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Shuo Cheng   Date

Investigating the Roles of *ACLY* in Multiple Myeloma Biology and Drug Sensitivity

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Investigating the Roles of *ACLY* in Multiple Myeloma Biology and Drug Sensitivity

By

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B.A., Emory University, 2019

Advisor: Lawrence H. Boise, Ph.D.

An abstract of
a thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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Master of Science
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Cancer Biology and Translational Oncology
2020.

Abstract

Investigating the Roles of *ACLY* in Multiple Myeloma Biology and Drug Sensitivity

By Shuo Cheng

The gene *ACLY* codes for the cytosolic protein ATP citrate lyase (ACLY), the protein responsible for the conversion of citrate into acetyl-coenzyme A (acetyl CoA) and oxaloacetate (OAA). Acetyl CoA is used in the fatty acid synthesis pathway, the mevalonate pathway, and acetylation reactions. Many cancerous tumor cells deregulate cellular metabolism and display upregulation of ACLY and increased fatty acid synthesis, which is important for endogenous formation of saturated fatty acids. These fatty acids are used in the formation of cell membranes and membrane bound organelles, such as the endoplasmic reticulum (ER), a critical component of the secretory pathway. In multiple myeloma, the myeloma cells secrete copious amounts of monoclonal antibody, so we reasoned that the myeloma cells could be functionally dependent on *ACLY*. An analysis of the MMRF CoMMpass database showed that patients who expressed high quantities of *ACLY* mRNA had significantly worse progression free and overall survival compared to patients who expressed low quantities of *ACLY* mRNA. In the Broad Institute's Cancer Dependency map, we found that myeloma cells are more dependent on *ACLY* than cell lines derived from other cancer types. To date, no studies have demonstrated the function of ACLY in multiple myeloma. The present study uses CRISPR-Cas9 single-guide RNA (sgRNA) lentiviral vectors to create *ACLY* knockout (KO) myeloma cell lines as well as the ACLY chemical inhibitor SB-204990 to investigate the impact of ACLY loss of function on myeloma cell proliferation, survivability, and response to treatment. of the greatest hindrances to multiple myeloma treatment is drug resistance, so it is critical to continue researching new ways to target myeloma's plasma cell and cancer biology to improve standard of care.

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Introduction

Multiple Myeloma Epidemiology

Multiple myeloma is a rather rare disease, accounting for 1.8% of all cancers. Multiple myeloma is the second most common hematological malignancy, following non-Hodgkin lymphoma, with an estimated 32,270 newly diagnosed cases and 12,830 deaths in 2020.[1] The lifetime risk of myeloma is 1 in 132 (0.76%).[2] The median age of patients at diagnosis is 69 years.[2] The majority of new cases are diagnosed in individuals between the ages of 65-74, with 37% of cases diagnosed in individuals younger than 65. Multiple myeloma is extremely rare in individuals younger than 35 years of age (0.5%) and is more common in males than females. Interestingly, multiple myeloma is more than two times more likely to occur in African Americans than their Caucasian counterparts and this trend is observed in both males and females.[2] Due to advances in detection and modern therapies, the five-year survival rate has improved to 52.2% for the 2009-2015 year period compared to 25% for 1975-1977.[1]

Clinical Characteristics of Multiple Myeloma

Dr. Samuel Solly first described multiple myeloma as *mollities ossium* in two women in a case study in 1844.[3,4] Solly noted that both patients suffered from significant pain, had substantial bone deformities, and their urine contained high concentrations of "phosphate of lime" (calcium phosphate).[3] During their post-mortem examinations, he noticed that the bones were "nearly absorbed", and the bone marrow was filled with dark red matter with "nucleated cells of malignant disease".[3] Dr. William MacIntyre described similar findings in the case of Thomas Alexander McBean in 1845.[4,5] A urine sample from McBean was sent for analysis to Dr. Henry Bence Jones, a well-respected chemical pathologist. The urine sample contained a mysterious substance that liquified when boiled, precipitated with nitric acid, and resumed its original state once cooled. Jones determined the protein in the urine was "hydrated deutoxide of

albumen", later named Bence Jones protein. Jones noted that the hydrated deutoxide of albumen in urine should be used to diagnose future cases of *mollities ossium.*[4,6]

Multiple myeloma is a disease of the terminally differentiated B-lymphocytes called plasma cells. Multiple myeloma falls on a spectrum of plasma cell dyscrasias starting with monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic myeloma to symptomatic myeloma with related organ or tissue impairment.[7] MGUS is one of the most common premalignant conditions and is primarily characterized by clonal proliferation of plasma cells (<10% of bone marrow), elevated levels of serum monoclonal proteins (<30g/L), and the absence of end organ damage or evidence of other lymphoproliferative disorders.[8,9] Asymptomatic myeloma or smoldering myeloma is defined as the presence of ≥30g/L serum monoclonal protein and ≥10% clonal plasma cells in the bone marrow, but there is still no evidence of end organ damage.[8] In addition to clonal bone marrow plasma cells and elevated monoclonal antibody or immunoglobulin light chain, symptomatic myeloma is primarily characterized by end organ damage, commonly manifested as hypercalcemia, renal insufficiency, anemia, and bone lesions (CRAB).[8] Myeloma cells secrete osteoclast-activating factors, causing patients severe bone pain or pathological fractures.[10] The degradation of bone causes calcium buildup and is a major cause of renal insufficiency and excess serum creatinine.[8] Normally, plasma cells make up between 1-3% of the cells in the bone marrow, but in multiple myeloma plasma cells are >10% of the bone marrow. Overcrowding of the bone marrow by abnormal plasma cells results in decreased hemoglobin, causing patients to experience fatigue and weakness, and decreased white blood cells, rendering patients vulnerable to recurrent infections. Other symptoms can include the presence of plasmacytomas, hyperviscosity, amyloidosis.[8]

B-cell Development and Plasma Cell Differentiation

During B-cell development, the cells progress through a series of defined stages before a select population become terminally differentiated, long-lived plasma cells. The early stages of B-cell development take place in the bone marrow. The recombination activating genes (RAG1 and RAG2) initiate somatic recombination of the variable (V), diversity (D), and joining (J) sequences of immunoglobulin heavy chain genes.[11,12] When this recombination results in a viable allele, the membrane bound μ heavy chain is expressed on the surface of pre-B cells with a surrogate light chain consisting of VpreB and λ5 proteins.[12,13] Next, the cells undergo clonal expansion and somatic recombination of the V and J segments (light chain does not have D regions) of the kappa (κ) light chain, then, if no viable allele occurs, of lambda (λ) light chain. The coexpression of heavy and light chains forms the B-cell receptor (BCR). Autoreactive clones are deleted, and the remaining immature B-cells leave the bone marrow and migrate to secondary lymphoid organs. As they leave the bone marrow to secondary lymphoid organs, they express membrane bound δ heavy chain.[14]

In the secondary lymphoid organs, mature naïve B-cells encounter antigen, endocytose the antigen, present it on their surface by the major histocompatibility complex II (MHC-II), and receive a costimulatory signal from T-helper cells. This results in the release of B-cell stimulating cytokines IL-4, IL-6, and IL-21, proliferation of B-cells, and the formation of a germinal center, in which B-cells will undergo somatic hypermutation, clonal expansion, and class-switch recombination of the immunoglobulin heavy chains, ultimately producing high-affinity antibody-secreting plasma cells and memory B-cells. Somatic hypermutation and class-switch recombination are mediated by the activation-induced cytidine deaminase (AID), which

deaminates cytosine residues in DNA, altering it to uracil.[13] This process can cause double strand breaks, increasing the possibility of translocations.

Transcriptional mechanisms shift drastically during B-cell development and plasma cell differentiation. B-cells require the transcription factors BCL-6, MTA3, PAX5, and MITF. Plasma cells require the transcription factors BLIMP1, XBP1, and IRF4.[13,15] Interestingly, the transcription programs of B-cells and plasma cells are mutually exclusive and repress each other. BCL-6, MTA3, PAX5, and MITF all promote genes that maintain B-cell lineage and suppress other lineage-specific genes. Together, they are responsible for germinal center formation, proliferation, modulation of DNA damage repair during the class switch recombination and somatic hypermutation, and prevention of B-cells from prematurely differentiating into plasma cells.[13] BLIMP1, XBP1, and IRF4 all promote genes that maintain plasma cells and suppress B-cell lineage genes. They are necessary for plasma cell secretion of antibodies, cessation of the cell cycle, and homing to the bone marrow.[13]

Genetics of Multiple Myeloma

Multiple myeloma is a genetically heterogeneous disease but share some common features. Primary or founding genetic events occur early in myelomagenesis. Most, if not all, of the primary translocations occur from errors in DNA damage repair during class-switch recombination and somatic hypermutation. Because these events occur early in B-cell development, these translocations are observed clonally and can also be found in MGUS.[12,16] Primary genetic events usually result in juxtaposition of an oncogene next to an immunoglobulin heavy chain enhancer, specifically cyclins D1 and D3 (much less frequently D2), MMSET and FGFR3, and C-MAF and MAFB.[12,16,17] Cyclin D dysregulation results from the translocations t(11;14), t(12;14), or t(6;14), and occurs in ~20-25% of tumors.[12,16] Cyclin D normally binds to

CDK4/6, leading to phosphorylation of Rb, the release and activation of the transcription factor

E2F, and subsequent cell cycle progression. Surprisingly, myeloma cells have a slow

proliferation rate, but cyclin D dysregulation or Rb inactivation appears to be an early and

unifying trait in multiple myeloma.[18] MMSET and FGFR3 dysregulation results from t(4;14)

translocations, and portends poor prognosis. This translocation occurs in ~15% of tumors.[16]

Approximately 30% of patients with t(4;14) do not overexpress FGFR3, indicating that MMSET

is the oncogenic driver in this subset of patients.[19] MMSET is a histone methyltransferase that

catalyzes the dimethylation of histone 3 lysine 36, resulting in more open chromatin and

transcriptional dysregulation. MAFB and C-MAF activation results from t(14;20) and t(14;16)

respectively, and occurs in ~10% of tumors.[16] Patients with t(14;16) tend to have poor overall

and progression free survival. MAFB and C-MAF are both b-ZIP transcription factors. Although

their role in myeloma is not well characterized, it has been observed that CCND2 and ITGB7 are

often upregulated by MAF activation. ITGB7 codes for integrin-β7, which interacts with the

extracellular matrix and impacts myeloma cell migration and adhesion.[20] Additionally, using an

inducible overexpression system, van Stralen et al. identified 11 target genes downstream from

MAF, including the HES genes and NOTCH2, implicating activation of the Notch signaling

pathway.[21] Hyperdiploidy is also considered to be a primary event, resulting from errors in

chromosome segregation during clonal expansion, and is characterized by trisomies of

chromosomes 3, 5, 7, 9, 11, 15, 19 and 21.[12,22] Hyperdiploidy occurs in ~40-50% of patients and

hyperdiploid patients usually have better prognosis than patients with heavy chain enhancer

translocations.[22] It is difficult to pinpoint the role of hyperdiploidy in initiating myelomagenesis,

as it is not frequently observed in human myeloma cell lines, which are immortalized cells from

extramedullary myeloma. This suggests that hyperdiploidy is sufficient to initiate monoclonal gammopathy, but secondary genetic events are required to progress to symptomatic myeloma.[12]

Secondary genetic events are associated with disease progression. Secondary events are observed subclonally and does not usually involve B-cell specific DNA rearrangement mechanisms. Secondary events often cooccur with heavy chain translocations. Some examples of secondary events include chromosomal del(13q), amp(1q), del(1p), del(17p), immunoglobulin light chain translocations, MYC structural variants, dysregulated nuclear factor (NF) κB signaling, and KRAS and NRAS mutations.[12] Deletion of 13q often results in loss of Rb (13q14) and dysregulation of the cell cycle.[12,23,24] Amplification of 1q predicts poor prognosis in a dose dependent manner. Most notably, upregulation of MCL1 (1q21) contributes to myeloma cell survival.[12,25] Deletion of 1p results in the loss of the cyclin dependent kinase inhibitor CDKN2C (1p32) and dysregulation of the cell cycle.[12,26] Additionally, loss of the non-canonical poly-(A) polymerase FAM46C (1p12) contributes to myeloma growth and survival.[12,27] Deletion of 17p often results in loss of the well-established tumor suppressor TP53 (17p13), and is associated with anoikis resistance and development of extramedullary disease. Nearly all HMCLs have deficient TP53 function; when TP53 is overexpressed in (HMCLs), they exhibit increased levels of apoptosis.[12,28,29] MYC structural variants are another common secondary genetic event in newly diagnosed myeloma. MYC alterations occur in a variety of ways and effect many pathways, including NF-κB and MAPK signaling, which activate oncogenes that enhance myeloma progression.[12,30,31] The IgL-MYC, but not IgK-MYC, translocation predicts particularly poor prognosis. IgL-MYC translocations usually cooccur with hyperdiploidy, so this subset of patients may be mistakenly classified as standard risk. Additionally, patients with IgL-MYC translocations do not respond to IMiDs.[32] Understanding the genetic background of patients is

critical for the proper classification of risk and the path of treatment. However, the major caveat to using cytogenetics as a prognostic biomarker is the clonal heterogeneity of myeloma. Additionally, the identification of a prognostic biomarker does not necessarily correspond to a druggable target. Successful treatment of multiple myeloma will need to target both the genetic features of myeloma and plasma cell biology.[33]

