acs.jmedchem.9b00455
- Peddaboina, C., Jupiter, D., Fletcher, S., Yap, J. L., Rai, A., Tobin, R. P., . . . Cao, X. (2012). The downregulation of Mcl-1 via USP9X inhibition sensitizes solid tumors to Bcl-xl inhibition. *BMC Cancer*, 12, 541. doi:10.1186/1471-2407-12-541

- Peperzak, V., Vikstrom, I., Walker, J., Glaser, S. P., LePage, M., Coquery, C. M., . . . Tarlinton, D. M. (2013). Mcl-1 is essential for the survival of plasma cells. *Nat Immunol*, 14(3), 290-297. doi:10.1038/ni.2527
- Perciavalle, R. M., & Opferman, J. T. (2013). Delving deeper: MCL-1's contributions to normal and cancer biology. *Trends Cell Biol*, 23(1), 22-29. doi:10.1016/j.tcb.2012.08.011
- Perciavalle, R. M., Stewart, D. P., Koss, B., Lynch, J., Milasta, S., Bathina, M., . . . Opferman, J. T. (2012). Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. *Nat Cell Biol*, 14(6), 575-583. doi:10.1038/ncb2488
- Peterson, L. F., Sun, H., Liu, Y., Potu, H., Kandarpa, M., Ermann, M., . . . Donato, N. J. (2015). Targeting deubiquitinase activity with a novel small-molecule inhibitor as therapy for B-cell malignancies. *Blood*, 125(23), 3588-3597. doi:10.1182/blood-2014-10-605584
- Petros, A. M., Medek, A., Nettesheim, D. G., Kim, D. H., Yoon, H. S., Swift, K., . . . Fesik, S. W. (2001). Solution structure of the antiapoptotic protein bcl-2. *Proc Natl Acad Sci U S A*, 98(6), 3012-3017. doi:10.1073/pnas.041619798
- Ploner, C., Rainer, J., Niederegger, H., Eduardoff, M., Villunger, A., Geley, S., & Kofler, R. (2008). The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia. *Leukemia*, 22(2), 370-377. doi:10.1038/sj.leu.2405039
- Popgeorgiev, N., Jabbour, L., & Gillet, G. (2018). Subcellular Localization and Dynamics of the Bcl-2 Family of Proteins. *Front Cell Dev Biol*, 6, 13. doi:10.3389/fcell.2018.00013
- Potu, H., Peterson, L. F., Pal, A., Verhaegen, M., Cao, J., Talpaz, M., & Donato, N. J. (2014). Usp5 links suppression of p53 and FAS levels in melanoma to the BRAF pathway. *Oncotarget*, 5(14), 5559-5569. doi:10.18632/oncotarget.2140
- Print, C. G., Loveland, K. L., Gibson, L., Meehan, T., Stylianou, A., Wreford, N., . . . Cory, S. (1998). Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. *Proc Natl Acad Sci U S A*, 95(21), 12424-12431. doi:10.1073/pnas.95.21.12424
- Raemy, E., Montessuit, S., Pierredon, S., van Kampen, A. H., Vaz, F. M., & Martinou, J. C. (2016). Cardiolipin or MTCH2 can serve as tBID receptors during apoptosis. *Cell Death Differ*, 23(7), 1165-1174. doi:10.1038/cdd.2015.166
- Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*, 8(11), 2281-2308. doi:10.1038/nprot.2013.143
- Rathmell, J. C., Lindsten, T., Zong, W. X., Cinalli, R. M., & Thompson, C. B. (2002). Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. *Nat Immunol*, 3(10), 932-939. doi:10.1038/ni834
- Rechsteiner, M., & Rogers, S. W. (1996). PEST sequences and regulation by proteolysis. *Trends Biochem Sci*, 21(7), 267-271.
- Renstrom, F., Payne, F., Nordstrom, A., Brito, E. C., Rolandsson, O., Hallmans, G., . . . Franks, P. W. (2009). Replication and extension of genome-wide association study results for obesity in 4923 adults from northern Sweden. *Hum Mol Genet*, 18(8), 1489-1496. doi:10.1093/hmg/ddp041
- Rinkenberger, J. L., Horning, S., Klocke, B., Roth, K., & Korsmeyer, S. J. (2000). Mcl-1 deficiency results in peri-implantation embryonic lethality. *Genes Dev*, 14(1), 23-27.

