MOLECULAR ANALYSIS OF COMBINED MTOR/HDAC INHIBITION IN MULTIPLE MYELOMA

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ABSTRACT

The molecular pathogenesis of many cancer types, including multiple myeloma (MM), involves alterations in the PI3K/AKT/mTOR (mTOR) and cyclin/CDK/Rb (CDK/Rb) pathways, often through activation of the mTOR pathway and epigenetic silencing of tumor suppressors in the CDK/Rb pathway. Previous studies have identified HDAC and mTOR inhibitors as effective in combating these molecular alterations in the CDK and mTOR pathways, respectively. Evaluating molecular synergy of combinations is challenging, yet identification of cooperatively responding, biologically-relevant targets is potentially useful for defining patient subsets for which the combination would be active. Previous work took an integrated, systems-level approach to distill the core synergistic consequence of combining MS-275 (Class I HDACi) with rapamycin (mTORi). Transcriptional co-expression analysis of multiple myeloma (MM) cells treated individually and in combination was used to define the contribution of each drug to an overall response network. Five highly connected transcriptional modules were identified, of which one distinct module of 126 genes was cooperatively affected by both drugs. Of the cooperatively affected genes, 37 were found to be differentially expressed in MM and predictive of survival ($p<0.01$). The pharmacodynamic response of the signature to the drug combination was examined by Western Blot and NanoString in a large number of MM cell lines before and after treatment. Ingenuity transcription factor enrichment

testing identified MYC as one of the regulators of the synergistic transcriptional response. Inducible MYC cell lines have shown diminished protein, but not mRNA, expression in response to the drug combination. Likewise, MYC is required for combination sensitivity and for the synergistic response of the gene signature to combined mTOR/HDAC inhibition. This analysis identified an alternative route to MYC inhibition and a systems-level approach for defining the molecular underpinnings of drug combinations which can be applied to many disease states.

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INTRODUCTION

Cancer

Cancer is a broad, multifaceted term for a variety of diseases ranging from adenocarcinomas to multiple myeloma. Through a progressive, multistep process, normal cells are transformed to a neoplastic state, resulting in tumor growth and metastatic dissemination (Hanahan and Weinberg 2011). Currently, there are six hallmarks attributed to this transformation: chronic proliferation, resistance to tumor suppressors, invasion and metastasis, replicative immortality, angiogenesis, and resistance to cellular death (Figure1).

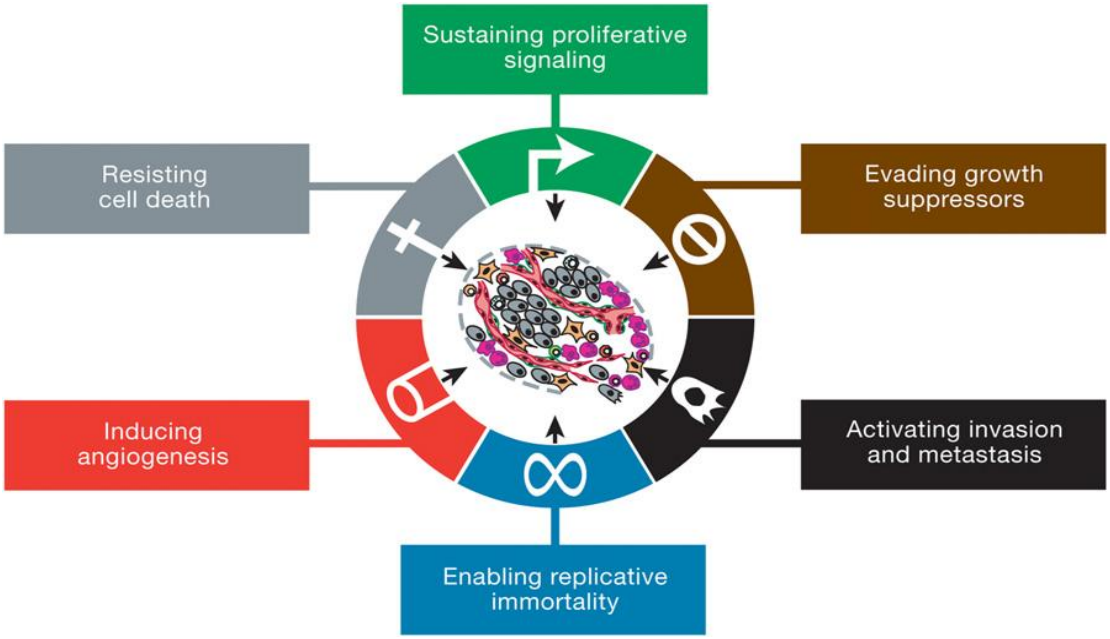


Figure 1. The Six Hallmarks of Cancer (Hanahan and Weinberg 2011). Cells must undergo a progressive, multistep process to transform to a neoplastic state.

Arguably the most fundamental hallmark of neoplastic growth is unchecked cellular proliferation largely due to the dysregulation of oncogenes and tumor suppressor genes. Through this dysregulation, cell cycle checkpoints are bypassed and neoplastic cellular immortality ensues. Recent studies have shown, however, that dysregulation of oncogenes such as RAS and MYC induce cells to enter the nonproliferative but viable state of senescence, suggesting some intrinsic cellular mechanism engineered to protect against unchecked proliferation (Hanahan and Weinberg 2011). Regardless, the chronically proliferative hallmark of cancer cells has lead to the identification of several pathways involved in cancer progression; two key pathways commonly dysregulated in cancer are the PI3K/AKT/mTOR/p53 (mTOR/p53) and the cyclin/CDK/CDKI/Rb (CDK/Rb) pathways, involved in protein translation, growth, and proliferation and cell cycle progression, respectively. Due to their high mutability and frequent dysregulation in cancer, the mTOR/p53 and CDK/Rb pathways offer enticing targets for tumor therapy. While many targeted therapies have led to improved clinical outcomes, targeting with single agents often eventually leads to drug resistant neoplastic cells. Therefore, combination therapies may provide an attractive alternative to further develop long-lasting treatment options as lower doses of the combined single agents are often effective.

Multiple Myeloma

Multiple myeloma is a type of cancer that affects antibody producing plasma cells (B-cells). With 20,000 new cases and 11,000 deaths a year in the United States alone, this neoplasia has a survival time of 5-10 years with current standard of care treatment

options (Palumbo 2011). mTOR and CDK/Rb pathways are often dysregulated in B-cell neoplasias, including Burkitt's lymphoma (Klangby, Okan et al. 1998; Sanchez-Beato, Saez et al. 2001) and multiple myeloma (Dilworth, Liu et al. 2000; Zhan, Huang et al. 2006; Harvey and Lonial 2007; Peterson, Laplante et al. 2009).

Recently, it has been found that virtually all multiple myeloma cases are preceded by monoclonal gammopathy of undetermined significance (MGUS) with a 1% annual average risk of MGUS progression to MM (Landgren 2009). MGUS is classified by the presence of monoclonal immunoglobulin proteins in persons without symptomatic evidence of multiple myeloma (Group 2002). Progression to MM is identified symptomatically in concert with M-protein levels over 30g/l, although this level is not universally accepted. Neoplastic proliferation of monoclonal plasma cells producing M-protein along with bone lesions, anemia, hypercalcaemia, and renal insufficiency denote transformation of MM from MGUS (Group 2002). Similar to MGUS, smoldering multiple myeloma (SMM) is a more aggressive asymptomatic plasma cell disease that has a greater risk of progression to MM (10% per year for the first 5 years) (Kyle, Remstein et al. 2007).

Multiple myeloma in particular arises from premalignant proliferation of monoclonal plasma cells that transform into malignant neoplasms from multistep genetic and microenvironmental changes (Palumbo 2011). Characterized by malignant plasma cells in the bone marrow, monoclonal blood proteins, and organ dysfunction, this form of Non-Hodgkin’s lymphoma accounts for approximately 1% of all neoplastic diseases and 13% of hematologic cancers (Palumbo 2011). Translocations are a common feature of

many hematologic tumors: Burkitt's lymphoma and mouse plasmacytomas frequently carry translocations involving the MYC locus on human chromosome 8 and mouse chromosome 15, respectively and the IgH locus on human chromosome 14 and mouse chromosome 12, respectively (Dalla-Favera and al. 1982; Kanungo and al. 2005; McClure and al. 2005). In contrast to these malignancies, multiple myeloma patients have promiscuous translocations, all involving the immunoglobulin heavy chain locus on chromosome 14 coupled with a variety of other loci on different chromosomes (Kuehl 2002). Primary early chromosomal translocations occurring at the immunoglobulin switch regions on chromosome 14 (q32.33) often result in the dysregulation of two adjacent genes (MMSET, a histone methyltransferase, and FGFR3, a factor in mitogenesis and differentiation) in 30% of all cases (Kuehl 2002; Bergsagel 2005). Late-onset secondary translocations and mutations are further implicated in MM progression and often lead to abnormalities in MYC, NRAS and KRAS activation, FGFR3 and TP53 mutations, and CDKN2A (p16, p19) and CDKN2C (p18) inactivation (Kuehl 2002); in addition to promiscuous translocations, nearly all MM tumors have been identified with dysregulation of cyclins D1, D2, and/or D3 (Hideshima, Bergsagel et al. 2004). These various abnormalities have been used to develop a risk-classifier for MM subtypes.

Cell Cycle and the CDK/Rb Pathway

Under normal conditions, cells must go through the cell cycle in order to proliferate and grow. The cell cycle is comprised of four distinct phases: G_1, S, G_2 (collectively known as interphase) and M-phase. M-phase is the process of mitosis and

involves chromosomal division and cytokinesis (cell division). Interphase denotes the time between mitotic events and consists of gap phases and DNA replication (S-phase). Multiple checkpoints are incorporated throughout the cell cycle in order to prevent aberrant cells from progressing to the next phase (Figure 2). If unable to pass a particular checkpoint due to environmental conditions or cellular mutations, the cell will remain in quiescence (G_0 cell cycle arrest) until ready to divide or in senescence, a permanently halted yet metabolically active state. Conversely, highly damaged or irreparable cells are marked for programmed cell death (apoptosis). These mechanisms are essential for the prevention of unchecked cellular growth, a hallmark of cancerous tumors.