Current Treatments for Multiple Myeloma

Modern therapies have advanced substantially from the infusion of orange peel, rhubarb pill, and opiates that Dr. Samuel Solly prescribed to Sarah Newbury in 1844.[3] Standard treatment options include hematopoietic stem cell transplantation, corticosteroids, proteasome inhibitors, alkylating agents, histone deacetylase (HDAC) inhibitors, BCL-2 homology domain-3 (BH3) mimetics, immunotherapy, and immunomodulatory agents (IMiDs). Patients under the age of 65 years who are transplant eligible undergo myeloablative conditioning followed by an autologous or allogeneic stem cell transplant to repopulate the bone marrow with healthy cells. Then they are given a combination of drugs for maintenance therapy. Corticosteroids like dexamethasone and prednisone bind to cytosolic glucocorticoid receptors, downregulate interleukin 6, and modulate NF-κB to activate intrinsic apoptosis. Generally, they promote an anti-inflammatory response.[34] Proteasome inhibitors like bortezomib and carfilzomib inhibit the 26S proteasome, preventing it from degrading ubiquitinated proteins. IκB accumulates and inhibits NF-κB, subsequently activating intrinsic apoptosis. Additionally, the accumulation of misfolded proteins in the cell induces a cellular stress response called the unfolded protein response (UPR), resulting in cell cycle arrest, and ultimately apoptosis if the stress is not resolved.[35] Alkylating agents like melphalan, cyclophosphamide, and temozolomide disrupt the cell cycle by adding alkyl groups onto guanine residues in DNA, resulting in adduct and crosslink formation, and

ultimately DNA double strand breaks.[36] If cells are unable to adequately repair these breaks, they arrest or undergo apoptosis. Histone deacetylases remove acetyl groups from lysine residues in histone and non-histone proteins, making these regions less accessible for transcription. Pan-HDAC inhibitors like panobinostat upregulate proteins like p21 and p53, resulting in cell cycle arrest.[37] BH3-mimetics like venetoclax bind to and inhibit function of the anti-apoptotic Bcl-2 like proteins to activate intrinsic apoptosis.[38] Immunotherapies such as the monoclonal antibody (mAb) daratumumab (anti-CD38) and elotuzumab (anti-SLAMF7) target specific surface markers and enhance NK-cell mediated antibody-dependent cell cytotoxicity.[39] Additionally, a B-cell maturation antigen (BCMA) targeting chimeric antigen receptor (CAR) T-cell therapy and bispecific antigen-directed CD3 T-cell engager (BiTE) are currently being tested in clinical trials.[40] IMiDs like lenalidomide and pomalidomide exhibit pleiotropic anti-myeloma activity. IMiDs inhibit tumor necrosis factor (TNF)-α, promote anti-tumor Th1 response, inhibit T-regulatory cells, and possess anti-angiogenic, anti-inflammatory, and anti-osteoclastogenic properties.[41–43] In an in vitro study, Fedele et al. (2018) reported that IMiDs induced degradation of Ikaros and Aiolos, resulting in upregulation of CD38, suggesting IMiDs could prime myeloma cells for daratumumab treatment.[44] Most regimens utilize a combination of tools to target different aspects of the disease. For example, one clinical trial used the combination of venetoclax with bortezomib and dexamethasone. Bortezomib has been shown to upregulate the pro-apoptotic sensitizer protein Noxa, which inhibits Mcl-1, so in theory, the combination therapy would result in dual inhibition of Mcl-1 and Bcl-2 and activation of intrinsic apoptosis.[45,46] Unfortunately, even when patients respond favorably to initial treatment, patients often relapse and become refractory, highlighting the need to continue developing novel therapeutics.

Cancer Metabolism

Cancer cells display a number of defining characteristics collectively termed the "hallmarks of cancer".[47] We will focus on the deregulation of cellular energetics and evasion of apoptosis. An overview of cellular metabolism is shown in Figure 1. Cancer cells commonly dysregulate metabolic pathways to coopt biosynthesis, bioenergetics, and oxidation/reduction (redox) homeostasis.[48] It is known that cancer cells frequently upregulate the glucose transporter GLUT1, increasing glucose uptake into the cells.[49] First, the glucose is routed through glycolysis, then to the pentose phosphate pathway (PPP), producing NADPH and ribose, which are necessary for reductive biosynthesis, detoxification of reactive oxygen species, production of pyruvate, and ultimately ATP.[50] In the mitochondria, pyruvate is converted to acetyl-CoA and continues to the tricarboxylic acid (TCA) cycle, which produces NADH, $FADH_2$, and ATP. NADH and $FADH_2$ carry electrons to the electron transport chain where ATP is generated by oxidative phosphorylation (OXPHOS).[48] Although OXPHOS is much more efficient at producing ATP (34 ATP/glucose) than aerobic glycolysis (2 ATP/glucose), cancer cells often preferentially upregulate aerobic glycolysis, a phenomenon known as the Warburg effect.[51] Overexpression of the oncogenes AKT, RAS, and MYC and loss of the tumor suppressor TP53 support aerobic glycolysis. AKT directly promotes glycolysis, and constitutively active AKT signaling is associated with a more aggressive phenotype.[52,53] RAS signaling upregulates GLUT1 through activation of HIF1-α.[48,54] MYC activation promotes a shift to dependency on glutamine metabolism, producing α-ketoglutarate, which feeds into the TCA to produce citrate, and ultimately acetyl moieties for fatty acid metabolism.[48,55]

Cancer cells can also upregulate fatty acid metabolism to provide biomolecular intermediates for downstream synthesis of saturated long chain fatty acids required for energy

storage and biosynthesis of cell membranes and signaling molecules, conferring a proliferation and survival advantage to the growing tumor.[56] The increased concentration of saturated fatty acids also protects cancer cells from reactive oxygen species and chemotherapeutics.[57] Normally, citrate is converted to acetyl-CoA, which can be directed to the mevalonate pathway for the production of sterol isoprenoids and protein prenylation, or to fatty acid synthesis for the production of palmitate and other complex fatty acids.[58] The key enzymes in fatty acid synthesis are ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACACA), and fatty acid synthase (FASN), all of which have been observed to be overexpressed in a variety of cancers.[56,59,60] ACLY uses ATP to catalyze the conversion of citrate into acetyl-CoA and oxaloacetate. ACACA uses ATP to catalyze the carboxylation of acetyl-CoA to malonyl-CoA, a substrate of FASN. FASN is responsible for the final step of the NADPH-dependent condensation of acetyl-CoA or malonyl-CoA to palmitate. FASN hyperactivity is a pan-cancer indicator of high-risk disease.[56,60]
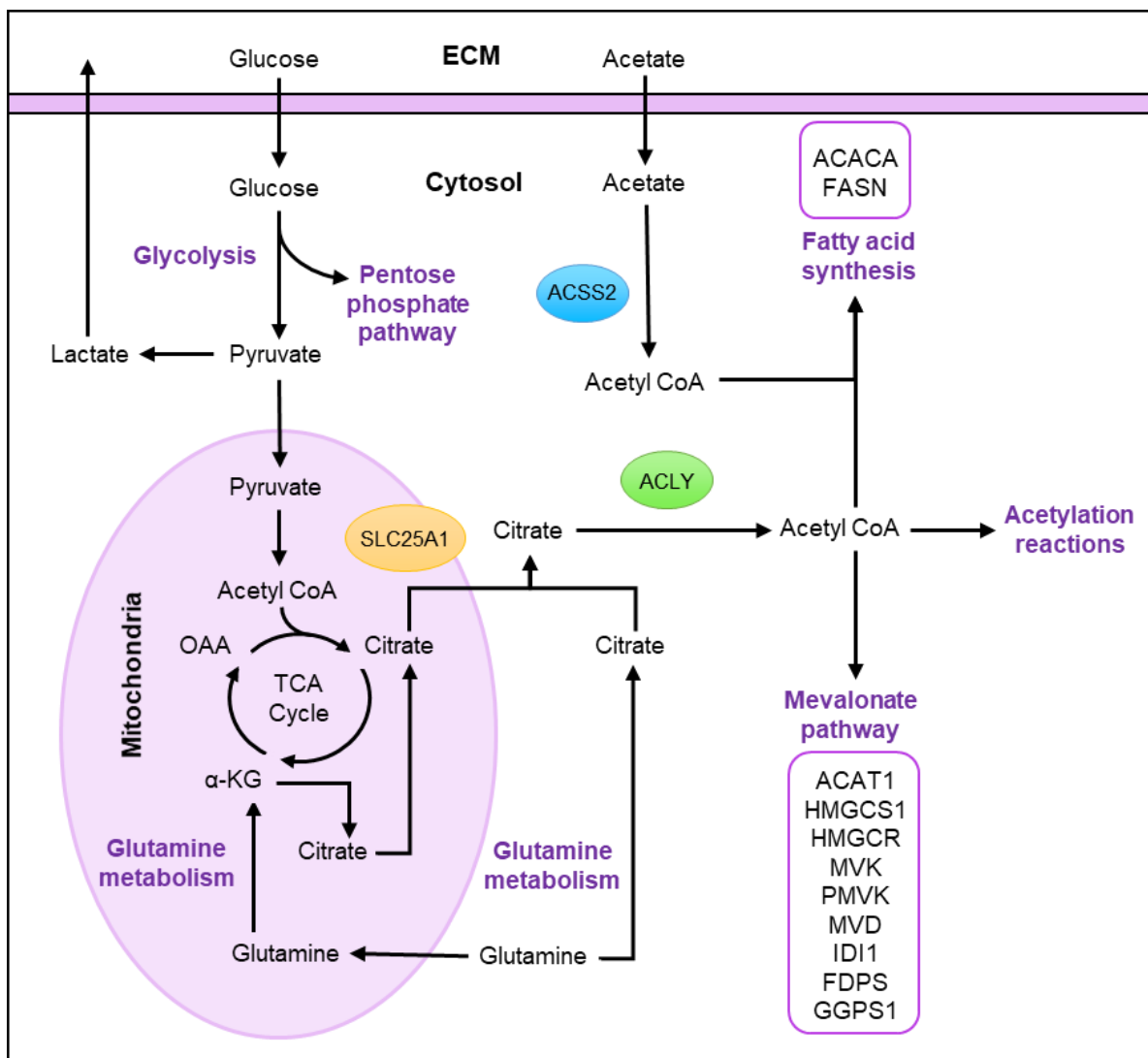
Figure 1 ACLY catalyzes the ATP dependent conversion of citrate to acetyl-CoA and oxaloacetate. ACLY links glycolysis and glutaminolysis and lipogenesis. OAA: Oxaloacetate, α-KG: α-Ketoglutarate, SLC25A1: mitochondrial citrate carrier, ACLY: ATP citrate lyase, ACSS2: Acyl-coenzyme A synthetase, ACACA: Acetyl-CoA carboxylase-α, FASN: Fatty acid synthase, ACAT1: Acetyl-CoA acetyltransferase, HMGCS1: HMG-CoA synthase, HMGCR: HMG-CoA reductase, MVK: Mevalonate kinase, PMVK: Phosphomevalonate kinase, MVD: Mevalonate diphosphate decarboxylase, IDI1: Isopentenyl diphosphate delta isomerase, FDPS: Farnesyl diphosphate synthase, GGPS1: Geranylgeranyl pyrophosphate, ECM: Extracellular matrix.

Metabolic alterations in cancer cells provide unique targets to improve cancer therapeutics. Myeloma cells are addicted to glutamine metabolism, which is indicated by the increased levels of secreted ammonium, the nitrogen waste product of glutaminolysis.[61,62]

Glutamine deprivation induces BIM binding to BCL-2, which is subsequently released upon treatment with venetoclax, inducing apoptosis.[61] Additionally, it has been shown that inhibition of the electron transport chain increases dependence on BCL-2, thus sensitizing myeloma cells to venetoclax treatment.[63] Myeloma cells are also uniquely dependent on GLUT4, GLUT8, and GLUT11 for proliferation and survival, indicating that glucose transport inhibitors such as ritonavir may be a useful therapeutic tool.[64] Myeloma cells can also utilize the lactate from their environment and utilize it in cellular metabolism, a phenomenon called the reverse Warburg effect.[65–67] Interestingly, lactate dehydrogenase (LDH) serum levels appear to increase during disease progression in patients.[68] Inhibition of the lactate importer monocarboxylate transporter (MCT) 1 results in apoptosis due to insufficient fuel, while inhibition of the lactate exporter MCT4 results in acidosis due to lactate accumulation.[67,69] Treatment with the FASN inhibitor orlistat and the carnitine acyltransferase (CPT1) inhibitor etomoxir inhibited myeloma cell proliferation and orlistat sensitized cells to induction of apoptosis by bortezomib.[70]

