THE GENETIC PROGRAM OF MYC-POTENTIATED APOPTOSIS: SYSTEMS DEVELOPMENT

by

Andrew Christopher Rust

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Medical Biophysics
University of Toronto

© Copyright by Andrew Christopher Rust (2009)
The Genetic Program of Myc-potentiated apoptosis: Systems Development

Master of Science

2009

Andrew Christopher Rust

Graduate Department of Medical Biophysics

University of Toronto

Abstract

Myc is a powerful oncogene frequently deregulated in cancer yet deregulated Myc alone does not lead to cellular transformation due to the intrinsic safety mechanism of deregulated Myc potentiating apoptosis. The mechanism by which Myc potentiates apoptosis remains unclear, however, because the regions of Myc essential for apoptosis are also required for Myc to function as a regulator of gene transcription, it is thought that Myc’s role in apoptosis is a function of its regulation of an apoptotic genetic program. We hypothesize that under apoptotic conditions, Myc differentially binds and/or regulates a specific cohort of genes to potentiate apoptosis. The foremost approach to addressing this hypothesis is the employment of ChIP-chip coupled with expression microarray analyses. Here, using the MCF10A breast epithelial and SHEP neuroblastoma cell lines, we developed and characterized two independent human systems for subsequent ChIP-chip and expression array analyses to elucidate the genetic program of Myc-potentiated apoptosis.
Acknowledgements

To my supervisor, Dr. Linda Penn. Thank you for your endless guidance and mentorship; thank you for challenging me to think critically and look beyond the numbers and thank you for giving this wonderful opportunity.

To my committee members: Dr. Jim Woodgett and Dr. Rod Bremner. Thank you for taking time out of your busy schedules to offer me the knowledge and suggestions necessary to keep me on track.

To the Penn lab members past and present: Paul Boutros, Christina Bros, James Clendening, Carolyn Goard, Fereshteh Khosravi, Sam Kim, Anna Martirosyan, Stefanie Oliveri, Aleksandra Pandyra, Romi Ponzielli, Angelina Stojanova, Grace Trentin, Amanda Wasylishen and Tessa Young. Thank you for all of your help and support, this would not have been possible without it. And thank you most for the friendships.

To Sam. Thank you for never hesitating to come in on weekends or late at night to lend me a hand and for always providing me with your genius perspective on my work.

To Amanda. Thank you for taking me under your wing from day one and seeing me all the way to the end. Thank you for always helping me when I needed it and always doing so with a smile. Never underestimate the utility of your motivational optimism.

To my family and friends. Thank you for all of your support and encouragement.
<table>
<thead>
<tr>
<th>Table of Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract .................................................................</td>
</tr>
<tr>
<td>Acknowledgements .........................................................</td>
</tr>
<tr>
<td>Table of Contents ................................................................</td>
</tr>
<tr>
<td>List of Figures and Tables ................................................</td>
</tr>
<tr>
<td>List of Abbreviations .......................................................</td>
</tr>
<tr>
<td>Introduction ........................................................................</td>
</tr>
<tr>
<td>Overview .............................................................................</td>
</tr>
<tr>
<td>Myc expression ...............................................................</td>
</tr>
<tr>
<td>Myc structure and function ...............................................</td>
</tr>
<tr>
<td>Myc transcriptional activity .......... ...................................</td>
</tr>
<tr>
<td>Apoptosis .................................................................</td>
</tr>
<tr>
<td>Myc and Apoptosis .........................................................</td>
</tr>
<tr>
<td>The genetic program of Myc-potentiated apoptosis ..................</td>
</tr>
<tr>
<td>ChIP-chip .................................................................</td>
</tr>
<tr>
<td>Hypothesis and Purpose ...................................................</td>
</tr>
<tr>
<td>Results .............................................................................</td>
</tr>
<tr>
<td>Optimization of ChIP for Myc in the MCF10A human breast cell line</td>
</tr>
<tr>
<td>Optimization and characterisation of MCF10A apoptotic condition</td>
</tr>
<tr>
<td>Optimization of ChIP of Myc in the SHEP human neuroblastoma cell line</td>
</tr>
<tr>
<td>Optimization and characterisation of SHEP apoptotic condition</td>
</tr>
<tr>
<td>Discussion .......................................................................</td>
</tr>
<tr>
<td>The MCF10A cell system .....................................................</td>
</tr>
<tr>
<td>The SHEP cell system ......................................................</td>
</tr>
<tr>
<td>Future work .......................................................................</td>
</tr>
<tr>
<td>Conclusion ........................................................................</td>
</tr>
<tr>
<td>Materials and Methods .....................................................</td>
</tr>
<tr>
<td>Cell culture .................................................................</td>
</tr>
<tr>
<td>Apoptotic treatments and Assays .........................................</td>
</tr>
<tr>
<td>ChIP-qPCR .................................................................</td>
</tr>
<tr>
<td>Immunoblotting ...........................................................</td>
</tr>
<tr>
<td>References .......................................................................</td>
</tr>
<tr>
<td>Appendix ..........................................................................</td>
</tr>
</tbody>
</table>
List of Figures and Tables

Figure 1  Working model of Myc differential binding and transcriptional regulatory activity under apoptotic vs. growth conditions ................................................................. 19
Figure 2  Schematic of ChIP-chip ........................................................................ 23
Figure 3  Optimization of Myc ChIP in the MCF10A cell line ............................ 27
Figure 4  Myc potentiates apoptosis in response to doxorubicin in combination with serum withdrawal ........................................................................................................... 32
Figure 5  Ectopic Myc is rapidly downregulated in response to doxorubicin in MCF10A cells .......................................................... 39
Figure 6  Schematic of inducible ectopic Myc expression system .......................... 41
Figure 7  Schematic of treatment regiment for MCF10A cells with inducible Myc expression system ........................................................................................................... 42
Figure 8  Myc induction potentiates apoptosis in response to tunicamycin and serum withdrawal in growth arrested MCF10A cells ......................................................... 45
Figure 9  Myc can be induced under apoptotic conditions to potentiate apoptosis as indicated by PARP-cleavage in MCF10A cells .................................................. 50
Figure 10 Optimization of Myc ChIP in the SHEP cell line ................................. 54
Figure 11 Optimization and validation of tunicamycin apoptotic treatment in SHEP cells ........................................................................................................... 58
Figure 12 Ectopic Myc potentiates apoptosis induced by tunicamycin as indicated by PARP-cleavage in SHEP cells and Myc expression is downregulated over time .................................................. 62
Figure 13 Optimization and validation of thapsigargin apoptotic treatment in SHEP cells .......................................................... 64
Figure 14 Ectopic Myc potentiates apoptosis induced by thapsigargin as indicated by PARP-cleavage in SHEP cells and Myc expression is downregulated over time .................................................. 68

Table 1 Various treatments tested to maximize Myc potentiated apoptosis in the MCF10A cell line ........................................................................................................... 35

Supplemental Figure 1 MCF10A cells arrest after 24h of serum withdrawal and enter cell cycle similar to normal growth within 24h to 32h upon mitogen stimulation .................................................. 103
Supplemental Figure 2 Myc induction is sufficient for cell cycle entry from under serum withdrawal conditions similar to mitogen stimulation .................................................. 104
Supplemental Figure 3 Myc can be induced under apoptotic conditions to potentiate apoptosis as indicated by PARP-cleavage in MCF10A cells .................................................. 105
Supplemental Figure 4 Ectopic Myc potentiates apoptosis induced by tunicamycin as indicated by PARP-cleavage in SHEP cells and Myc expression is downregulated over time .................................................. 107
Supplemental Figure 5 Ectopic Myc potentiates apoptosis induced by thapsigargin as indicated by PARP-cleavage in SHEP cells and is downregulated .................................................. 108
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAF1</td>
<td>apoptotic peptidase activating factor 1</td>
</tr>
<tr>
<td>APEX</td>
<td>apurinic/apyrimidinic endonuclease</td>
</tr>
<tr>
<td>ARF</td>
<td>alternate reading frame</td>
</tr>
<tr>
<td>ATF6</td>
<td>activating transcription factor 6</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL2-antagonist of cell death</td>
</tr>
<tr>
<td>BAK</td>
<td>BCL2-antagonist/killer</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
</tr>
<tr>
<td>BH1-4</td>
<td>BCL2 homology domains 1-4</td>
</tr>
<tr>
<td>bHLH-LZ</td>
<td>basic, helix-loop-helix, leucine zipper domain</td>
</tr>
<tr>
<td>BID</td>
<td>BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>BIK</td>
<td>BCL2-interacting killer</td>
</tr>
<tr>
<td>BIN-1</td>
<td>bridging integrator 1</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell lymphoma protein 2</td>
</tr>
<tr>
<td>BS</td>
<td>binding site</td>
</tr>
<tr>
<td>CAD</td>
<td>carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase</td>
</tr>
</tbody>
</table>
CASP8  caspase 8
Ch   chromosome
ChIP  chromatin-immunoprecipitation
CHOP  C/EBP homologous protein
CTD  C-terminal domain
CUL7  cullin 7
DD  death domain
DED  death effector domain
DISC  death-inducing signalling complex
Dnmt3a  DNA methyltransferase 3a
DR5  death receptor 5
E-box  enhancer box
EGFR  epidermal growth factor receptor
ER  endoplasmic reticulum
ERK  extracellular signal-regulated kinase
ERSE  ER stress response elements
ERα  estrogen receptor-α
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADD</td>
<td>Fas (TNFRSF6)-associated via death domain</td>
</tr>
<tr>
<td>FasR</td>
<td>Fas receptor</td>
</tr>
<tr>
<td>FBS/ HS</td>
<td>foetal bovine/horse serum</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE inhibitory protein</td>
</tr>
<tr>
<td>GADD34/45/153</td>
<td>growth arrest and DNA damage-inducible protein 34/45/153</td>
</tr>
<tr>
<td>GAS1</td>
<td>growth arrest-specific 1</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GRP78</td>
<td>glucose-regulated protein 78kDa</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HIF-1</td>
<td>hypoxia inducible factor 1</td>
</tr>
<tr>
<td>HK2</td>
<td>hexokinase 2</td>
</tr>
<tr>
<td>HNT</td>
<td>neurotrimin</td>
</tr>
<tr>
<td>HSP27</td>
<td>heat shock protein 27</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>Inr</td>
<td>initiator element</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IRE1</td>
<td>inositol-requiring enzyme 1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>MAX</td>
<td>MYC associated factor X</td>
</tr>
<tr>
<td>MBI-IV</td>
<td>Myc homology boxes I – IV</td>
</tr>
<tr>
<td>Mdm2</td>
<td>transformed mouse 3T3 cell double minute 2</td>
</tr>
<tr>
<td>MIZ-1</td>
<td>MIZ-type zinc finger transcription factor</td>
</tr>
<tr>
<td>MOMP</td>
<td>mitochondrial outer membrane permeabilisation</td>
</tr>
<tr>
<td>MYC</td>
<td>myelocytomatosis</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NOL6</td>
<td>nucleolar protein family 6</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP)ribose polymerase</td>
</tr>
<tr>
<td>PDK1</td>
<td>pyruvate dehydrogenase kinase, isozyme 1</td>
</tr>
<tr>
<td>PERK</td>
<td>(PKR)-like ER kinase</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinases</td>
</tr>
</tbody>
</table>
PKB  protein kinase B
P-TEFb  Positive Transcription Elongation Factor b
PUMA  p53 upregulated modulator of apoptosis
qPCR  quantitative real-time PCR
RAS  rat sarcoma
RIP  receptor-interactive protein
RNAPII  RNA polymerase II
Sp1/Sp2  specificity protein 1/2
SWI/SNF  SWItch/Sucrose NonFermentable
TCERG  transcription elongation regulator 1
TIP48/TIP49  TBP-interacting protein
TNF  tumour necrosis factor
TRADD  TNFRSF1A-associated via death domain
TRAIL  TNF-related apoptosis-inducing ligand
TRRAP  transformation/transcription domain-associated protein
TRB3  Tribbles-related protein 3
TUNEL  terminal dUTP nick-end labelling
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>XBPI</td>
<td>X box-binding protein 1</td>
</tr>
</tbody>
</table>
Introduction

Overview

The Myc oncogene is frequently deregulated in human cancers and has been associated with poor patient prognosis and aggressive disease progression; playing a role in various biological functions including cell growth, cell cycle, cell mass, angiogenesis and proliferation (1-4). In its normal proto-oncogenic state, Myc protein acts as the “intracellular sentinel of the extracellular milieu,” reflecting the growth state of the cell such that protein levels are elevated during growth and minimal during quiescence (4-6). Appropriately, proto-oncogenic Myc is highly regulated in normal cells through several mechanisms including mRNA and protein stability, as well as Myc gene transcription (7). This highly regulated state is lost in tumour cells. Deregulation can take a number of forms including overexpression through gross chromosomal abnormalities and constitutive activation by impaired upstream signalling pathways (8-10). Increased mRNA and protein stability can also contribute to deregulation (11).

Although deregulation of Myc plays a pivotal role in driving transformation, deregulated Myc also potentiates apoptosis (12-14). Whereas cells with regulated Myc and otherwise normal genetic backgrounds will undergo cell cycle arrest when encountering an anti-proliferative stimulus, such as serum deprivation or exposure to chemotherapeutics, cells harbouring deregulated Myc will be sensitized to such anti-proliferative stimuli and undergo apoptosis. This is thought to act as a sort of intrinsic safety mechanism (13). By deregulated Myc itself potentiating apoptosis, it is removing the cell from the population and thus preventing subsequent oncogenic development.
Because structure function analysis shows that the regions of Myc essential for apoptosis are also required for Myc to function as a regulator of gene transcription, it is thought that Myc's role in apoptosis is a function of its regulation of specific target genes. Identifying these apoptotic gene targets will not only provide further insight into the mechanism by which Myc potentiates apoptosis but will also provide potential targets for the development of anti-cancer therapeutics and perhaps allow for the possible exploitation of the deregulated Myc seen in many cancers.

**Myc expression**

The myelocytomatosis (MYC) gene was first identified as the transduced v-myc gene of the transforming avian myelocytomatosis retrovirus (15). The MYC family includes three transforming members: MYC, MYCN and MYCL1 (1). Whereas the protein products of all three transforming MYC gene family members (c-Myc, N-Myc and L-Myc) are all expressed during fetal development, in distinct tissue compartments, only the c-Myc (Myc) protein of the MYC gene is expressed in normal adult tissues (3, 15).

In non-transformed cells, Myc expression correlates with the growth state of the cell (16). Expression is regulated both positively and negatively by numerous proliferative and anti-proliferative signalling pathways. As such, levels are minimal during quiescence but increase rapidly upon mitogen stimulation as MYC is an immediate-early response gene (17). This expression of Myc is required and sufficient for G1/S transition, driving proliferation (18-20). Appropriately, in non-transformed cells, Myc expression is tightly regulated and responsive to environmental cues. Consequently, anti-proliferative signals, such as growth factor withdrawal, lead to downregulation of Myc expression (21). This occurs at the
transcriptional and translational level, as well as through mRNA and protein stability, both of which undergo rapid turnover with short half-lives of 20-30min (22, 23).

This tight regulation of Myc expression, however, is often lost in tumour cells. Deregulation of Myc leading to constitutive overexpression and lack of response to regulatory signals can occur through a number of mechanisms. Translocation of the \textit{MYC} gene downstream of a strong immunoglobulin enhancer drives mRNA and protein expression in Burkitt’s lymphoma (24). Amplification of the \textit{MYC} gene is frequently seen in primary breast tumours while \textit{MYCN} amplification is a hallmark of neuroblastoma and \textit{MYCL1} is amplified in ovarian cancer (25-27). All three transforming \textit{MYC} gene family members have been shown to be amplified separately, in small cell lung carcinoma (3). In addition to gross chromosomal abnormalities, Myc can be deregulated through the disruption of direct or indirect upstream signalling pathways (5). It is therefore evident that loss of Myc regulation is a feature common to many cancers types and can result from mutations at several levels of regulation.

\textbf{Myc structure and function}

Myc functions as a transcription factor, capable of both transcriptional activation and repression and is a member of the basic helix-loop-helix leucine zipper (b-HLH-LZ) superfamily (28). The N-terminal domain (NTD) is involved in both transactivation and transrepression and is essential for the assembly of transcriptional complexes (5). Contained within it are four conserved Myc boxes: MBI, MBII, MBIII and MBIV (4, 15, 29). MBI contributes to transactivation whereas MBII is essential for transrepression and has been shown to play a more critical role in transformation (4). The C-terminal domain (CTD) contains the basic helix-loop-helix leucine zipper as well as the nuclear localization signal
The basic motif is involved in site specific DNA binding at the major groove while the helix-loop-helix leucine zipper domain is required for Myc heterodimerization with its binding partner, Max (31, 32).

Sites of DNA binding by the Myc-Max heterodimer can be influenced by several factors. Myc-Max binds Enhancer boxes (or E-boxes), DNA elements with the canonical sequence of 5’-CACGTG-3’ (31, 33). At E-boxes, the Myc/Max heterodimers align head-to-tail to form a bivalent heterotetramer (34). This is thought to allow for both the binding of multiple and distant E-boxes by the heterotetramer through DNA looping as well as providing a substantial platform for interacting proteins. In addition to sequence specificity, Myc binding is also directed by chromatin modifications and accessibility. DNA methylation of the CpG dinucleotide, a transcriptionally repressive modification, within E-boxes can prevent N-Myc binding at the promoters of \textit{EGFR} and \textit{CASP8} (35). Moreover, in an allele-specific manner, Myc binds and regulates the active unmethylated maternal \textit{H19} promoter but does not bind nor alter expression of the silenced methylated paternal promoter (36). Additionally, histone H3 and H4 acetylation correlates positively with Myc binding to E-boxes, suggesting a preference for pre-acetylated chromatin (37).

**Myc transcriptional activity**

Although Myc can both activate and repress transcription, the mechanisms by which it achieves both functions are distinct.

For Myc to activate gene transcription, the Myc/Max heterodimer first binds directly to the DNA. Once bound, transcriptional activation can be achieved through several regulatory mechanisms such as chromatin remodelling or promoter clearance. One mechanism by which Myc is involved in chromatin remodelling is through its MBII-
dependent interaction with TRRAP. TRRAP forms a multi-protein complex with histone acetyltransferase (HAT) activity including GCN5 which has been shown to interact with Myc when mediated by TRRAP (38). Interaction with TRRAP is essential as inhibition of TRRAP abrogates Myc-dependent transformation (39). Independent of TRRAP, Myc also interacts with other molecules involved in chromatin remodelling including TIP48 and TIP49 (40). Another mechanism by which Myc contributes to chromatin remodelling is through ATP-dependent processes. Myc recruits the SWI/SNF complex which repositions nucleosomes to activate transcription. This recruitment is achieved by Myc directly interacting with INI and BAF53, two key components of the SWI/SNF complex (41, 42). Myc can also activate gene transcription through promoter clearance of RNA polymerase II (RNAPII). Myc has been shown to recruit P-TEFb which allows for phosphorylation of RNAPII and activation of elongation (43).