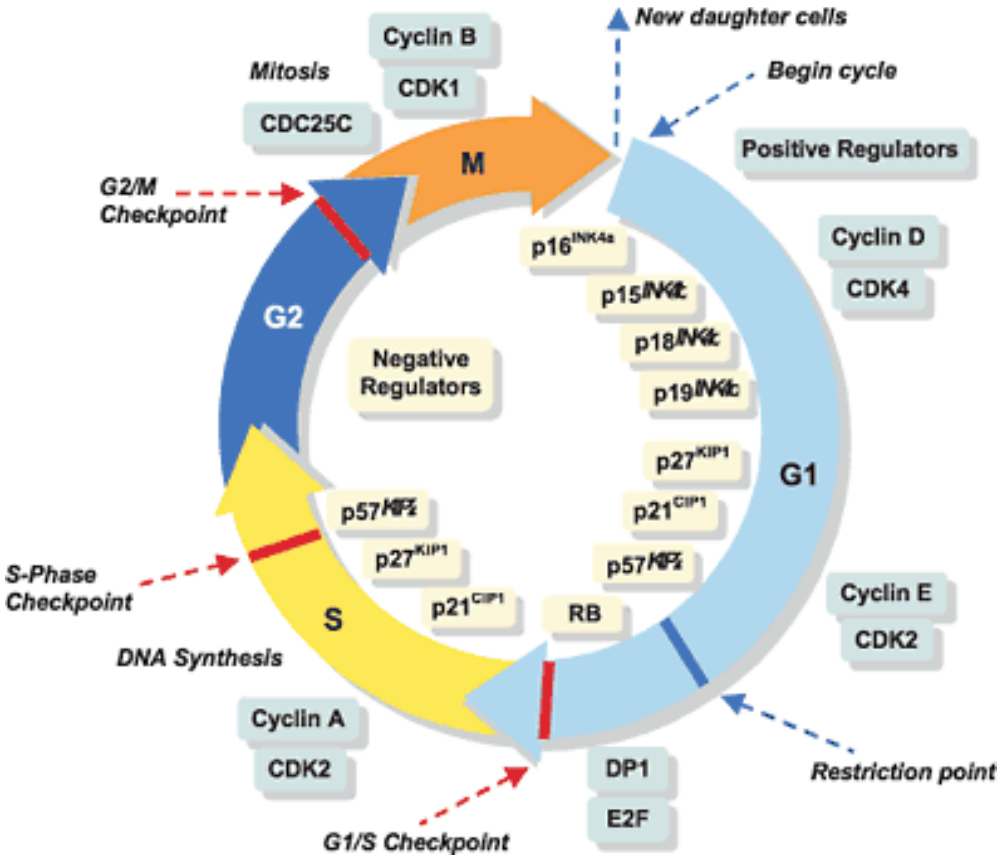


Figure 2. Cell cycle and Associated CDK/Rb Pathway (Kong, Fotouhi et al. 2003). Under normal conditions, the CDK/Rb pathway regulates the cell cycle to keep aberrant cellular growth in check through the use of positive and negative regulators.

The cell cycle is controlled by a set of regulatory molecules – cyclins and cyclin dependent kinases (CDKs) and tumor suppressors (Figure 2). In recent years it has been established that progression from one phase to the next is controlled by CDKs in all eukaryotic cells with associated cyclin proteins required for their activation (Nigg 1995). CDK activity is regulated by two inhibitor families (CDKIs): INK4 proteins (INK4A-D) and the Cip and Kip family (p21, p27, and p57) (Malumbres and Barbacid 2009). When functioning normally, cyclins and associated kinases typically promote progression through the cell cycle; conversely, negative regulators often act as tumor suppressors and inhibit further progression if DNA damage is detected. Due to their critical role in cell cycle regulation, it is not surprising that mutations and dysregulation of this class of proteins have been strongly defined in many human tumors (Malumbres and Barbacid 2009).

The CDK/Rb pathway is highly dysregulated in multiple myeloma. Essential in progression through the cell cycle during proliferation, CDK/Rb pathway subunits, such as RB, p21, p130, and p107, bind to the E2F family of proteins to govern DNA replication events, metabolism, and synthesis (Sherr and McCormick 2002). Mutations of the CDK/Rb pathway, however, are highly frequent in cancer cells and peg this pathway as an attractive target for therapeutic agents. p16 (INK4a, Cdkn2a), a member of the CDK/Rb pathway, is frequently mutated and silenced in human cancers. This protein is responsible for the control of cyclin D/Cdk4 kinase activity, which in turn affects Rb

phosphorylation and E2F accumulation (Nevins 2001). Likewise, pRb appears to exert some translational control over cyclin D1 and p16 expression (Li and al. 1994; Muller and al. 1994). p53 is also a major tumor suppressive transcription factor mutated in more than 50% of human cancers by acting upstream of the CDK/Rb pathway via regulation of p21 (Sherr and McCormick 2002). Previous studies have shown that p53-deficient mice spontaneously develop tumors and succumb to cancer-induced deaths early in life (Jacks 1996; Kamijo, Bodner et al. 1999). Because of the frequent dysregulation seen in the CDK/Rb pathway in human tumors, novel therapeutic approaches to restoring its regulatory function are critical.

PI3K/mTOR/p53 Pathway

The phosphoinositide 3-kinases (PI3Ks) are members of a conserved family of lipid kinases and are highly involved in cellular growth, proliferation and survival signaling due to phosphorylation of AKT and subsequent activation of mTOR signaling (Naegele and Morley 2004; Yang and al. 2007; Topisirovic and Sonenberg 2011). The most well-characterized of these is PIP_3, an essential secondary messenger that recruits AKT for activation and is negatively regulated by dephosphorylation by the tumor suppressor, PTEN (Yuan 2008). While only class IA, a catalytic and regulatory subunit, PI3Ks have been implicated in human cancer thus far, numerous genetic alterations in these enzymes and nearly all of their major elements in the pathway have classified PI3K as one of the most frequently dysregulated pathways in cancer (Yuan 2008). Combined with the fact that numerous tumor types have mutated or amplified PI3K elements, often

leading to changes in mTOR expression, PI3K specific inhibitors have become attractive targets for the treatment of cancer. Previous research has suggested that PI3K has a major role in the control of proliferation and apoptosis of growth factor-independent multiple myeloma cell lines, often with constitutive activation of AKT and more recently, mTOR (Peterson, Laplante et al. 2009; Hoang, Frost et al. 2010); inhibition of the PI3K pathway leads to caspase-dependent apoptosis (Pene 2002).

The kinase mechanisitic target of rapamycin (mTOR) is the catalytic component of two distinct multiprotein complexes called mTORC1 and mTORC2. mTORC1 drives cellular growth by regulating protein synthesis and degradation; mTORC2 directly acts on AKT, downstream of PI3K, and functions in regulation of cell growth, proliferation, and survival (Naegele and Morley 2004; Guertin and Sabatini 2007; Yang et al. 2007; Topisirovic and Sonenberg 2011). Originally identified by the inhibitory drug (rapamycin) after which it is named, a growing body of evidence suggests that the connection between AKT and mTOR is a critical step in PI3K-mediated tumorigenesis. For instance, tumors arising in mouse models harboring either PTEN deletion or constitutively active AKT are sensitive to rapamycin (Guertin and Sabatini 2005). Additionally, somatic mutations in PIK3CA, a catalytic encoding subunit, have been identified in several tumors (Samuels 2004) and preclinical studies have shown that breast cancer lines with this mutation are sensitive to PI3K-mTOR and AKT inhibitors (Serra 2008; She 2008).

Translation of RNA into functional protein products is tightly regulated by a number of factors, most notably by the eukaryotic translation initiation factor (eIF)

family of proteins. As cancer cells are continuously proliferating, it is not surprising that functional alterations of eIFs have been linked in tumorigenesis, particularly eIF4E (Topisirovic and Sonenberg 2011). Translation initiation is regulated by 4E-binding proteins (4E-BPs) that competitively bind to the eIF4E binding site. Control of 4E-BPs are regulated via phosphorylation by the mammalian target of rapamycin (mTOR) protein complex (Figure 3). One subunit, mTORC1, is involved in a number of cellular processes including translation, proliferation, growth, metabolism, and autophagy due to its action on 4E-BPs and ribosomal protein S6 kinases (S6Ks); mTORC2 is involved in cell survival and directly acts on AKT, an upstream effector and kinase for mTORC1 (Topisirovic and Sonenberg 2011). Due to its regulatory action on translation of mRNA involved in cell proliferation and growth, mTOR dysregulation is a common characteristic in human tumors (Mamane, Petroulakis et al. 2006; Petroulakis, Mamane et al. 2006).

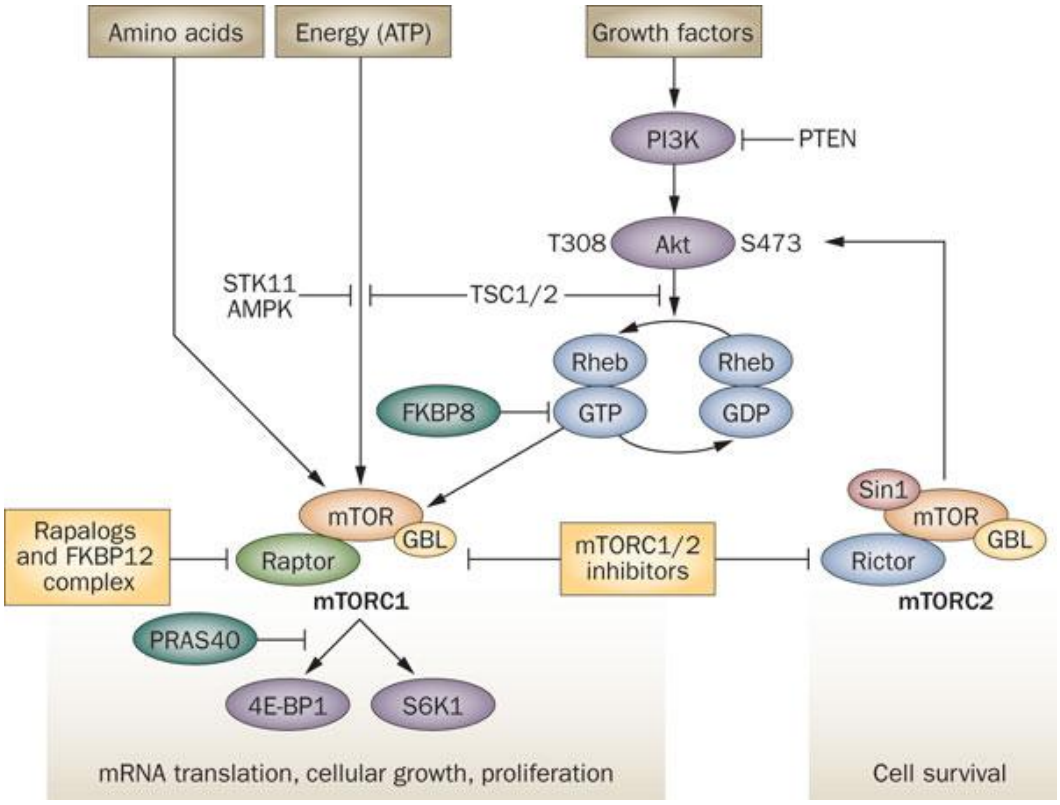


Figure 3. mTOR/p53 Pathway (Dancey 2010). Involved in cellular translation, growth, proliferation, and survival, the mTOR/p53 pathway is often dysregulated in cancers such as multiple myeloma. Sirolimus (rapamycin) and its analogs selectively inhibit mTORC1, leading to diminished cellular growth and proliferation in tumors.