- Roberts, A. W., Davids, M. S., Pagel, J. M., Kahl, B. S., Puvvada, S. D., Gerecitano, J. F., . . . Seymour, J. F. (2016). Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. *N Engl J Med*, 374(4), 311-322. doi:10.1056/NEJMoa1513257
- Rodriguez, J., & Lazebnik, Y. (1999). Caspase-9 and APAF-1 form an active holoenzyme. *Genes Dev*, 13(24), 3179-3184. doi:10.1101/gad.13.24.3179
- Roos, W. P., & Kaina, B. (2006). DNA damage-induced cell death by apoptosis. *Trends Mol Med*, 12(9), 440-450. doi:10.1016/j.molmed.2006.07.007
- Rosebeck, S., Sudini, K., Chen, T., & Leaman, D. W. (2011). Involvement of Noxa in mediating cellular ER stress responses to lytic virus infection. *Virology*, 417(2), 293-303. doi:10.1016/j.virol.2011.06.010
- Ross, A. J., Waymire, K. G., Moss, J. E., Parlow, A. F., Skinner, M. K., Russell, L. D., & MacGregor, G. R. (1998). Testicular degeneration in Bclw-deficient mice. *Nat Genet*, 18(3), 251-256. doi:10.1038/ng0398-251
- Roux, K. J., Kim, D. I., & Burke, B. (2013). BioID: a screen for protein-protein interactions. *Curr Protoc Protein Sci*, 74, Unit 19.23. doi:10.1002/0471140864.ps1923s74
- Roux, K. J., Kim, D. I., Raida, M., & Burke, B. (2012). A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *J Cell Biol*, 196(6), 801-810. doi:10.1083/jcb.201112098
- Ryan, J. A., Brunelle, J. K., & Letai, A. (2010). Heightened mitochondrial priming is the basis for apoptotic hypersensitivity of CD4⁺ CD8⁺ thymocytes. *Proc Natl Acad Sci U S A*, 107(29), 12895-12900. doi:10.1073/pnas.0914878107
- Scheffold, A., Jebaraj, B. M. C., & Stilgenbauer, S. (2018). Venetoclax: Targeting BCL2 in Hematological Cancers. *Recent Results Cancer Res*, 212, 215-242. doi:10.1007/978-3-319-91439-8_11
- Schenk, R. L., Tuzlak, S., Carrington, E. M., Zhan, Y., Heinzl, S., Teh, C. E., . . . Herold, M. J. (2017). Characterisation of mice lacking all functional isoforms of the pro-survival BCL-2 family member A1 reveals minor defects in the haematopoietic compartment. *Cell Death Differ*, 24(3), 534-545. doi:10.1038/cdd.2016.156
- Schinzl, A., Kaufmann, T., & Borner, C. (2004). Bcl-2 family members: integrators of survival and death signals in physiology and pathology [corrected]. *Biochim Biophys Acta*, 1644(2-3), 95-105. doi:10.1016/j.bbamcr.2003.09.006
- Schwickart, M., Huang, X., Lill, J. R., Liu, J., Ferrando, R., French, D. M., . . . Dixit, V. M. (2010). Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival. *Nature*, 463(7277), 103-107. doi:10.1038/nature08646
- Sieghart, W., Losert, D., Strommer, S., Cejka, D., Schmid, K., Rasoul-Rockenschaub, S., . . . Wacheck, V. (2006). Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. *J Hepatol*, 44(1), 151-157. doi:10.1016/j.jhep.2005.09.010
- Simonetta, K. R., Taygerly, J., Boyle, K., Basham, S. E., Padovani, C., Lou, Y., . . . Saha, A. (2019). Prospective discovery of small molecule enhancers of an E3 ligase-substrate interaction. *Nat Commun*, 10(1), 1402. doi:10.1038/s41467-019-09358-9
- Skowrya, A., Allan, L. A., Saurin, A. T., & Clarke, P. R. (2018). USP9X Limits Mitotic Checkpoint Complex Turnover to Strengthen the Spindle Assembly Checkpoint and Guard against Chromosomal Instability. *Cell Rep*, 23(3), 852-865. doi:10.1016/j.celrep.2018.03.100

- Song, L., Coppola, D., Livingston, S., Cress, D., & Haura, E. B. (2005). Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. *Cancer Biol Ther*, 4(3), 267-276.