The mechanism by which Myc represses gene transcription is less well understood, however Myc represses as many genes as it activates and Myc transrepression has been shown to be more essential to transformation than transactivation (4, 44). Several growth arrest genes have been identified as Myc repressed targets including GAS1, p15, p21, p27, GADD34, GADD45 and GADD153 (45). One characteristic of Myc transrepression that has emerged is that Myc does not appear to directly bind the promoters of repressed target genes but rather interacts indirectly through protein:protein interactions. The interaction between Myc and Miz-1 demonstrates two mechanisms by which Myc can repress transcription: passive functional inference of transcriptional activators and/or active recruitment of transcriptional repressors. At the p15 core-promoter, Miz-1 directly binds the DNA and recruits the histone acetyltransferase, p300, activating transcription (46). Myc has been
shown to directly bind Miz-1, its binding surface of Miz-1 overlapping with that of p300 thereby blocking p300 recruitment and subsequent transcription activation. At the $p21$ promoter, Myc and Miz-1 form a ternary complex with the DNA methyltransferase Dnmt3a, a transcriptional repressor (47). This recruitment leads to methylation and silencing of $p21$. Myc may also mediate repression of $p21$ through its interaction with Sp1/Sp3 but how this interaction represses $p21$ is not yet clear (48). It does not involve histone deacetylation but because Sp1 is required for transcription, Myc may be mediating repression through the sequestration of Sp1. Initiator (Inr) elements, weak consensus sequences YYCAYYYYYY, within the promoter were thought to be required for Myc transrepression (45). However, deletion of this element in the $p21$ promoter has no effect on repression by Myc, as well, Inr elements are absent in other Myc-repressed targets including $GADD34$, $GADD45$ and $GADD153$ (45, 48). As with transactivation, Myc repression requires Max. It has been shown that Max binds all loci bound by Myc and that Myc-Max heterodimerization is required for repression (49).

**Apoptosis**

Paradoxically, in addition to Myc's ability to drive proliferation, Myc also potentiates apoptosis, that is, it sensitizes cells to undergo apoptosis under exposure to anti-proliferative stimuli. Apoptosis is a non-inflammatory form of programmed cell death, which is both coordinated and energy-dependent, involving the activation of a series of caspases leading to the "death" of the cell (50). Morphologically, apoptosis is characterised by nuclear fragmentation and condensation, membrane bebbing and non-inflammatory phagocytosis (51). There are two distinct apoptotic pathways: the intrinsic and extrinsic pathway, although mounting evidence suggests extensive cross-talk between the two.
The intrinsic pathway is triggered by stimuli that produce intracellular signals that act directly on apoptotic machinery. These stimuli include DNA damage, hypoxia, limited growth factors and oncogene activation (52). All of these signals converge at the mitochondria, resulting in mitochondrial outer membrane permeabilisation (MOMP) and the release of various pro-apoptotic molecules including cytochrome \( c \) and Smac/DIABLO (53). Upon release, Smac/DIABLO binds and blocks IAP (inhibitors of apoptosis proteins) which antagonize caspase activation (54). Cytochrome \( c \) binds and activates Apaf-1 and caspases 9, forming the apoptosome (55). The apoptosome activates caspases 3 which cleaves key substrates such as poly(ADP-ribose)polymerase (PARP), to produce the morphological changes characteristic of apoptosis (55).

This permeabilisation of the mitochondria is regulated largely by the BCL2 family of proteins. The decision between survival and death is largely a product of the teetering balance between pro-apoptotic and anti-apoptotic family members (56). A healthy cell contains all molecules necessary to execute apoptosis but is kept below the "apoptotic threshold" by pro-survival signals (52). Anti-apoptotic BCL2 proteins include BCL2 and BCLXL, which contain all four BCL2 homology domains (BH1-4). Pro-apoptotic members include multi-domain (BH1-3) pore-forming proteins, such as BAX and BAK, and smaller BH-3 only proteins, such as BID, BIM, BAD, BIK, NOXA and PUMA. "Sensitizer" domains, such as those of BAD and BIK, displace "activator" domains, such as those from BID and BIM, so that they can activate pore-forming BAX and BAK (52). At the mitochondrial outer membrane, BAX and BAK homo-oligomerize, forming pores and permeabilise the outer mitochondrial membrane for pro-apoptotic molecule release.
The extrinsic pathway initiates apoptosis via transmembrane receptor mediated interactions. Death receptors are members of the tumour necrosis factor (TNF) receptor family and share similar cytoplasmic domains of approximately 80 amino acids called "death domains" (DD) (57). Such death receptors include CD95/FasR, tumour necrosis factor receptor (TNFR) and death receptor DR5. In the case of CD95 and TNFR, upon ligand binding, receptors cluster, bringing together their corresponding death domains (DD) to interact with the DD of adaptor molecules (52). Adaptor molecules, such as FADD and TRADD (in the case of TNFR), then, through their death effector domains (DED), interact with the DED of pro-caspase 8. This results in the formation of the death-inducing signalling complex (DISC) and allows for the auto-catalytic activation of pro-caspase 8. Caspase 8 directly activates caspases 3 and caspases 7, triggering the execution phase of apoptosis while circumventing the mitochondria. However, BID is also a substrate of caspases 8, which, upon activation, activates BAX and BAK for MOMP, thus revealing a source of cross-talk between the intrinsic and extrinsic pathway of apoptosis (58).

Another apoptotic pathway that is thought to be distinct from both the intrinsic and extrinsic pathways is endoplasmic reticulum (ER) stress-induced apoptosis. The ER is the site of synthesis and folding of secreted and membrane-bound proteins. Proper ER function requires optimal protein folding, ATP, Ca^{2+} and an oxidizing environment (59). Disruption of this environment leads to the accumulation and aggregation of unfolded proteins; this is toxic to the cell and termed ER stress. The unfolded protein response (UPR) is a strategy used by the cell to overcome this burden. Upon ER stress, GRP78, an ER chaperone, dissociates from and thereby activates PERK, ATF6 and IRE1. PERK inhibits general translation to lessen the load of nascent proteins (60). ATF6 translocates to the nucleus and
induces genes with ER stress response elements (ERSE) including \textit{XBPI} and \textit{CHOP} (61). IRE1 splices XBPI mRNA and the XBPI protein, a transcription factor, activates genes of several functions, including protein degradation (60). The UPR is a pro-survival response in that if the accumulation of unfolded proteins is reduced, the ER will be restored to normal function. However, if the accumulation of protein is not resolved, the UPR will trigger apoptosis as it switches from pro-survival to pro-apoptotic. Although much of how the UPR triggers apoptosis remains unclear, several findings have added to our understanding. CHOP (GADD-153) is a transcription factor that has been shown to induce apoptosis (59). It downregulates anti-apoptotic BCL2 and also induces TRB3 which may bind and antagonize pro-survival Akt (59, 60, 62). Furthermore, IRE1 can activate JNK which phosphorylates ER localized BCL2, antagonizing its anti-apoptotic function and JNK phosphorylates and activates pro-apoptotic BIM (60). Pro-apoptotic BH3-only proteins, PUMA and NOXA also play a role in ER stress-induced apoptosis (63). Collectively, these events allow for the activation of BAX and BAK at the mitochondria, subsequent MOMP and cytochrome c release.

\textbf{Myc and Apoptosis}

In the early 1990's, three groups reported Myc as a potentiator of apoptosis. Myc was first shown to potentiate apoptosis when 32D myeloid cells expressing ectopic Myc rapidly underwent apoptosis when deprived of IL-3 cytokine, in contrast to the normal response of downregulation of Myc, G1 arrest and eventual cell death (12). Similarly, Rat1 fibroblasts expressing constitutive Myc, unable to arrest growth under low serum, were not increasing in numbers due to an increase in cell death (13). The cell death observed was shown to depend on the overexpression of Myc and was in fact apoptosis. Furthermore, in T cell hybridomas,
activation-induced apoptosis requires Myc as it is disrupted by antisense oligonucleotides corresponding to Myc (14). Myc can potentiate apoptosis at any stage in the cell cycle and in some systems, the absence of Myc renders the cell resistant to diverse apoptosis-inducing stimuli (64, 65). A "dual signal" model proposes that Myc constitutively activates two separable pathways: cell cycle progression and apoptosis, and that the apoptotic phenotype is rescued by pro-survival factors such as cytokines (44, 66).

Although the ability of deregulated Myc to potentiate apoptosis acts as a safety mechanism to prevent uncontrolled proliferation, this function can be lost owing to anti-apoptotic mutations thus abrogating Myc-potentiated apoptosis but leaving intact Myc-driven proliferation. In Rat1/MycER cells, ectopic expression of anti-apoptotic BCL2 abrogates Myc-potentiated apoptosis but has no effect on its mitogenic function (67). In the Eµ-Myc mouse model for human Burkitt lymphoma, MYC is coupled to the immunoglobulin heavy chain μ enhancer, leading to B-cell specific overexpression and subsequent clonal lymphoma (68). Cotransfection of Eµ-Myc with Eµ-BCL2 decreases the latency of disease development (69). CUL7, a member of the cullin family of E3 ligases, has also been shown to block Myc-potentiated apoptosis through the binding of p53 and inhibition of p53-dependent apoptosis (70). The anti-apoptotic function of CUL7 can cooperate with Myc to drive transformation. Myc-potentiated apoptosis can be circumvented by anti-apoptotic lesions and it is, in fact, a critical function of cooperating mutations to abrogate the apoptotic function of oncogenic Myc (52).

The mechanism by which Myc potentiates apoptosis remains unclear, although it has been shown to act in both a p53-dependent and p53-independent manner. p53 is a tumour suppressor known widely as the "guardian of the genome" (71). It responds to diverse anti-
proliferative stimuli, such as DNA damage, hypoxia and aberrant oncogene activation, to mediate cell cycle arrest and apoptosis, both through transcription-dependent mechanisms as a transcription factor and transcription-independent mechanisms (72, 73). During cellular stress, p53 induces apoptosis transcriptionally by directly modulating the expression of pro-apoptotic genes such as PUMA, NOXA, BAX, BIM, Fas and DR5 (74). There is also evidence that p53 can translocate from the nucleus to the mitochondria to promote apoptosis, although the nature of this transcription-independent mechanism is less clear. Findings indicate that cytoplasmic p53 directly activates BAX at the mitochondria to promote MOMP (75). Furthermore, cytoplasmic p53 can cooperate with PUMA, whose gene is upregulated by nuclear p53, to induce apoptosis (76). The mechanism by which this is thought to occur is cytoplasmic p53 is bound and inhibited by BCLXL but in response to cellular stress, nuclear p53 activates PUMA that then binds BCLXL to free and activate cytoplasmic p53 (73).

Myc promotes p53-dependent apoptosis through a well understood mechanism: oncogene activation of the ARF-Mdm2-p53 axis. Mdm2 (the human homologue being Hdm2) is a negative regulator of p53 that binds and mono-ubiquitinates p53, resulting in its translocation to the cytoplasm and subsequent poly-ubiquitination and degradation by the proteasome (77). ARF, the gene product of the Ink4a locus, which also encodes the p16^{Ink4a} cell cycle inhibitor, is a negative regulator of Mdm2 by either directly binding or sequestering it in the nucleolus, both acting to prevent p53 mono-ubiquitination by Mdm2 (78, 79). Although the exact mechanism is unclear, Myc deregulation increases ARF expression (80). And so, through this axis, deregulated Myc increases p53 levels and consequently, promotes subsequent p53-dependent apoptosis. However, Myc can accelerate apoptosis in cells deficient of p53 through p53-independent mechanisms (81).
Myc-potentiated apoptosis independent of p53 is firmly established, yet the precise mechanism through which it acts is unclear. Because Myc potentiates apoptosis in response to a variety of anti-proliferative stimuli, Myc's most significant apoptotic function may be at the point of convergence of the numerous apoptotic pathways: the mitochondria, altering the balance of pro-apoptotic and anti-apoptotic BCL2 family proteins and shifting the apoptotic threshold (52). Myc has been shown to suppress expression of both anti-apoptotic BCL2 and BCLX\textsubscript{L}, although not likely as direct transcriptional targets (82, 83). Myc also plays a critical role in stimulating BAX activity at the mitochondria. Although translocation of BAX to the mitochondria is observed in both in the presence and absence of Myc, BAX is only activated and cytochrome \textit{c} released when Myc is present (65). This is independent of BAX expression as mRNA and protein levels are not altered by Myc. Furthermore, Clusterin, which can bind and inhibit conformation-altered BAX, antagonising subsequent oligomerization, blocks Myc-potentiated apoptosis (84). BIM, a pro-apoptotic BH3-only protein that antagonises BCL2, shows increased expression in B-cells derived from E\textsubscript{\mu}-Myc mice and mediates Myc-potentiated apoptosis (85). Furthermore, apoptosis-deficient T58A Myc mutants fail to induce BIM expression (86). While there are two canonical E-boxes located within the \textit{BIM} locus, direct activation by Myc has not been established (85).

Myc has been shown to extend to receptor-mediated apoptosis as it potentiates apoptosis via TNFR, CD95 and DR5. Rat1 fibroblasts that express CD95 but are refractory to CD95-induced apoptosis are sensitized to undergoing apoptosis by the CD95 ligand upon induction of MycER (87). However, it has been proposed that rather than activating death receptors, Myc synergizes with death receptors to drive apoptosis through the amplification of FADD and caspase 8-mediated death receptor signals (88). Myc increases the apoptotic
activity of both FADD and caspase 8, which are depended upon by TNFR and CD95. Furthermore, Myc is able to augment the apoptotic activity of RIP, a death receptor adaptor protein that functions through FADD and caspase 8 and this is contingent on Myc's modulation of the mitochondria (88). The mechanism by which Myc potentiates apoptosis through DR5 is more direct. Myc upregulates the expression of the TRAIL receptor DR5 and thus stimulates caspase 8 processing (89). Additionally, in SHEP cells, overexpression of N-Myc upregulates both DR5 mRNA and protein (90). The DR5 promoter contains two noncanonical E-boxes and N-Myc is able to activate transcription from the DR5 promoter in luciferase reporter assays. Collectively, this suggests that perhaps DR5 is a direct transcriptional target of Myc. Moreover, Myc directly binds the promoter of FLIP, a TRAIL-signalling inhibitor, and represses transcription, subsequently enhancing TRAIL-induced apoptosis (91).

ER-stress-induced apoptosis is also potentiated by Myc. Ectopic Myc sensitizes cells to apoptosis induced by ER-stressors taxol, ceramide and serum withdrawal as well as DNA damaging agents etoposide and doxorubicin (65). However, the apoptotic pathways triggered by the ER-stressors and the DNA damaging agents are spatially distinct in that ER-stressor-induced apoptosis is blocked by BCL2 targeted to the ER (BCL2-cb5) whereas the DNA damage-induced apoptosis is not. Nevertheless, both pathways converge at the mitochondria as both are blocked by BCL2 targeted to the mitochondria (BCL2-acta). Therefore, it seems that as with the intrinsic and extrinsic pathways, the mitochondria are a critical convergence node for ER-stress-induced apoptosis and likely where Myc plays a role. Two BH3-only proteins that have been strongly implicated in ER-stress-induced apoptosis are NOXA and PUMA. Traditionally, these have been thought to be mediated primarily by p53 but there is
evidence that under ER-stress, Myc may play a partial, if not exclusive role in modulating these pro-apoptotic proteins. Myc directly binds the promoter and upregulates expression of NOXA in response to the 26S proteasome inhibitor bortezomib (Velcade), increasing the apoptotic response (92, 93). This activation of NOXA is independent of p53 as well as E2F-1 and HIF-1 (93). PUMA is induced by both p53-dependent apoptotic pathways, such as DNA damage, as well as p53-independent pathways, such as glucocorticoid exposure and serum withdrawal (94). Furthermore, there is mounting evidence that suggests ER stress plays a negative role in p53 activation. Several ER stressors, including tunicamycin and thapsigargin, have been shown to both increase cytoplasmic localization of p53 and enhance destabilization of p53, both mediated by phosphorylation by GSK3β (95, 96). It has not been shown that Myc directly activates PUMA transcription in response to ER-stress, however, Myc has been shown to bind the PUMA promoter under normal growth conditions and PUMA and NOXA to a lesser extent, are suppressors of Myc-induced lymphomagenesis (37, 97).

The genetic program of Myc-potentiated apoptosis

The abilities of Myc to induce cell cycle progression and to potentiate apoptosis are two separable functions that can be uncoupled (44). Furthermore, it is thought that these function are achieved through Myc's transcriptional regulation of a specific cohort of genes which mediate these disparate functions. Several findings suggest that Myc's transrepression function correlates with Myc's ability to potentiate apoptosis while its transactivation function is involved primarily in growth and proliferation. Deletions made in MBI reduce expression of the Myc activated gene target ODC but have no effect on apoptosis, whereas deletions made in MBII reduce repression of the Myc repressed target GADD45 and abrogate
apoptosis (44). The threonine at amino acid 58 of Myc is a mutational hotspot in Burkitt lymphoma and in highly transforming v-myc is substituted for a nonphosphorylatable residue (98, 99). The T58A mutant, in which the threonine is substituted for an alanine, shows a marked decrease in its ability to potentiate apoptosis as well as to repress $GADD45$ yet it transcriptionally activates $ODC$ as efficiently as wild-type (44). Omomyc is a Myc-derived b-MLH1-LZ in which substitutions were made at four amino acids shown to prevent Myc homodimerization (100). Omomyc acts as a dominant negative by dimerizing with Myc and forming a complex unable to bind to E-boxes thus inhibiting Myc transcriptional activator function. Although inhibiting Myc's transactivation function, Omomyc had little or no effect, apart from marginal enhancement on Myc transrepression (101). Moreover, Omomyc enhanced Myc-potentiated apoptosis in cells expressing elevated levels of Myc. It is likely that Myc transrepression plays a major role over transactivation in potentiating apoptosis but not an exclusive role. Myc mutants deficient in Miz-1-dependent repression but capable of transactivation still potentiate apoptosis (102). Additionally, Myc directly binds and transcriptionally activates $NOXA$ in response to ER-stress to drive apoptosis (93).