While inhibition of these pathways by single agents have shown some clinical utility, these treatments are often met with eventual resistance by tumors. Therefore, due to the high prevalence, mortality, and chemoresistance displayed by multiple myeloma, research into the development of novel therapeutics is warranted.

Transcription Factor MYC

In somatic cells, progression through the cell cycle requires cellular growth and is transcriptionally controlled by MYC. MYC, a proto-oncogenic transcription factor (TF),

is regarded as a key TF for approximately 10-15% of all human transcribed genes. MYC functions primarily to facilitate entry into the cell cycle by targeting cyclins and CDKs and to promote cellular growth by heterodimerizing with MYC-associated protein X (MAX) (Figure 4) (Bonke, Turunen et al. 2013; Cattoretti 2013).

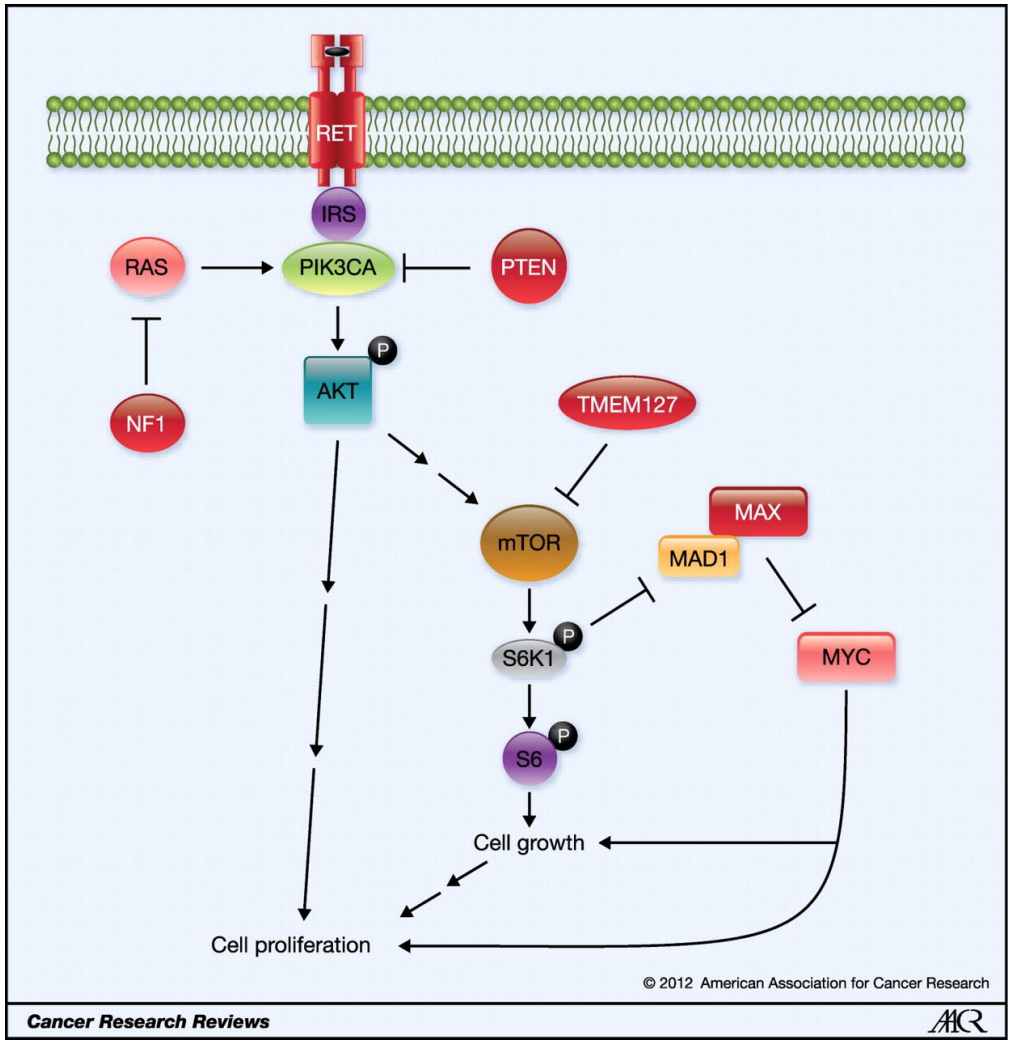


Figure 4. MYC Action on Cell Growth and Proliferation (Cascon and Robledo 2012).

In normal cells, internal and external signals leads to a rapid and fleeting overexpression of MYC mRNA and protein that promote cell cycle progression while additionally inducing apoptotic responses in cells devoid of growth-factor signals,

suggesting a potentially protective role against its tumorigenic capabilities (Cascon and Robledo 2012).

Due to its highly ubiquitous and functional nature, however, it is not surprising that many human tumors exhibit elevated levels of MYC which correlates with tumor aggression and poor clinical outcome. Through multiple mechanisms, including gene amplification, chromosomal translocation, mutations of upstream signaling pathways, and stability-enhancing mutations, MYC can be a potent oncogene that can promote tumorigenesis in a variety of tissues (Lin, Loven et al. 2012). Likewise, many of the hallmarks of tumor progression, including proliferation, cell adhesion, and angiogenesis, are affected by enhanced MYC expression (Cascon and Robledo 2012). It has recently been suggested that MYC accumulates in the promoter region of its target genes in tumor cells which express high levels of MYC, leading to transcriptional amplification of the cell's existing gene expression profile as opposed to binding to and regulating a new set of genes (Lin, Loven et al. 2012; Nie, Hu et al. 2012). This amplifying role may provide an explanation for the widely diverse effects seen by MYC in different tumor cells.

mTOR/HDAC Inhibition

Currently, rapamycin and its analogs have been the primary pharmaceuticals to inhibit mTOR action (Figure 5). While promising results are seen in certain cancers such as mantle cell lymphoma, endometrial cancer, and renal cell carcinoma, rapamycin's results are often unpredictable and highly variable in many tumor types (Guertin and Sabatini 2007). Although rapamycin has been previously shown to act preferentially on

mTORC1, thereby inhibiting S6K1 phosphorylation, there is also a strong negative feedback loop from S6K1 to AKT signaling, leading to subsequent loss of feedback inhibition of AKT and the promotion of cell survival (Guertin and Sabatini 2007). Therefore, while rapamycin shows promising anti-tumor activity initially, neoplastic chemoresistance can ensue.

Histone deacetylases (HDACs) and acetyltransferases (HATs) regulate gene expression through the removal/addition of acetyl groups on core nucleosomal histones and their balance is essential for normal cell differentiation and function (Mitsiades, Mitsiades et al. 2003). Previous research has shown that HDAC inhibition promotes differentiation, cell-cycle arrest, and/or apoptosis of tumor cells *in vitro* (Glick, Swendeman et al. 1999; Butler, Agus et al. 2000) and *in vivo* (Richon, Emiliani et al. 1998; Butler, Agus et al. 2000). Likewise, HDAC inhibition in multiple myeloma cell lines has been shown to negatively regulate the CDK/Rb pathway by decreasing the expression of cyclin D1 and E2F1 and causing the re-expression of the tumor suppressor, p16 (Mitsiades, Mitsiades et al. 2003; Mitsiades, Mitsiades et al. 2004; Lee, Wang et al. 2010) (Figure 5). MS-275 (entinostat), an orally active synthetic pyridyl benzylcarbamate, is a selective Class I HDAC inhibitor (Saito, Yamashita et al. 1999). This selective cytotoxicity promotes gene expression favoring growth arrest and differentiation with increased expression of antiproliferative genes such as p21 (Saito, Yamashita et al. 1999; Park, Lee et al. 2002), and *in vivo* tumor reduction through reactivation of tumor suppressor pathways (Saito, Yamashita et al. 1999; Lee, Park et al. 2001).

With rapamycin's ability to diminish cellular growth via inhibition of mTOR complexes and MS-275's ability to reactivate tumor suppressor pathways, combined mTOR/HDAC inhibition offers a promising therapeutic agent in treating human cancers.

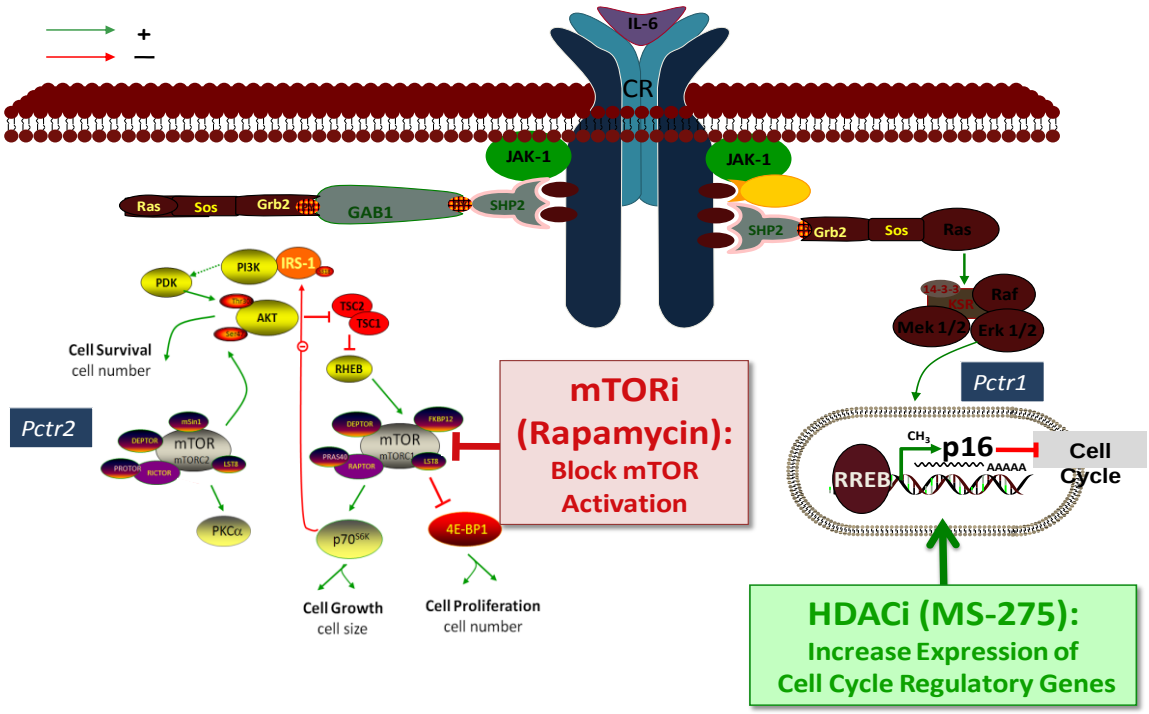
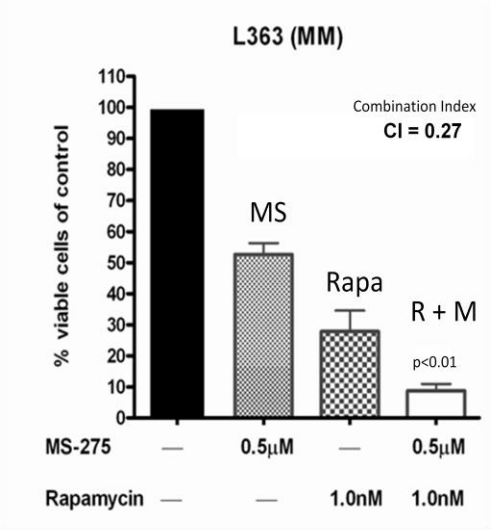


Figure 5. Rapamycin/MS-275 Inhibition on mTOR/HDAC Pathways, Respectively (Zhang, Readinger et al. 2011). Rapamycin selectively inhibits mTORC1, leading to inhibited cellular growth, translation, and proliferation. Entinostat (MS-275), an orally active synthetic pyridyl carbamate, is a selective Class I HDAC inhibitor that leads to increased expression of tumor suppressors such as p16 and p21 and decreased expression of positive cellular regulators such as cyclin D1 and E2F1.