- Song, L., & Rape, M. (2008). Reverse the curse--the role of deubiquitination in cell cycle control. *Curr Opin Cell Biol*, 20(2), 156-163. doi:10.1016/j.ceb.2008.01.012
- Sorenson, C. M. (2004). Bcl-2 family members and disease. *Biochim Biophys Acta*, 1644(2-3), 169-177. doi:10.1016/j.bbamcr.2003.08.010
- Souers, A. J., Levenson, J. D., Boghaert, E. R., Ackler, S. L., Catron, N. D., Chen, J., . . . Elmore, S. W. (2013). ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med*, 19(2), 202-208. doi:10.1038/nm.3048
- Stefani, F., Zhang, L., Taylor, S., Donovan, J., Rollinson, S., Doyotte, A., . . . Woodman, P. (2011). UBAP1 is a component of an endosome-specific ESCRT-I complex that is essential for MVB sorting. *Curr Biol*, 21(14), 1245-1250. doi:10.1016/j.cub.2011.06.028
- Stevenson, L. F., Sparks, A., Allende-Vega, N., Xirodimas, D. P., Lane, D. P., & Saville, M. K. (2007). The deubiquitinating enzyme USP2a regulates the p53 pathway by targeting Mdm2. *Embo j*, 26(4), 976-986. doi:10.1038/sj.emboj.7601567
- Stewart, D. P., Koss, B., Bathina, M., Perciavalle, R. M., Bisanz, K., & Opferman, J. T. (2010). Ubiquitin-independent degradation of antiapoptotic MCL-1. *Mol Cell Biol*, 30(12), 3099-3110. doi:10.1128/mcb.01266-09
- Strasser, A., Cory, S., & Adams, J. M. (2011). Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. *Embo j*, 30(18), 3667-3683. doi:10.1038/emboj.2011.307
- Subramanian, A., Andronache, A., Li, Y. C., & Wade, M. (2016). Inhibition of MARCH5 ubiquitin ligase abrogates MCL1-dependent resistance to BH3 mimetics via NOXA. *Oncotarget*, 7(13), 15986-16002. doi:10.18632/oncotarget.7558
- Sun, Y., & Leaman, D. W. (2005). Involvement of Noxa in cellular apoptotic responses to interferon, double-stranded RNA, and virus infection. *J Biol Chem*, 280(16), 15561-15568. doi:10.1074/jbc.M412630200
- Suzuki, M., Youle, R. J., & Tjandra, N. (2000). Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell*, 103(4), 645-654. doi:10.1016/s0092-8674(00)00167-7
- Swatek, K. N., & Komander, D. (2016). Ubiquitin modifications. *Cell Res*, 26(4), 399-422. doi:10.1038/cr.2016.39
- Tagawa, H., Karnan, S., Suzuki, R., Matsuo, K., Zhang, X., Ota, A., . . . Seto, M. (2005). Genome-wide array-based CGH for mantle cell lymphoma: identification of homozygous deletions of the proapoptotic gene BIM. *Oncogene*, 24(8), 1348-1358. doi:10.1038/sj.onc.1208300
- Tahir, S. K., Smith, M. L., Hessler, P., Rapp, L. R., Idler, K. B., Park, C. H., . . . Lam, L. T. (2017). Potential mechanisms of resistance to venetoclax and strategies to circumvent it. *BMC Cancer*, 17(1), 399. doi:10.1186/s12885-017-3383-5
- Tan, C., Byrne, E. F. X., Ah-Cann, C., Call, M. J., & Call, M. E. (2019). A serine in the first transmembrane domain of the human E3 ubiquitin ligase MARCH9 is critical for down-regulation of its protein substrates. *J Biol Chem*, 294(7), 2470-2485. doi:10.1074/jbc.RA118.004836

- Tao, Z. F., Hasvold, L., Wang, L., Wang, X., Petros, A. M., Park, C. H., . . . Souers, A. J. (2014). Discovery of a Potent and Selective BCL-XL Inhibitor with in Vivo Activity. *ACS Med Chem Lett*, 5(10), 1088-1093. doi:10.1021/ml5001867
- Thomas, L. W., Lam, C., & Edwards, S. W. (2010). Mcl-1; the molecular regulation of protein function. *FEBS Lett*, 584(14), 2981-2989. doi:10.1016/j.febslet.2010.05.061
- Thomas, R. L., Roberts, D. J., Kubli, D. A., Lee, Y., Quinsay, M. N., Owens, J. B., . . . Gustafsson, A. B. (2013). Loss of MCL-1 leads to impaired autophagy and rapid development of heart failure. *Genes Dev*, 27(12), 1365-1377. doi:10.1101/gad.215871.113