Post-translational modifications also appear to play a critical role in modulating Myc-potentiated apoptosis. This is largely mediated by two Ras pathways. Ras activation of PI3K suppresses Myc potentiated apoptosis (103). Through the PI3K and PKB/AKT pathway, GSK3, which phosphorylates Myc at T58 and targets it for subsequent ubiquitin-mediated degradation, is inhibited (104). This further emphasizes the importance of T58 phosphorylation in Myc-potentiated apoptosis which when blocked through substitution with a nonphosphorylatable alanine, abrogates apoptosis (44). Through the Ras, Raf and ERK pathway, ERK phosphorylates Myc at S62, stabilizing the protein and promoting apoptosis
Interestingly, the tumour suppressor Bin-1 has been shown to interact with Myc and to mediate Myc-potentiated apoptosis (106, 107). However, phosphorylation of S62, which is achieved through the pro-apoptotic arm of Ras, inhibits Bin-1 interaction with Myc. Although it has not been shown that S62 phosphorylation abrogates Bin-1-mediated apoptosis through Myc, this finding highlights that regulation of Myc-potentiated apoptosis by post-translational modifications is shrouded in complexities.

Many questions regarding Myc's ability to potentiate apoptosis remain. If Myc is in fact potentiating apoptosis through the regulation of a specific cohort of genes distinct from that of driving proliferation, how is this distinction achieved? Does Myc acquire binding targets upon exposure to anti-proliferative stimuli or is Myc already bound to these apoptotic gene targets during normal growth, incorporating anti-proliferative signals to modulate the apoptotic genetic program? Furthermore, if a differential in binding observed at a particular gene is not all-or-nothing, to what do we attribute an increase in binding? Is Myc binding at additional sites in the promoter or being stabilized at an already bound site? Finally, does the cohort of genes bound by Myc under apoptotic conditions change in its deregulated state? Although these questions have still not been answered, a model can be constructed based on previous findings.

Estrogen receptor-α (ERα) is a transcription factor that mediates the biological effects of estrogen, including transactivation of Myc. Myc is a positive regulator of ERα-mediated transcriptional activation (108). When cells are deprived of hormone and then treated with 17β-estradiol (E2), Myc and ERα binding increases at activated ERα-responsive promoters and this is met by a subsequent increase in expression. The Myc:ERα complex co-immunoprecipitates in greater amounts and nuclear protein levels of both Myc and ERα are
augmented. As well, at some of the promoters, TRRAP is recruited and physically interacts with both Myc and ERα. Interestingly, Myc is bound to the activated ERα-responsive promoters prior to E2 treatment and binding increases with E2 treatment, there is no all-or-nothing binding. This suggests that perhaps, in this system, Myc is already bound to the promoters and upon E2 treatment, ERα interacts with Myc and recruits cofactors such as TRRAP, thus stabilizing Myc and increasing the level of binding at the promoter.

HIF-1 is a transcription factor induced by hypoxia and activates genes involved in metabolic adaptation such as HK2, PDK1 and VEGF (109). All three genes HK2, PDK1 and VEGF possess non-overlapping Myc and HIF-1 binding sites and all are upregulated by hypoxia alone. Using an inducible Myc system it was shown that Myc can upregulate both HK2 and VEGF under normoxia and synergize with HIF-1 under hypoxia. In contrast, Myc has little effect on the expression of PDK1 under normoxia but enhances the effect of HIF-1 under hypoxia. These findings suggest that, in this system, Myc is already bound to the promoter prior to the stimulus, either activating transcription or having no apparent effect. Upon treatment, Myc integrates the signal at the chromatin level and synergizes with or enhances the transcription activating functions of co-regulators.

Bortezomib, a 26S proteasome inhibitor, increases Myc binding at the NOXA promoter which drives apoptosis (93). The NOXA promoter contains three sites that are bound by Myc, one shows strong binding (BS III) and the other two are minimal (BS II and IV). Myc is bound to the promoter even under non-treatment conditions and upregulates its expression. When treated, Myc binding increases only at the strong binding site BS III and NOXA expression is further upregulated. Furthermore, bortezomib treatment increases Myc protein levels but has no effect on Myc mRNA levels, suggesting that Myc is being
stabilized. Collectively, these findings indicate that under growing conditions Myc is bound at the NOXA promoter and upregulating NOXA expression. The apoptotic treatment stabilizes Myc at the promoter which has the effect of further upregulating NOXA expression and driving apoptosis. This study also clarifies the nature of what an "increase in promoter binding" in fact is. It was shown that Myc binding was increasing at only one of the Myc binding sites indicating that an increase in binding may not be the acquisition of additional binding sites within the promoter. The fact that Myc protein is increased after the apoptotic treatment without an increase in mRNA suggests that Myc is being stabilized and that this stabilization is also occurring at the NOXA promoter, whether through protein:protein interactions or post-translational modifications. Assuming Myc binding at the NOXA promoter is transient, an increase in Myc stability at the promoter would increase the latency at which it occupies the promoter. Therefore, an "increase in promoter binding" may in fact be an increase in the duration of time at which Myc occupies the promoter due to increased stability, increasing the likelihood that this occupancy will be captured by the binding assay resulting in a measurement of increased binding.

Briefly, it should be noted that although these aforementioned studies show that even when Myc is minimally bound to its targets prior to signalling, there is at least some regulation of transcription, Myc binding does not necessarily have modulating effects on expression. Some genes that are efficiently bound by Myc show no induction, however, no repressed Myc targets were included in this study (37).

Our model proposes that under growing conditions, Myc is already bound to its apoptotic gene targets, both repressed and activated (Figure 1). In the absence of any apoptotic signals, Myc binding may have a modulating effect on the expression of gene
Figure 1: Working model of Myc differential binding and transcriptional regulatory activity under apoptotic vs. growth conditions

Under growing conditions, Myc is already bound to its apoptotic gene targets and regulating transcription to some degree, whether it be activation or repression. In response to anti-proliferative stimuli, Myc is stabilized at the promoter of its apoptotic gene targets through protein:protein interactions and/or post-translational modifications. This increases Myc binding at the gene promoter as well as enhances Myc transcription regulatory function, further increasing activation and repression of the apoptotic gene cohort and potentiating apoptosis.
targets. Upon exposure to an apoptotic signal, Myc forms a regulatory complex and through protein:protein interactions and/or post-translational modifications, Myc is stabilized at the promoter and its regulator activity is enhanced. Collectively, this induces a genetic program that drives Myc-potentiated apoptosis.

A major caveat of this model is that many of the findings from which it is derived are based on Myc-activated genes and not Myc-repressed genes. As described above, the mechanism of Myc transcriptional activation and Myc transcriptional repression vary greatly and it may, in fact, be erroneous to extrapolate what is known of the dynamics of Myc binding at activated promoters in response to signalling to Myc repression. However, given the limited data on the dynamics and mechanisms of Myc transcriptional repression, some generalisation has been made to construct a comprehensive working model.

Another distinction that remains unclear is how Myc becomes such a potent promoter of apoptosis in its deregulated state. It is not known whether Myc in its deregulated state acquires additional apoptotic gene targets to drive apoptosis or whether the deregulated state merely leads to altered regulation of the same cohort of genes. Myc is predicted to bind over 4000 target loci, however, in serum-stimulated human fibroblasts there are predicted to be 3000-6000 Myc molecules per cell (mpc) and in cycling cells this number drops to 1000-3000mpc (6, 37, 110). What this suggests is that there may not be enough Myc protein in a cell at a given time to bind and regulate all possible Myc targets. Furthermore, Myc target promoters bind Myc at varying affinities and it is only with forced Myc expression that binding is achieved at the lower affinity targets (37). It is possible that through Myc deregulation, because Myc protein levels are augmented, Myc begins to bind these lower affinity targets that are otherwise not bound by regulated Myc. This in essence would be
gain-of-function binding by deregulated Myc. H19 is an oncogene reactivated in several
types of cancer including breast cancer and has been shown to play a role in transformation
(36). In MCF10A non-transformed breast epithelial cells, H19 is marginally expressed and
shows minimal Myc binding. However, with ectopic Myc expression, there is a robustly
large increase in Myc binding to the H19 promoter met with a substantial induction of H19
expression. It is possible that when Myc is deregulated, it binds and regulates low affinity
pro-apoptotic gene targets not otherwise regulated by Myc when in its regulated state. This
gain-of-function apoptotic genetic program of deregulated Myc would further sensitize the
cell to apoptotic stimuli beyond that of regulated Myc. It is also possible that with
deregulated Myc the same apoptotic gene cohort as that of the regulated state is bound but a
proportion of these genes are regulated beyond physiological levels. Myc protein has a short
half-life of 20-30 min and so it can be presumed that Myc bound at a particular promoter is
turned over rapidly. If Myc levels are elevated as a result of deregulation then perhaps Myc
binding at that promoter will be replenished more rapidly thus elevating the activation or
repression of that gene. If this is occurring at genes of the apoptotic cohort then this could
result in the sensitization of the cell to apoptotic stimuli. Because Myc binding affinity is a
continuum rather than all-or-nothing measure, Myc deregulation leading to gain-of-function
binding to low-affinity targets and Myc deregulation leading to non-physiological regulation
of previously bound targets are essentially the same phenomenon. They are simply different
perspectives on how a cell with regulated Myc exposed to an anti-proliferative stimulus will
undergo cell cycle arrest but that same cell with deregulated Myc will undergo apoptosis.
The most promising approach to finally answering this longstanding question is ChIP-chip.
**ChIP-chip**

ChIP-chip is the combination of chromatin-immunoprecipitation (ChIP) and high-throughput DNA microarray technology (chip) (111). Through this method, the DNA-bound protein of interest along with the sheared DNA fragments to which it is bound are precipitated using specific antibodies and the protein-bound DNA is isolated (Figure 2). The DNA is then labelled and hybridized to genomic microarrays, allowing for *in vivo* mapping of protein-DNA interactions. Using this method it is possible to identify genes whose regulatory region is bound by a particular transcription factor both through protein-DNA interactions and protein-protein interactions. ChIP-chip coupled with expression array analysis allows for the identification of the genetic program specifically regulated by the transcription factor and the subsequent effect of binding on expression of direct targets as well as the regulatory effects on downstream secondary targets.

**Hypothesis and Purpose**

We hypothesize that under apoptotic conditions, Myc differentially binds and/or regulates a specific cohort of gene targets to potentiate apoptosis. The foremost approach to addressing this hypothesis is the employment of ChIP-chip coupled with expression array analyses. This thesis focuses on the development of systems for performing high-throughput ChIP-chip assays of Myc genomic binding and expression array analysis of Myc transcriptional regulation under apoptotic conditions. We sought to develop two independent human cell systems which have previously been shown to exhibit MBII-dependent cell cycle progression: the MCF10A non-transformed human breast epithelial cell line and the SHEP human neuroblastoma cell line. This required the optimization of Myc ChIP as well as the
Figure 2: Schematic of ChIP-chip
Protein:protein and protein:DNA interactions in live cells are cross-linked using formaldehyde. The cells are lysed and DNA is sheared through sonication for fragments of 100-1,000bp. An antibody against the protein of interest is used for immunoprecipitation of the protein of interest (e.g. Myc) along with bound DNA fragments. The cross-linking is reversed and the protein-bound DNA is isolated and purified. DNA fragments bound by the protein of interest can be identified by qPCR or microarray analysis. The entire reaction is similarly processed using IgG as a negative control.
development and characterization of apoptotic conditions that specifically maximize levels of Myc-potentiated apoptosis.
Results

Optimization of ChIP for Myc in the MCF10A human breast cell line

Myc deregulation is found in both early and late-stage breast tumours and is often associated with poor prognosis (112, 113). Myc overexpression through amplification and other genetic abnormalities is frequently seen in breast tumours (25, 114-116). Apart from its pathological role in tumorigenesis, Myc plays a physiological role in apoptosis in breast tissue. For example, during mammary gland involution, Myc expression is elevated and promotes apoptosis of alveolar cells (117). However, breast cancer is often associated with anti-apoptotic lesions such as increased expression of anti-apoptotic BCL2, Hsp27 and Clusterin (118, 119). The MCF10A cell line is a non-transformed immortalized human breast epithelial cell line (120, 121). We identified this cell line as an appropriate system for studying the mechanisms of Myc-potentiated apoptosis because of its non-transformed characteristics that provide an approximate model of normal breast tissue and lacks any known apoptotic lesions, in contrast to the MCF7 human breast line often used in Myc growth and transformation studies that has been shown to be caspase 3 deficient (122). Furthermore, in previous reports the MCF10A cell line has been used as a model for the study of apoptosis, although these were not in the context of Myc (123-125).

Optimization of cell number and antibody concentration for Myc ChIP in the MCF10A cell line

ChIP for Myc in the MCF10A cell line had been under development in the Penn lab, such as the optimization of sonication using the Bioruptor by Amanda Wasylishen, although no ChIP-chip studies using this cell line had yet been conducted by the Penn lab or published by any other group. Two parameters that remained to be optimized were antibody
concentration and the number of cells per ChIP reaction. An approach that has been taken previously in the Penn lab has been to perform parallel ChIP reactions with a range of both antibody concentrations and cell numbers. The conditions are evaluated based on their signal-to-noise ratio, that is, maximizing the signal of ChIP using the N262 Myc-specific antibody and minimizing the noise using the IgG non-specific control antibody. Typically, biological replicates of this optimization experiment are not required as the selected condition is subsequently validated for reproducibility.

ChIP reactions were performed in parallel in the MCF10A cell line expressing ectopic Myc using 0.7µg, 1.5µg and 5µg of Myc specific antibody, and the equivalent IgG control antibody, with either 5x10^6 or 10x10^6 cells. The efficiency of each Myc ChIP was quantified using quantitative real-time PCR (qPCR) (Figure 3A). Chromosome 6 (Ch6) contains an E-box which, previously, has been shown not to be bound by Myc and was used as a negative control (111). Briefly, negative control targets were identified by Dr. Paul Boutros by mining Myc gene targets databases for four classes of E-boxes: those downstream of CpG islands, in CpG islands, in coding regions and in non-coding regions. Appropriately, at all antibody concentrations and cell numbers, there was no signal enrichment of Myc ChIP compared to IgG control, at Ch6. CAD possesses an E-box which is known to be bound by Myc and served as the positive control (126). All the Myc ChIP conditions assayed showed strong signal enrichment at CAD. As a general trend, antibody concentration and cell number were positively correlated with Myc signal. For 5x10^6 cells, 1.5µg and 5µg of antibody showed the highest Myc ChIP signals and for 10x10^6 cells, all antibody concentrations had similar signals with 1.5µg and 5µg having the highest. Although these conditions could have been evaluated based on their signal-to-noise ratios, this would
Figure 3: Optimization of Myc ChIP in the MCF10A cell line

A) ChIP reactions were conducted using a range of cell numbers and antibody concentrations of the Myc specific (N262) and the non-specific IgG (normal rabbit) control antibodies. Efficiencies of the ChIP reactions were assessed using qPCR and absolute quantification was used to represent the amount of DNA isolated in the ChIP reactions in ng of DNA. CAD and an E-box on chromosome 6 (Ch6) are the positive and negative controls, respectively. qPCR
was conducted in triplicate and the bars represent the mean. Error bars ±1SD. B) The optimized Myc ChIP condition of 1.5 µg of antibody and 10x10^6 cells was validated for reproducibility. Fold enrichment of Myc ChIP at each target is expressed as the mean log2 ratio of Myc ChIP signal to IgG noise. E-boxes on chromosome 6 and 21 (Ch6 and Ch21, respectively) and HNT were pooled as the negative control (neg). *CAD, TCERG1, GADD45, NOL6 and APEX* are positive controls. Four independent biological replicates were conducted and qPCR was conducted in triplicates. Error bars ±1SD, *indicates p<0.05, **indicates p<0.001 vs. pooled negative controls by t-test.
not be greatly indicative of the efficiency of each condition as ChIP requires replicates for true measure. The 1.5µg of antibody and 10x10⁶ cells ChIP condition was chosen as the optimal condition since it yielded high Myc ChIP signals but required less antibody than the 5µg condition which yielded a similar signal, thereby lowering costs.

**Validation of optimized Myc ChIP conditions in the MCF10A cell line**

The optimized Myc ChIP condition of 1.5µg of antibody and 10x10⁶ cells was validated, with biological replicates, at additional Myc target sites again using qPCR (Figure 3B). In addition to chromosome 6, chromosome 21 and HNT contain E-boxes that have been shown not to be bound by Myc and served as the pooled negative control (49, 111). The positive controls were genes with E-boxes shown to be bound by Myc including CAD, TCERG1, GADD45, NOL6 and APEX (49, 111, 126, 127). Signal-to-noise is expressed as the mean log2 fold increases of Myc ChIP signal over IgG noise. Appropriately, there was little signal enrichment for Myc at the pooled negative controls. CAD, GADD45, NOL6 and APEX showed robust signal enrichments for Myc ChIP that were significantly higher (p<0.001) than that of the pooled negative controls. Signal enrichment for Myc ChIP at TCERG1 was not significantly higher than the pooled negative controls, which was made apparent by the large error bars. This was due to one value that was much lower than the other three and added much variability. Therefore, the optimized condition of 1.5µg of antibody and 10x10⁶ cells in the MCF10A cell line was both efficient, as it showed high levels of signal enrichment for Myc ChIP at various known Myc binding targets, and was reproducible.
Optimization and characterisation of MCF10A apoptotic condition

In identifying and optimizing an apoptotic condition, we sought to maximize levels of Myc-potentiated apoptosis while minimizing basal levels of apoptosis. This required an agonist that yields high levels of apoptosis in cells expressing ectopic Myc and low levels in control cells. As such, we established criteria for evaluating each apoptotic treatment as to whether it is appropriate for our purposes. We sought to develop apoptotic conditions that induce less than 10% apoptotic levels in control cells and greater than 25% apoptotic levels in cells expressing ectopic Myc. Essentially, treatments meeting these criteria would typically yield apoptotic levels at least 3-fold higher in the ectopic Myc cell lines as compared to the control cell lines.

Serum withdrawal synergizes with doxorubicin to induce Myc-potentiated apoptosis in the MCF10A cells expressing constitutive Myc in a 6h exposure

Since these apoptotic systems were being developed for subsequent ChIP-chip and expression array analysis, we were interested in capturing the apoptotic genetic program prior to the execution of apoptosis. However, the time point for capturing this program is unknown. By developing a short apoptotic treatment that yields high levels of Myc-potentiated apoptosis, the window for capturing the Myc genetic program would be presumably narrowed, increasing the likelihood of capturing the shift in Myc binding and/or transcription regulation. Therefore, we first attempted to develop a relatively short 6h apoptotic treatment.