Previous Studies with Rapamycin/MS-275

To test the effects of the novel rapamycin/MS-275 drug combination *in vitro*, 17 multiple myeloma patient cell lines were treated with the drugs individually and in combination (Figure 6).



Chou-Talalay Combination Index Calculations for Drug Synergy		
Cell Lines	% lines tested	Combination Index (CI Value)
7/17	41%	0.1-0.3 (Strong **Synergy**)
7/17	41%	0.3-0.85 (**Synergy**)
1/17	6%	0.85-0.9 (Slight **Synergy**)
2/17	12%	0.9-1.1 (Nearly Additive)
MS/Rapa Synergistic in 88% of MM Cell Lines Tested		

Figure 6. Multiple Myeloma Patient Cell Lines Treated with Rapamycin/MS-275 Individually and in Combination. Of the seventeen lines tested, 88% displayed some degree of synergy, indicating the relevance of the drug combination in MM cell lines.

Of the 17 lines tested, 15 lines (88%) had synergistic responses to the combination treatment which meant that their combined action was greater than the sum of their individual effects. This is depicted by the sensitive MM cell line L363, which had an enhanced decrease in cellular viability treated with the drug combination as compared to individual treatments ($p < 0.01$). The remaining 2 cell lines experienced nearly additive effects of the drug combination.

To delineate the underlying cellular mechanisms of the synergistic response to mTOR/HDAC inhibition, gene expression profiles (GEP) were completed on multiple

myeloma cell lines treated with each respective inhibitor individually and in combination to identify any significant expression changes among each group as determined by ANOVA (Simmons et al., unpublished). Weighted gene co-expression network analyses (WGCNA) were completed on the 1647 genes identified by the GEP. WGCNA is a systems level approach for defining the correlation patterns among genes; in this case, genes identified from MM cell line GEPs. This approach identified 901 correlated genes and separated them into distinct modules based on their response to rapamycin and MS-275 individually and in combination (Figure 7). Five highly correlated gene sets (modules) were identified: of these five modules, two were identified as primarily affected by MS-275 alone (springgreen and darkgreen), one by rapamycin alone (red), and two affected by both drugs (orange and blue), as can be seen by the expression changes in the heatmap (Figure 7A). In the orange module, each drug causes a counteractive response with respect to expression, leading to no net change in expression. The blue module was *synergistically* affected by both drugs and became the "cooperative" module, narrowing down 126 genes of interest (Figure 7).

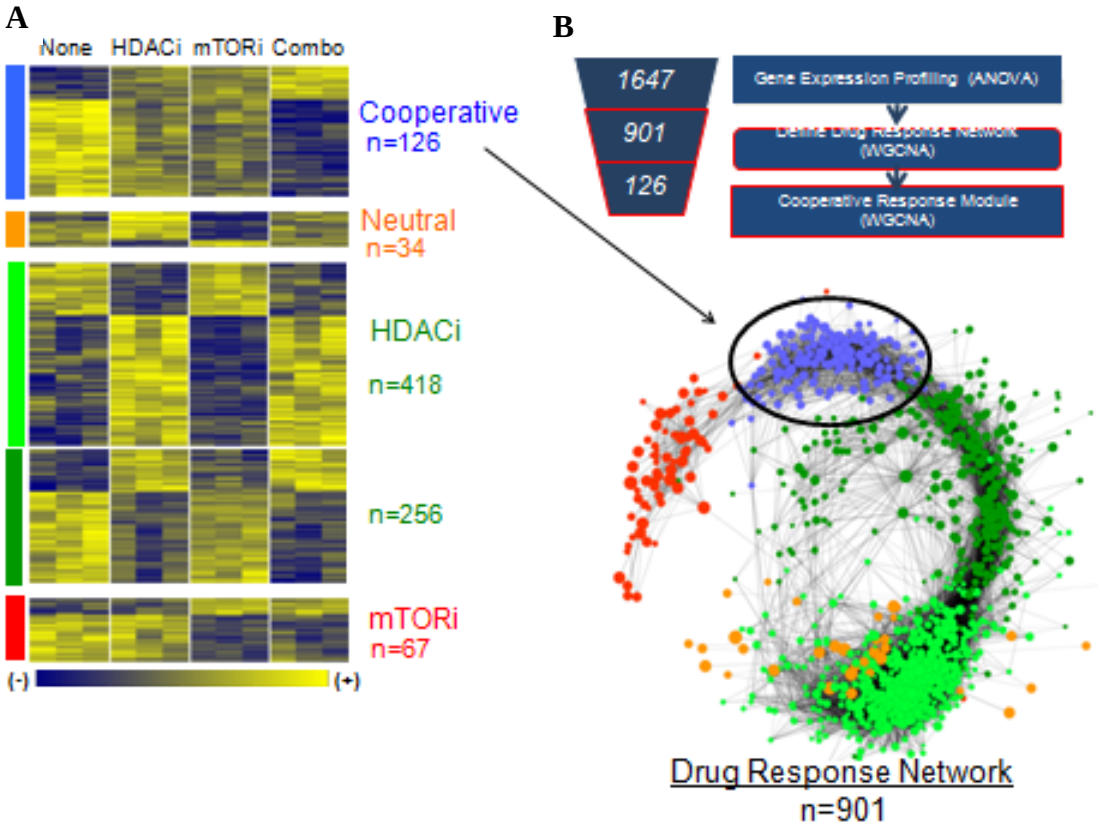


Figure 7. **Weighted Gene Co-Expression Network Analysis.** A. Heatmaps of networks by module, corresponding to drug-specific effects (yellow, up-regulated; blue, down-regulated): Cooperative Combination (blue), Neutral Combination (orange), entinostat (springgreen), entinostat (darkgreen), and sirolimus (red). Expression values are mean centered by rows. B. Network of the 901 most connected nodes (genes) from the drug-specific modules (Cytoscape edge-weighted, spring-embedded layout algorithm). Nodes are colored by module assignment, and sizes are proportional to within-module connectivity (Simmons et al., unpublished.)

Using the cooperative response signature (blue module), Simmons et al. (unpublished) examined the correlation of the expression of these genes with patient survival to elucidate possible clinical utility of the drug combination. This was completed with a multivariate predictor analysis of GEPs from 207 MM patients by combining both gene expression data and clinical data to identify which genes in the cooperative

signature correlate with patient prognosis and survival (Bair and Tibshirani 2004). From this gene set they identified significant dysregulation in the expression patterns of 37 genes in patients with poor prognosis (Figure 8).

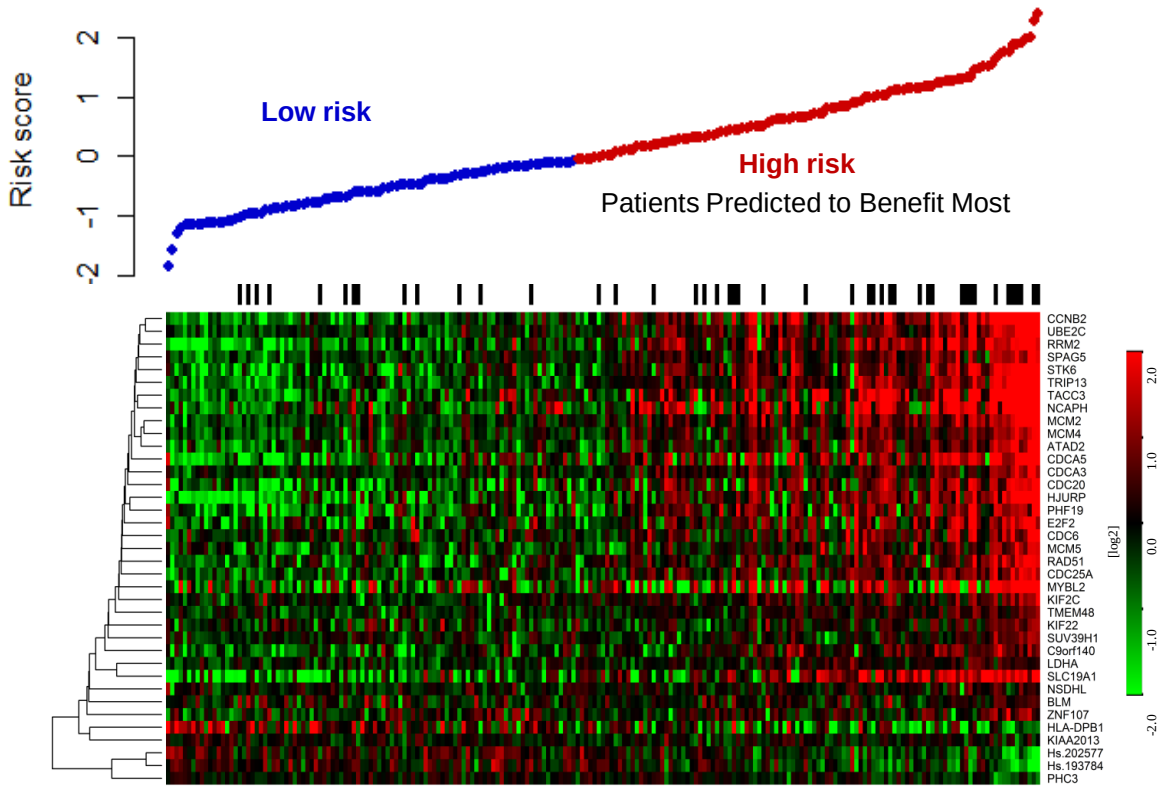


Figure 8. Expression of Cooperative (Blue) Module Genes Correlates with Survival in Multiple Myeloma Patients. Survival predictor gene expression (median centered) heatmap of 207 patients in test set. Samples are ordered by increasing risk score from the survival classifier and plotted above the heatmap. Black bars indicate death.

Interestingly, many of the genes which were over-expressed in patients were down-regulated and genes which were under-expressed were up-regulated by the drug

combination. This helped to identify a subset of multiple myeloma patients that would be more sensitive to the combination drug treatment.