ATP Citrate Lyase

ACLY (17q21.2) is a 480kDa homotetrameric protein that converts citrate to oxaloacetate and acetyl-CoA, which is used for fatty acid synthesis, steroid synthesis, and acetylation reactions.[71–74] The structure of ACLY is shown in Figure 2. ACLY contains six domains: an N-terminal citryl-CoA synthetase (CCS) domain, consisting of CCSβ and CCSα, a CoA binding domain, a His760 catalytic phosphorylation site, an ATP binding domain, a citrate binding domain, and a C-terminal citryl-CoA lyase (CCL) domain.[71,73] ACLY is only active as a homotetramer.[74] ACLY is expressed ubiquitously, but is more strongly expressed in the liver and adipose tissue and is found in the nucleus and the cytoplasm.
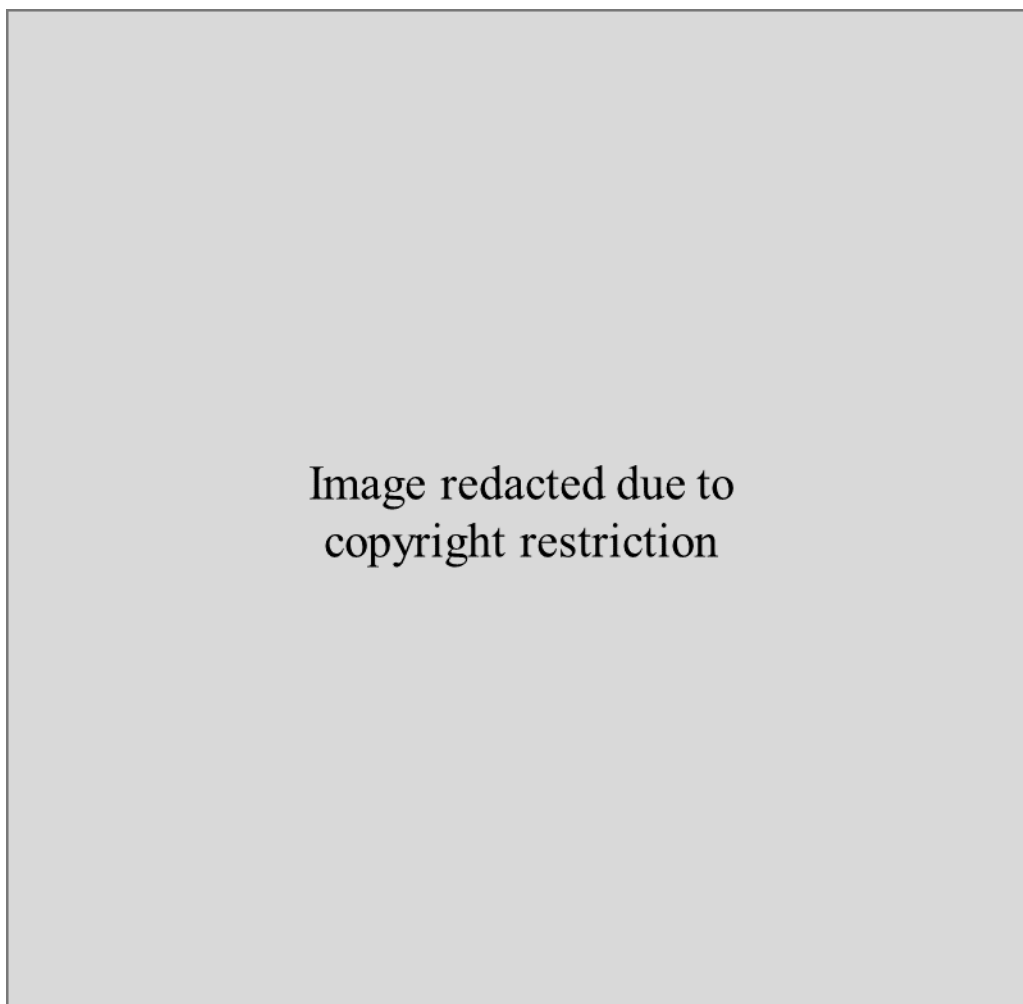
Figure 2 Structure of ACLY. a. domain organization of the human ACLY gene. ACLY-A is the ancestral part consisting of residues 1-425. ACLY-B consists of residues 487-1101. ACLY-A/B is joined by a linker region. **b.** Crystal structure of human ACLY. The colors used in the crystal structure correspond to the colors in **a.** Citrate, CoA and ADP are shown in their respective binding sites as colored spheres. Image is figure 1 from https://doi.org/10.1038/s41586-019-1095-5. Image removed due to copyright restriction.

ACLY has been implicated in many cancers. ACLY upregulation affects proliferation, survival, and chemosensitivity. Zaidi et al. (2012) silenced *ACLY* in prostate, lung, and liver cancer cell lines and found conditional growth arrest depending on their environment. Under conditions of lipid starvation, ACLY knockdown induced cell cycle arrest, which was rescued by fatty acid and/or cholesterol supplementation. Additionally, ACLY knockdown induced

expression of acyl-CoA synthetase short-chain family member 2 (ACSS2), which catalyzes the conversion of acetate to acetyl-CoA, indicating a compensatory mechanism for fatty acid synthesis in the absence of ACLY.[75] Wen et al (2019) showed that in colorectal cancer cell lines ACLY interacts with β-catenin, stabilizing it and translocating to the nucleus. Additionally, ACLY KO cells expressed more E-cadherin, and less N-cadherin, β-catenin, vimentin, and snail, indicating that ACLY plays a role in the epithelial mesenchymal transition (EMT). ACLY KO also attenuates metastasis and invasion in vitro and in vivo.[76] Nuclear ACLY promotes DNA damage repair and homologous recombination.[77] Interestingly, in head and neck squamous cell carcinoma, ACLY inhibition conferred radiosensitivity, due to a diminished homologous recombination.[78]

Outside of cancer, ACLY has been implicated in development and chronic disease. Rhee et al (2019) showed that inhibition of ACLY causes cell cycle arrest in myeloid progenitor cells as they differentiate into macrophages, and acetyl-CoA or acetate supplementation were able to rescue cell cycle arrest.[79] Calejman et al (2020) showed that mTORC2/AKT signaling activates ACLY and ACSS2, thus upregulating nuclear acetyl-CoA production, resulting in the epigenetic modifications necessary for the differentiation of brown adipocytes.[80] Chu et al (2010) showed that increased fatty acid palmitate and/or ACLY knockdown increased pancreatic β-cell apoptosis and endoplasmic reticulum (ER) stress, implicating it in progression of type 2 diabetes mellitus.[81]

ACLY's unique role in cancers and chronic diseases makes it a potential molecular target. One ACLY inhibitor is (-)-hydroxycitrate (Figure 3.a) ($K_i = 0.15$ μM).[82,83] It is a competitive inhibitor and was first isolated from *Garcinia cambogia*, the Malabar tamarind fruit and common weight loss supplement. (-)-Hydroxycitrate reduced cholesterol synthesis by 27%

in HepG2 cells. NDI-091143 (Figure 3.c) is unique in that it allosterically inhibits ACLY.[72] NDI-091143 binds to a hydrophobic cavity near the citrate binding domain, and causes conformational changes to the enzyme to disrupt citrate binding.[72] BMS-303141 (Figure 3.b) is a 2-hydroxy-N-arylbenzenesulfonamide with potent inhibitory effects in vitro ($IC_{50} = 0.13\mu M$). Lastly, the compound SB-204990 (Figure 3.e) is the cell-penetrant lactone prodrug of the ACLY inhibitor SB-201076 (Figure 3.d) ($K_i = 1\mu M$).[84] SB-204990 was shown to reduce cholesterol and fatty acid synthesis in HepG2 cells by 91% and 82% respectively.[84] Hatzivassiliou et al (2005) demonstrated that chemical inhibition of ACLY using SB-204990 reduced lipid synthesis in a dose dependent manner, delayed cell cycle progression, reduced cell survival, and decreased tumor mass in vivo using A549, PC3, and SKOV3 cell lines.[85]
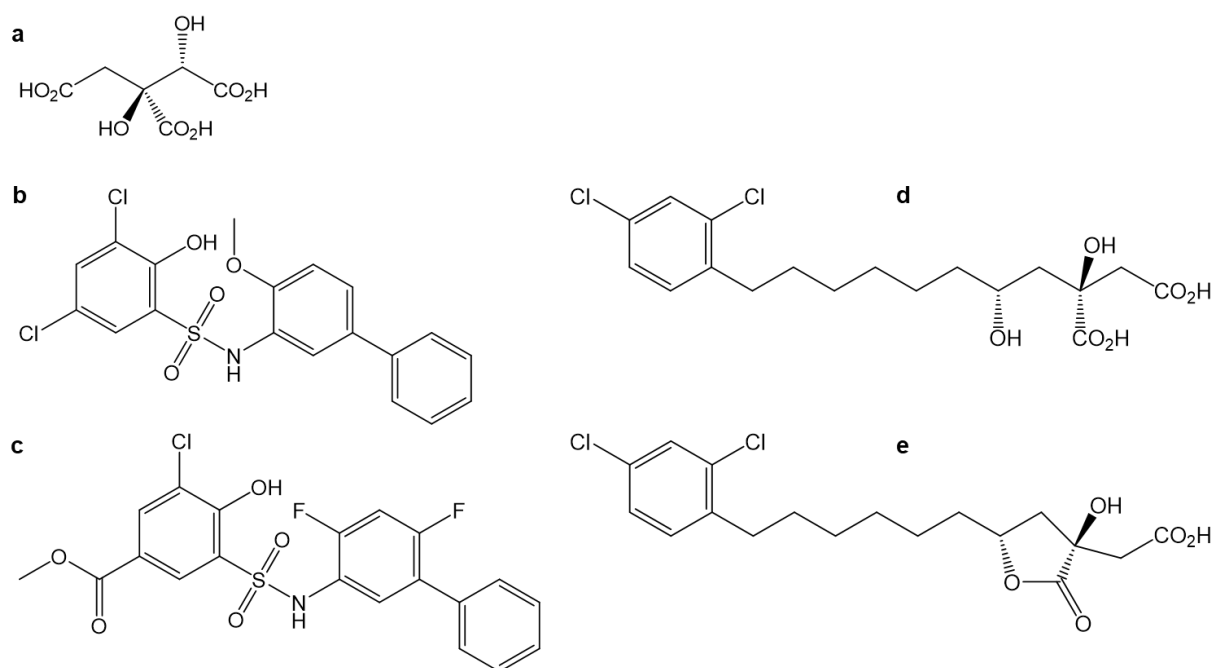
Figure 3 Chemical structures of ACLY inhibitors found in the literature.
a. (-)-hydroxycitrate **b.** BMS-303141 **c.** NDI-091143 **d.** SB-201076 **e.** SB204990.

Scope of Thesis

Though ACLY has been shown to regulate cell proliferation and survival in a myriad of cancers, its role in multiple myeloma has yet to be studied. Looking at the CoMMpass dataset, we know that high mRNA expression of ACLY is associated with significantly worse patient prognosis (Figure 4). The goal of this thesis is to begin to investigate the role of ACLY in multiple myeloma biology and drug sensitivity.

The action of ACLY results in a major source of cytoplasmic acetyl-CoA, a crucial biomolecule for formation of cell membranes and membrane bound organelles. Given that multiple myeloma cells are secretory in nature, we hypothesized that multiple myeloma cells would depend on ACLY for growth and to maintain organellar homeostasis of the secretory pathway, such as the ER, by fueling lipogenesis. Our approach to address this hypothesis was two-fold. We aimed to develop stable ACLY knockout cells using a doxycycline inducible CRISPR/Cas9 system in a HMCL that is not dependent on ACLY. We also aimed to study HMCLs treated with the ACLY chemical inhibitor SB-204990. With these two approaches, we aimed to investigate the impact of ACLY on proliferation, cell death, and chemosensitivity to induction of apoptosis by bortezomib.
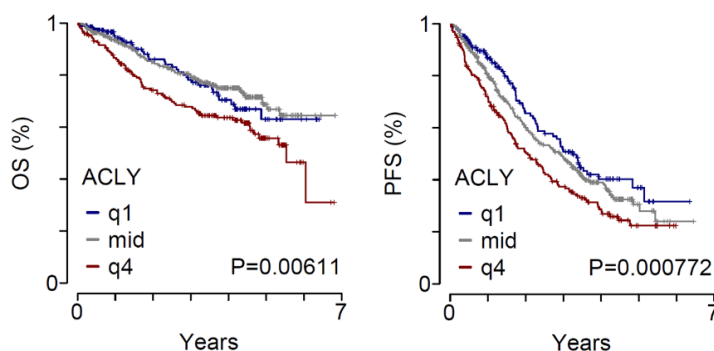
Figure 4 Kaplan-Meier curves of overall survival (OS) and progression free survival (PFS) in the CoMMpass dataset. Patients with the highest quartile of *ACLY* transcript expression is shown in red and patients with the lowest quartile of *ACLY* transcript expression is shown in blue. Ben Barwick generated these Kaplan-Meier curves through the MMRF Research Gateway (https://research.themmrf.org/)