MCF10A cells expressing ectopic pMN-GFP constructs were generated: empty vector control (MCF10A-GFP) and Myc (MCF10A-Myc), providing a regulated and deregulated Myc model, respectively. It had previously been shown by Amanda Wasylissen
that under growing conditions (5% horse serum, 10µg/mL insulin and additional supplements) MCF10A-GFP and MCF10A-Myc cells express comparable levels of Myc protein (Figure 4A). However, following 1h of serum withdrawal (0.05% horse serum, 10µg/mL insulin minus additional supplements) Myc levels in the MCF10A-GFP cells are rapidly diminished while Myc levels in MCF10A-Myc cells are sustained to a greater degree. This is expected as Myc is deregulated and constitutively expressed in the MCF10A-Myc cells.

These data show that in these MCF10A cells lines, a differential in Myc protein can be achieved through serum withdrawal. It was predicted that this difference in Myc protein could further drive the subsequent differential in apoptosis. It was, therefore, investigated as to whether serum withdrawal prior to and during drug treatment could further increase the apoptotic differential between the two cell lines. The drug used was doxorubicin, a topoisomerase II inhibitor that induces apoptosis through DNA damage. MCF10A cells were treated with every combination of three treatment elements: i) a 6h doxorubicin treatment; ii) a 1h pre-treatment serum withdrawal prior to drug treatment; and iii) a 6h treatment serum withdrawal that is in conjunction with the 6h doxorubicin treatment. Serum withdrawal was modified to media free of all horse serum, insulin and additional supplements to lower endogenous Myc levels as rapidly as possible. Apoptosis was measured using TUNEL which detects exposed 3’-hydroxy ends of DNA breaks by endonucleases characteristic of apoptosis (Figure 4B).

The 1h pre-treatment serum withdrawal alone (Figure 4B, treatment 2) had little effect on inducing apoptosis and the 6h treatment of serum withdrawal alone (Figure 4B, treatment 3) only marginally increased apoptosis levels in both cell lines above that of the no
Figure 4: Myc potentiates apoptosis in response to doxorubicin in combination with serum withdrawal

A) Myc expression visualized by immunoblotting. Lysates were harvested from MCF10A-GFP (G) and MCF10A-Myc (M) cells under normal growing conditions and after 1h of serum withdrawal (SW) (0.05% horse serum, 10μg/mL insulin and no additional supplements). Kindly provided by Amanda Wasylishen.

B) MCF10A-Myc and MCF10A-GFP cells were treated with every combination of i) 1h pre-treatment serum withdrawal (SW) (no horse serum, insulin or additional supplements) prior to drug treatment, ii) a 6h 1μM doxorubicin treatment and iii) 6h treatment serum withdrawal (during doxorubicin treatment if treated with doxorubicin). Apoptosis (TUNEL positive) levels were assayed using TUNEL. Two independent biological replicates were performed. Error bars +1SD. *indicates p<0.05, MCF10A-GFP vs. MCF10A-Myc of same treatment by t-test.
treatment condition (Figure 4B, treatment 1). The 1h pre-treatment and 6h treatment of serum withdrawal combined (Figure 4B, treatment 5), essentially a 7h serum withdrawal condition, on average showed a strong increase in apoptosis in the MCF10A-Myc cells and a 3-fold differential. However, the large error bars suggest that there may be little apoptotic differential between the two cell lines under these conditions, this is furthered by the low levels seen under 6h of serum withdrawal (Figure 4B, treatment 3). Therefore, serum withdrawal in the absence of additional agonists does not induce apoptosis in such a short time of treatment. To that end, doxorubicin alone (Figure 4B, treatment 4) only marginally induced apoptosis with only a slight apoptotic differential between the MCF10A-GFP and MCF10A-Myc cells. Doxorubicin was only effective at inducing apoptosis when in combination with serum withdrawal. Doxorubicin with the 1h pre-treatment serum withdrawal (Figure 4B, treatment 5) slightly increased apoptosis in both MCF10A-GFP and MCF10A-Myc over doxorubicin alone (Figure 4B, treatment 6). Doxorubicin synergized with the 6h treatment serum withdrawal (Figure 4B, treatment 7) as high levels of apoptosis were achieved in the MCF10A-Myc cells and low levels in MCF10A-GFP cells and a strong 3-fold differential. Apoptosis levels in both cell lines were further increased when doxorubicin and the 6h treatment serum withdrawal were combined with the 1h pre-treatment serum withdrawal (Figure 4B, treatment 8) with apoptosis levels in both MCF10A-GFP and MCF10A-Myc cells but the differential was reduced to 2-fold.

Doxorubicin synergized with the treatment serum withdrawal to yield high levels of apoptosis in the MCF10A-Myc cells while maintaining a strong 3-fold differential over the MCF10A-GFP. Nevertheless, this did not meet the established criteria of greater than 25% apoptotic levels in the ectopic Myc cell line. This poses the problem of creating excessive
background, noise that may mask the apoptotic signature in subsequent ChIP-chip and expression array analysis. Although the pre-treatment serum withdrawal greatly increased apoptosis levels in the MCF10A-Myc cells, it also increased basal apoptotic levels in the MCF10A-GFP cells to greater than 10%, again not meeting the established criteria. 

*Alteration of the serum withdrawal condition does not improve selectivity for Myc-potentiated apoptosis with a 6h doxorubicin exposure in MCF10A cells expressing constitutive Myc*

The treatment regiment of withdrawing serum from the cells 1h prior to drug treatment and maintaining serum withdrawal thereafter effectively induced Myc-potentiated apoptosis; however, it also triggered basal apoptosis. It was thought that perhaps by using a less severe serum withdrawal condition, basal apoptotic levels could be lessened while maintaining adequate levels of Myc-potentiated apoptosis. The serum withdrawal condition was subsequently investigated. Various combinations of titrating horse serum and insulin concentrations were used for the serum withdrawal condition (Table 1). All results showed either low levels of Myc-potentiated apoptosis that were not in excess of 25% or a poor apoptotic differential between the MCF10A-GFP and MCF10A-Myc cells owing to high basal levels of apoptosis of greater than 10%.

*Various apoptotic agents combined with serum withdrawal do not selectively induce Myc-potentiated apoptosis in a 6h exposure in MCF10A cells expressing constitutive Myc*

All the aforementioned experiments employed doxorubicin and did not meet the required criteria. Therefore, different agonists were tested in hopes that one or more would show superior Myc-selectivity. Agonists of various apoptosis-inducing mechanisms were employed. These included latrunculin-A (an inhibitor of actin polymerization), thapsigargin
<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>Mechanism</th>
<th>Duration</th>
<th>1h Pre-treatment SW</th>
<th>Treatment SW</th>
<th>Withdrawal Condition</th>
<th>% Apoptosis</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>5% HS 0% insulin</td>
<td>7.2</td>
<td>6.6</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0.5% HS 0% insulin</td>
<td>8.5</td>
<td>19</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0.05% HS 0% insulin</td>
<td>9.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>5% HS 0.1% insulin</td>
<td>2.8</td>
<td>9.9</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0.5% HS 0.1% insulin</td>
<td>1.6</td>
<td>7.1</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0.5% HS 0.05% insulin</td>
<td>2.9</td>
<td>8.6</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0.5% HS 0.025% insulin</td>
<td>1.5</td>
<td>9.5</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0.5% HS 0.0125% insulin</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>Latrunculin-A</td>
<td>0.5µM</td>
<td>Actin polymerization inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>7.8</td>
<td>11.7</td>
</tr>
<tr>
<td>Latrunculin-A</td>
<td>1µM</td>
<td>Actin polymerization inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>1.1</td>
<td>11.3</td>
</tr>
<tr>
<td>Latrunculin-A</td>
<td>2µM</td>
<td>Actin polymerization inhibitor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>9.5</td>
<td>18</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>0.5µM</td>
<td>ER stressor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>2.6</td>
<td>13.5</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>1µM</td>
<td>ER stressor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>5.6</td>
<td>15.9</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>2µM</td>
<td>ER stressor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>0.5</td>
<td>13.7</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>0.1µg/mL</td>
<td>ER stressor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>4.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>1µg/mL</td>
<td>ER stressor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>1.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>10µg/mL</td>
<td>ER stressor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>6.5</td>
<td>11.6</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>25nM</td>
<td>ER stressor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>3.2</td>
<td>14.2</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>50nM</td>
<td>ER stressor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>3.1</td>
<td>5.7</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>100nM</td>
<td>ER stressor</td>
<td>6h</td>
<td>yes</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>14.2</td>
<td>9.7</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.5µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>yes</td>
<td>0.05% HS 0.1% insulin</td>
<td>1.7</td>
<td>3.3</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>yes</td>
<td>0.05% HS 0.1% insulin</td>
<td>8.1</td>
<td>28.2</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>2µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>yes</td>
<td>0.05% HS 0.1% insulin</td>
<td>46.8</td>
<td>54.3</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.25µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>0.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.5µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>0.7</td>
<td>4.5</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>10.3</td>
<td>21.2</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>2µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>yes</td>
<td>0% HS 0% insulin</td>
<td>63.3</td>
<td>64.5</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.125µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>no</td>
<td>N/A</td>
<td>0.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.25µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>no</td>
<td>N/A</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.5µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>no</td>
<td>N/A</td>
<td>2.3</td>
<td>3.9</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1µM</td>
<td>DNA replication inhibitor</td>
<td>24h</td>
<td>no</td>
<td>no</td>
<td>N/A</td>
<td>35.9</td>
<td>71.2</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>0.5µM</td>
<td>ER stressor</td>
<td>24h</td>
<td>no</td>
<td>yes</td>
<td>0.05% HS 0.1% insulin</td>
<td>14.7</td>
<td>19</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>1μM</td>
<td>ER stressor</td>
<td>24h</td>
<td>no</td>
<td>yes</td>
<td>0.05% HS 0.1% insulin</td>
<td>14.8</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 1: Various treatments tested to maximize Myc-potentiated apoptosis in the MCF10A cell line
(a SERCA inhibitor that causes cytosolic Ca\(^{2+}\) accumulation), tunicamycin (an inhibitor of N-linked glycoprotein synthesis) and bortezomib (a 26S proteasome inhibitor). All agonists were combined with serum withdrawal (media free of horse serum, insulin and additional supplements) 1h prior to and during the 6h drug treatment. Apoptosis was assayed with either TUNEL or fixed PI, which measures DNA degradation associated with cell death (Table 1). All the conditions displayed low levels of Myc-potentiated apoptosis of less than 25% or poor apoptotic differentials owing to high basal death of greater than 10%.

Extended exposure to 24h does not selectively induce Myc-potentiated apoptosis in MCF10A cells expressing constitutive Myc

In general, all of the 6h treatments resulted in either low levels of Myc-potentiated apoptosis as indicated by the MCF10A-Myc cells or a poor apoptotic differential owing to high levels of basal apoptosis in the MCF10A-GFP cells. A possible explanation for these results could be that due to the relative short treatment period, there is not sufficient time for the agonists to induce Myc-potentiated apoptosis and the conditions that are able to induce Myc-potentiated apoptosis in such a rapid manner are too severe and are triggering basal apoptosis. To find a middle ground, a longer treatment was investigated. It was thought that with longer treatments, less harsh apoptotic treatments could be employed, sufficient to induce Myc-potentiated apoptosis but not sufficient to drive basal apoptosis.

To investigate longer treatment times, dose ranges of doxorubicin were used to treat MCF10A cells for 24h with and without serum withdrawal (the two main types of serum withdrawal previously discussed) (Table 1). The results were typical of the previous treatments: either low levels of death of less than 25% were achieved in the MCF10A-Myc cells or high levels of Myc-potentiated apoptosis were met with high levels, greater than
10%, of basal death. Results were similar when a different agonist, thapsigargin, was used in combination with serum withdrawal.

*Constitutively expressed ectopic Myc is downregulated in response to doxorubicin exposure in MCF10A cells*

To gain more insight as to why a poor apoptotic differential was often yielded between the MCF10A-GFP and MCF10A-Myc cells, a 12h condition using 1µM doxorubicin with full serum was employed. Under these conditions high levels of death were induced in both cell lines (Figure 5A). Under these same conditions, lysates of both MCF10A-GFP and MCF10A-Myc were harvested every two hours up to ten hours during the treatment. Immunoblots probing Myc protein and PARP-cleavage were performed for each of the time points to observe the kinetics of the treatment (Figure 5B). As previously established, under growing conditions, or in this case 0h, Myc protein levels were similar between the two cells lines. Myc levels in the MCF10A-GFP cells decreased rapidly and were almost undetectable by 6h of treatment. Similarly, Myc levels in MCF10A-Myc cells decreased as the treatment progressed, although at a slightly slower rate. By 8h, Myc levels were near undetectable in the MCF10A-Myc cells and levels were similar to that of MCF10A-GFP cells. With respect to PARP-cleavage, which is characteristic of apoptosis, levels of the large cleaved fragment of PARP increased gradually in both cell lines as the doxorubicin treatment progressed. Levels increased at a slightly faster rate in the MCF10A-Myc cells but by 8h and 10h, when Myc protein levels were similar, PARP-cleavage was similar between MCF10-GFP and MCF10A-Myc cells.

What these data suggest is that ectopic Myc was being down regulated in response to doxorubicin in the MCF10A cells. Myc protein levels were having comparable response in
Figure 5: Ectopic Myc is rapidly downregulated in response to doxorubicin in MCF10A cells
MCF10A-GFP (G) and MCF10A-Myc (M) cells were treated with doxorubicin. A) Cells were harvested after 12h and levels of cell death (pre-G1) levels were assayed using fixed PI. Two independent biological replicates were performed. Error bars +1SD. *indicates p<0.05, MCF10A-GFP vs. MCF10A-Myc of same treatment by t-test. B) Myc expression and PARP-cleavage were visualized by immunoblotting. Lysates were harvested every two hours for up to ten hours. Two independent biological replicates were conducted. Representative blot shown.
the regulated Myc model (MCF10A-GFP) and the deregulated Myc model (MCF10A-Myc).

This helps to explain why such poor apoptosis differentials were observed between the two cells lines and suggests that the MCF10A-Myc cell line is not an ideal model for the potentiation of apoptosis by Myc deregulation. Consequently, an alternative system was evaluated.

*Serum withdrawal followed by tunicamycin exposure selectively triggers Myc-potentiated apoptosis in MCF10A cells with inducible ectopic Myc*

To keep ectopic levels sustained and deregulated, we tested an inducible expression system to continually drive *de novo* Myc expression. MCF10A cells expressing a 4-hydroxy tamoxifen (4-OHT)-inducible lentiviral expression system were generated (Figure 6) (128). In this system, cells are co-infected with Myc downstream of an upstream activating sequence and the GEV16 transcription factor. A ubiquitin promoter constitutively drives expression of the GEV16 transcription factor. The fusion protein contains an estrogen-receptor ligand binding domain so that it is retained in the cytoplasm in the absence of 4-OHT. In the presence of 4-OHT, the transcription factor translocates to the nucleus where the GAL4-DNA binding domain directs DNA binding to GAL4 upstream activating sequences and the VP16 transactivation domain upregulates transcription of ectopic Myc.

Using this system, an alternative approach to studying the differential Myc genetic program of growth and of apoptosis was designed. Rather than comparing cells simply under growing and apoptotic conditions, a system was designed to study how Myc responds to mitogenic and anti-proliferative signals with such disparate biological functions from a common point: cell cycle arrest (Figure 7). Serum was withdrawn from MCF10A cells (0.05% horse serum, 10µg/mL insulin and no additional supplements) for 24h in the absence
Figure 6: Schematic of inducible ectopic Myc expression system
Cells are co-infected with Myc downstream of an upstream activating sequence (5xUAS) and the GEV16 transcription factor. A ubiquitin promoter constitutively drives expression of the GEV16 transcription factor. The fusion protein contains an estrogen-receptor ligand binding domain so that it is retained in the cytoplasm in the absence of 4-OHT. In the presence of 4-OHT, the transcription factor translocates to the nucleus where the GAL4-DNA binding domain directs DNA binding to GAL4 upstream activating sequences (5xUAS) and the VP16 transactivation domain upregulates transcription of ectopic Myc.
Growing Cell cycle arrest Apoptosis

Figure 7: Schematic of treatment regiment for MCF10A cells with inducible Myc expression system

MCF10A cells under normal growing conditions with full media (5% horse serum, 10µg/mL insulin and additional supplements) are serum deprived for 24h (0.05% horse serum, 10µg/mL insulin and no additional supplements) to induce cell cycle arrest. Ectopic Myc is induced through the addition of 4-hydroxytamoxifen (4-OHT) and cells are either mitogen stimulated to force cell cycle entry or serum withdrawal is maintained and tunicamycin is added to induce apoptosis.
of 4-OHT. This served to both minimize endogenous Myc levels and to induce cell cycle arrest. At this point, ectopic Myc was induced with 4-OHT and cells were either treated with mitogen stimulation or an apoptotic signal of sustained serum withdrawal and the addition of tunicamycin, triggering cell cycle entry and growth or apoptosis, respectively. Using the aforementioned MCF10A cells expressing constitutive ectopic Myc and MCF10A-GFP control line, preliminary data had already shown that maximum levels of cell cycle arrest could be achieved in the MCF10A cells through 24h hours of serum withdrawal, as indicated by G1 accumulation, levels which remained constant for 48h and 72h (Supplemental Figure 1A). Furthermore, upon mitogen stimulation following either 48h or 72h of serum withdrawal, MCF10A cells entered the cell cycle and returned to normal growth states within 24h to 32h, as indicated by the S phase population (Supplemental Figure 1B). The behaviour of the MCF10A-GFP and MCF10A-Myc cell lines in response to serum withdrawal and subsequent mitogen stimulation were similar in that both achieved similar levels of cell cycle arrest upon serum withdrawal and similar levels of cell cycle entry upon mitogen stimulation. The MCF10A-Myc cells did, however, respond to mitogen stimulation with cell cycle entry more rapidly.

Using the MCF10A with inducible ectopic Myc and the treatment regiment described above, cells were harvested under normal growing conditions and after 24h of serum withdrawal to show the induction of cell cycle arrest. For each subsequent treatment, cells were harvested after 48h (following the 24h serum withdrawal). Ethanol served as the vehicle control for 4-OHT.

Cell cycle analysis using fixed PI showed that after only 24h, cell cycle arrest was rapidly induced as over 90% of the cells accumulated in G1 while the S population was
significantly (p<0.05) diminished to minimal levels, as compared to growing cells (Figure 8A). When the cells were mitogen stimulated, the cells entered cell cycle as the proportion of cells in G1 decreased and that of G2 and S increased. The responses were comparable between the 4-OHT and the ethanol control with both showing a significant (p<0.05) increase in S phase populations as compared to 24h of serum withdrawal. Although the cells did enter cell cycle, the cell cycle profiles did not return to the previous state, that is, under normal growing conditions prior to serum withdrawal. This was likely because the plates became confluent after 48h of mitogen stimulation, dampening the proliferative response through contact inhibition. This was furthered by the fact that there was an increase in the pre-G1 population when ectopic Myc was induced by 4-OHT. The deregulated Myc may have sensitized the cells to the anti-proliferative cues of contact inhibition, potentiating apoptosis. No increase in pre-G1 was seen when cells were treated with the ethanol control.