- Thomenius, M. J., & Distelhorst, C. W. (2003). Bcl-2 on the endoplasmic reticulum: protecting the mitochondria from a distance. *J Cell Sci*, 116(Pt 22), 4493-4499. doi:10.1242/jcs.00829
- Tromp, J. M., Geest, C. R., Breij, E. C., Elias, J. A., van Laar, J., Luijckx, D. M., . . . Eldering, E. (2012). Tipping the Noxa/Mcl-1 balance overcomes ABT-737 resistance in chronic lymphocytic leukemia. *Clin Cancer Res*, 18(2), 487-498. doi:10.1158/1078-0432.Ccr-11-1440
- Tron, A. E., Belmonte, M. A., Adam, A., Aquila, B. M., Boise, L. H., Chiarparin, E., . . . Hird, A. W. (2018). Discovery of Mcl-1-specific inhibitor AZD5991 and preclinical activity in multiple myeloma and acute myeloid leukemia. *Nat Commun*, 9(1), 5341. doi:10.1038/s41467-018-07551-w
- Tse, C., Shoemaker, A. R., Adickes, J., Anderson, M. G., Chen, J., Jin, S., . . . Elmore, S. W. (2008). ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res*, 68(9), 3421-3428. doi:10.1158/0008-5472.Can-07-5836
- Tsujimoto, Y., Cossman, J., Jaffe, E., & Croce, C. M. (1985). Involvement of the bcl-2 gene in human follicular lymphoma. *Science*, 228(4706), 1440-1443. doi:10.1126/science.3874430
- Tze, L. E., Horikawa, K., Domaschenz, H., Howard, D. R., Roots, C. M., Rigby, R. J., . . . Goodnow, C. C. (2011). CD83 increases MHC II and CD86 on dendritic cells by opposing IL-10-driven MARCH1-mediated ubiquitination and degradation. *J Exp Med*, 208(1), 149-165. doi:10.1084/jem.20092203
- Ui-Tei, K. (2013). Optimal choice of functional and off-target effect-reduced siRNAs for RNAi therapeutics. *Front Genet*, 4, 107. doi:10.3389/fgene.2013.00107
- Uren, R. T., Iyer, S., & Kluck, R. M. (2017). Pore formation by dimeric Bak and Bax: an unusual pore? *Philos Trans R Soc Lond B Biol Sci*, 372(1726). doi:10.1098/rstb.2016.0218
- van Delft, M. F., Wei, A. H., Mason, K. D., Vandenberg, C. J., Chen, L., Czabotar, P. E., . . . Huang, D. C. (2006). The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell*, 10(5), 389-399. doi:10.1016/j.ccr.2006.08.027
- van Wijk, S. J., de Vries, S. J., Kemmeren, P., Huang, A., Boelens, R., Bonvin, A. M., & Timmers, H. T. (2009). A comprehensive framework of E2-RING E3 interactions of the human ubiquitin-proteasome system. *Mol Syst Biol*, 5, 295. doi:10.1038/msb.2009.55
- Vaux, D. L., Cory, S., & Adams, J. M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*, 335(6189), 440-442. doi:10.1038/335440a0
- Veis, D. J., Sorenson, C. M., Shutter, J. R., & Korsmeyer, S. J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell*, 75(2), 229-240. doi:10.1016/0092-8674(93)80065-m

- Vick, B., Weber, A., Urbanik, T., Maass, T., Teufel, A., Krammer, P. H., . . . Schulze-Bergkamen, H. (2009). Knockout of myeloid cell leukemia-1 induces liver damage and increases apoptosis susceptibility of murine hepatocytes. *Hepatology*, 49(2), 627-636. doi:10.1002/hep.22664
- Vikstrom, I., Carotta, S., Luthje, K., Peperzak, V., Jost, P. J., Glaser, S., . . . Tarlinton, D. M. (2010). Mcl-1 is essential for germinal center formation and B cell memory. *Science*, 330(6007), 1095-1099. doi:10.1126/science.1191793
- Vucic, D., Dixit, V. M., & Wertz, I. E. (2011). Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. *Nat Rev Mol Cell Biol*, 12(7), 439-452. doi:10.1038/nrm3143
- Wang, B., Xie, M., Li, R., Owonikoko, T. K., Ramalingam, S. S., Khuri, F. R., . . . Deng, X. (2014). Role of Ku70 in deubiquitination of Mcl-1 and suppression of apoptosis. *Cell Death Differ*, 21(7), 1160-1169. doi:10.1038/cdd.2014.42