Materials and Methods

a. Overlap extension PCR
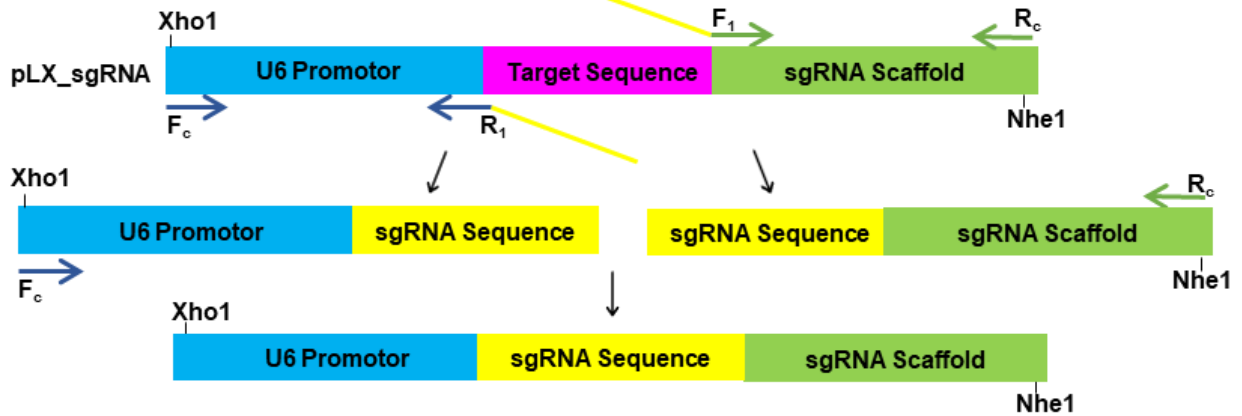
b. Restriction enzyme double digestion and ligation
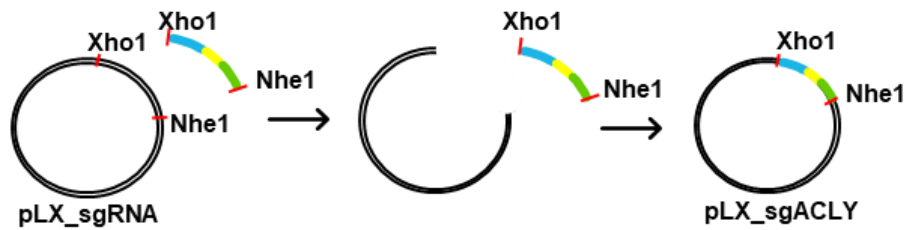
c. Competent cell transformation and cloning plasmid purification
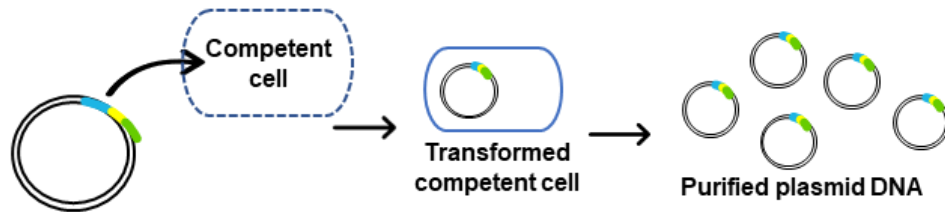
d. Transfection of HEK293T cells and collection of virus
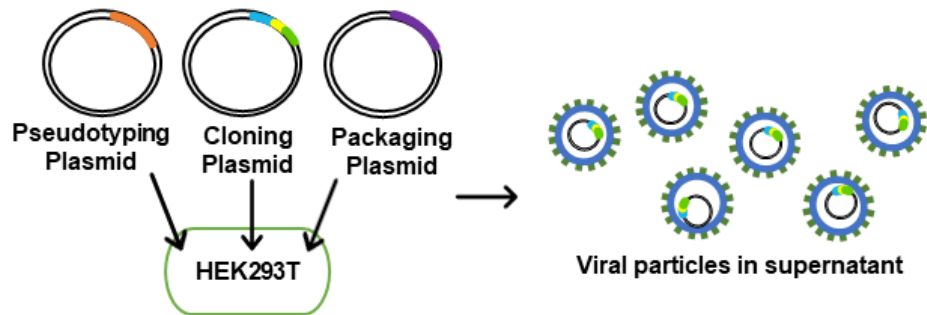
e. Infection of human myeloma cells
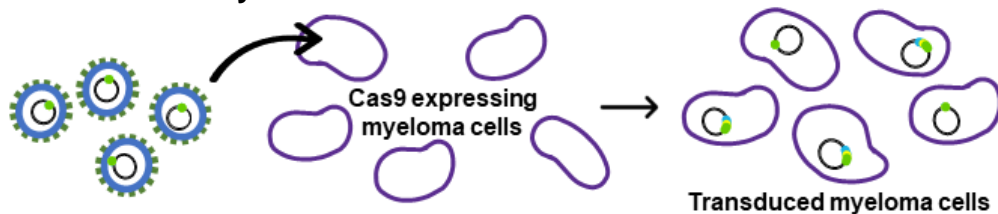
Figure 5 Methodology for creating doxycycline-inducible Cas9 expressing ACLY^{-/-} **myeloma cells. a.** sgRNA-specific forward and reverse primers were designed. Overlap extension PCR was used to clone the sgRNA into the target sequence. F_c and R_c are the common forward and reverse primers. F_1 and R_1 are the sgRNA-specific forward and reverse primers. **b.** Restriction enzyme double digestion of pLX_sgRNA and the PCR product and ligation were performed to create pLX_sgACLY. **c.** Competent cells (JM109) were transformed and grown on an agar plate with carbenicillin. Plasmid DNA was purified from transformed cells. Sequencing confirmed that the plasmids contained the sgRNA. **d.** Transfection of HEK293T cells was performed to produce a lentivirus containing pLX_sgACLY. Lentivirus was harvested by collecting the supernatant of the HEK293T cells. **e.** Dox-inducible Cas9 expressing myeloma cells were infected with lentivirus. Blasticidin selection was used to isolate transduced cells. Doxycycline was added to turn on the inducible Cas9, resulting in an ACLY knockout cell line.

Cell Lines and Culture Conditions

      LP1 and OCI-My5 myeloma cell lines were obtained from Dr. Jonathan Keats (Translational Genomics Research Institute, Phoenix, AZ). HEK293T cells were purchased from the American Type Culture Collection (Manassas, VA). LP1 and OCI-My5 cell lines containing a doxycycline inducible cas9 system were developed using the pCW-Cas9 plasmid [86] (Addgene, plasmid #50661, Watertown, MA), deposited by Eric Lander and David Sabatini's lab.[86] (Addgene, plasmid #50661, Watertown, MA), deposited by Eric Lander and David Sabatini's lab. Myeloma cell lines were cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% HEPES buffer, 100 U/mL penicillin/streptomycin, and 2mM L-glutamine (all from Cellgro, Mediatech, Manassas, VA). HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated FBS, 100 U/mL penicillin/streptomycin, 2mM L-glutamine, 1% non-essential amino acids, and 1mM sodium pyruvate (all from Cellgro, Mediatech). All cells were cultured at 37°C with 5% carbon dioxide.

sgRNA Design

      Six sgRNA sequences were selected for *ACLY* (located on chromosome 17q21.2) from the protein coding sequence using http://crispor.tefor.net based on the specificity score, predicted efficiency, and out-of-frame score. Forward and reverse primer oligonucleotides were designed for the six sgRNAs and adapter sequences were added (see Appendix II for sequences). PCR with the common forward (F_c) and reverse (R_c) primers was used to individually clone and ligate the sgRNA sequences into the pLX_sgRNA plasmid[86] (Addgene, plasmid #50662) between the Xho1 and Nhe1 restriction sites (Figures 1A and 1B). The pLX_sgRNA plasmid containing the *ACLY* sgRNAs will hereby be referred to as pLX_sgACLY.

Xho1 and Nhe1 restriction enzymes were purchased from New England Biolabs (Ipswich, MA). PCR was performed using the Herculase II fusion DNA Polymerase kit from Agilent Technologies (Santa Clara, CA) according to the manufacturer's instructions on the S1000 Thermal Cycler from Bio-Rad (Hercules, CA). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Competent Cell Transformation and Plasmid Purification

JM109 competent cells (Promega, Madison, WI) were transformed with the pLX_sgACLY constructs and plated on Lysogeny broth (LB) agar plates with carbenicillin. The plates were incubated at 37°C overnight. Colonies were selected from each plate, deposited in Falcon tubes with LB broth and carbenicillin, and incubated at 37°C. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit from Qiagen (Hilden, Germany). Plasmid DNA and the F_c samples were sent to Psomagen (Rockville, MD) for sequencing to confirm the successful cloning of each pLX_sgACLY construct (Figure 1C).

Transfection of HEK293T Cells

HEK293T cells were co-transfected with the pCMV-dR8.2 dvpr (Addgene, plasmid #8455) packaging plasmid[87], pCMV-VSV-G (Addgene, plasmid #8454) envelope plasmid[87] and pLX_sgACLY cloning plasmids using lipofectamine (Thermo Fisher Scientific, Waltham, MA) (Figure 1D). Media was changed after 6 and 24 hours post-transfection. Viral supernatant was collected 72 hours post-transfection after filtering through a 0.45µm syringe filter (Pall, Port Washington, NY). The viral supernatant was stored in 1mL aliquots at -80°C.

Lentiviral Transduction

LP1[cas9] and OCI-My5[cas9] cells were infected with each of the six viruses in 24-well cell culture plates using polybrene medium (Figure 1E). Spinoculation was performed by

centrifuging the cell mixture at 2,250rpm for 1.5 hours at 37°C. After centrifugation, virus was removed, and cells were resuspended in complete myeloma media. After 24 hours, blasticidin was added to the media to select for the transduced cells. Note that the amount of blasticidin required for each cell line differs as they have different levels of blasticidin sensitivity (20µg/mL for LP1 Cas9 and 7.5µg/mL for OCI-My5 Cas9). After the cells were selected in blasticidin for two weeks, the cells were resuspended in media with 1µg/mL doxycycline to induce cas9 expression. Cas9 was induced for one week before downstream analysis.

Western Blot Analysis

Whole cell lysates were prepared from cultured cells after washing twice in phosphate buffered saline (PBS). Cells were resuspended in lysis buffer consisting of RIPA buffer, PMSF, and protease inhibitor cocktail. Samples were centrifuged for 10 minutes at 4°C spinning at 15,000rpm and the supernatant was collected. Protein concentration was determined by the BCA assay. Western samples were prepared by adding β-mercaptoethanol and 6x Laemmli buffer to 25µL of sample. Samples were boiled and centrifuged. Approximately 20µg of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred from the gel to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK), and membranes were blocked in 5% nonfat milk in tris-buffered saline with 0.1% Tween 20 (TBS-T) for one hour at room temperature. Blots were incubated in primary antibody overnight at 4°C and washed in TBS-T. Membranes were probed with the corresponding secondary antibody conjugated to a horseradish peroxidase (HRP) for one hour at room temperature, then visualized using enhanced chemiluminescence. Blots were analyzed by densitometry using Fiji (ImageJ) software.

Primary antibodies used were as follows: rabbit anti-ATP citrate lyase pAb (Cell Signaling Technology, #4332, Danvers, MA), mouse anti-β-actin mAb (Santa Cruz Biotechnology, Dallas, TX), rabbit anti-DYKDDDDK Tag pAb (Thermo Fisher Scientific, #PA1-984B). Secondary antibodies used were as follows: anti-mouse IgG1- HRP (GE Healthcare, #NA931), anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-45040).

Single Cell Cloning

Cells were diluted to a concentration of ~1.3 cells/mL in 20% FBS media. Cells were aliquoted into 96-well plates, plates were wrapped in aluminum foil, and incubated at 37°C with 5% carbon dioxide. Colony growth was assessed after 4 weeks and subsequently grown out for downstream analysis.

Quantitative Real-Time PCR

RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Complementary DNA was generated using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the TaqMan gene expression master mix (Thermo Fisher Scientific, #4369016) with the CFX96 Real Time System (Bio-Rad) according to the manufacturer's instructions. Fold change of ACLY mRNA was calculated. The Hs00982738_m1 probe (Thermo Fisher Scientific, # 4331182) was used to detect ACLY mRNA and the Hs99999905_m1 GAPDH probe (Thermo Fisher Scientific, # 4331182) was used as a control.

Sequencing of Genomic DNA

Genomic DNA was extracted from single cell clones using the Blood and Tissue Kit from Qiagen (#69504) according to the manufacturer's instructions. PCR was used to amplify the

region of DNA that contained the sgRNA. The TOPO TA cloning kit (Thermo Fisher Scientific, #K4575J10) was used to ligate the desired DNA fragment into the TOPO TA vector. One Shot TOP10 chemically competent cells were transformed, plated with X-gal, and incubated overnight at 37°C. From each plate, ten colonies were selected, miniprepped, and sent to Psomagen for sequencing.

PicoProbe Acetyl-CoA Fluorometric Assay

Myeloma cells were cultured in 24 well plates, 1 million cells per well in 1mL of medium treated with various concentrations of the ACLY chemical inhibitor SB-204990 (Cayman Chemical, #15245, Ann Arbor, MI). The cells were incubated for the designated amount of time. This assay was performed using the PicoProbe Acetyl-CoA Fluorometric Assay Kit (Biovision, #K317, Palo Alto, CA) according to the manufacturer's instructions. Briefly, free CoA is quenched, acetyl-CoA is converted to CoA, CoA is reacted to form NADH, which interacts with the PicoProbe to generate fluorescence that can be measured at Ex/Em = 535/587nm.

Cell Death Assay

Myeloma cells were cultured in 24 well plates, 250,000 cells per well in 1mL of medium, and treated with various concentrations of SB-204990, bortezomib or combinations of SB-204990 and bortezomib. The cells were incubated for the designated amount of time. Cells were collected, washed with PBS, resuspended in Annexin buffer (containing 10mM HEPES, 140mM NaCl, and 2.5mM $CaCl_2$) with Annexin V-FITC (BioVision, #1001-1000, Palo Alto, CA) and propidium iodide (PI) (Sigma-Aldrich, St Louis, MO). Cell death was measured with the BD FACSymphony A3 flow cytometer analyzer and data were analyzed using FlowJo (BD).

Cell Proliferation Assay

Myeloma cells were cultured in 24 well plates, 250,000 cells per well in 1mL of medium, and treated with various concentrations of SB-204990, bortezomib or combinations of SB-204990 and bortezomib. The cells were incubated for the designated amount of time. Cells were collected, mixed volumetrically with AccuCheck Counting Beads (Thermo Fisher Scientific, #PCB100), cell counts were measured with the BD FACSymphony A3, and data were analyzed using FlowJo.

Results

Cancer Dependency Map analyses show most HMCLs exhibit dependency on *ACLY*

We wanted to identify genes that were essential in myeloma cells, but not in other cell types. Through the Broad Institute's Cancer Dependency Map (DepMap) portal, we identified *ACLY* as a candidate gene. *ACLY* codes for the cytosolic protein ATP citrate lyase (ACLY), an enzyme that catalyzes the formation of acetyl-CoA, a critical substrate for lipogenesis, cell growth, and membrane maintenance. We reasoned that myeloma cells could be dependent on ACLY to maintain their plasma cell biology, including maintaining the endoplasmic reticulum and secreting proteins. We also included *SLC25A1, ACSS2, ACACA*, *FASN*, *ACAT1, HMGCS1, HMGCR, MVK, PMVK, MVD, IDI1, FDPS,* and *GGPS1* in the analysis. SLC25A1 transports citrate from the mitochondria to the cytoplasm. ACSS2 converts acetate to acetyl-CoA. ACACA and FASN are involved in fatty acid synthesis. All of the other genes in the analysis are components of the mevalonate pathway. Figure 6a shows a heat map summarizing the dependencies of HMCLs and non-HMCLs. The heat map was built using CERES scores from the DepMap Portal. The CERES score is a representation of data from a CRISPR screen in which enrichment or depletion of sgRNAs targeting a specific gene is calculated and normalized to the gene copy number. A CERES score of > 0 indicates that knockout of the gene resulted in enhanced growth, < -0.5 indicates dependency, and < -1.0 indicates essentiality of the given gene. The dark blue color indicates high dependency on the given gene, whereas the white indicates no dependency.