Additionally, preliminary data for which cells were harvested after 24h, 48h and 72h of the described treatment following 24h of serum withdrawal (Supplemental Figure 2) show that after 24h of mitogen stimulation, the cell cycle profiles achieved by cells treated with either 4-OHT or the ethanol control are similar to that of normal growing conditions in Figure 8A. By 48h, there is an increase in the G1 population and a decrease in the S and G2 populations, further supporting that at 48h, the proliferative response of the mitogen stimulated cells was lessened by contact inhibition owing to confluence.

For the apoptotic arm of the treatment, DMSO served as the vehicle control for tunicamycin. Assuming that the effects of the given DMSO concentration had little effect on the growth and induction of apoptosis of the MCF10A cells, the DMSO treatment was essentially a sustained serum withdrawal treatment. In the absence of 4-OHT, the cells
Figure 8: Myc induction potentiates apoptosis in response to tunicamycin and serum withdrawal in growth arrested MCF10A cells

MCF10A cells with inducible Myc expression system under normal growing conditions with full media (5% horse serum, 10µg/mL insulin and additional supplements) were serum deprived for 24h (SW) (0.05% horse serum, 10µg/mL insulin and no additional supplements). After 24h of serum withdrawal, ectopic Myc was induced through the addition of 100nM 4-hydroxytamoxifen (4-OHT) and ethanol served as the vehicle control (EtOH). As well, cells were either mitogen stimulated (FS) or serum withdrawal (SW) was maintained and cell were treated with 2µg/mL tunicamycin or the DMSO vehicle control. Cells were harvested under growing conditions (growing), after the 24h of serum withdrawal (24h SW) and after 48h of the subsequent treatments. A) Cell cycle analysis was performed using fixed PI. Three independent biological replicates were conducted. Error bars +1SD. Statistics discussed in Results section. B) Apoptosis (TUNEL positive) levels were assayed using TUNEL. Three independent biological replicates were conducted. Error bars +1SD. *indicates p<0.05, 4-OHT vs. ethanol control of same treatment by t-test.
remained in growth arrest and the cell cycle profile was comparable to that of the previous 24h serum withdrawal. When Myc was induced with 4-OHT, the cells entered cell cycle, consistent with previous findings showing that Myc is sufficient to drive cycle entry (18, 19, 129). Although the decrease in the G1 population and the increase in the G2 population were almost equal to those seen under mitogen stimulation conditions, it was met with a smaller but significant (p<0.05) increase in the S phase population when compared to 24h of serum withdrawal. It should be noted, however, that the S phase population when Myc was not induced, in the corresponding ethanol control, was also marginally but still significantly higher (p<0.05) than that of 24h serum withdrawal. This makes it difficult to interpret how real the increase in S phase was in response to ectopic Myc induction. Again, this was likely due to the cells reaching confluence as a result of 48h of growth and thus lessening the proliferative response. Preliminary data showed that after 24h of Myc induction following serum withdrawal, cells in serum deprived conditions enter cell cycle at levels similar to that of mitogen stimulation and that by 48h there is an increase in the G1 population and a decrease in the S and G2 populations (Supplemental Figure 2). Therefore, induction of Myc alone under conditions of serum deprivation is sufficient for cell cycle entry in the MCF10A cells. Also, consistent with deregulated Myc potentiating apoptosis in response to anti-proliferative stimuli, including serum withdrawal, there was a marked and almost significant (p=0.055) increase in pre-G1 population when Myc was induced as compared to the corresponding ethanol condition.

MCF10A cells treated with tunicamycin in combination with serum withdrawal and in the absence of 4-OHT, retained a high G1 accumulation. There was, however, a strong and significant (p<0.05) increase in the pre-G1 population as the cells began to die in response to
the addition of tunicamycin as compared to the DMSO condition. When Myc was induced under tunicamycin and serum withdrawal, the MCF10A cells responded with robust and significant (p<0.05) levels of cell death when compared to when Myc was not induced in the corresponding ethanol condition.

Although pre-G1 measurement by fixed PI is a reliable assay of cell death, it is not specific to apoptosis. Therefore, TUNEL was performed in parallel (Figure 8B). Overall, the TUNEL data was consistent with the fixed PI data. Under mitogen stimulation, the TUNEL positive levels were minimal, although there was a slight but insignificant increase when ectopic Myc was induced, consistent with the increase seen in the pre-G1 population when 4-OHT was added. When the DMSO vehicle control was combined with serum withdrawal, again TUNEL positive levels were low and again, when ectopic Myc was induced, there was a marginal and near significant (p=0.074) increase in TUNEL positivity, consistent with the increase in pre-G1. Finally, when tunicamycin was added with serum withdrawal, the TUNEL positive population increased slightly in absence of 4-OHT and was approaching significance (p=0.085) when compared to the corresponding DMSO treatment. When Myc was induced, there was a robust and significant (p<0.05) increase in TUNEL positivity with on average, the majority of the population being apoptotic. This indicated that the cell death being induced by these treatments and that increase seen in the pre-G1 populations is largely specific to apoptosis. Additionally, these conditions far exceed the set criteria of less than 10% and greater than 25% apoptotic levels in the control and in the ectopic Myc cell line, respectively.
**Ectopic Myc is induced within 12h under serum withdrawal conditions**

To gain insight into the kinetics of the treatment developed for the MCF10A cells with inducible ectopic Myc expression, lysates were harvested from MCF10A cells under growing conditions and after 24h of serum withdrawal. Additionally, proceeding the 24h serum withdrawal, lysates were harvested after 12h and 24h of either mitogen stimulation or tunicamycin treatment with sustained serum withdrawal, with and without induction of ectopic Myc by 4-OHT. Immunoblots probing Myc protein and PARP-cleavage were conducted (Figure 9A and Supplemental Figure 3). After 24h of serum withdrawal, there was a 2-fold decrease in Myc protein compared to growing MCF10A cells (Figure 9B). Following mitogen stimulation, there was a rapid 2- to 3-fold increase in Myc after 12h and these levels were maintained at 24h. Consistent with the cell cycle entry seen in the fixed PI data, Myc protein levels were similar with and without induction of ectopic Myc. MCF10A cells treated with tunicamycin combined with serum withdrawal but in the absence of 4-OHT showed a further 2-fold decrease in Myc protein by 12h and by 24h where levels dropped 3-fold. Under the same conditions with the addition of 4-OHT, Myc levels increased 2-fold above levels of 24h serum withdrawal and were maintained at the same level after 24h of treatment, significantly higher (p<0.05) than the ethanol equivalent at both time points. Although Myc levels increased by 12h and were maintained at the same level at 24h similar to the mitogen stimulation conditions, inducing Myc under the apoptotic conditions did not achieve as much expression as mitogen stimulation.

In growing MCF10A cells, PARP-cleavage was minimal but increased following 24h of serum withdrawal (Figure 9C). When treated with tunicamycin and continued serum withdrawal, there was no increase in PARP-cleavage in the absence of 4-OHT after 12h but
A

<table>
<thead>
<tr>
<th></th>
<th>12h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FS SW</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Growing 24h SW + subsequent 12h treatment

**Relative Myc protein levels (normalized to actin)**

B

![Image of Western blot analysis](image)

150kDa-

102kDa-

76kDa-

52kDa-

38kDa-

- α PARP
- Large cleaved PARP fragment
- α Myc
- α Actin

For detailed analysis, please refer to the figure.
Figure 9: Myc can be induced under apoptotic conditions to potentiate apoptosis as indicated by PARP-cleavage in MCF10A cells

A) Myc expression and PARP-cleavage visualized by immunoblotting. MCF10A cells with inducible Myc expression system under normal growing conditions with full media (5% horse serum, 10μg/mL insulin and additional supplements) were serum deprived for 24h (SW) (0.05% horse serum, 10μg/mL insulin and no additional supplements). After 24h of serum withdrawal, ectopic Myc was induced through the addition of 100nM 4-hydroxytamoxifen (4-OHT) and ethanol served as the vehicle control (EtOH). As well, cells were either mitogen stimulated (FS) or serum withdrawal (SW) was maintained and cell were treated with 2μg/mL tunicamycin. Lysates were harvested under growing conditions (growing), after the 24h of serum withdrawal (24h SW) and after 12h and 24h of the subsequent treatments. Representative blot shown. See Supplemental Figure 3 for additional two blots.

B) Myc and C) PARP-cleavage protein levels (normalized to actin) were quantified relative to the maximum observed levels. Three independent biological replicates were conducted. Error bars +1SD, *indicates p<0.05, ethanol control vs. 4-OHT for same treatment at given time point by t-test.
levels approximately doubled after 24h of treatment. When ectopic Myc was induced by 4-OHT, PARP-cleavage levels tripled following 12h of treatment and increased further after 24h of treatment at which point levels were significantly higher (p<0.05) than the corresponding ethanol treatment.

Collectively, the data characterising the apoptotic system involving the MCF10A cells with inducible Myc expression suggest that this is an appropriate system for studying the genetic program of Myc-potentiated apoptosis through subsequent ChIP-chip and expression array analyses. Using the aforementioned conditions, high levels of Myc-potentiated apoptosis can be achieved while minimizing basal levels. Furthermore, the cell death observed has been shown to indeed be apoptosis.

Optimization of ChIP of Myc in the SHEP human neuroblastoma cell line

Neuroblastoma is the most common childhood extracranial malignant solid tumour (130). Approximately 20% of neuroblastoma tumours have amplified MYCN and amplification is often associated with poor patient prognosis (26, 131). Furthermore, MYCN status is used as a marker for stratification of neuroblastoma cases with patients lacking N-Myc amplification fairing better (26). MYCN amplification is also frequently associated with disrupted apoptosis as complete caspase 8 inactivation occurs almost exclusively in neuroblastomas with amplified MYCN, rendering the cells resistant to receptor-mediated and doxorubicin-induced apoptosis (132). Myc and N-Myc have been shown to share much functional homology as N-Myc can functionally replace Myc in murine development and both Eµ-Myc and Eµ-N-Myc mice develop B-cell lymphomas (133, 134). With respect to neuroblastoma, tumours lacking MYCN amplification generally express Myc at levels above that of normal tissues (135, 136). The SHEP cell line used in this work is a human
neuroblastoma line expressing low endogenous and non-amplified MYCN with a Tet-Off inducible N-Myc expression system (137). ChIP-chip of both Myc and N-Myc in SHEP cells has since been published, although not under apoptotic conditions (130). The SHEP cell line was, however, used to study the potentiation of apoptosis by Myc (c-Myc) rather than that by N-Myc. The reason for this was to complement the developed system in the MCF10A cell line, that is, establishing a second independent cell system for the study of c-Myc-potentiated apoptosis. Furthermore, despite the functional homology demonstrated between Myc and N-Myc, it has been shown that a large number of genes targeted by both Myc and N-Myc in SHEP cells are less responsive to N-Myc than to Myc (130). Therefore, it may not be appropriate to validate or assume equivalency between the apoptotic genetic program of Myc in the MCF10A cell line and that of N-Myc in the SHEP cell line. To that end, SHEP cells expressing ectopic Myc (c-Myc) were generated and for all subsequent experiments N-Myc expression was repressed through 48h of doxycycline exposure.

*Optimization of cell number and antibody concentration for Myc ChIP in the SHEP cell line*

ChIP of Myc in the SHEP cell line had not been previously established in the Penn lab. As with the MCF10A cell line, ChIP parameters including cell number and antibody concentration required optimization. ChIP reactions were performed in parallel with the SHEP cells expressing ectopic Myc using 1µg, 2µg or 4µg of N262 Myc specific antibody and the equivalent concentration of the IgG non-specific control antibody, with either 5x10^6 or 10x10^6 cells. The efficiency of each Myc ChIP condition was quantified using qPCR where Ch6 was the negative control and CAD the positive control (Figure 10A). All ChIP conditions appropriately showed no signal enrichment of Myc ChIP over IgG at Ch6 but allvshowed strong enrichment at CAD. All three antibody concentrations had similar Myc
Figure 10: Optimization of Myc ChIP in the SHEP cell line

A) ChIP reactions were conducted using a range of cell numbers and antibody concentrations of the Myc specific (N262) and the non-specific IgG (normal rabbit) control antibody. Efficiencies of the ChIP reactions were assessed using qPCR and absolute quantification was used to represent the amount of DNA isolated in the ChIP in ng of DNA. *CAD and an E-box
on chromosome 6 (Ch6) are the positive and negative controls, respectively. qPCR was conducted in triplicate and the bars represent the mean. Error bars +1SD. B) The optimized Myc ChIP condition of 2µg of antibody and 10x10^6 cells was validated for reproducibility. Fold enrichment of Myc ChIP at each target is expressed as the mean log2 ratio of Myc ChIP signal to IgG noise. Two independent biological replicates were conducted and qPCR was conducted in triplicates. Error bars +1SD, *indicates p<0.05, vs. negative control by t-test.
ChIP signals at $5 \times 10^6$ cells and minimal IgG noise. Except for the 1µg antibody concentration, $10 \times 10^6$ cells increased the Myc ChIP signal over that of $5 \times 10^6$ cells. The IgG noise for $10 \times 10^6$ cells was only marginally higher when compared to the equivalent antibody concentrations at $5 \times 10^6$ cells. The 2µg of antibody and $10 \times 10^6$ cells conditions were chosen as the Myc ChIP condition in the SHEP cell line since it yielded high Myc signals and although similar to the 4µg condition of equivalent cell number, it required less antibody and thus would lower costs.

Validation of optimized Myc ChIP conditions in the SHEP cell line

The optimized Myc ChIP condition of 2µg of antibody and $10 \times 10^6$ cells in the SHEP cell line was validated using qPCR with biological replicates (Figure 10B). Again, the targets included the Ch6 negative control and the CAD positive control. Signal-to-noise is expressed as the mean log2 fold increases of Myc ChIP over IgG. Appropriately, there was little signal enrichment for Myc ChIP at the Ch6. Signal enrichment was significant higher than Ch6 at CAD with a 3- to 4-fold increase. Therefore, the optimized condition of 2µg of antibody and $10 \times 10^6$ cells in the SHEP cell line was both efficient, as it showed high levels of signal enrichment for Myc at the known Myc binding targets, and reproducible.

Optimization and characterisation of SHEP apoptotic condition

As with the MCF10A cell line, we sought to develop apoptotic conditions that induce less than 10% apoptotic levels in control cells and greater than 25% apoptotic levels in cells expressing ectopic Myc.

Tunicamycin induces Myc-potentiated apoptosis in SHEP cells expressing constitutive Myc

SHEP cells expressing ectopic pMN-GFP constructs were generated: empty vector control (SHEP-GFP) and Myc (SHEP-Myc) providing a regulated and deregulated Myc
model, respectively. Tunicamycin had previously been established by Sam Kim in the Penn lab to be a strong inducer of N-Myc-potentiated apoptosis in the SHEP cell line. Therefore, the SHEP-GFP and SHEP-Myc cells were treated with a dose range of tunicamycin: 0.1µg/mL, 1µg/mL and 10µg/mL, as well as the equivalent DMSO vehicle control. Cells were harvested at 24h, 48h and 72h and levels of cell death were measured as indicated by pre-G1 population using fixed PI (Figure 11A). Generally, there was a dose response and a clear time dependency in the tunicamycin treatments. After 24h, pre-G1 levels were low at all three doses of tunicamycin in both cell lines though there was a small increase at the two higher doses in the SHEP-Myc cells. At 48h, levels of cell death remained low at the 0.1µg/mL dose in both cell lines and increased only slightly by 72h. After 48h, there was a strong increase in pre-G1 in the SHEP-Myc cells treated with 1µg/mL and a smaller increase in the SHEP-GFP cells with an approximate 2-fold differential. Cell death levels increased further after 72h in the SHEP-Myc under this dose but there was a stronger increase in the SHEP-GFP cells and the differential was diminished. At the highest dose of 10µg/mL of tunicamycin, there was a robust increase in the pre-G1 population in the SHEP-Myc cells and a strong but lesser increase in the SHEP-GFP cells, yielding a 2- to 3-fold differential. By 72h, much of the differential was lost as there was strong increase in cell death in the SHEP-Myc cells but an even stronger increase in the SHEP-GFP cells. Overall, it appeared as though the best cell death differentials between SHEP-GFP and SHEP-Myc cells with tunicamycin were achieved at 48h although at this time points, as well as at the 24h and 72h time point, no dose of tunicamycin yields significantly more cell death in the SHEP-Myc cells than the SHEP-GFP cells.

Although 48h was shown to be the optimal time point for assaying the efficacy of the
Figure 11: Optimization and validation of tunicamycin apoptotic treatment in SHEP cells

A) SHEP-GFP and SHEP-Myc cells were treated with a dose range of tunicamycin and harvested at 24h, 48h and 72h. Levels of cell death (pre-G1) levels were assayed using fixed PI. Two independent biological replicates were conducted. Error bars +1SD. *indicates p<0.05, SHEP-GFP vs. SHEP-Myc for same treatment and time point by t-test. Cells were treated with 2.5μg/mL tunicamycin and harvested at 48h.

B) Levels of cell death (pre-G1) levels were assayed using fixed PI. Four independent biological replicates were conducted. Error bars +1SD. *indicates p<0.05, SHEP-GFP vs. SHEP-Myc for same treatment by t-test.

C) 48h treatment

% Annexin V positive population

% pre-G1 population
C) As a pilot experiment; apoptosis (Annexin V positive) levels were assayed using Annexin V. One assay was conducted.
treatments in inducing Myc-potentiated apoptosis in the SHEP cells, none of the doses in the tunicamycin dose range met the criteria of less than 10% apoptosis in the SHEP-GFP cells and greater than 25% in the SHEP-Myc cells. However, the optimal dose was narrowed to between 1µg/mL and 10µg/mL. Therefore, the tunicamycin dose was further optimized to 2.5µg/mL (data not shown). After 48h at this dose, pre-G1 levels in the SHEP-Myc cells were 3-fold and significantly (p<0.05) higher than that of the SHEP-GFP cells (Figure 11A). Cell death levels in the SHEP-Myc cells exceeded 25% and death in the SHEP-GFP cells was low at just under 10%. This condition was thus selected for further characterization. Interestingly, SHEP-Myc cells showed significantly higher (p<0.05) pre-G1 levels than SHEP-GFP cells under the DMSO control treatment.