CHAPTER 2

PURPOSE AND OBJECTIVES

Prior research has shown the combination of mTOR and HDAC inhibitors to be active and synergistic in multiple myeloma patient cell lines (Simmons et al., unpublished). The purposes of my research are to validate the cooperative signature and enriched transcription factors at the protein and RNA level as well as delineate a potential molecular mechanism for the efficacy of combined mTOR/HDAC inhibition in MM cell lines. The objectives of my research were as follows:

1) To validate the synergy-specific expression networks down-regulated by the rapamycin/MS-275 drug combination using mRNA and protein analysis.
2) To examine the predicted transcription factor activity related to the synergistic gene expression response induced by the drug combination and examine protein levels of transcription factor targets down-regulated by the combination.
3) To determine the relative contributions of rapamycin/MS-275 inhibited transcription factors identified by the drug synergy/response signature.

CHAPTER 3

MATERIALS AND METHODS

Materials

The following were used within this study: Human MM cell lines L363, U266, SKMM1, KMS20, KMS11LB, KMS28PE, KMS28BM, FR4, KMS12PE, EJM, KMS12BM, XG6, OCIMY5, MMM1, KMS18, MCF7, and KMS26, Plasmablast P-493-6 cell line, RPMI-1640 media, L-glutamine, 10% Fetal Bovine Serum, Penicillin, Streptomycin, MS-275 (Sigma-Aldrich), Sirolimus (Developmental Therapeutics Program, NCI), Dimethyl Sulfoxide, MTT Reagent, VERSAmax Microplate Reader (Molecular Devices, Sunnyvale, CA), Softmax Software, Phosphate Buffered Saline (PBS), 10x Tris Buffered Saline (TBS), Tween-20, 20x MOPS SDS Running Buffer, 5x Loading Dye, Instant Non-fat Dry Milk, Epitomics Primary Antibodies (MYC, MCM5, RAD51, TACC3, LDHA, SUV39H1, MCM2), Cell Signaling Primary Antibodies (CDC20, MCM4, E2F1), Santa Cruz Biotechnology Primary Antibody (RRM2), Millipore Primary Antibody (GAPDH), Ultra Pure Water, 70% Ethanol, Bovine Serum Albumin, 4-12% Bis-Tris Sodium Dodecyl Sulfate Polyacrylamide Pre-cast Gels (Novex, Invitrogen; Carlsbad, CA), Pierce BCA Protein Assay Reagent (Thermo Scientific; Rockford, IL), iBlot (Invitrogen; Carlsbad, CA), RNeasy Mini Kit (Qiagen Sample and Assay Technologies), NanoDrop 3300 Fluorospectrometer (Thermo Scientific; Rockford, IL), nCounter Analysis System (NanoString Technologies; Seattle, WA),

SuperSignalWest Dura Extended Signal Substrate (Thermo Scientific; Rockford, IL), G:Box and GeneSnap Software (Syngene).

Cell Lines

Human MM cell lines L363, U266, SKMM1, KMS20, KMS11LB, KMS28PE, KMS28BM, FR4, KMS12PE, EJM, KMS12BM, XG6, OCIMY5, MMM1, KMS18, MCF7, and KMS26 were derived and authenticated as previously described (Gabrea, Martelli et al. 2008). MM cell lines were cultured in RPMI-1640 (2 mM L-glutamine, 10% fetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin). All cell lines were maintained in a humidified incubator with 5% CO_2 at 37°C.

Drugs

For *in vitro* studies: MS-275 (Sigma-Aldrich) and sirolimus (Developmental Therapeutics Program (DTP), NCI) were dissolved in dimethyl sulfoxide (DMSO) at 10mM (stored at -20^{0}C). In culture medium, final concentrations for MS-275 ranged from 0.1-0.5 µM and 1-10 nM for rapamycin with a maximum of 0.2% DMSO.

Cell Proliferation (MTT) Assay

A colorimetric assay (CellTiter 96 Non-radioactive Cell Proliferation Assay; Promega; Madison, WI) to determine cell viability was used based on the cleavage of a tetrazolium component of the Dye solution into a formazan product. 50,000 cells per 200 µl of media per well were seeded in 96-well plates and incubated with rapamycin and/or MS-275 for 4-48 hours at 37°C and 5% CO_2. 20 µl of MTT reagent was added at the end

of treatment and incubated for 1.5-2 hours in the same conditions. Absorbance was measured against a blank background control at 490 nm using a VERSAmax microplate reader (Molecular Devices; Sunnyvale, CA) and Softmax software. The same assay was used to analyze both single (rapamycin or MS-275) and combination treatments.

Western Blot Analysis

MM cell lines were treated with rapamycin, MS-275, and/or the combination at various time points (4, 8, 18, 24, 48 hours) and cell pellets were collected by centrifugation and subsequent washing with PBS. Protein lysates were prepared by adding 75 – 300 µL RIPA lysis buffer containing phosphatase and protease inhibitors depending on the size of the cell pellet. Protein concentration was determined using Pierce BCA (bicinchoninic acid) Protein Assay Reagent (Thermo Scientific; Rockford, IL) based on the colorimetric reaction produced from the reduction of Cu^{2+} to Cu^{1+}. 2 µl lysate in 18 µl DI H_2O were added to a 96 well plate with albumin standards as controls. 200 µl BCA were added to each well and incubated for 30 minutes at 37°C and 5% CO_2. 15 µg protein in 20 µL prepped lysate was loaded and electrophoresed on 4-12% Bis-Tris sodium dodecyl sulfate polyacrylamide pre-cast gels (Novex, Invitrogen; Carlsbad, CA) at 130V until the dye front reached the terminal end of the gel. Gels were transferred to nitrocellulose membranes via the iBlot system (Invitrogen; Carlsbad, CA). Blots were washed in TBST, blocked for 1 hour in 10% milk/TBST, washed in TBST and incubated in primary antibody overnight at 4°C. All antibodies were obtained from Cell Signaling and Epitomics and were used at a 1:1000 dilution except for RRM2 (1:200), p-Rb (1:2000), GAPDH (1:25,000), MCM2 (1:10,000), and RAD51 (1:10,000) in a 5% bovine

serum albumin (BSA) blocking agent. Blots were then washed in TBST, incubated in 1:5000 anti-rabbit conjugated HRP secondary antibody in 5% milk/TBST for 1 hour, washed in TBST, and incubated in 1:1 SuperSignalWest Dura Extended Signal Substrate (Thermo Scientific; Rockford, IL) for 5 minutes. Blots were then imaged using G:Box and GeneSnap software (Syngene).

NanoString Analysis

MM cell lines were treated with rapamycin, MS-275, and/or the combination at various time points and cell pellets were collected. Total RNA was prepared using RNeasy Mini Kit (Qiagen Sample and Assay Technologies). RNA quantification was done using the NanoDrop 3300 Fluorospectrometer (Thermo Scientific; Rockford, IL). RNA expression was analyzed using the nCounter Analysis System (NanoString Technologies; Seattle, WA) with a custom color-coded oligo probe set for genes of interest.

CHAPTER 4

RESULTS

Validation of Down-Regulated Targets from the Cooperative Signature

RNA Expression Levels of Cooperative Signature Drop in Combined mTOR/HDAC Inhibition Drug Treatment Compared to Control in the Sensitive Multiple Myeloma Cell Line L363.

RNA lysates from untreated, individual and combination treated L363 and U266 MM cell lines were analyzed with the NanoString system using probes specific to genes from the cooperative signature. Thus far, RNA expression levels through NanoString analysis have validated 18 of the 32 down-regulated genes established by the cooperative gene signature in L363 cells and 15 of 32 in U266 MM cells (Figure 9). Therefore, at least 15 genes found to be over-expressed in patient datasets were down-modulated by the drug combination in two separate cell lines.

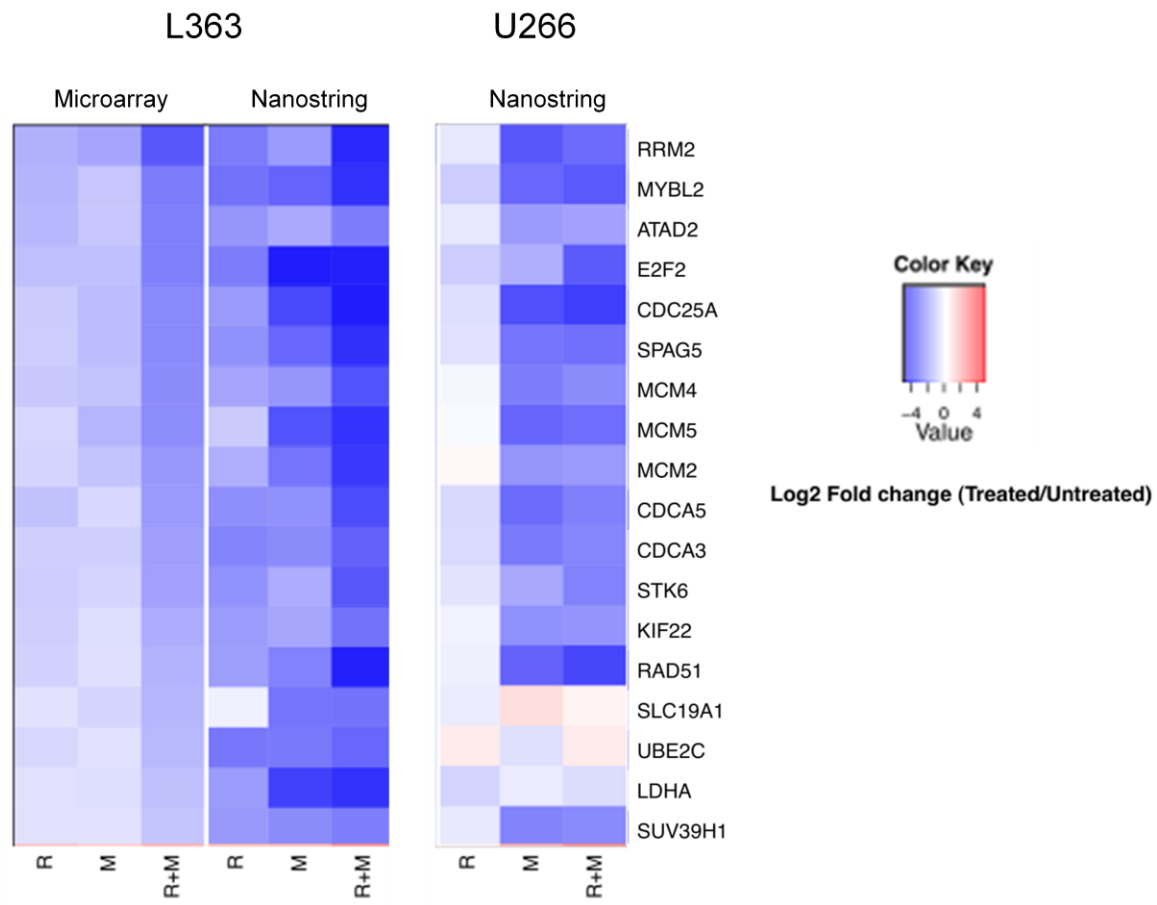


Figure 9. **NanoString Analysis of L363 and U266 MM Cell Lines.** Each gene was detected by hybridizing 100ng of total RNA with color-coded oligo probes specific for the gene of interest. Within each cell line, samples are either untreated, individual, or combination treated for 48 hours with rapamycin (10nM) and/or MS-275 (500nM). Colors represent relative fold change between treated/untreated (blue: down-modulated; red: up-modulated).