In this analysis, 14/19 of the HMCLs were dependent on *ACLY*, whereas 351/713 of the non-HMCLs were dependent on *ACLY*. According to a Welch's t-test, there is a significant difference between HMCLs' and non-HMCLs' dependency on *ACLY* (Figure 6c). Additionally, there is a significant difference between dependency on *MVK,* but nearly all cell lines were

dependent on *MVK*, so this was not unique to myeloma. Most cell lines were dependent on members of the mevalonate pathway and were not dependent on fatty acid synthesis. When we broke down the non-HMCLs by cell type, further analysis showed that *ACLY* dependency varies greatly across different cell types (Figure 6b) . Between HMCLs there is variance in expression and dependency on *ACLY* (Figure 7a, b), but there is no statistical correlation between expression and dependency on *ACLY* (Figure 7c).
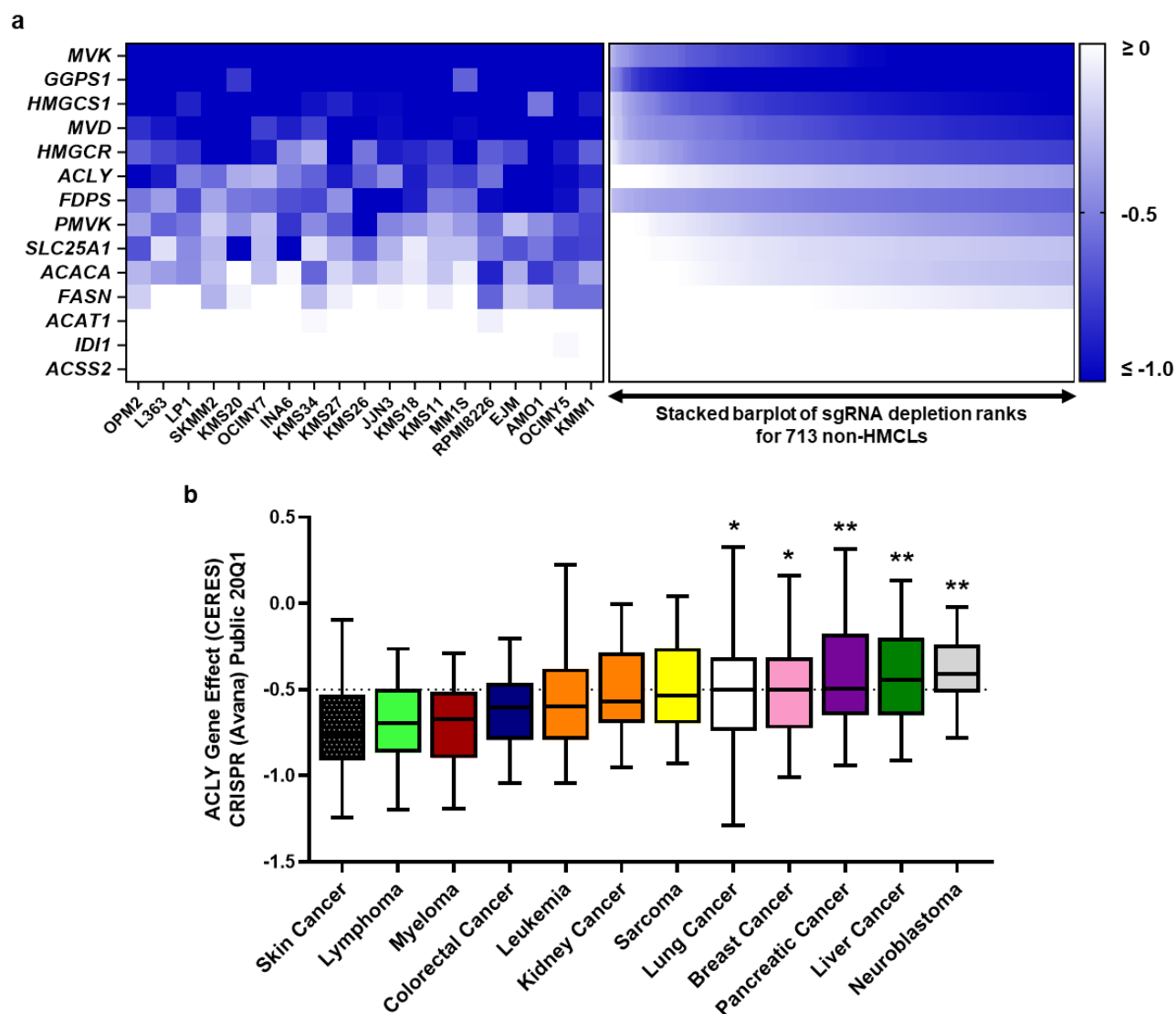
c

Sources of Acetyl-CoA
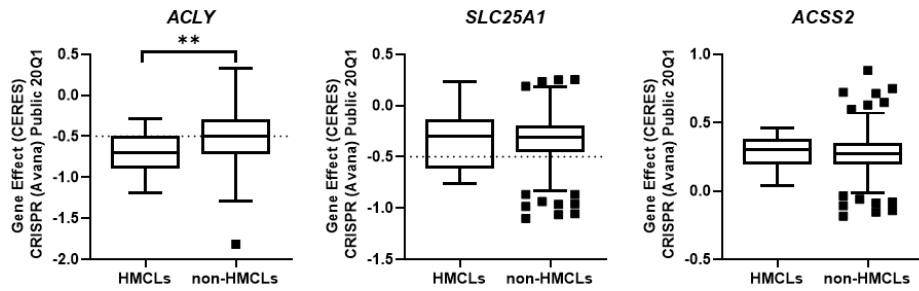
Fatty Acid Synthesis
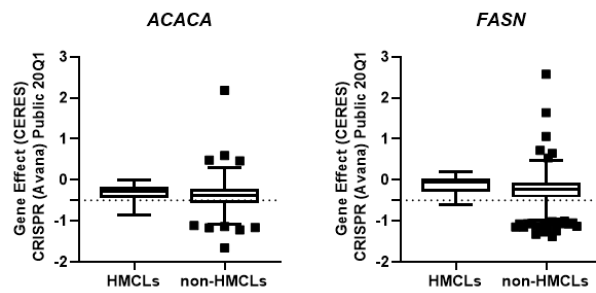
Mevalonate Pathway
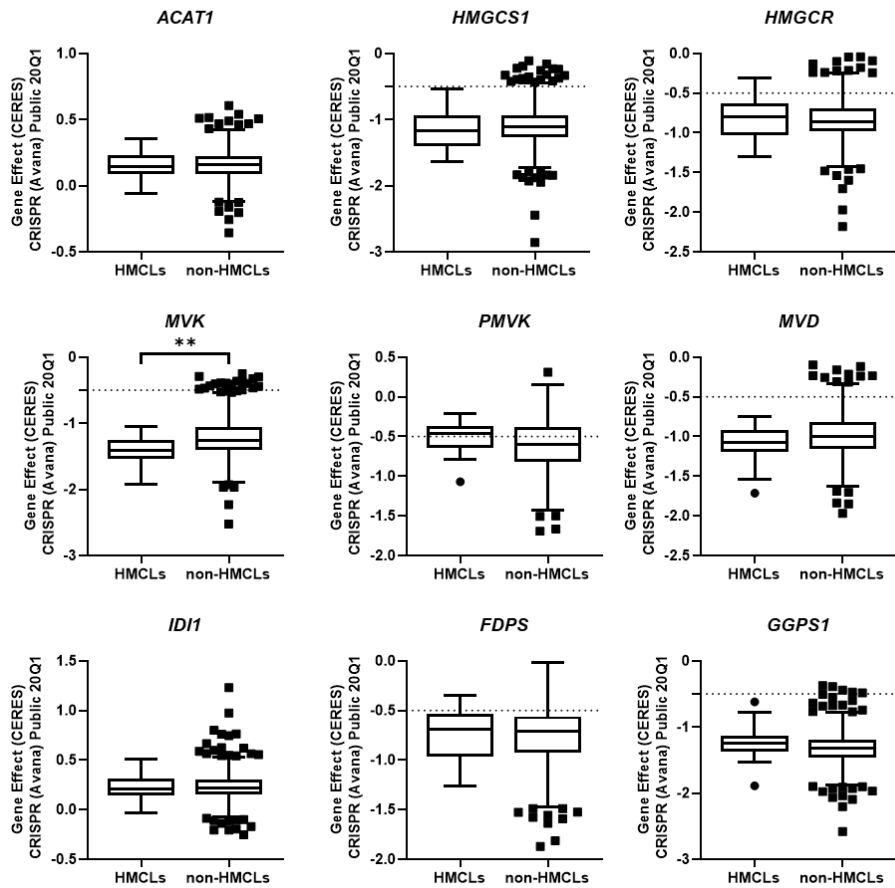
Figure 6 Cancer Dependency Map analysis of dependencies of HMCLs and 713 non-HMCLs on *ACLY*, genes involved in sourcing cytoplasmic acetyl-CoA, fatty acid synthesis, and the mevalonate pathway. a. The heat map on the left shows the dependencies of 19 HMCLs and the right shows the dependencies of more 713 non-HMCLs. This heat map was created based on CERES scores. In this analysis, white indicates no dependency, while dark blue indicates higher dependency on the given gene. The heat map is organized by greatest to least dependency of HMCLs. **b.** Box and whisker plots of CERES scores of cell lines from different cancer types. Each cancer cell type is represented in its own box and whisker plot. The statistical measures used were one-way ANOVA ($p<.001$), and Dunnett's multiple comparisons test to multiple myeloma (* $p < 0.05$, ** $p < 0.01$). **c.** The dependencies of HMCLs and combined non-HMCLs are shown in these box and whisker plots. The plots are organized by gene and categorized by function. Welch's two-tailed t-test was used to determine significance. (** $p < 0.01$). All data used in this figure comes from the Broad Institute's Depmap portal.[88–90] These data are published under the CC Attribution 4.0 license. DepMap, Broad (2020): DepMap 20Q1 Public. figshare. Dataset doi:10.6084/m9.figshare.11791698.v2.
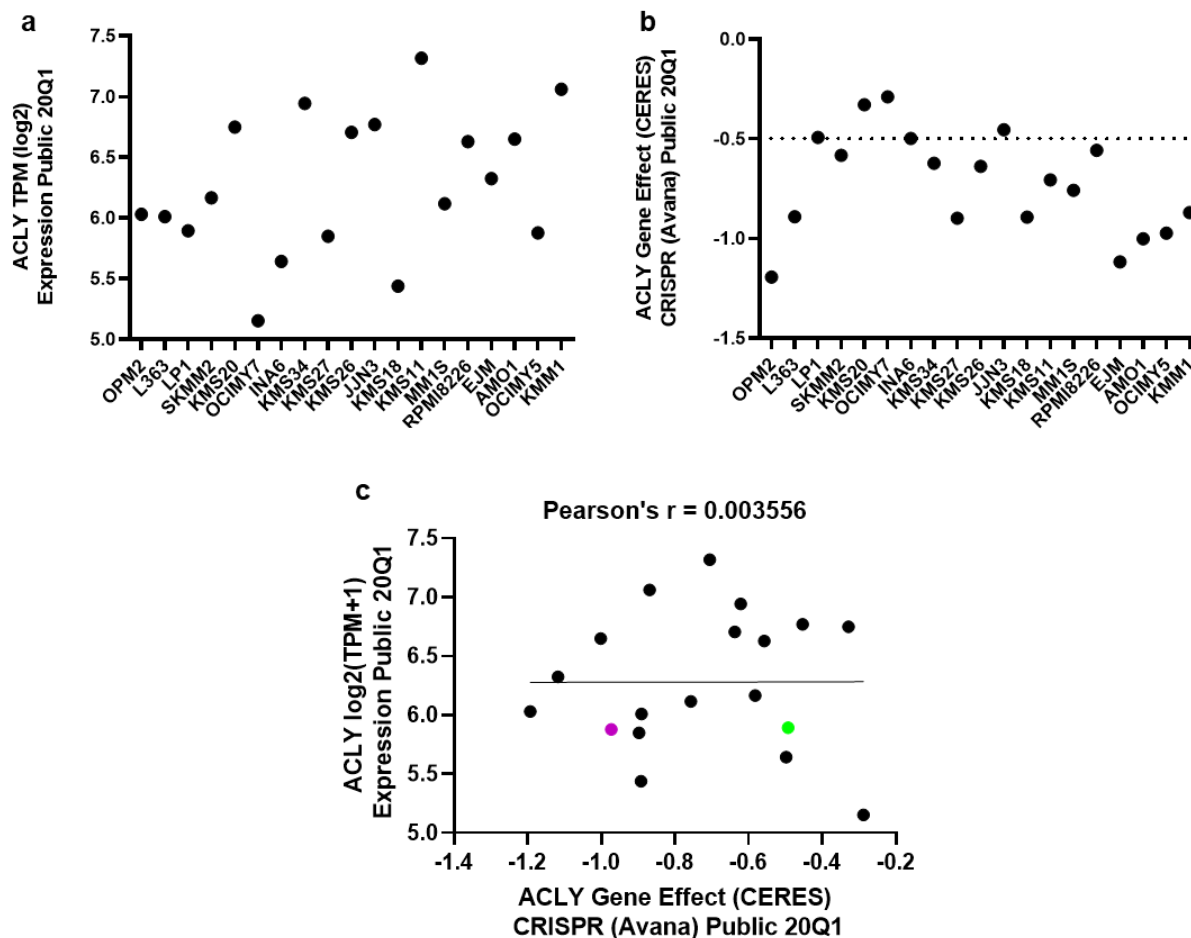
Figure 7 Cancer Dependency Map analyses of expression levels and dependency of HMCLs on ACLY. a. Scatter plot of expression levels of ACLY in 19 different HMCLs. TPM stands for transcripts per million and is based on RNAseq data. **b.** Scatter plot of HMCLs' dependency scores (CERES) on ACLY. **c.** Scatter plot of HMCLs' expression plotted against dependency. The Pearson's correlation test showed no correlation between expression and dependency. The pink point is OCI-My5 and the green point is LP1. All data used in this figure comes from the Broad Institute's Depmap portal.[86–88] These data are published under the CC Attribution 4.0 license. DepMap, Broad (2020): DepMap 20Q1 Public. figshare. Dataset doi:10.6084/m9.figshare.11791698.v2.