To ensure that the cell death being induced was in fact apoptosis, an apoptosis specific assay was needed. TUNEL assays were attempted in the SHEP cells under the tunicamycin treatment; however, apoptotic cells were not being adequately labelled as positive controls were not yielding previously established TUNEL positive population sizes (data not shown). For this reason, an alternative apoptosis detection assay was employed. Annexin V staining detects phosphatidylserine exposure at the outer-leaflet of the cell membrane, which is characteristic of apoptosis. A pilot experiment was performed to determine whether Annexin V is an appropriate apoptosis assay in this cell line. Annexin V staining was used to detect apoptosis in SHEP-GFP and SHEP-Myc cells treated for 48h with 2.5µg/mL of tunicamycin (Figure 11C). The Annexin V data was consistent with the fixed PI data in that the SHEP-Myc cells displayed a high Annexin V positive population and that of the SHEP-GFP was low. Although the values for Annexin V positivity were lower than the value for pre-G1 populations, the apoptotic differential between the two cell lines was almost
identical at 3-fold. Therefore, although this was a pilot experiment and requires further replicates, the death induced by tunicamycin in the SHEP cells appears to be apoptosis.

Constitutively expressed ectopic Myc is downregulated in response to tunicamycin treatment in SHEP cells

To gain insight into the kinetics of the tunicamycin treatment developed for the SHEP cells, lysates were harvested from SHEP-GFP and SHEP-Myc cells at 0h, 12h, 24h and 48h of 2.5µg/mL tunicamycin treatment. Immunoblots probing Myc protein and PARP-cleavage were performed to follow the kinetics of the treatments (Figure 12A and Supplemental Figure 4).

Under normal growing conditions, at 0h, the SHEP-Myc cells expressed significantly (p<0.05) and robustly more Myc protein than SHEP-GFP cells (Figure 12B). In the SHEP-Myc cells, Myc protein was downregulated in response to tunicamycin with levels diminishing 2-fold after 48h of treatment compared to the 0h time point. In the SHEP-GFP cells, there was a general trend of downregulation of Myc but Myc protein levels were so low that the trend was difficult to interpret. At every time point, the SHEP-Myc cells expressed significantly more (p<0.05) Myc protein than the SHEP-GFP cells.

Tunicamycin treatment induces higher levels of PARP-cleavage in SHEP cells expressing ectopic Myc

With respect to PARP-cleavage, SHEP-Myc cells displayed basal levels of PARP-cleavage under normal growing conditions, or 0h (Figure 12C). This was consistent with the increase in pre-G1 population seen in the SHEP-Myc cells under the DMSO vehicle control treatment. It appeared as though deregulation of Myc through ectopic expression in the SHEP cells potentiated basal apoptosis. Nonetheless, upon treatment with tunicamycin,
Figure 12: Ectopic Myc potentiates apoptosis induced by tunicamycin as indicated by PARP-cleavage in SHEP cells and Myc expression is downregulated over time

A) Myc expression and PARP-cleavage visualized by immunoblotting. SHEP-GFP (G) and SHEP-Myc (M) cells were treated with 2.5 μg/mL tunicamycin. Lysates were harvested at 0h, 12h, 24h and 48h. Representative blot shown. See Supplemental Figure 4 for additional two blots.

B) Myc and C) PARP-cleavage protein levels (normalized to actin) were quantified relative to the maximum observed levels. Three independent biological replicates were conducted. Error bars ±1SD, *indicates p<0.05, SHEP-GFP vs. SHEP-Myc controls at same time point by t-test.
PARP-cleavage levels increased substantially in the SHEP-Myc cells throughout the time course. In the SHEP-GFP cells, PARP-cleavage also increased in a time dependent manner in response to tunicamycin. Although the quantification of PARP-cleavage in the SHEP-Myc cells was variable due to variation in the rate of cleavage between replicates resulting in large error bars, there was still a clear trend of increasing PARP-cleavage in response to tunicamycin treatment at all time points following treatment and SHEP-Myc cells displayed significantly more (p<0.05) PARP-cleavage than the SHEP-GFP cells at every time point, except the 24h. At the 24h time point, the increased levels of PARP-cleavage displayed by the SHEP-Myc cells compared to the SHEP-GFP cells were approaching significance (p=0.096). This was consistent with the previous data showing that deregulated Myc was potentiating apoptosis in the SHEP cells in response to tunicamycin treatment.

*Thapsigargin induces Myc-potentiated apoptosis in SHEP cells expressing constitutive Myc*

It was also previously established by Sam Kim in the Penn lab that thapsigargin is a strong inducer of N-Myc-potentiated apoptosis in the SHEP cell line. Therefore, SHEP-Myc and SHEP-GFP cells were treated with a dose range of thapsigargin: 0.1µM, 1µM and 10µM, along with the corresponding DMSO vehicle controls. Cells were harvested at 0h, 12h, 24h and 48h and assayed for pre-G1 populations using fixed PI (Figure 13A). Overall, the thapsigargin treatments showed a clear dose response. After 24h, the two lower doses yielded low pre-G1 levels in both cell lines, although there was a small increase in SHEP-Myc cells. The higher dose of 10µM showed higher levels in both cell lines with a 3-fold cell death differential of SHEP-Myc cells over SHEP-GFP. At 24h, cell death levels increased substantially at all doses in both SHEP-Myc and SHEP-GFP cells. At 0.1µM and 1µM of thapsigargin, pre-G1 levels were 2-fold higher in the SHEP-Myc cells compared to SHEP-
Figure 13: Optimization and validation of thapsigargin apoptotic treatment in SHEP cells

A) SHEP-Myc and SHEP-GFP cells were treated with a dose range of thapsigargin and harvested at 24h, 48h and 72h. Levels of cell death (pre-G1) levels were assayed using fixed PI. Two independent biological replicates were conducted. Error bars ±1SD. *indicates p<0.05, SHEP-GFP vs. SHEP-Myc for same treatment and time point by t-test. Cells were treated with 0.25µM thapsigargin and harvested at 48h. B) Levels of cell death (pre-G1) levels were assayed using fixed PI. Four independent biological replicates were conducted.
Error bars +1SD. *indicates p<0.05, SHEP-GFP vs. SHEP-Myc for same treatment by t-test.
C) As a pilot experiment; apoptosis (Annexin V positive) levels were assayed using Annexin V. One assay was conducted.
GFP cells. At 72h, cell death levels were unchanged in the SHEP-Myc cells and increased in the SHEP-GFP cells to similar levels, eliminating the differential in cell death between the two cell lines. At the highest dose of 10µM, 48h of treatment induced high levels of pre-G1 in both cell lines, greatly diminishing the cell death differential seen at 24h. At 72h, cell death levels in the SHEP-GFP cells appeared to exceed those of SHEP-Myc cells but since the pre-G1 levels were likely erroneously low in the SHEP-Myc cells after 72h of 10µM tunicamycin treatment since they were higher at the 48h point, they were likely equivalent with no differential. Again, after 72h SHEP-Myc cells showed an increase in pre-G1 in all three control DMSO conditions. None of the treatments induced significantly higher levels of cell death in the SHEP-Myc cells compared to the SHEP-GFP cells at any of the assayed time points. Furthermore, none met the established criteria of less than 10% apoptosis in the SHEP-GFP cells and greater than 25% in the SHEP-Myc cells.

As with the tunicamycin treatment, 48h was shown to be the optimal time point for assaying the efficacy of the treatments in inducing Myc-potentiated apoptosis in the SHEP cells. Using the results derived from 0.1µM and 1µM treatments, the thapsigargin dose was further optimized to 0.25µM (data not shown). At this dose, pre-G1 levels in the SHEP-Myc cells were 4-fold higher than that of SHEP-GFP cells (Figure 13B). This condition narrowly missed the set criteria in that although as it yielded far greater than 25% cell death in the SHEP-Myc cells; cell death levels were just over the 10% upper-bound at about 11%. Nonetheless, this condition was further characterized.

To ensure that the cell death being induced by thapsigargin was in fact apoptosis, as a pilot experiment, Annexin V staining was used to detect apoptosis in SHEP-GFP and SHEP-Myc cells treated for 48h with 0.25µM of thapsigargin (Figure 13C). The SHEP-Myc cells
showed a large Annexin V positive population indicating that Myc potentiated apoptosis in response to the thapsigargin treatment. However, the SHEP-GFP cells also had a large increase in the Annexin V positive population though it was less than that of the SHEP-Myc cells. This suggests that the thapsigargin treatment is triggering both Myc-potentiated and basal apoptosis. Furthermore, the apoptotic differential between the two cells with respect to Annexin V positivity was less than that of pre-G1 population. This was, nevertheless, a pilot experiment and thus requires additional replicates before proper interpretation can be performed although it does indicate that the death observed under the optimized thapsigargin treatment is in fact apoptosis.

*Constitutively expressed ectopic Myc is downregulated in response to thapsigargin treatment in SHEP cells*

To gain insight into the kinetics of the thapsigargin treatment developed for the SHEP cells, lysates were harvested from SHEP-GFP and SHEP-Myc cells at 0h, 12h, 24h and 48h of 0.25µM thapsigargin treatment. Immunoblots probing Myc protein and PARP-cleavage were performed (Figure 14A and Supplemental Figure 5). As established above, SHEP-Myc cells under normal growing conditions, at 0h, expressed significantly (p<0.05) more Myc protein than SHEP-GFP cells (Figure 14B). In SHEP-Myc cells, Myc was downregulated in response to the thapsigargin treatment although this decrease was not seen until 24h treatment as levels were relatively unchanged at 12h. Myc levels decreased further after 48h, 2- to 3-fold less than growing levels. The response with respect to Myc protein levels in the SHEP-GFP cells under thapsigargin treatment was difficult to interpret, similar to the tunicamycin treatment, since Myc levels were already low under growing conditions and any trend was difficult to interpret. Nevertheless, at every harvest point under the optimized
Figure 14: Ectopic Myc potentiates apoptosis induced by thapsigargin as indicated by PARP-cleavage in SHEP cells and Myc expression is downregulated over time

A) Myc expression and PARP-cleavage visualized by immunoblotting. SHEP-GFP (G) and SHEP-Myc (M) cells were treated with 0.25 µM thapsigargin. Lysates were harvested at 0h, 12h, 24h and 48h. Representative blot shown. See Supplemental Figure 5 for additional two blots.

B) Myc and PARP-cleavage protein levels (normalized to actin) were quantified relative to the maximum observed levels. Three independent biological replicates were conducted. Error bars ±1SD, *indicates p<0.05, SHEP-GFP vs. SHEP-Myc controls at same time point by t-test.
thapsigargin treatment, SHEP-Myc cells expressed significantly more (p<0.05) Myc protein than SHEP-GFP cells.

*Thapsigargin treatment induces higher levels of PARP-cleavage in SHEP cells expressing ectopic Myc*

Again, basal levels of PARP-cleavage were observed in the SHEP-Myc cells under normal growing conditions, at 0h, and this was consistent with the increase in pre-G1 seen in the SHEP-Myc cells under the three DMSO vehicle control treatments after 48h (Figure 14C). Thapsigargin treatment increased PARP-cleavage in a time dependent manner in both cell lines although levels were higher in the SHEP-Myc cells. SHEP-Myc PARP-cleavage levels were significantly higher (p<0.05) than in SHEP-GFP cells only at the 48h point, however, the increase was near or approaching significance at the 12h and 24h time point (p=0.054 and p=0.084, respectively). Unlike the 0h time point under tunicamycin exposure, SHEP-Myc cells did not display significantly more PARP-cleavage than the SHEP-GFP cells at 0h of thapsigargin exposure (p=0.146), even though conditions are essentially equivalent to growing conditions. This can be attributed to one biological replicate in which PARP-cleavage was detected at higher levels in the SHEP-GFP cells and lower levels in the SHEP-Myc cells when compared to the other two independent biological replicates, which is evident from the large error bars for the SHEP-Myc cells at the 0h time point of the thapsigargin treatment. Collectively, this was consistent with the fixed PI and Annexin V data that deregulated Myc was potentiating apoptosis in response to the optimized thapsigargin dose.
Discussion

Myc is a powerful oncogene, yet deregulated Myc alone does not lead to cellular transformation due to the intrinsic safety mechanism of deregulated Myc potentiating apoptosis. Because structure function analysis shows that the regions of Myc essential for apoptosis are also required for Myc to function as a regulator of gene transcription, it is thought that Myc's role in apoptosis is a function of its regulation of an apoptotic genetic program. We hypothesize that Myc differentially binds and/or transcriptionally regulates a specific cohort of targets genes under anti-proliferative conditions to potentiate apoptosis. The foremost approach to addressing this hypothesis is through ChIP-chip coupled with expression array analysis under apoptotic conditions and growth conditions to determine differential Myc binding and/or transcriptional regulatory activity, respectively. In this study, we optimized and characterized two independent human cell systems in which to conduct these high-throughput assays. For each cell system, this required the optimization of Myc ChIP as well as the development and characterization of apoptotic conditions that maximize levels of Myc-potentiated apoptosis while minimizing basal levels.

The MCF10A cell system

Optimization of Myc ChIP

The methodology of ChIP for Myc has long been established in the Penn lab and although many of the parameters had already been optimized in the MCF10A cell line, further optimization was required. Therefore, parallel Myc ChIP reactions were carried out in MCF10A cells using a range of cell numbers and antibody concentrations. Conditions were assessed for efficiency based on maximizing Myc ChIP signal and minimizing IgG non-specific binding noise. This approach to optimizing ChIP conditions has been taken in the
past in the Penn lab and often biological repetition of the experiments was not required as the
selected condition would be further validated. Based on the optimization experiment, the
condition of 1.5µg of antibody and 10x10^6 cells per reaction was selected. This condition
yielded high Myc ChIP signal and low IgG noise, furthermore, it required less antibody than
the 5µg condition which displayed similar results. This condition was validated with several
biological replicates at five known Myc binding sites, four of which showed highly
significant fold enrichment for Myc ChIP when compared to the pooled negative controls. As
further support for the efficiency of these conditions and their use in future Myc apoptosis
ChIP-chip experiments, these Myc ChIP conditions were since successfully used in a large-
scale ChIP-chip experiment investigating the genetic targets of Myc transformation by
Amanda Wasylishen of the Penn lab (unpublished).

*Optimization and characterisation of apoptotic conditions with constitutive ectopic Myc*

Previous studies have shown that apoptosis could be induced in the MCF10A cell line
using various agonists including staurosporine, latrunculin-A and TRAIL, however, none of
the studies were in a Myc context (123-125). The MCF10A cell line has not yet been used as
a system for studying Myc-potentiated apoptosis. Therefore, we had to identify and optimize
conditions for maximizing Myc-potentiated apoptosis in the MCF10A cell line while
minimizing basal apoptotic levels. We sought to develop apoptotic conditions that induce
less than 10% apoptotic levels in control cells and greater than 25% apoptotic levels in cells
expressing ectopic Myc. A relatively short treatment of 6h was first investigated. The
reasoning was that by inducing high levels of apoptosis within a short time period, the
window to study the mechanism of apoptosis would be focused. For subsequent Myc ChIP
under the chosen apoptotic treatment, cells would not be harvested at the end of the time
course when apoptosis has been executed but rather prior to the execution of apoptosis when Myc is modulating the genetic program. Since the point at which Myc would shift its binding and/or transcription regulatory activity from growth to apoptotic function is not yet known, by rapidly inducing apoptosis, the window in which to investigate further is narrowed.

It was previously shown that MCF10A cells with ectopic Myc (MCF10A-Myc) express comparable levels to those with the empty vector control (MCF10A-GFP) under growing conditions. Expression analyses performed in the Penn lab have shown that when ectopic Myc is introduced into the MCF10A cells, mRNA of endogenous Myc is lowered, although not significantly (data not shown). When deprived of serum, within an hour, Myc protein levels decrease in the MCF10A-GFP cells but are sustained at a higher level in the MCF10A-Myc cells, which is appropriate as this cell line was intended to mimic deregulated Myc. For the 6h treatment, serum withdrawal and doxorubicin alone had little effect in inducing apoptosis in the MCF10A cells; however, we showed that they synergized to induce strong levels of Myc-potentiated apoptosis and low basal levels. When the cells were deprived of serum for 1h prior to the combinatory treatment to create a differential in Myc protein between the two cell lines, apoptotic levels in both cell lines increased dramatically. Although high levels of Myc-potentiated apoptosis, above 25%, were achieved, basal levels were too high, above 10%. These experiments showed that serum withdrawal in combination with drug treatment plus withdrawing serum for 1h prior to treatment could induce high levels of Myc-potentiated apoptosis thus various other drugs and serum withdrawal conditions were evaluated in an effort to find conditions that were more Myc-selective and would minimize basal apoptosis. None of the conditions were effective in that either they only induced low levels of Myc-potentiated apoptosis (less than 25%) or when levels were
high, basal levels were high as well (greater than 10%), thus not meeting the set criteria. We rationalized that perhaps inducing apoptosis within the short time span of 6h required treatments too severe for triggering Myc-potentiated apoptosis without activating other apoptotic pathways. We therefore, extended the treatments to 24h and experimented with lower doses. This simply had the effect of elevating both Myc-potentiated apoptosis and basal apoptosis with none of the conditions yielding a large and reproducible differential between the two cell lines.

Due to the consistent result of high levels of Myc-potentiated apoptosis being met with high basal levels we determined that perhaps it was not the conditions employed that were yielding the poor differentials but rather something inherent of the system used. When we looked at Myc protein levels and PARP-cleavage every two hours for ten hours in MCF10A-Myc and MCF10A-GFP cells in response to doxorubicin, we found that both ectopic and endogenous Myc were downregulated to near undetectable levels within 10h. It was not surprising that ectopic Myc, that was meant to mimic Myc deregulation, was downregulated in response to doxorubicin; in the MCF7 breast line which harbours amplified Myc, Myc is also downregulated in response to doxorubicin (138). What was surprising was that the kinetics between the MCF10A-GFP and MCF10A-Myc cells were very similar except that in the ectopic line, Myc was downregulated at a slightly slower rate and PARP-cleavage increased at a slightly faster rate. Nonetheless, by ten hours the state of Myc protein and PARP-cleavage were similar between the two cell lines. This was consistent with poor overt cell death differential between the two cell lines by 12h. Collectively, these experiments indicated that MCF10A cells expressing constitutive ectopic Myc were not an
appropriate model for Myc deregulation for the purpose of investigating the mechanism of Myc-potentiated apoptosis.