- Wang, X., Bathina, M., Lynch, J., Koss, B., Calabrese, C., Frase, S., . . . Opferman, J. T. (2013). Deletion of MCL-1 causes lethal cardiac failure and mitochondrial dysfunction. *Genes Dev*, 27(12), 1351-1364. doi:10.1101/gad.215855.113
- Wang, Z., He, N., Guo, Z., Niu, C., Song, T., Guo, Y., . . . Zhang, Z. (2019). Proteolysis Targeting Chimeras for the Selective Degradation of Mcl-1/Bcl-2 Derived from Nonselective Target Binding Ligands. *J Med Chem*, 62(17), 8152-8163. doi:10.1021/acs.jmedchem.9b00919
- Wei, G., Twomey, D., Lamb, J., Schlis, K., Agarwal, J., Stam, R. W., . . . Armstrong, S. A. (2006). Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. *Cancer Cell*, 10(4), 331-342. doi:10.1016/j.ccr.2006.09.006
- Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., . . . Korsmeyer, S. J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*, 292(5517), 727-730. doi:10.1126/science.1059108
- Weng, C., Li, Y., Xu, D., Shi, Y., & Tang, H. (2005). Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. *J Biol Chem*, 280(11), 10491-10500. doi:10.1074/jbc.M412819200
- Wensveen, F. M., Derks, I. A., van Gisbergen, K. P., de Bruin, A. M., Meijers, J. C., Yigitop, H., . . . van Lier, R. A. (2012). BH3-only protein Noxa regulates apoptosis in activated B cells and controls high-affinity antibody formation. *Blood*, 119(6), 1440-1449. doi:10.1182/blood-2011-09-378877
- Wensveen, F. M., van Gisbergen, K. P., Derks, I. A., Gerlach, C., Schumacher, T. N., van Lier, R. A., & Eldering, E. (2010). Apoptosis threshold set by Noxa and Mcl-1 after T cell activation regulates competitive selection of high-affinity clones. *Immunity*, 32(6), 754-765. doi:10.1016/j.immuni.2010.06.005
- Wertz, I. E., Kusam, S., Lam, C., Okamoto, T., Sandoval, W., Anderson, D. J., . . . Dixit, V. M. (2011). Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. *Nature*, 471(7336), 110-114. doi:10.1038/nature09779
- Willer, C. J., Speliotes, E. K., Loos, R. J., Li, S., Lindgren, C. M., Heid, I. M., . . . Hirschhorn, J. N. (2009). Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. *Nat Genet*, 41(1), 25-34. doi:10.1038/ng.287
- Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., . . . Huang, D. C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2,

- until displaced by BH3-only proteins. *Genes Dev*, 19(11), 1294-1305. doi:10.1101/gad.1304105
- Willis, S. N., Fletcher, J. I., Kaufmann, T., van Delft, M. F., Chen, L., Czabotar, P. E., . . . Huang, D. C. (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science*, 315(5813), 856-859. doi:10.1126/science.1133289
- Wilson, W. H., O'Connor, O. A., Czuczman, M. S., LaCasce, A. S., Gerecitano, J. F., Leonard, J. P., . . . Humerickhouse, R. A. (2010). Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. *Lancet Oncol*, 11(12), 1149-1159. doi:10.1016/s1470-2045(10)70261-8
- Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G., & Youle, R. J. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol*, 139(5), 1281-1292. doi:10.1083/jcb.139.5.1281
- Wong, R. S. (2011). Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res*, 30, 87. doi:10.1186/1756-9966-30-87
- Xu, S., Cherok, E., Das, S., Li, S., Roelofs, B. A., Ge, S. X., . . . Karbowski, M. (2016). Mitochondrial E3 ubiquitin ligase MARCH5 controls mitochondrial fission and cell sensitivity to stress-induced apoptosis through regulation of MiD49 protein. *Mol Biol Cell*, 27(2), 349-359. doi:10.1091/mbc.E15-09-0678
- Yamamura, K., Kamada, S., Ito, S., Nakagawa, K., Ichihashi, M., & Tsujimoto, Y. (1996). Accelerated disappearance of melanocytes in bcl-2-deficient mice. *Cancer Res*, 56(15), 3546-3550.