Custom sgRNA sequences were cloned into pLX_sgRNA between the Xho1 and Nhe1 restriction sites.

Six 20bp sgRNA sequences, with specificity scores greater than 90, were chosen to selectively bind to *ACLY* and generate a double strand break at the target site. The PCR products were resolved using gel electrophoresis. (Figure 8a) The gel showed that the PCR amplified the correct sequences. Overlap extension (OE) PCR was used to anneal the two DNA fragments and join the U6 promoter, target, and sgRNA scaffold sequences. The PCR products were resolved using gel electrophoresis. (Figure 8b) The gel showed that OE-PCR amplified the correct sequences. The DNA fragment containing the U6 promoter, sgRNA sequence, and sgRNA scaffold and the empty pLX_sgRNA were digested using the restriction enzymes Xho1 and Nhe1. The DNA fragment and linearized vector were ligated. Plasmid DNA was grown and purified from JM109 cells, the presence of the guide was confirmed by sequencing, and lentivirus was produced using transfected HEK293T cells.
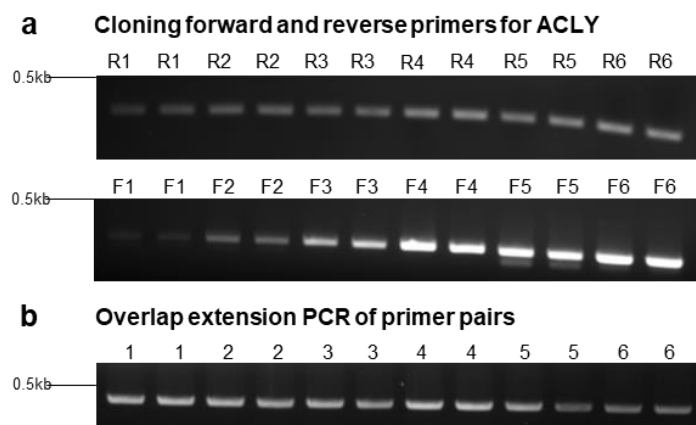
Figure 8 Gel electrophoresis was used to resolve PCR products. a. Top image is the gel used to resolve the PCR product containing the sgRNA sequence and the U6 promotor. This PCR product was expected to be 261bp. The bottom image shows the gel used to resolve the PCR product containing the sgRNA sequence and the sgRNA scaffold. This PCR product was expected to be 96bp. **b.** Image of gel used to resolve the overlap extension PCR product, amplified using the common forward and reverse primers. This PCR product was expected to be 337bp. The sequences of the common and custom primers are found in Appendix II.

ACLY was knocked out in OCI-My5 Cas9 cells after one week of Cas9 induction, but at two weeks ACLY expression increased.

OCI-My5 Cas9 cells were infected with each of the six lentiviruses. Post-infection the cells were cultured in media with blasticidin to select for cells that received the plasmid. After one week of selection, they were cultured in media with doxycycline to induce Cas9 expression. Western blotting was performed to determine when ACLY was effectively knocked out (Figure 9). After 1 week in doxycycline, ACLY expression decreased in cells infected with the sgACLY2 and sgACLY3 constructs. At week 2, ACLY expression appeared to return to the same levels as the uninfected control.
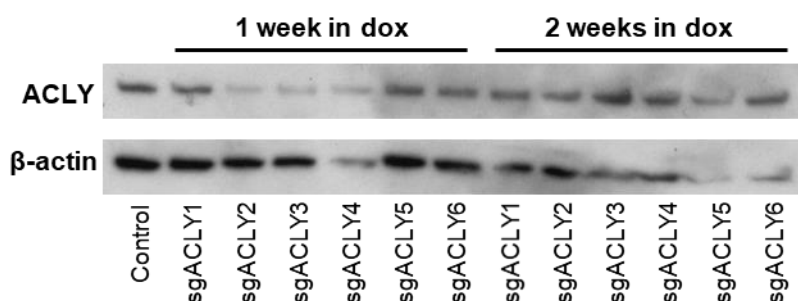
Figure 9 Western blot of infected and control OCI-My5 Cas9 cells. OCI-My5 Cas9 cells containing six custom sgRNA constructs were cultured in media with doxycycline for one week or two weeks. The first lane is OCI-My5 Cas9 control. We probed for ACLY and β-actin was used as a loading control.

ACLY was knocked out in OCI-My5 Cas9 and LP1 Cas9 cells after one week of Cas9 induction.

OCI-My5 Cas9 and LP1 Cas9 cells were infected with each of the six lentiviruses. After one week in selection in blasticidin and another week in doxycycline, infected cells were lysed and ACLY expression was determined by western blotting (Figure 10). In OCI-My5 Cas9 cells, ACLY expression decreased in cells infected with the sgACLY1 and sgACLY2 constructs. In LP1 Cas9 cells, ACLY expression decreased in cells infected with the sgACLY2, sgACLY5, and sgACLY6 constructs. We chose to continue downstream experiments using the cells infected with the sgACLY2 and sgACLY5 constructs.
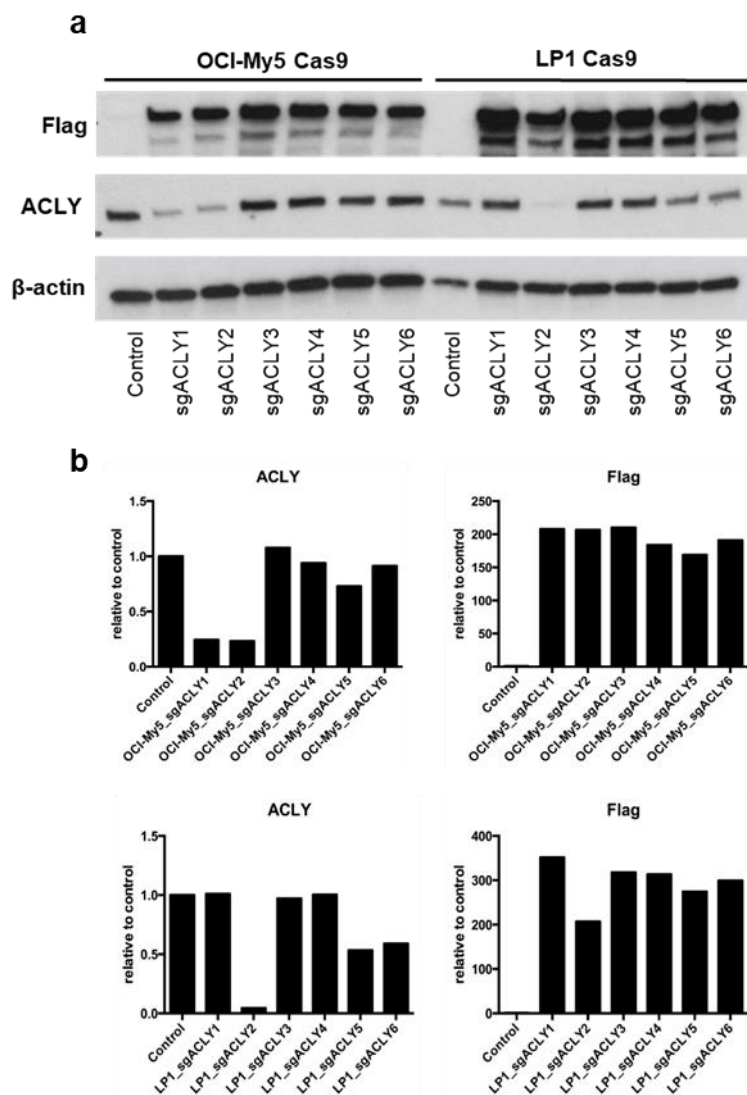
Figure 10 Western blots and quantifications of infected and control OCI-My5 Cas9 and LP1 Cas9 cells. a. After one week cultured in doxycycline, infected OCI-My5 Cas9 and LP1 Cas9 cells were lysed and probed for Flag, ACLY, and β-actin. **b.** Western blots were quantified and as a ratio of ACLY/β-actin or Flag/β-actin and then normalized to the uninfected control. OCI-My5 Cas9 quantification is on the top and LP1 Cas9 quantification is on the bottom.

LP1 sgACLY2 and LP1 sgACLY5 cells were single cell cloned. One line appeared to have almost complete knockout, while three others expressed about half the protein of the control sample.

OCI-My5 sgACLY2 and OCI-My5 sgACLY5 single cell clones never grew out, but LP1 sgACLY2 and LP1 sgACLY5 single cell clones grew out after about 4 weeks. The single cell clones were moved to individual flasks and allowed to grow for another week before western blotting to determine which cell lines knocked out ACLY (Figure 11). L5-1 appeared to have almost no ACLY, while L2-4, L5-3, and L5-8 showed decreased expression of ACLY.
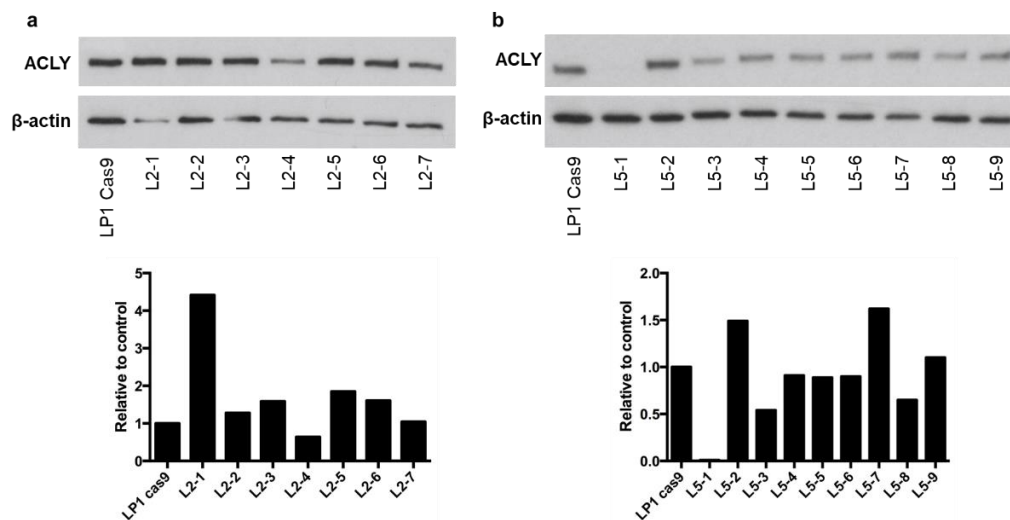
Figure 11 Western blots and quantifications of LP1 sgACLY single cell clones. a. Western blot and quantifications for LP1 sgACLY2 single cell clones and **b.** LP1 sgACLY5 single cell clones. Blots were probed for ACLY and β-actin, quantified and as a ratio of ACLY/β-actin and then normalized to the uninfected control. The single cell clones will be hereby abbreviated; e.g. L2-4: LP1 sgACLY2 clone 4.

Quantitative real-time PCR on LP1 sgACLY2 and LP1 sgACLY5 single cell clones showed no decrease in mRNA expression in any of the single cell clones.

To try to confirm knockout of ACLY, qRT-PCR was performed on LP1 sgACLY2 and LP1 sgACLY5 single cells. Fold change of ACLY was calculated with reference to GAPDH and then normalized to the fold change of ACLY in the LP1 Cas9 control (Figure 12). All of the single cell clones had similar if not higher fold change of ACLY compared to the LP1 Cas9 control samples.
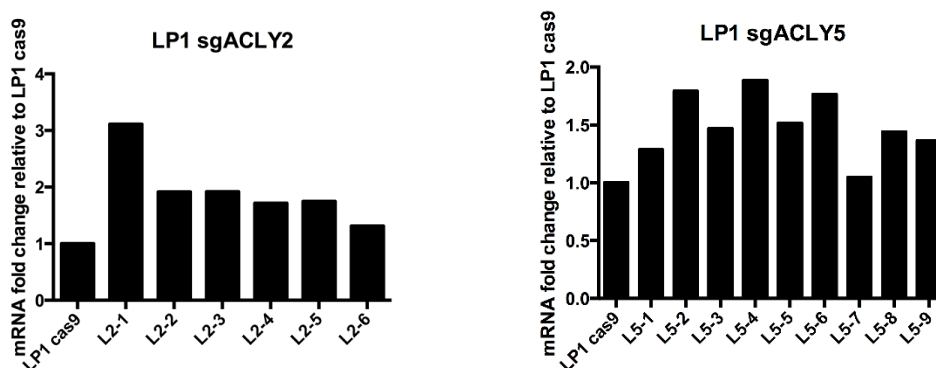
Figure 12 Fold change of ACLY relative to control of LP1 sgACLY2 (left) and sgACLY5 (right) single cell clones. GAPDH was used as the reference. Fold change was calculated with respect to GAPDH and normalized to the fold change of ACLY in the LP1 Cas9 control sample.

Genomic DNA did not show any appreciable alterations in regions targeted by the sgRNA in any of the single cell clones.