_Optimization and characterisation of apoptotic conditions with inducible ectopic Myc expression_

The inducible Myc system proved much more effective at maximizing Myc-potentiated apoptosis and minimizing basal apoptosis than the constitutive Myc system in the MCF10A cell line. Based on previous cell cycle work in the Penn lab and the data provided by Amanda Wasylishen, it was known that in the MCF10A cells, through serum withdrawal, within 24h cell cycle arrest could be achieved and endogenous Myc protein levels could be minimized. When tunicamycin was added without induction of ectopic Myc, Myc protein levels continued to decrease and modest levels of about 5% apoptosis were yielded, indicating that basal levels were well below the set 10% upper-bound. However, when Myc was induced, within 12h there was a 4- to 5-fold increase in Myc protein and apoptosis levels increased 10-fold to about 53%, far in excess of the 25% minimum criterium. The inducible Myc expression system combined with the apoptotic treatment thus provides an ideal system for studying Myc-potentiated apoptosis. Because the high levels of apoptosis are largely dependent on the induction of Myc, we can be confident in deeming the apoptosis as Myc-potentiated and not basal. Furthermore, it is our goal to perform subsequent ChIP-chip and expression array analysis under both growing and apoptotic conditions so that any differentials may be identified and this system allows for the investigation of how Myc can integrate disparate signals to either induce cell cycle entry and growth or to potentiate apoptosis from the common point of growth arrest. We showed that after inducing growth arrest in the MCF10A cells, mitogen stimulation rapidly increases Myc protein levels and
drives cell cycle entry and growth, regardless of the induction of ectopic Myc. This system, therefore, provides both the apoptotic as well as the growing conditions for ChIP-chip and expression array analysis in determining the genetic program of Myc-potentiated apoptosis.

Aside from apoptosis, the MCF10A cell line with the inducible Myc expression system provides a system for studying how Myc alone can drive cell cycle entry. It has long been established that Myc is sufficient to drive G1/S transition. Here we showed that induction of ectopic Myc in growth arrested cells could drive cell cycle entry under serum withdrawal conditions and that the cell cycle profile was similar to that of the mitogen stimulation conditions, except for that the S-phase population was smaller than expected due to confluence and there was an accumulation of pre-G1 cells as Myc was still potentiating apoptosis under these anti-proliferative conditions. However, at 24h prior to confluence, Myc induction under serum deprived conditions led to cell cycle entry and growth states similar to that of mitogen stimulation. This system thus provides a tool for studying Myc induction of cell cycle entry in the absence of proliferative stimuli.

The SHEP cell system

Optimization of Myc ChIP

When we began our optimization of Myc, ChIP of Myc had not been reported in SHEP cells, although since, ChIP of Myc and N-Myc in the SHEP cell line has been reported (130). Much of the protocol for Myc ChIP in the SHEP cells carried over from that in the MCF10A cell line although optimization of sonication (data not shown), cell number and antibody concentration had to be performed. Therefore, as with the MCF10A cell line, parallel Myc ChIP reactions were carried out in SHEP cells using a range of cell numbers and antibody concentrations. Conditions were assessed for efficiency based on maximizing
Myc ChIP signal and minimizing IgG non-specific binding noise. The condition of 2µg of antibody and 10x10^6 cells per reaction was selected. This condition yielded high Myc ChIP signal and low IgG noise and it required less antibody than the 4µg condition which displayed similar results. This condition was validated at a known Myc binding site where significant enrichment for Myc ChIP was seen when compared to the negative controls.

**Optimization and characterisation of apoptotic conditions**

Previous studies in which a condition was designed to induce apoptosis in SHEP cells in a Myc-dependent manner were in an N-Myc context. In the Penn lab, it was shown by Sam Kim that N-Myc strongly potentiates apoptosis induced by tunicamycin and thapsigargin. For this work, tunicamycin and thapsigargin were optimized and characterized as apoptotic agonists of Myc-potentiated (c-Myc) apoptosis in the SHEP cells. SHEP cells were treated with a range of doses of both tunicamycin and thapsigargin. The treatments were further optimized to 2.5µg/mL tunicamycin and 0.25µM of thapsigargin. After 48h of both these treatments, cell death levels as indicated by pre-G1 populations were 3-fold higher in SHEP-Myc cells than SHEP-GFP cells, both meeting the criteria of greater than 25% apoptotic levels in cells expressing ectopic Myc. The tunicamycin treatment met the criteria of less than 10% apoptotic levels in control cells but the thapsigargin treatment narrowly missed with 11%, nonetheless, the 10% upper-bound was well within the error bars. Because pre-G1 is not specific to apoptosis and can include necrosis, another apoptosis specific assay had to be employed (51). Although TUNEL, which detects DNA breaks characteristic of apoptosis, is often hailed as the "gold standard" of apoptosis assays, attempts at using this assay were unsuccessful as positive controls known to be apoptotic were not sufficiently TUNEL positive and this issue could not be resolved by extending labelling steps. Annexin
V, which detects exposure of phosphotidylserine at the outer leaflet of the cell membrane, characteristic of apoptosis, was employed in a pilot experiment. Although the experiments have not yet been repeated, pilot experiments showed that apoptosis was being induced by these treatments in the SHEP cells. The apoptotic differential was consistent with the pre-G1 data for the tunicamycin treatment but was lessened for the thapsigargin treatment.

Additional support for these treatments inducing apoptosis in the SHEP cells came from the PARP-cleavage data. In both the thapsigargin and tunicamycin treatment, after 48h, the PARP-cleavage level was significantly higher, at least 3-fold, in the SHEP-Myc cells than the SHEP-GFP.

Ectopic Myc was introduced into the SHEP cells using the same constitutive expression vector used to introduce constitutive Myc into the MCF10A cells. Whereas MCF10A-GFP and MCF10A-Myc cells expressed similar levels of Myc under growing conditions, SHEP-Myc cells expressed at least 7-fold more Myc protein under growing conditions than did SHEP-GFP cells. The downregulation of ectopic Myc upon introduction of ectopic Myc in the MCF10A cells appears to be absent in the SHEP cells. In response to either tunicamycin or thapsigargin, ectopic Myc was downregulated approximately 2-fold over 48h of treatment. Although constitutive ectopic Myc was downregulated in the MCF10A cells in response to doxorubicin, the downregulation of ectopic Myc seen in the SHEP cells was not as rapid and/or as severe. Furthermore, at all points assayed during the tunicamycin and thapsigargin treatments, Myc protein levels were significantly higher in the SHEP-Myc cells when compared to SHEP-GFP cells. In the SHEP cells systems, ectopic Myc expression sufficiently mimicked Myc deregulation and the optimized conditions efficiently induced Myc-potentiated apoptosis while minimizing basal levels. Therefore, the
SHEP cell line expressing constitutive ectopic Myc with the optimized tunicamycin and thapsigargin treatments has been established as an independent system for ChIP-chip and expression array analysis of the genetic program of Myc-potentiated apoptosis.

Future work

The primary objective of this project was to develop independent systems for determining the genetic program of Myc-potentiated apoptosis by high-throughput ChIP-chip and expression array analyses. Therefore, ultimately our main goal in the near future is to in fact perform these analyses using the systems developed here, that is, harvesting cells with deregulated Myc under growing and apoptotic conditions and determining the differential in Myc binding and transcriptional regulatory function by ChIP-chip and expression array analyses, respectively, and thus identifying the distinct Myc apoptotic genetic program. Although the work presented here shows the development and optimization of many of the parameters required for the subsequent microarray studies including identification of appropriate cell lines, optimization and characterization of Myc-potentiated apoptosis inducing treatments and optimization of the Myc ChIP conditions; one critical parameter that needs to be addressed is the time point proceeding the apoptotic treatments at which to harvest the cells for both ChIP-chip and expression array analyses.

Unfortunately, to date there are no established positive controls for detecting an apoptotic shift in Myc binding or direct transcriptional regulatory activity that we could employ for determining an optimal point for harvest. One target which does have potential as a control is NOXA. In HeLa cells, there is a 2- to 3-fold increase in Myc binding at the promoter following 8h of treatment with bortezomib, a 26S proteasome inhibitor and subsequent ER stressor (93). Furthermore, luciferase reporter assays using the specific Myc
binding site in the NOXA promoter showed that luciferase activity increased by ~20% following 6h of bortezomib treatment and ~40% after 8h. Unfortunately, the report did not indicate the levels of apoptosis achieved in the cells under this dose of bortezomib. As NOXA has often been implicated in several forms of ER stress-induced apoptosis, it is possible that ER stressors tunicamycin, thapsigargin and serum withdrawal may also modulate Myc-dependent activation of the NOXA promoter (63). The approach that we will take to determine the most appropriate time points for assaying binding and expression will be to assay Myc binding and expression of several known Myc binding targets through ChIP-qPCR and qRT-PCR, respectively, at different time points following the apoptotic treatment and evaluate any changes. We will look at Myc activated genes, such as CAD and CyclinD2, Myc repressed genes, such as GADD45 and p21, and putative apoptotic Myc gene targets including PUMA and NOXA. Although the time point at which we will see changes in Myc binding and transcriptional regulator activity following the apoptotic treatment is unknown, it is likely to occur within just a few hours. As mentioned above, in response to bortezomib Myc binding increased by 6h and increases in transcription at the NOXA promoter were detected by 6h and even greater changes at 8h. Although not apoptotic but signal integration nonetheless, in MCF7 cells deprived of hormones, Myc binding increases 2- to 4-fold at the promoters of ERα-responsive genes 3h following estradiol stimulation (108). With respect to transcription, activation of these genes, as indicated by Ac-H3-K9 marks, is maximal at 3h following estradiol treatment and returns to basal levels by 12h and 24h. Therefore, it is predicted that we will detect any changes in Myc binding and transcriptional regulatory function within the first few hours following treatment.
With respect to technical issues, it will be critical that we monitor the cells to ensure that they are sub-confluent at our determined harvest points. This was an issue with respect to the MCF10A cells with inducible ectopic Myc expression after 48h of either mitogen stimulation and/or induction of ectopic Myc under serum deprived conditions. It is expected that the determined time points for harvest for both ChIP-chip and expression array analysis will be much earlier than 48h and at time points at which the cells will be still very much sub-confluent. However, if cells reaching confluence remains an issue than it will be necessary to compensate and re-evaluate an appropriate number for the seeding of cells.

One challenge we may encounter in subsequent ChIP-chip and expression analyses is population heterogeneity. In all of our apoptosis experiments, within a cell population there were always variable responses to the treatment in that some cells underwent apoptosis faster than others and by the end of the treatments there were always large population of cells, often the majority, that had not yet undergone apoptosis. This was the case for both synchronous and asynchronous cells. There are two possible scenarios for explaining these observations. The first is that only some cells are reacting to the agonists with an apoptotic response and the remainder simply are not. If this were the case, the large population of cells not responding with apoptosis may create background noise to the apoptotic signal provided by the cells that actually undergo apoptosis. The second scenario would be that all the cells show some perturbation to the agonists but for many it may be insufficient to induce apoptosis. This scenario would be more ideal in that all cells would be providing some degree of an apoptotic signal. For some time this variability would have been attributed to stochastic events, however, more recent findings suggest that it is not random and is even, to some degree, predictable. It was shown that the variable response of MCF10A cells to
TRAIL-induced apoptosis was a function of the difference in the state or levels of proteins involved in receptor mediated apoptosis (139). Essentially, the variability would rest in the apoptotic thresholds. The protein states are transmitted from mother to daughter cells, but these states and the cell's response to the apoptotic stimuli diverge with time. Furthermore, it was shown that when protein synthesis was inhibited using cycloheximide, all cells would eventually undergo apoptosis but when uninhibited a fraction would always survive, presumably due to the induction of pro-survival pathways. What this suggests is that in all of the cells, apoptotic pathways are engaged although overt apoptosis may not follow. Therefore, it is possible that in fact all the cells are responding to the apoptotic stimuli but for many it may be insufficient to induce apoptosis. Rather than a dichotomy of apoptotic and non-apoptotic cells, this would provide a spectrum of cells in different apoptotic states as well as perhaps the Myc apoptotic genetic program at different stages of progression.

Another challenge that we may encounter is that although there is likely no apoptotic pathway that is completely independent of Myc, we may have trouble distinguishing pathways directly activated by Myc from those activated directly by other pro-apoptotic molecules such as p53. ChIP-chip would largely prevent this by identifying only direct Myc targets and then correlating Myc binding with the subsequent transcriptional effects provided by expression array analyses, however, some problems could still arise. For example, \( p21 \), a cell cycle inhibitor, is a direct target of both Myc and p53. Myc represses \( p21 \) to promote cell cycle and p53 activates \( p21 \) to promote cell cycle arrest. If another gene, similar to \( p21 \) only by the fact that it is directly repressed by Myc and directly activated by p53, was shown to be increasingly bound by Myc as indicated by the Myc ChIP-chip following the apoptotic treatment and expression array analysis showed that it was upregulated, this alone would
suggest that Myc was directly activating the gene in response to apoptotic stimuli. Although subsequent validation and functional work could eventually discount this, because of the vast amount of data provided by the high-throughput approaches, misinterpretation could occur.

Conclusion

We have developed two independent cell systems using the MCF10A human breast epithelial and SHEP human neuroblastoma cell lines. These systems were established for the purpose of determining the genetic program of Myc-potentiated apoptosis using ChIP-chip and expression array analysis. As such, we optimized and characterized apoptotic conditions that maximize levels of Myc-potentiated apoptosis while minimizing basal apoptotic levels for each cell system. Furthermore, for each cell line, we optimized conditions of Myc ChIP that will be employed for subsequent ChIP-chip analysis.

The two independent systems developed here for studying Myc-potentiated apoptosis are novel in that they offer a human context for studying the role of Myc in apoptosis whereas the non-human Rat1 system has predominantly been used in the past. Although the findings made in the Rat1 system have been instrumental in our understanding of Myc and apoptosis, human systems will likely provide more relevance to cancer in humans.

To date, the mechanism by with Myc potentiates apoptosis remains largely unclear although previous findings suggest that Myc’s role in apoptosis is a function of its regulation of an apoptotic genetic program. It is our hope that the identification of genes bound and/or regulated by Myc under apoptotic conditions using the systems developed here for ChIP-chip and expression array analyses will provide not only insight into how it is that Myc can sensitize cells to such a wide-spectrum of anti-proliferative stimuli but also make a contribution to the development of novel cancer therapeutics.
Materials and Methods

Cell culture

Reagents

Media, supplemented with penicillin and streptomycin, and phosphate-buffered saline (PBS) were supplied by the UHN Tissue Culture Media Facility. Fetal bovine serum (FBS) was from HyClone (#CA16777-531). Horse serum (HS) was purchased from Gibco (#16050-122). Trypsin-EDTA was purchased from Gibco (#15400-054) at 10x concentration and diluted to 1x concentration in 1XPBS.

MCF10A cells

MCF10A cells, a kind gift from Senthil Muthuswamy (Ontario Cancer Institute, Toronto, Canada) were grown in 1:1 DMEM H21/HAM F12 growth media supplemented with 5% [v/v] horse serum, 20ng/mL EGF (R&D Systems #236-EG), 0.5µg/mL hydrocortisone (Sigma, cat. no. H-0888), 0.1µg/mL cholera toxin (Sigma #C-8052), 10µg/mL insulin (Sigma #I9278).

SHEP cells

SHEP cells, a kind gift from Manfred Schwab (Deutsches Krebsforschungszentrum, Heidelberg, Germany) were grown in RPMI 1640 growth media supplemented with 10% [v/v] fetal bovine serum.

Generation of retroviral particles

Ecotropic retroviral particles were generated using the Phoenix Ecotropic packaging cell system, generated by Gary Nolan and obtained from ATCC, grown in DMEM H21 supplemented with 10% FBS. Phoenix Eco cells were seeded at approximately 7x10^5 cells per 10cm tissue culture plate, allowed to adhere overnight, then transfected by calcium phosphate co-precipitation. 30µg of PEG-prepped plasmid DNA (pMN-GFP and pMN-GFP-)
Myc) was mixed with 375µL of 0.5M calcium chloride (Sigma #C7902), and sterile water to bring the volume to 750µL. 750µL of 2X HEPES-buffered saline (2X-HBS, 140mM NaCl, 1.5mM Na2HPO4, 50mM HEPES, pH 7.0) was then added drop wise with bubbling from a pipettor. DNA precipitates were incubated on ice for 10 minutes, and then added to Phoenix Eco cells with 8.5mL of H21+10% FBS and 25µM chloroquine (Sigma #C6628, dissolved in 1XPBS). Transfection reagents were incubated on cells overnight and media was exchanged 16h later for 5mL fresh H21+10% FBS media. 24h and 48h after transfection, virus was harvested and filtered through a 0.45µM filter unit to remove any Phoenix cells, and was frozen at -70°C in 2mL aliquots for future use.

**Retroviral infection of tissue culture cells**

Target cells (MCF10A and SHEP) were seeded at approximately 5x10^5 cells per 10cm dish, allowed to adhere overnight, and then infected with virus. A 2mL aliquot of frozen virus stock was mixed with 1mL of fresh media and 30µL of 0.8mg/mL polybrene. Cells were incubated with virus overnight and media was replaced. Cells were sorted by fluorescence-activated cell sorting a minimum of 48hrs post infection.

**Generation of lentiviral particles**

Ecotropic lentiviral particles were generated using the 293TV packaging cell system grown in DMEM H21 supplemented with 10% FBS. 293TV cells were seeded at approximately 7x10^5 cells per 10cm tissue culture plate, allowed to adhere overnight, then transfected by calcium phosphate co-precipitation. 10µg of pspax2 and 4µg of pMDG1.vsvg PEG-prepped plasmid DNA added to each 5µg of pFU-GEV16, pF-5xUAS-Myc or pF-5xUAS empty vector of PEG-prepped plasmid DNA and were mixed with 375µL of 0.5M calcium chloride (Sigma #C7902), and sterile water to bring the volume to 750µL. 750µL of 2X HEPES-
buffered saline (2X-HBS, 140mM NaCl, 1.5mM Na2HPO4, 50mM HEPES, pH 7.0) was then added drop wise with bubbling from a pipettor. DNA precipitates were incubated on ice for 10 minutes, and then added to separate 293TV cells with 8.5mL of H21+10% FBS and 25µM chloroquine (Sigma #C6628, dissolved in 1XPBS). Transfection reagents were incubated on cells overnight and media was exchanged 16h later for 5mL fresh H21+10% FBS media. 24h and 48h after transfection, virus was harvested and filtered through a 0.45µM filter unit to remove any 293TV cells, and was frozen at -70°C in 2mL aliquots for future use.

**Lentiviral infection of tissue culture cells**

Target cells (MCF10A) were seeded at approximately 5x10^5 cells per 10cm dish, allowed to adhere overnight, and then infected with virus. A 1mL aliquot of frozen pFU-GEV16 and 1mL aliquot of frozen pF-5xUAS (Myc or empty vector) virus stock was mixed with 1mL of fresh media and 30µL of 0.8mg/mL polybrene. Cells were incubated with virus overnight and media was replaced. Cells were co-selected in 100µg/mL hydromycin and 1µg/mL puromycin. Selected transfected cells were pooled.