Gene Signature Protein Validation and Enriched Transcription Factor Analysis

Gene Signature Protein Expression Levels Drop in L363 Cells in Response to Combined mTOR/HDAC Inhibition. Predicted Enriched Transcription Factors Correlated with the Cooperative Signature are Synergistically Affected by the Drug Combination

We hypothesized that signature genes found to be down-regulated by the combination treatment at the RNA level would also be down-regulated at the protein level. Of the 19 genes examined by NanoString, 10 had primary antibodies available for analysis. Of the 10 tested, all but LDHA were validated at the protein level (Figure 10). These data are consistent with Figure 9 in that LDHA mRNA did not validate in U266.

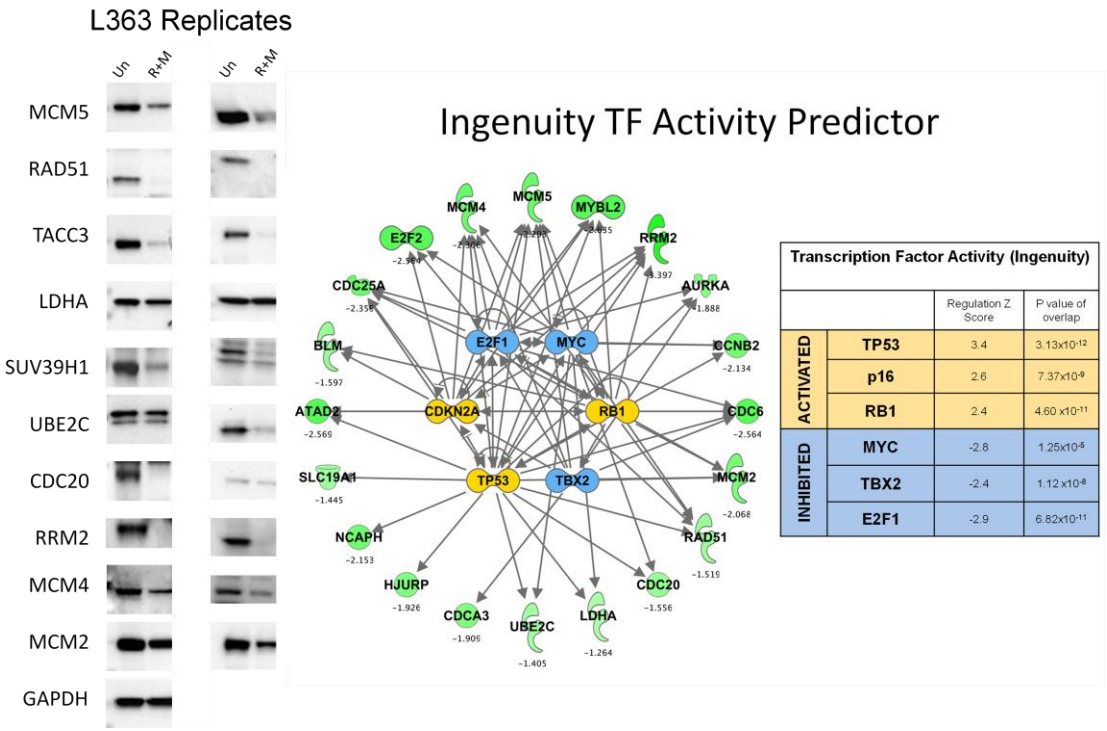


Figure 10. Gene Signature Protein Expression Validation and Enriched Transcription Factor Prediction. Examined genes from the cooperative signature, aside from LDHA, were validated at the protein level in the MM cell line L363 (two replicates shown). Transcription factors predicted from the cooperative gene signature were evaluated with Ingenuity's TF activity tool. Z-scores were determined by a proprietary enrichment testing algorithm as part of the Ingenuity analysis which used the amount of relative fold-change when cells were treated with the drug combination.

To determine whether transcription factor (TF) modulation is acting on the synergistically-affected gene set, Ingenuity TF activity predictor was utilized to identify

particular transcription factors predicted to be active in our gene set based on enrichment testing of published TF transcriptional networks. When the 37 genes in our signature were interrogated with the Ingenuity TF predictor, a core of 20 genes were identified that predicted involvement of 6 TFs (Figure 10). Of the 10 genes validated at the protein level, 8 were found to be highly correlated with the enriched TFs. Of those TFs identified to be enriched, three were predicted to be activated (p53, p16, and Rb) and three to be inhibited (E2F1, TBX2, and MYC). Once identified, inhibited TF expression was analyzed at RNA and protein levels using NanoString and Western blot, respectively.

NanoString analysis was used to examine transcript levels of predicted inhibited TFs in total RNA from the MM cell line L363. As predicted, E2F1 total RNA drops with combination treatment at 48 hours whereas, surprisingly, MYC total RNA increases (Figure 11). As TBX2 was not found to be expressed above background levels established by the NanoString system (Figure 11), it was omitted from further analysis. E2F1 and MYC, therefore, became our two down-regulated TFs targeted for further study.

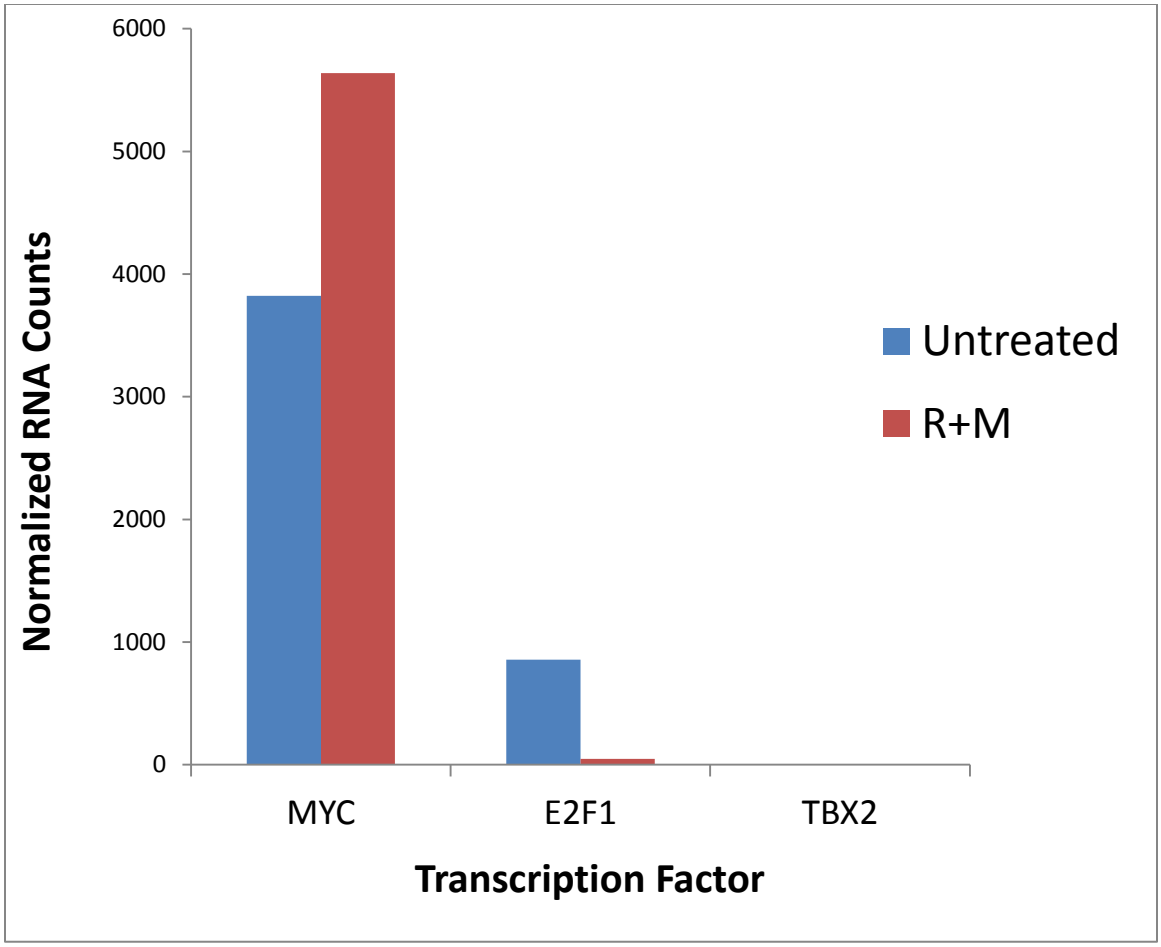


Figure 11. **Total RNA Levels of MYC, E2F1 and TBX2 in the MM Cell Line L363.** Cells were either untreated or treated with rapamycin/MS-275 in combination at 48 hours. While both MYC and E2F1 responded to the combination treatment, TBX2 was not found to be expressed above background levels.

To further examine the difference between MYC and E2F1 responses to the drug treatment, RNA fold change was examined in various MM cell lines by looking at expression when treated with the rapamycin/MS-275 combination compared to expression in untreated control cells (Figure 12, left).