TOPO TA cloning was performed to prepare samples for genome sequencing. Samples were sent to Psomagen for sequencing using the M13.R sequencing primer. L2-4, L5-1, and L5-3 showed no alterations compared to the reference sequence in the regions that guides target (Figure 13). sgACLY2 lies between base 41,912,505 and 41,912,524; sgACLY5 lies between bases 41,913,778 and 41,913,797. L5-8 had one point mutation from an A→G resulting in the amino acid leucine instead of phenylalanine. Sequences were aligned using the http://genome.ucsc.edu.[91]
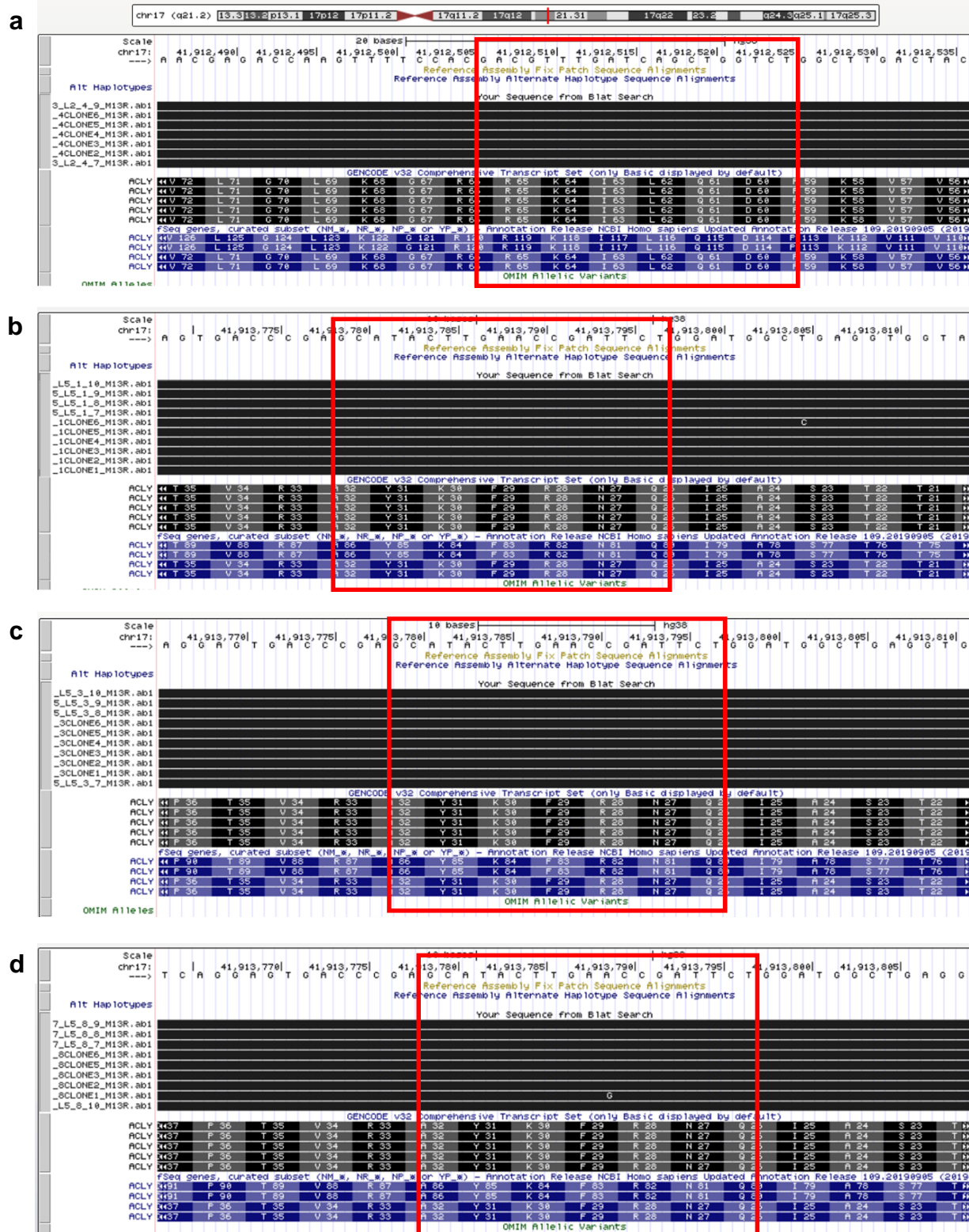
Figure 13 Sequencing of genomic DNA of L2-4 (a), L5-1 (b), L5-3 (c), and L5-8 (d). A red box is drawn around the region that the single guide RNA targets. Figures were made using http://genome.ucsc.edu.

LP1 cells treated with various concentrations of SB-204990 showed very little change in acetyl-CoA concentration.

LP1 cells were treated with SB-204990 for 24 or 48 hours and the concentrations of acetyl-CoA were determined by a fluorometric acetyl-CoA assay (Figure 14). In the assay, free CoA is quenched, acetyl-CoA is converted to CoA, and CoA is reacted to form NADH, which interacts with the PicoProbe to generate fluorescence. The acetyl-CoA concentration is calculated based on a standard curve. There were slight changes in acetyl-CoA concentrations.
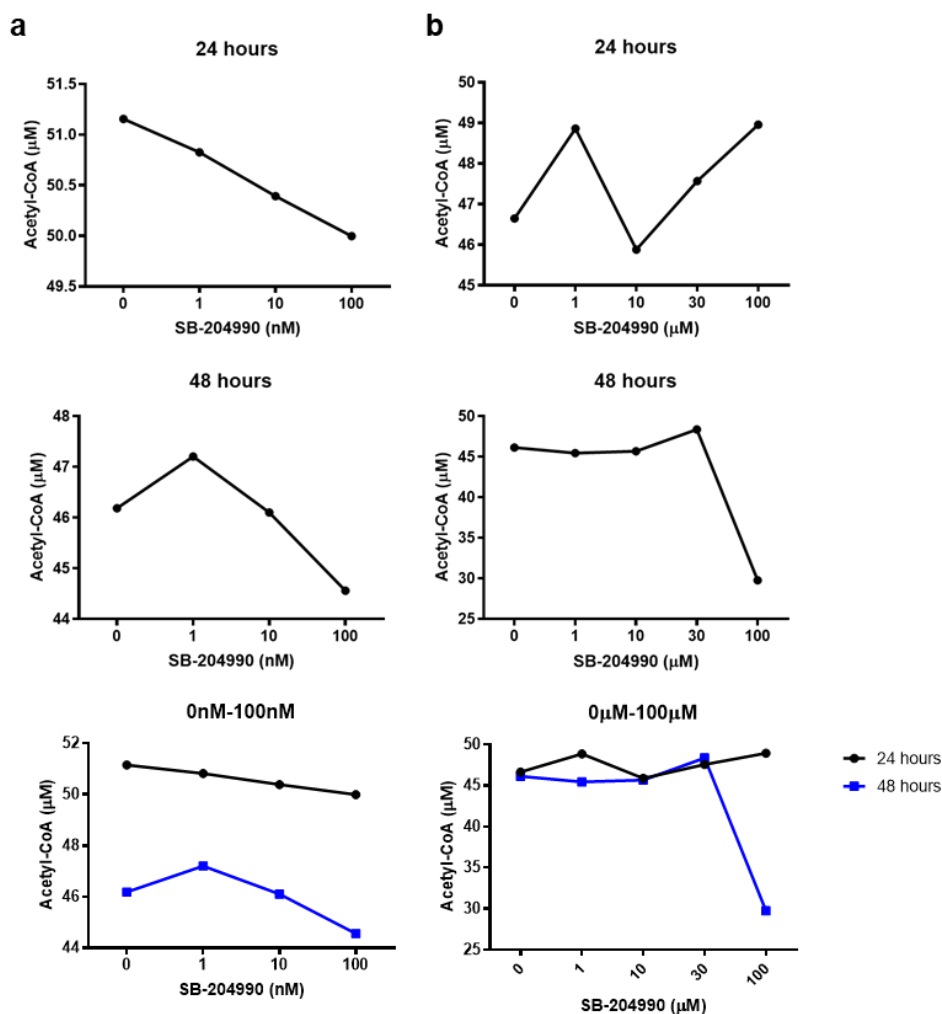
Figure 14 Concentration of acetyl-CoA in LP1 after treatment with various concentrations of SB-204990 for 24 and 48 hours. a. Cells were treated with 0, 1, 10, and 100nM SB-204990. **b.** Cells were treated with 0, 1, 10, 30, and 100µM SB-204990. Concentrations of acetyl-CoA were interpolated from a standard curve.

SB-204990 slowed cell growth and induced apoptosis in LP1, MM.1s, and OCI-My5 cells.

LP1, MM.1s, and OCI-My5 were treated with 0, 1, 10, 30, and 100µM SB-204990 in 1mL complete myeloma media for three days. There was greater percent cell death in the cells treated with 100µM drug at all three time points (Figure 15). The greatest percent cell death in LP1, MM.1s, and OCI-My5 was 14.8% at 72 hours, 19.1% at 24 hours, and 16.2% at 24 hours respectively.

This experiment was repeated as a six day experiment. On day three, one more milliliter of complete media with the appropriate dose of SB-204990 was added to the wells for days 4 through 6. There was greater percent cell death in the cells treated with 100µM drug in LP1 and MM.1s, but in OCI-My5, percent cell death decreased on days 4 through 6 (Figure 16).The greatest percent cell death in LP1, MM.1s, and OCI-My5 was 6.6% on day 5, 10.4% on day 4, and 15.6% on day 1 respectively.

In a third experiment, cells were treated with 0, 10, 25, 50, 75, and 100µM SB-204990 in 1mL complete myeloma media for four days. There was greater percent cell death in the cells treated with 100µM drug at all four time points (Figure 17). The greatest percent cell death in LP1, MM.1s, and OCI-My5 was 8.1% on day 4, 11.1% on day 3, and 14.1% on day 3 respectively. Interestingly, a number of doses had less cell death than the control condition.