**Apoptotic treatments and Assays**

**Drugs**

Doxorubicin (Sigma #D1515), latrunculin-A (Biomol #T119), thapsigargin (Sigma #T4557), tunicamycin (Sigma #T7765) and bortezomib (a kind gift from the Trudel lab) were dissolved in DMSO and stored at -70°C and aliquot were made. Individual aliquots were thawed for one time use immediately prior to treatments.
**MCF10A apoptotic treatments**

MCF10A cells were seeded at a density of $1 \times 10^6$ cells on 10cm plates and allowed to adhere and grow overnight. Media was removed, plates were washed twice with 1XPBS and treatment media was added.

**SHEP apoptotic treatments**

For fixed PI assays, SHEP cells were seeded at a density of $0.25 \times 10^6$ cells on 10cm plates and allowed to adhere and grow overnight. Media was removed, plates were washed twice with 1XPBS and media with 5ng/mL doxycyclin (Sigma #D9891) was added to turn off N-Myc (Tet-off system). After 48h, media was removed, plates were washed twice with 1XPBS and treatment media was added with 5ng/mL doxycyclin.

For Annexin V assays, the protocol was identical except that SHEP cells were seeded at a density of $6 \times 10^3$ cells on 24-well plates.

**Fixed PI and TUNEL**

After apoptotic treatment, cells were harvested from 10cm dishes. All media and 1XPBS washes were collected in Falcon tubes so that both adherent and floating cells were counted. Plates were washed once with 2mL 1XPBS and 1mL 1X or 2x trypsin was added for SHEP or MCF10A cells, respectively. After 10min or 20min trypsinisation for SHEP or MCF10A cells, respectively, residual trypsin was used to wash cells down the plate and collected in to the Falcon tubes. Plates were rinsed twice with 1mL 1XPBS. All cell pelleting was carried out by centrifugation at 4ºC and 2000rpm. Cells were pelleted, washed once with 1mL 1XPBS and pelleted again. Cells were then fixed in 2mL of 70% ethanol for one hour on ice, or placed at -20ºC for future analysis.
For fixed PI, after fixing, cells were pelleted, and washed with 5mL 1XPBS. Cells were then resuspended in 50µL 1XPBS with 5µL of 2µg/mL DNAse-free RNAse (Roche #11119915001). RNA was degraded for 1 hour at 37°C. Cells were then stained using 0.5mL PI buffer: 1x 1XPBS, 0.1% NP-40 (Sigma #I8896 “Igepal”), and 1:100 dilution of 5µg/µL stock propidium iodide (Sigma #P4170). Cells and stain were incubated on ice for at least 15 minutes before analysing on the FACScalibur cytometer (Becton Dickinson) to determine the amount of apoptotic cells that were present in a pre-G1 peak and well as for cell cycle analysis.

For TUNEL, the APO-BrdU apoptosis detection kit was used (Phoenix #AU-1001). The protocol followed was that of the manufacturer except for two modifications. First, the DNA labelling step was increased from 1h to 3h. Second, rather than centrifuging cells from 5min at 300xg, cells were centrifuged for 2min at 1000xg, rotated 180° and centrifuged for an additional 2min at 1000xg.

*Annexin V*

After apoptotic treatment, cells were harvested from 24-well plates. All media and 1XPBS washes were collected in Falcon tubes so that both adherent and floating cells were counted. Plates were washed once with 200µL 1XPBS and 200µL 1X trypsin was added. After 10min trypsinisation, residual trypsin was used to wash cells down the plate and collected into the Falcon tubes. Plates were rinsed twice with 200µL 1XPBS. All cell pelleting was carried out by centrifugation at 4°C and 2000rpm. Cells were pelleted, washed once with 200µL 1XPBS and pelleted again. Annexin V staining was carried out using Annexin V-FITC reagent
ChIP-qPCR

Chromatin-immunoprecipitation

Exponentially growing cells (MCF10A and SHEP) were cross-linked with 1% formaldehyde for 15 min at 37°C. The cross-linking reaction was quenched by addition of glycine to a final concentration of 0.125M for 15 min, followed by two washes with phosphate-buffered saline (1XPBS). Cells were resuspended in cell lysis buffer (5mM PIPES pH 8, 85mM KCl, 0.5% [v/v] NP40, 1 mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin) for 10 min on ice and then pelleted (5000rpm., 5min, 4°C). The pellet was resuspended in 1 ml of nuclei lysis buffer (50mM Tris–HCl pH 8.1, 10mM EDTA, 1% SDS, 1mM PMSF, 10µg/mL aprotinin, 10µg/mL leupeptin) for 10 min on ice and then sonicated, MCF10A cells with 11 pulses and SHEP cells with 9 pulses (setting high, 30s per pulse, 30s on ice between pulses) from a BioRuptor Sonicator (Diagenode, BioRuptor 200, UCD-200 TM-EX) to generate fragments between 100 and 1,000bp. Lysates were centrifuged for 10 min at 1,000 rpm at 4°C.

Supernatants were diluted into an equal volume with IP dilution buffer (0.01% SDS, 1.1%
Triton-X100, 1.2mM EDTA, 16.7mM Tris HCl pH8.1, 0.2% Sarkosyl, 1mM PMSF, 10µg/mL aprotinin, 10µg/mL leupeptin) and precleared for 30min at 4°C with protein G-PLUS agarose beads (Santa Cruz Biotechnology #sc-2002). Prior to use, G-PLUS agarose beads were blocked with salmon sperm DNA at a final concentration of 50µg/mL and rotated overnight at 4°C. Diluted and cleared extracts corresponding to 10 x 10⁶ MCF10A or SHEP cells were incubated and rotated at 4°C overnight with each 1.5µg for MCF10A and 2µg for SHEP cells of normal rabbit IgG (Santa Cruz Biotechnology #sc-2027), or home-made purified N262. 50µL of salmon sperm DNA pre-blocked Protein G-PLUS agarose beads were added to each sample, incubated on a rotating platform at 4°C for 3h. Each pellet was washed once with 1.4 mL of sonication buffer and then twice with 1.4mL of high salt buffer (0.1% [v/v] SDS, 1% [v/v] Triton X-100, 1 mM EDTA, 50mM HEPES, 500mM NaCl, 0.1% [w/v] sodium deoxycholate) and then once with 1.4mL LiCl Buffer (250mM LiCl, 1% [v/v] NP 40, 1% [w/v] sodium deoxycholate, 1mM EDTA, 1mM Tris pH8) and finally twice with 1.4 mL TE pH 8 (10 mM Tris pH8, 1 mM EDTA). For each wash, the pellets were mixed for 5min at room temperature then pelleted (3000rpm, 30s, room temperature). After the last wash, the pellets were eluted in 300µL of Elution buffer (1% [w/v] SDS, 10mM Tris pH8, 5mM EDTA), incubated at 65°C for 15 min, and then pelleted (3000rpm, 3min, room temperature). Cross-links were reversed in the presence of 200mM NaCl at 65°C over-night and samples were treated with RNase A (Sigma #R5500). After ethanol precipitation, the samples were resuspended in 100µL of TE (10mM Tris, pH7.5, 1mM EDTA), 25µL of 5x proteinase K buffer (1.25% SDS, 50mM Tris, pH7.5, 25mM EDTA), and 1.5µL of proteinase K (Roche #1413783) and incubated at 42°C for 2h. Samples were then column
purified sample using Qiagen Qiaquick PCR purification Kit (#28104), and eluted in 30uL of Buffer EB.

Real-time quantitative PCR (Q-PCR)

Real-time quantitative PCR amplification was conducted using the SYBR Green assay in the ABI PRISM 7900-HT (Applied Biosystems). Each 12µL quantitative PCR reaction was composed of the following: 2µL of the DNA isolated in the ChIP, 1x PCR Buffer, 2.5mM MgCl₂, 0.17mM dNTP mix, sterile water, 0.25x SYBR Green (Sigma #S9430), 0.2µL Rox reference dye (Invitrogen #12223-012) and 0.05µL Platinum Taq DNA polymerase (Invitrogen #10966-034) and was performed in triplicate in a 384-well plate. The reactions began at 50°C for 2min and then were activated at 95°C for 10min followed by 40 cycles of 95°C for 15sec and 60°C for 1min. Human male genomic DNA (Novagen #70572) was used as standard real-time quantitative PCR (Q-PCR). The Q-PCR data was analyzed by calculating the Myc/IgG ratios for each target gene, and the negative control(s). A symmetrical distribution was then obtained by log₂-transformation of each individual ratio. For pooled negative controls, the average of the mean log2 ratio of each individual negative control target was calculated. Statistical testing involved comparison of the ratios for each target gene to the ratios of the negative control(s) using a two-tailed paired t-test.

**Primers**

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward (5’ - 3’)</th>
<th>Reverse (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAD</td>
<td>ACGTGGACCGACTCCGG</td>
<td>CCATGGGAAGGGAACTCAGA</td>
</tr>
<tr>
<td>Ch21</td>
<td>AAAACGCAGCACCACCCGGATGAGT</td>
<td>GCAGAAGTGGCGGTTCATCAG</td>
</tr>
<tr>
<td>Ch6</td>
<td>CATTCATCATAGTTATGCCCCAA</td>
<td>CAAACACAGAAATTTTCTGGTTGGTGA</td>
</tr>
<tr>
<td>CyclinD2</td>
<td>CTTGGACTCAAAGGATGCCTAGGA</td>
<td>GAGCCGACTGCGTTGAAAGT</td>
</tr>
<tr>
<td>GADD45</td>
<td>TGGGCTATGCAAATCAGCCT</td>
<td>GCGTGCTCCTCCCTTTTT</td>
</tr>
<tr>
<td>HNT1C</td>
<td>TGCTGTACTGCGTTGGTTG</td>
<td>CCATCTCTATGCTGGGAATG</td>
</tr>
<tr>
<td>NOL6</td>
<td>ACTCGGGCCCAAGTCCAG</td>
<td>GCGAGTAATCGACTGATAATCCAA</td>
</tr>
</tbody>
</table>
Immunoblotting

Whole cell extracts

After apoptotic treatment, cells were harvested from 10cm dishes. All media and 1XPBS washes were collected in 15mL Falcon tubes so that dead and floating cells were counted. Plates were washed once with 2mL 1xPBS and 1mL 1X or 2x trypsin was added for SHEP or MCF10A cells, respectively. After 10min or 20min trypsinisation for SHEP or MCF10A cells, respectively, residual trypsin was used to wash cells down the plate and collected in to the Falcon tubes. Plates were rinsed twice with 1mL 1xPBS. All cell pelleting was carried out by centrifugation at 4°C and 2000rpm for 2min. Cells were pelleted, washed once with 1mL 1xPBS and pelleted again. Cells pellets were resuspended in 300µl 1x SDS loading buffer (1%SDS [w/v] SDS, 11% [v/v] glycerol 0.1M Tris pH6.8) and boiled for 5min. Lysates were syringed to fragment chromatin with a 22½ gauge needle until viscosity was minimal. Lysates were centrifuged at room temperature and 14,000rpm for 2min and transferred to new microfuge tubes. 10% [v/v] β-mercaptoethanol was added to lysates, boiled for 5min and placed at -20°C.

SDS-PAGE

SDS-PAGE gels were 8% polyacrylamide. Gels were electrophoresed and transferred to nitrocellulose membranes (Whatman #NBA083C). Membranes were blocked for 1 hour in 5% milk in 1xPBS with 0.01% TWEEN-20 (Sigma #P5927) at room temperature. Primary antibodies were applied overnight at 4°C in 5% milk in 1xPBS with 0.01% TWEEN-20. Membranes were washed in 1xPBS + 0.01% TWEEN-20 for 5min three times. Secondary antibodies were applied for one hour at room temperature in 5% milk in 1xPBS with 0.01%
TWEEN-20. Membranes were washed in 1xPBS + 0.01% TWEEN-20 three times for 5min and once with 1xPBS for 5min.

**Antibodies**

Primary antibodies were used as follows: 1/1000 anti-Myc (9E10 – monoclonal, mouse, hybridoma obtained from Gerard Evan and maintained by Fereshteh Khosravi), 1/1000 anti-PARP (Cell signalling #9542), 1/3000 anti-actin (Sigma #A2066). Secondary antibodies were used as follows: 1/20,000 IRDye 680 conjugated goat (polyclonal) anti-rabbit (LICOR #926-32221) and 1/20,000 IRDye 800 CW conjugated goat (polyclonal) anti-mouse (LICOR #926-32210).

**Scanning**

Imaging was carried out using an Odyssey IR Imaging system (LICOR) and densitometry was carried out using ImageJ software (bundled version from MacBiophotonics, originally developed by NIH).
References


80. Zindy, F., Eischen, C. M., Randle, D. H., Kamijo, T., Cleveland, J. L., Sherr, C. J.,
and Roussel, M. F. (1998) Myc signaling via the ARF tumor suppressor regulates
p53-dependent apoptosis and immortalization. *Genes Dev* 12, 2424-2433

apoptosis associated with c-Myc-mediated block in myeloid cell differentiation.
*Oncogene* 19, 2967-2977

82. Eischen, C. M., Packham, G., Nip, J., Fee, B. E., Hiebert, S. W., Zambetti, G. P., and
Cleveland, J. L. (2001) Bcl-2 is an apoptotic target suppressed by both c-Myc and
E2F-1. *Oncogene* 20, 6983-6993

J. L. (2003) c-Myc augments gamma irradiation-induced apoptosis by suppressing
Bcl-XL. *Mol Cell Biol* 23, 7256-7270

Clusterin inhibits apoptosis by interacting with activated Bax. *Nat Cell Biol* 7, 909-
915

85. Egle, A., Harris, A. W., Bouillet, P., and Cory, S. (2004) Bim is a suppressor of Myc-

86. Hemann, M. T., Bric, A., Teruya-Feldstein, J., Herbst, A., Nilsson, J. A., Cordon-
p53 tumour surveillance network by tumour-derived MYC mutants. *Nature* 436, 807-
811

Requirement for the CD95 receptor-ligand pathway in c-Myc-induced apoptosis.
*Science* 278, 1305-1309

activity of cytosolic death receptor signaling proteins by engaging the mitochondrial
apoptotic pathway. *J Biol Chem* 277, 43224-43232

(2004) Synthetic lethal targeting of MYC by activation of the DR5 death receptor
pathway. *Cancer Cell* 5, 501-512

90. Cui, H., Li, T., and Ding, H. F. (2005) Linking of N-Myc to death receptor machinery
in neuroblastoma cells. *J Biol Chem* 280, 9474-9481

91. Ricci, M. S., Jin, Z., Dew, M., Yu, D., Thomas-Tikhonenko, A., Dicker, D. T., and
El-Deiry, W. S. (2004) Direct repression of FLIP expression by c-myc is a major

92. Nawrocki, S. T., Carew, J. S., Maclean, K. H., Courage, J. F., Huang, P., Houghton,
aggresome formation, the induction of Noxa, and apoptosis in response to the
combination of bortezomib and SAHA. *Blood* 112, 2917-2926

Y., Verhaegen, M., Varambally, S., Chinnaiyan, A. M., Jakubowiak, A. J., and
Soengas, M. S. (2007) Tumor cell-selective regulation of NOXA by c-MYC in

94. Han, J., Flemington, C., Houghton, A. B., Gu, Z., Zambetti, G. P., Lutz, R. J., Zhu,
L., and Chittenden, T. (2001) Expression of bbc3, a pro-apoptotic BH3-only gene, is
regulated by diverse cell death and survival signals. *Proc Natl Acad Sci U S A* 98, 11318-11323


Supplemental Figure 1: MCF10A cells arrest after 24h of serum withdrawal and enter cell cycle similar to normal growth within 24h to 32h upon mitogen stimulation

MCF10A-GFP and MCF10A-Myc cells were serum deprived for 24h, 48h and 72h (SW) (0.05% horse serum, 10µg/mL insulin and no additional supplements). After 48h and 72h of serum withdrawal, cells were mitogen stimulated and harvested every 8h for up to 40h. Cell cycle analysis was performed using fixed PI and proportions of A) G1 and B) S phase populations were determined.
Supplemental Figure 2: Myc induction is sufficient for cell cycle entry from under serum withdrawal conditions similar to mitogen stimulation

MCF10A cells with inducible Myc expression system were serum deprived for 24h (0.05% horse serum, 10µg/mL insulin and no additional supplements). After 24h of serum withdrawal, ectopic Myc was induced through the addition of 100nM 4-hydroxytamoxifen (4-OHT) and ethanol served as the vehicle control (EtOH). As well, cells were either mitogen stimulated (FS) or serum withdrawal (SW) was maintained and cell were treated with 2µg/mL tunicamycin or the DMSO vehicle control. Cells were harvested after 24h, 48h and 72h of the subsequent treatments. Cell cycle analysis was performed using fixed PI.
Supplemental Figure 3: Myc can be induced under apoptotic conditions to potentiate apoptosis as indicated by PARP-cleavage in MCF10A cells

A) and B) Myc expression and PARP-cleavage visualized by immunoblotting. MCF10A cells with inducible Myc expression system under normal growing conditions with full media (5% horse serum, 10µg/mL insulin and additional supplements) were serum deprived for 24h
(SW) (0.05% horse serum, 10µg/mL insulin and no additional supplements). After 24h of serum withdrawal, ectopic Myc was induced through the addition of 100nM 4-hydroxytamoxifen (4-OHT) and ethanol served as the vehicle control (EtOH). As well, cells were either mitogen stimulated (FS) or serum withdrawal (SW) was maintained and cells were treated with 2µg/mL tunicamycin. Lysates were harvested under growing conditions (growing), after the 24h of serum withdrawal (24h SW) and after 12h and 24h of the subsequent treatments. Three independent biological replicates were conducted.
Supplemental Figure 4: Ectopic Myc potentiates apoptosis induced by tunicamycin as indicated by PARP-cleavage in SHEP cells and Myc expression is downregulated over time

A) and B) Myc expression and PARP-cleavage visualized by immunoblotting. SHEP-GFP (G) and SHEP-Myc (M) cells were treated with 2.5µg/mL tunicamycin. Lysates were harvested at 0h, 12h, 24h and 48h. Three independent biological replicates were conducted.
Supplemental Figure 5: Ectopic Myc potentiates apoptosis induced by thapsigargin as indicated by PARP-cleavage in SHEP cells and is downregulated

A) and B) Myc expression and PARP-cleavage visualized by immunoblotting. SHEP-GFP (G) and SHEP-Myc (M) cells were treated with 0.25µM thapsigargin. Lysates were harvested at 0h, 12h, 24h and 48h. Three independent biological replicates were conducted.