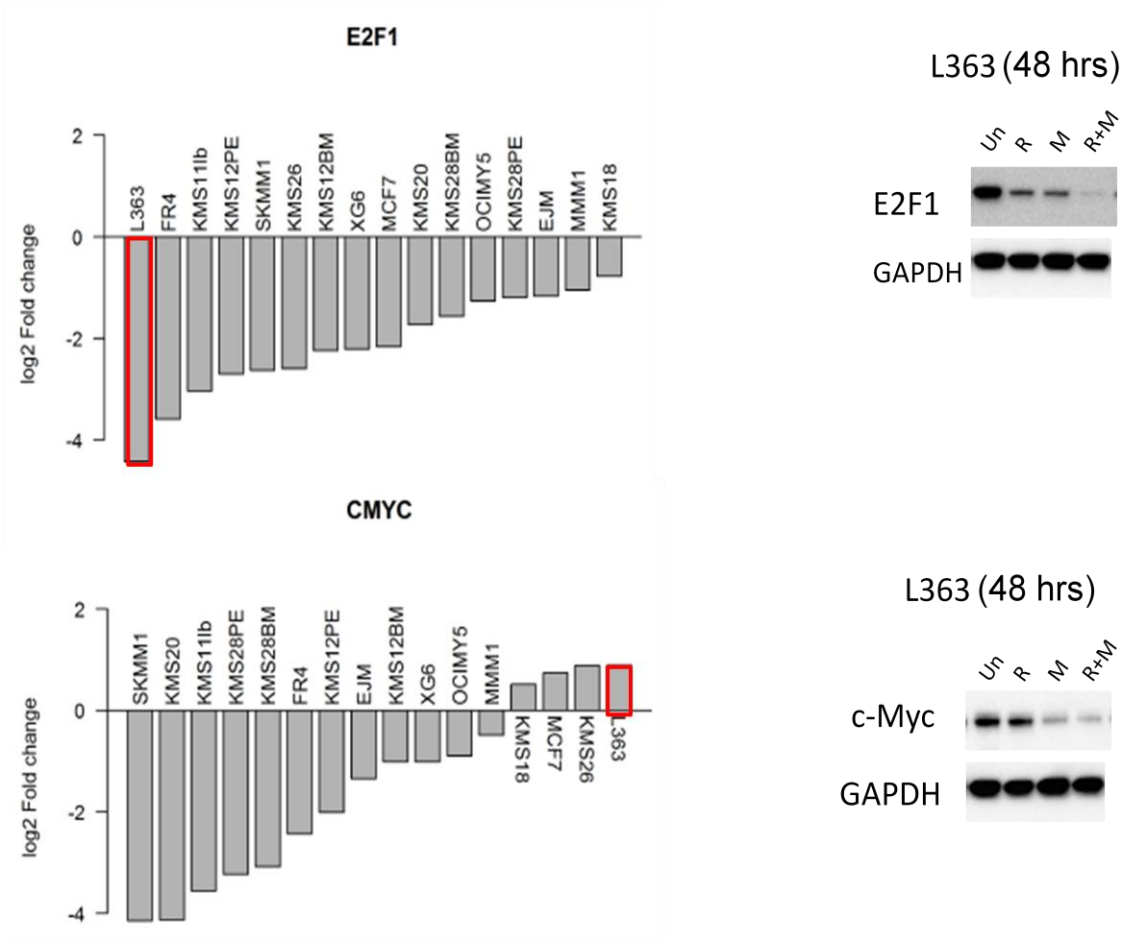


Figure 12. **RNA (left) and Protein (right) Analysis of E2F1 and MYC in Cell Lines Treated with Rapamycin/MS-275 at 48 Hours.** E2F1 mRNA (top left) dropped when treated with the drug combination compared to untreated control cells. MYC mRNA (bottom left) followed no discernible pattern related to viability response to the combination or amount of protein. Both E2F1 and MYC protein (top right and bottom right, respectively) expression dropped when treated with the drug combination as compared to individual treatments.

As expected, E2F1 mRNA levels dropped in our sensitive MM cell line L363 when compared to E2F1 mRNA in untreated cells. Surprisingly, MYC mRNA levels *increased* in L363. To examine the effect of our combination on MYC and E2F1 protein levels at 48 hours, Western blot analyses were performed. Protein expression of both MYC and E2F1 decreased with the combination compared to individual treatments

(Figure 12, right). The fact that RNA results do not match protein results for MYC suggests a feedback loop such that low protein levels provide a signal to transcribe more MYC. Overall, the amount of E2F1 and MYC varied among the 16 cell lines examined.

Relative Contributions of Identified Transcription Factors to Drug Synergy

MYC Protein Expression Drops Prior to E2F1 and is Required for Combination Response and Sensitivity

To further examine the relative contribution of E2F1 and MYC to the drug synergy/response signature, time-courses of protein expression following treatment were performed. By treating the MM cell line L363 with rapamycin and MS-275, individually and in combination, at 4, 8, 18, 24, and 48 hours we determined that E2F1 protein expression levels begin to drop between 24 and 48 hours post combination treatment (Figure 13). MYC protein expression levels, however, begin to drop as early as 18 hours, at least 6 hours prior to E2F1 (Figure 13). These data suggest that MYC may play a larger role in driving the response to drug treatment (O'Donnell, Wentzel et al. 2005).

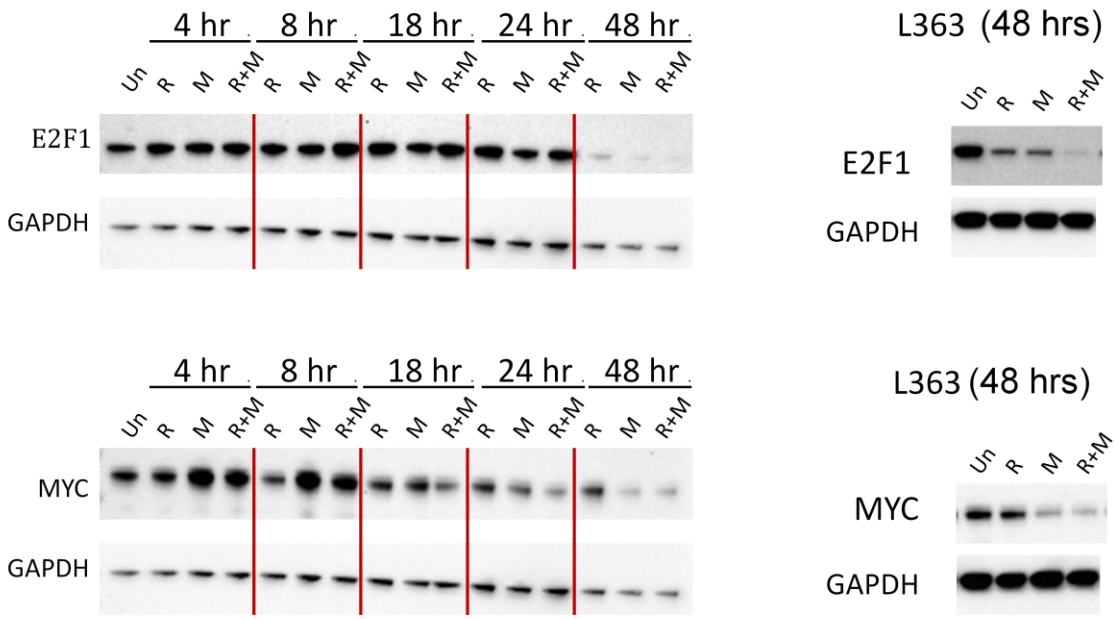


Figure 13. Time Course of E2F1 and MYC Protein Expression in the MM Cell Line L363. MYC protein expression levels drop at approximately 18 hours in response to combination treatment, which precedes the drop in E2F1 expression by at least 6 hours.

In order to further investigate the role of MYC in our drug combination response, an immortalized plasmablast cell line stably transfected with a tetracycline-repressible MYC expression construct (P-493-6) was used. When tetracycline is added to these cells, MYC levels drop below normal physiological levels. However, when tetracycline is removed, MYC levels recover fully by approximately 24 hours. This construct allowed us to test our response signature in the relative absence/presence of MYC. To first verify these cells were indeed inducible, RNA and protein expression was examined in both tetracycline on (MYC off) and tetracycline off (MYC on) conditions (Figure 14A/B). As expected, both RNA and MYC levels were high when MYC is on and absent when turned off. Once confirmed, we examined viability of P-493 cells treated with our rapamycin/MS-275 combination under both conditions (Figure 14C).

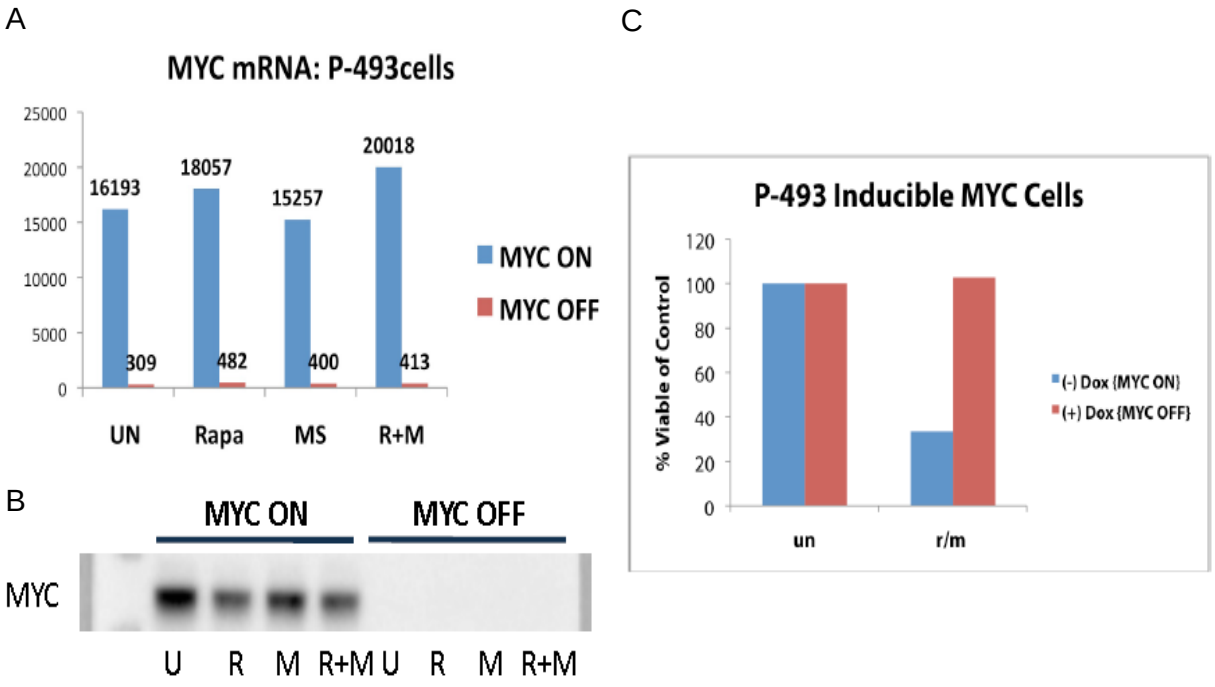


Figure 14. Response to Rapamycin/MS-275 Treatment in P-493 Cells. MYC RNA (A) and protein (B) levels drop in the presence of tetracycline (MYC off) and recover when tetracycline is removed (MYC on). When MYC is present, P-493 viability drops dramatically when treated with the drug combination (C). When MYC is absent, however, no response is seen.

When MYC is present, there is a dramatic drop in viability in the combination treatment group when compared to untreated control cells (Figure 14C). When MYC is absent, however, no response is seen (Figure 14C). These results establish MYC as an essential driver for our response signature/drug synergy. As such, our gene signature was examined in response to MYC on/MYC off in the P-493 cell line.

The importance of MYC is highlighted by the expression change of the gene signature when MYC is on in the P-493 cells (Figure 15A). A heatmap was created to show the Log_2 fold change of expression for our cooperative signature genes in both combination treated L363 versus untreated cells when MYC was "on" in P-493 cells (Figure 15B).