- Yamashita, M., Kuwahara, M., Suzuki, A., Hirahara, K., Shinnaksu, R., Hosokawa, H., . . . Nakayama, T. (2008). Bmi1 regulates memory CD4 T cell survival via repression of the Noxa gene. *J Exp Med*, 205(5), 1109-1120. doi:10.1084/jem.20072000
- Yan, J., Zhong, N., Liu, G., Chen, K., Liu, X., Su, L., & Singhal, S. (2014). Usp9x- and Noxa-mediated Mcl-1 downregulation contributes to pemetrexed-induced apoptosis in human non-small-cell lung cancer cells. *Cell Death Dis*, 5, e1316. doi:10.1038/cddis.2014.281
- Yang, T., Buchan, H. L., Townsend, K. J., & Craig, R. W. (1996). MCL-1, a member of the BCL-2 family, is induced rapidly in response to signals for cell differentiation or death, but not to signals for cell proliferation. *J Cell Physiol*, 166(3), 523-536. doi:10.1002/(sici)1097-4652(199603)166:3<523::Aid-jcp7>3.0.Co;2-r
- Yang, T., Kozopas, K. M., & Craig, R. W. (1995). The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. *J Cell Biol*, 128(6), 1173-1184.
- Yoo, Y. S., Park, Y. Y., Kim, J. H., Cho, H., Kim, S. H., Lee, H. S., . . . Cho, H. (2015). The mitochondrial ubiquitin ligase MARCH5 resolves MAVS aggregates during antiviral signalling. *Nat Commun*, 6, 7910. doi:10.1038/ncomms8910
- Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*, 9(1), 47-59. doi:10.1038/nrm2308
- Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W., & Vogelstein, B. (2001). PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell*, 7(3), 673-682.
- Zaltsman, Y., Shachnai, L., Yivgi-Ohana, N., Schwarz, M., Maryanovich, M., Houtkooper, R. H., . . . Gross, A. (2010). MTCH2/MIMP is a major facilitator of tBID recruitment to mitochondria. *Nat Cell Biol*, 12(6), 553-562. doi:10.1038/ncb2057

- Zhang, H., Guttikonda, S., Roberts, L., Uziel, T., Semizarov, D., Elmore, S. W., . . . Lam, L. T. (2011). Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines. *Oncogene*, 30(16), 1963-1968. doi:10.1038/onc.2010.559
- Zhang, S., Zhang, M., Jing, Y., Yin, X., Ma, P., Zhang, Z., . . . Zhuang, G. (2018). Deubiquitinase USP13 dictates MCL1 stability and sensitivity to BH3 mimetic inhibitors. *Nat Commun*, 9(1), 215. doi:10.1038/s41467-017-02693-9
- Zhao, X., Ren, Y., Lawlor, M., Shah, B. D., Park, P. M. C., Lwin, T., . . . Tao, J. (2019). BCL2 Amplicon Loss and Transcriptional Remodeling Drives ABT-199 Resistance in B Cell Lymphoma Models. *Cancer Cell*, 35(5), 752-766.e759. doi:10.1016/j.ccell.2019.04.005
- Zhong, Q., Gao, W., Du, F., & Wang, X. (2005). Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell*, 121(7), 1085-1095. doi:10.1016/j.cell.2005.06.009
- Zhou, P., Levy, N. B., Xie, H., Qian, L., Lee, C. Y., Gascoyne, R. D., & Craig, R. W. (2001). MCL1 transgenic mice exhibit a high incidence of B-cell lymphoma manifested as a spectrum of histologic subtypes. *Blood*, 97(12), 3902-3909. doi:10.1182/blood.v97.12.3902
- Zong, W. X., Lindsten, T., Ross, A. J., MacGregor, G. R., & Thompson, C. B. (2001). BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev*, 15(12), 1481-1486. doi:10.1101/gad.897601
- Zou, H., Henzel, W. J., Liu, X., Lutschg, A., & Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell*, 90(3), 405-413. doi:10.1016/s0092-8674(00)80501-2
- Zou, H., Li, Y., Liu, X., & Wang, X. (1999). An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem*, 274(17), 11549-11556. doi:10.1074/jbc.274.17.11549