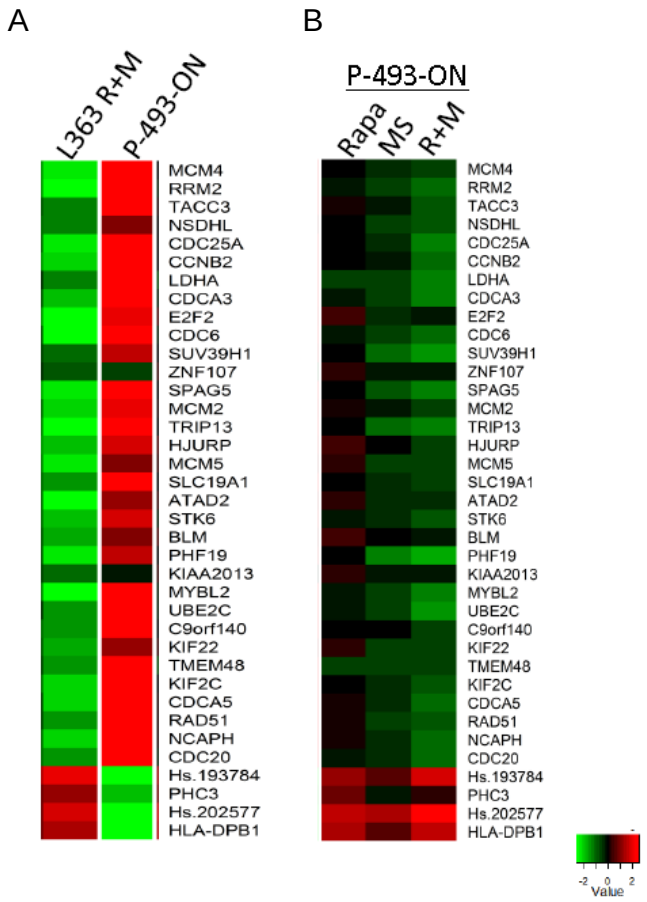


Figure 15. Gene Signature Response in L363 and P-493 Cell Lines. A. When MYC expression is on in P-493 cells, gene expression is largely the opposite of combination treated L363 cells. B. When P-493 cells (MYC on) were treated with each drug individually and in combination, gene expression was affected similarly to combination treated L363 cells.

P-493 cells were then treated with rapamycin and MS-275 individually and in combination in cells expressing MYC (Figure 15B). While gene expression levels dropped in both individual treatments, the most dramatic response seen was in the combination treatment. Nearly all genes tested, except NSDHL, ZNF107, and KIAA2013, exhibited an inverse response in the combination treatment when compared to untreated, MYC on P-493 cells, indicating the importance of MYC expression to the rapamycin/MS-275 drug synergy.

CHAPTER 5

DISCUSSION

Currently, single agent drugs targeting mTOR and HDACs have shown modest efficacy in multiple myeloma. While standard treatment options have prolonged overall survival, novel therapeutics are still needed as nearly all multiple myeloma patients will develop disease resistance to current therapies. Previous studies have shown efficacy of the mTOR and HDAC inhibitors rapamycin and MS-275, respectively, in concert with other drugs but little research has been completed on their combined action. After screening the viability of seventeen multiple myeloma patient cell lines treated with rapamycin, MS-275 and the drug combination, 15 of the 17 lines (88%) had synergistic responses, indicating an active and functional drug combination *in vitro*. In-depth analysis of one MM cell line, L363, highlights the efficacy of the drug combination, displaying a significant decrease in cell viability when compared to individual treatments (Figure 6).

To delineate gene expression changes involved in the synergistic response, gene expression profiling of untreated and treated L363 cells was performed (Simmons et al., unpublished). Weighted gene co-expression network analyses (WCGNA) identified a cooperative module of highly connected genes that respond to the drug combination in MM patient cell lines (Figure 7). Further multivariate predictor analyses, combining gene expression profiles and clinical patient data, honed down the cooperative module to 37

genes highly correlated with disease prognosis and patient survival (Figure 8). This was designated as our drug-responsive gene signature.

To validate the gene signature, total RNA was isolated from the sensitive MM cell lines, L363 and U266 after treating the cells with rapamycin/MS-275 individually and in combination for 48 hours. Gene expression was determined by NanoString analysis. While some genes had a greater response to the drug combination, the overall net expression of the gene signature dropped in the combination treatment when compared to control at 48 hours post treatment (Figure 9). At least three genes (LDHA, UBE2C, and SLC19A1) were found to be less consistent in their responses to drug treatment when examined in a separate cell line (U266).

To further validate our gene signature at the protein level, Western blot analysis was used. Proteins with available antibodies were examined in L363 at 48 hours post rapamycin/MS-275 combination treatment (Figure 10). It was found that protein expression was largely down-regulated in the combination compared to untreated control cells, validating our signature at the protein level. Of those genes examined, many are directly involved in cell cycle and DNA repair: RAD51, for example, plays a major role in homologous recombination during DNA double stranded break repair and has been shown to be correlated in malignancies such as breast cancer (Sassi, Popielarski et al. 2013); siRNA inhibition of RRM2, involved in the catalysis of deoxyribonucleotides from ribonucleotides, has been shown to decrease viability in head and neck squamous cell carcinomas and non-small cell lung cancer cell lines (Rahman, Amin et al. 2012); TACC3, essential for mitotic spindle dynamics and centrosome integrity during mitosis, has been shown to be dysregulated in a number of cancers (Ha, Park et al. 2013); MCM

protein complexes (including MCM2, 4, and 5) are involved in both the initiation and elongation phases of eukaryotic DNA replication and MCM2 overexpression has been tied with poor prognosis in gastric cancer (Yang, Wen et al. 2012) and multiple myeloma through activation of E2F1 and suppression of p53 (Teoh, Urashima et al. 1997); CDC20 is an essential regulatory molecule of cellular division and its overexpression has been tied to numerous neoplastic diseases (Yuan, Xu et al. 2006; Chang, Ma et al. 2012; Kato, Daigo et al. 2012). Taking into consideration the vast dysregulation of our signature genes in various malignancies, inhibition of these genes by combination treatment suggests clinical utility.

To investigate transcription factor enrichment associated with our gene signature, Ingenuity transcription factor predictor analysis was used. By mining published datasets and microarray analyses to determine TF overrepresentation, two TFs of interest were shown to be significantly inhibited: E2F1 and MYC (Figure 10). A third inhibited TF, TBX2, was not found to have transcript levels above background and was therefore omitted from future analyses (Figure 11). As with the gene signature, TF validation was done at the protein and RNA levels for E2F1 and MYC. Protein and RNA isolation were completed using the same methods for the gene signature and individual and combination treatments were completed at multiple time points (4, 8, 18, 24, and 48 hours). E2F1 protein and RNA expression dropped significantly in the combination treatment at 48 hours when compared to individual treatments and control (Figure 11). Involved in cell cycle regulation and DNA synthesis in eukaryotic cells, E2F1 is under the control of the tumor suppressor, Rb. In normal cells, Rb is bound to E2F1, inhibiting the G1/S phase transition and suppressing transcription. Once phosphorylated by CDKs, Rb releases

from E2F1 and cell cycle progression is allowed to continue. Conversely, E2F1 accumulation can also lead to cellular apoptosis, suggesting E2F1 also acts as a regulator of Rb-dependent cell growth and survival (Matsumura, Tanaka et al. 2003). Due to its role in the cell cycle, particularly the G1/S transition, dysregulation of E2F proteins are commonly seen in human tumors (Nevins 2001). While some studies suggest elevated levels of E2F1 and subsequent cyclin D levels in multiple myeloma (Lai, Medeiros et al. 1998), others have shown E2F1-induced apoptosis on neoplastic cells when levels are elevated (Pan, Yin et al. 1998). While paradoxical in tumorigenic environments, abrogation of E2F1 by the rapamycin/MS-275 combination results in diminished levels of signature genes, suggesting E2F1 has a role in the cooperative response.

MYC protein levels diminish in a time-dependent fashion in the combination treatment group (Figure 13). MYC levels begin dropping at approximately 18 hours, preceding the drop in E2F1 levels by at least 6 hours. This suggests MYC may contribute more to the synergistic response seen in the mTORi/HDACi combination, although both TFs certainly play a role. Likewise, diminished E2F1 levels may also have a feedback mechanism on MYC levels as previous studies have shown that E2F activity is essential for survival of human cancer cells over-expressing MYC (Santoni-Rugiu, Duro et al. 2002). Previous studies have displayed MYC translocations in 19/20 cell lines and 50% of primary advanced MM tumors (Shou, Martelli et al. 2000), often leading to drastically elevated MYC levels and increased cell proliferation. Thus, due to MYC's ubiquitous and modulatory action on large fractions of the genome, significant dysregulation of many genes is observed. Therefore, synergistic inhibition of both E2F1 and MYC protein levels

with the rapamycin/MS-275 combination may ameliorate tumorigenic capabilities in MM patients responsive to the combination.

Surprisingly, MYC RNA levels displayed an *increase* in expression in the combination treatment (Figure 12). While counterintuitive, an increase in mRNA with a concurrent decrease in protein can potentially be explained by a change in the half-life or synthesis of the MYC protein. While transcripts may not be affected, translational effects can lead to greater MYC degradation or instability. To examine this paradoxical phenomenon further, pulse-chase experiments will be conducted using various MM cell lines to determine if MYC synthesis, degradation or both is affected by the drug combination.

To further elucidate the molecular action of MYC on the synergistic response to the rapamycin/MS-275 combination, Epstein Barr Virus (EBV) transformed tonsilar B-cells (P-493-6) containing an inducible MYC tetracycline-off expression construct was used. When exposed to tetracycline, MYC levels drop to very low endogenous levels and recover when tetracycline is removed (Figure 14). This inducible system allows for manipulation of MYC levels to determine the overall contribution of this TF to the synergistic response. To confirm the line is indeed inducible, RNA and protein expression analyses were done on cells treated with rapamycin/MS-275 individually and in combination (Figure 14). As expected, both RNA and protein expression diminished when tetracycline was added to the cells (MYC off) and raised when removed (MYC on). Of significant importance, however, was the cellular response to the drug combination when MYC is absent. While the viability responds in a similar fashion as our sensitive MM cell lines when MYC is present, no change is seen in combination treated cells

compared to untreated control cells in the absence of MYC (Figure 14). Now established as an important driver of viability, we wanted to examine our gene signature response in the presence and absence of MYC. Signature gene expression was shown to be largely up-regulated in untreated, MYC on, P-493 cells (Figure 15). When treated with rapamycin/MS-275 in combination, however, an inverse relationship was seen, resulting in down-regulation of up-regulated signature genes and vice versa. These results underscore the importance of MYC in the synergistic response to the drug combination, indicating MYC as an important molecular driver of the drug response. Likewise, this may provide an alternative approach to MYC inhibition in cancer therapy. As such, future studies to divulge the mechanistic underpinnings of MYC on the gene signature and combination sensitivity are warranted.

Our studies, based on gene expression profiling and cell viability of combination treated cell lines, used a series of biological filters based on patient datasets to provide a novel and systematic approach in identifying the synergistic underpinnings of drug combinations which may be applicable to any disease setting.

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