Overall, in each experiment in all of these cell lines, the cells treated with 100µM drug had a lower absolute cell count compared with the control sample. However, the effect of SB-204990 on induction of apoptosis was different in each trial.
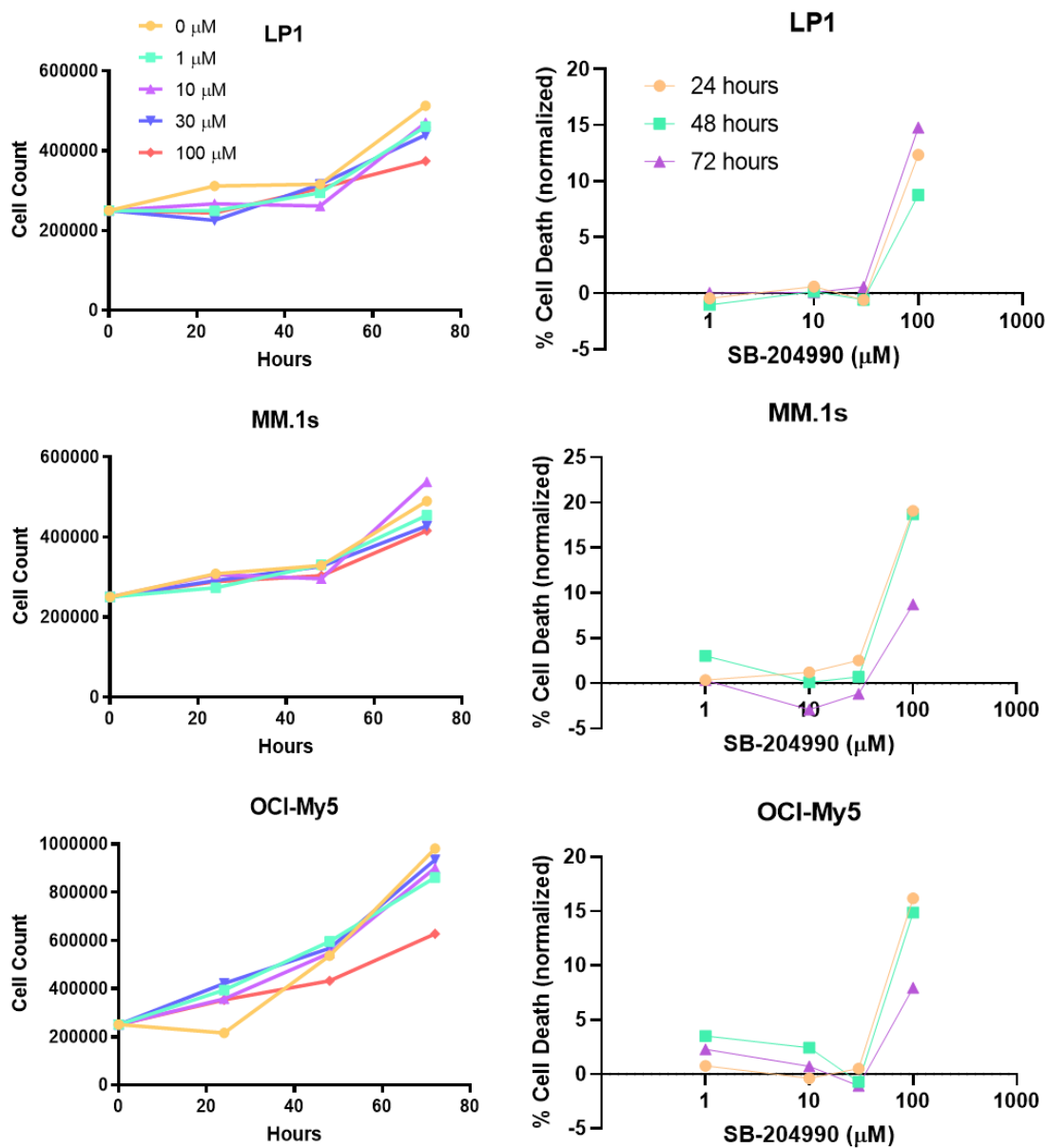
Figure 15 Cell counts (left) and cell death (right) after 3-day treatment with SB-204990. LP1, MM.1s, and OCI-My5 were treated with 0, 1, 10, 30, and 100µM SB-204990 for three days. Cell count was measured using FITC-counting beads and cell death was measured by Annexin V-FITC and PI staining. Cell death was normalized to the untreated control samples.
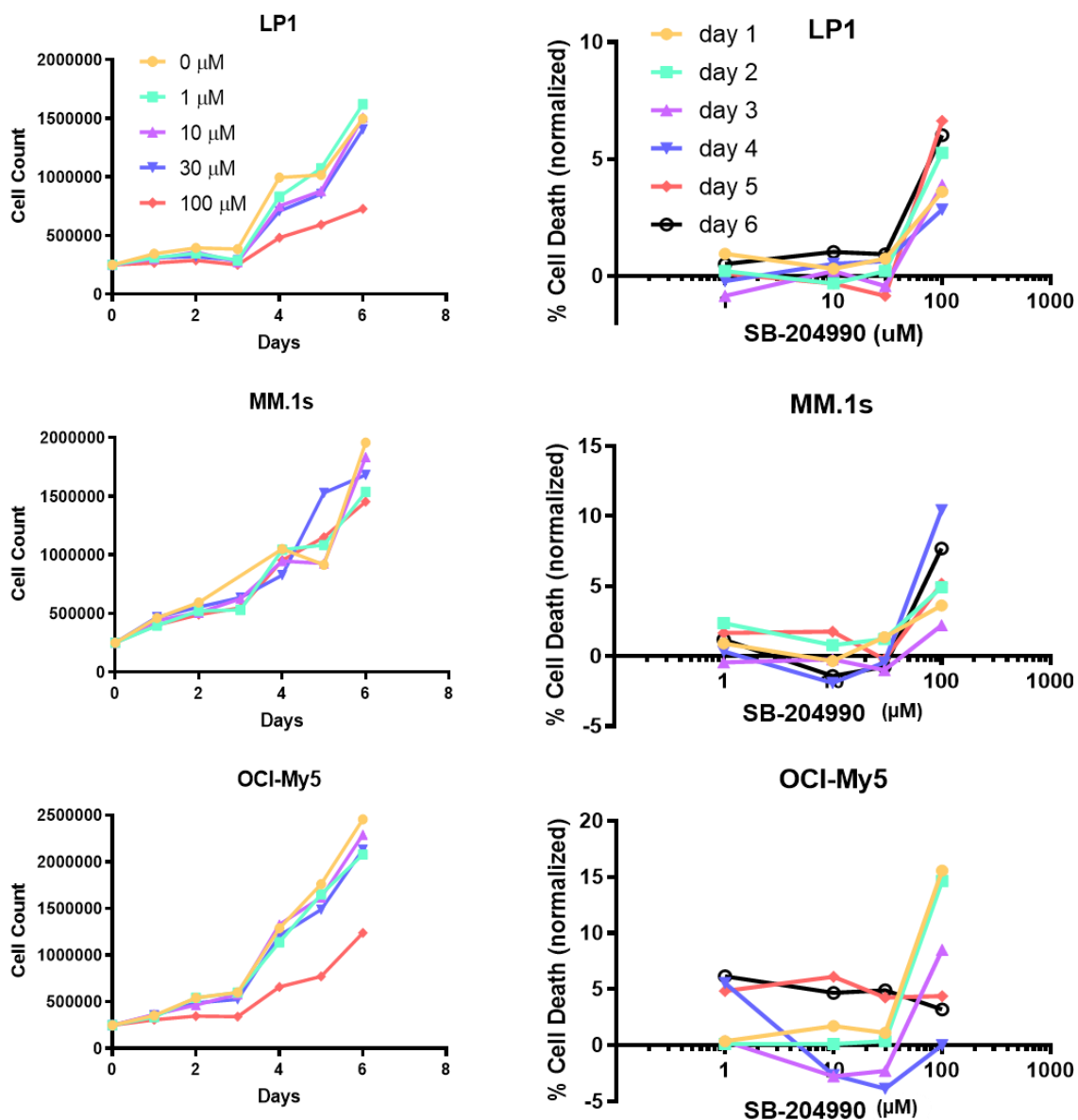
Figure 16 Cell counts (left) and cell death (right) after 6-day treatment with SB-204990.
LP1, MM.1s, and OCI-My5 were treated with 0, 1, 10, 30, and 100µM SB-204990 for six days.
Cell count was measured using FITC-counting beads and cell death was measured by Annexin
V-FITC and PI staining. Cell death was normalized to the untreated control samples.
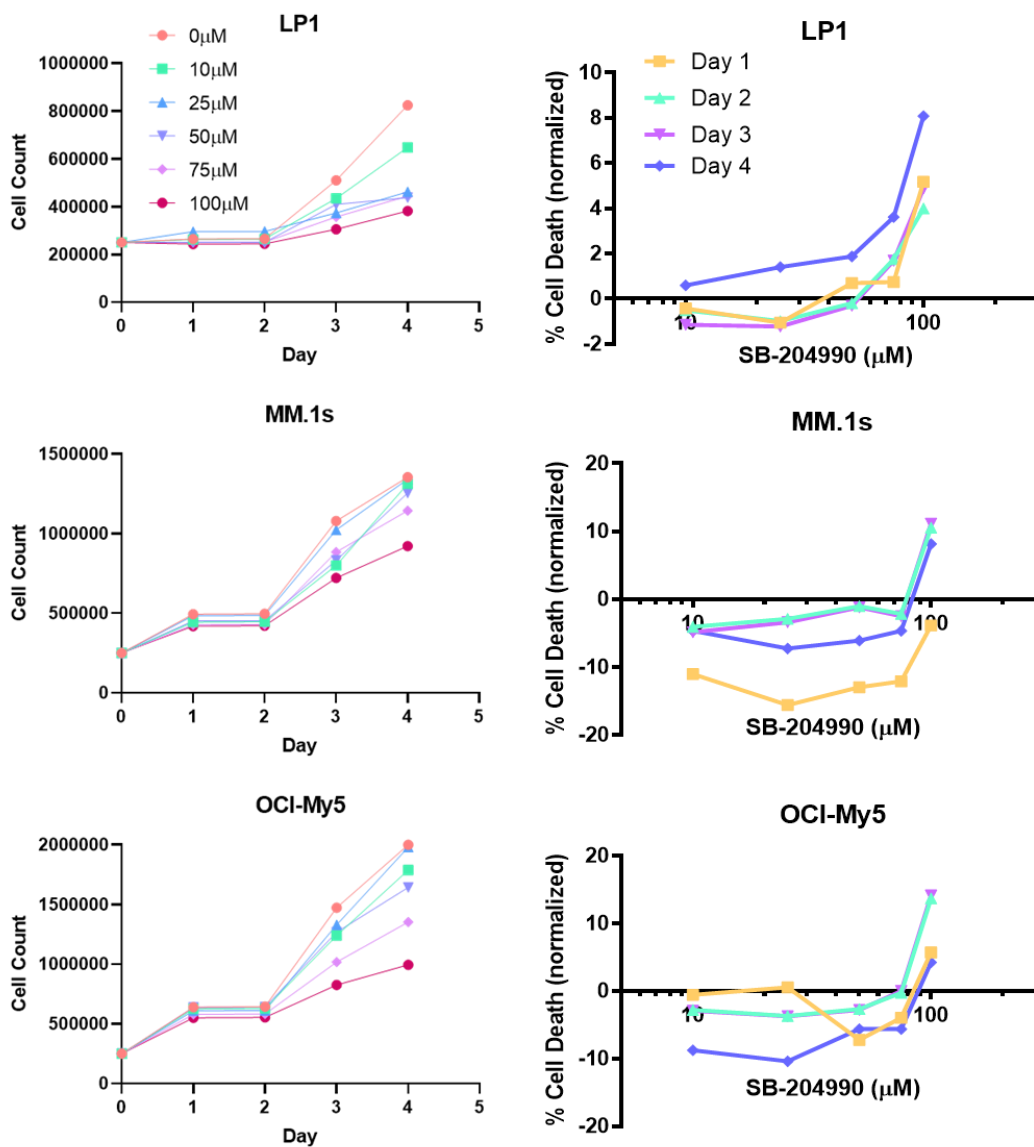
Figure 17 Cell counts (left) and cell death (right) after 4-day treatment with SB-204990. LP1, MM.1s, and OCI-My5 were treated with 0, 1, 10, 30, and 100μM SB-204990 for four days. Cell count was measured using FITC-counting beads and cell death was measured by Annexin V-FITC and PI staining. Cell death was normalized to the untreated control samples.

Combination treatment of SB-204990 and bortezomib induced more cell death in LP1 and KMS27, and at lower doses of bortezomib in MM.1s and OCI-My5.

LP1, MM.1s, and KMS27 cells were treated with 0, 1, 10, and 100nM of bortezomib and 100μM SB-204990, or bortezomib alone in 1mL complete myeloma media for 24 hours. In all of these cell lines, the cells treated with 100μM SB-204990 induced more cell death than conditions using bortezomib alone. (Figure 18a). When this experiment was repeated, and included OCI-My5, similar results were observed, except in LP1, cell death was ~10% higher in LP1 treated with 100nM bortezomib alone compared to 100nM bortezomib with 100μM SB-204990. In MM.1s and OCI-My5, at 10 and 100nM of bortezomib, there was almost no difference in cell death between the cells treated with and without SB-204990 (Figure 18b). This was also observed in the case of MM.1s in the first experiment. The percent cell death in LP1 and KMS27 was higher in the first experiment than the second experiment in conditions where SB-204990 was added, but they were similar in the conditions of bortezomib alone.
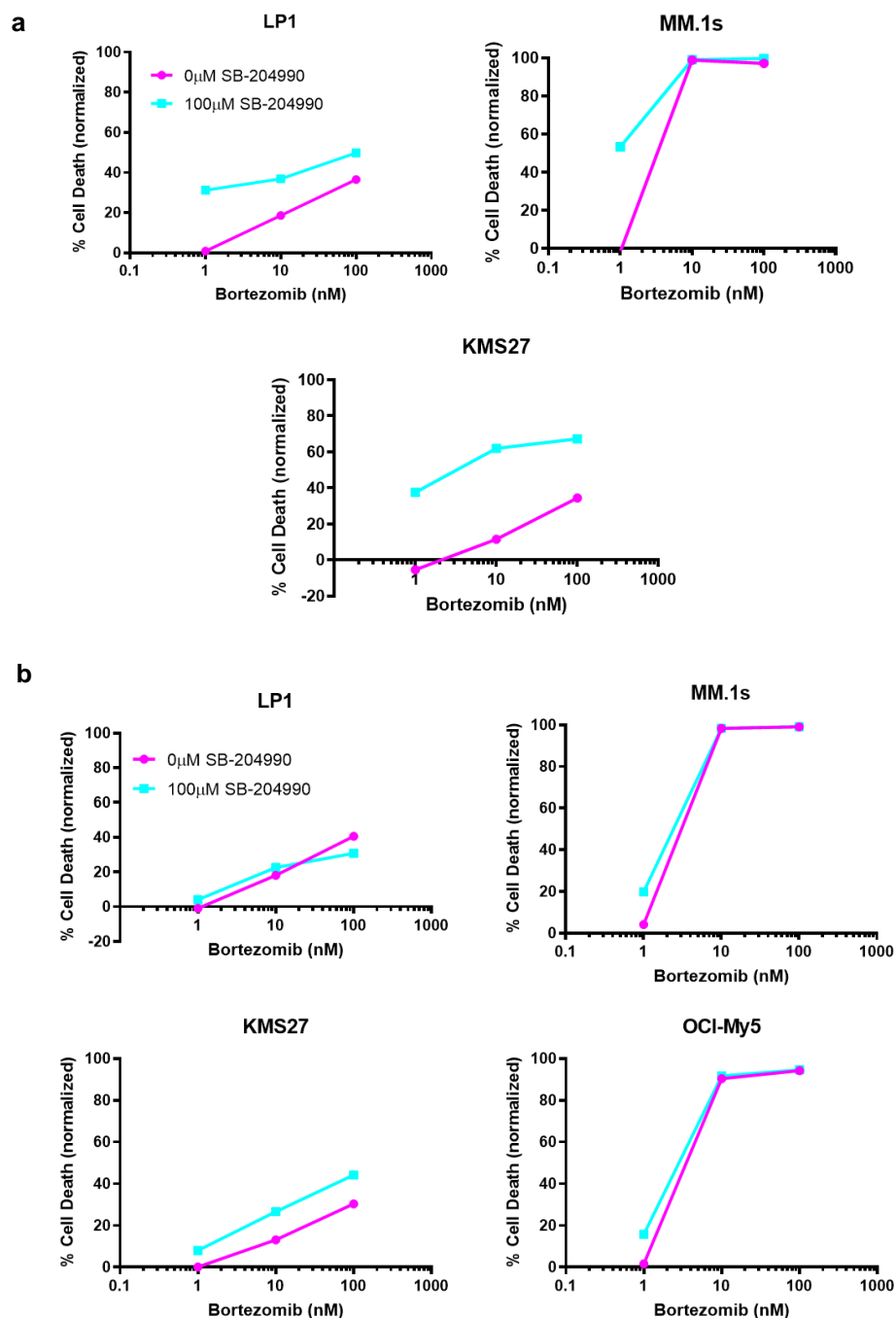
Figure 18 Cell death after treatment with bortezomib or SB-204990 and bortezomib. a.
LP1, MM.1s, and KMS27 were treated with 0, 1, 10, 100nM bortezomib alone or 0, 1, 10,
100nM bortezomib with 100µM SB-204990 for 24 hours. **b.** The same experiment was repeated
but included OCI-My5. Cell death was measured by Annexin V-FITC and PI staining. Cell death
was normalized to the untreated control samples.

Discussion

This thesis was a preliminary study of the role of ACLY in multiple myeloma biology and drug sensitivity. ACLY is the cytosolic enzyme responsible for producing a major source of cytosolic acetyl-CoA, which is required for fueling lipogenesis. Lipids and sterols serve as secondary signaling molecules and components of cell membranes. Because myeloma cells are secretory, we hypothesized that ACLY was essential to myeloma cell proliferation, survival and organellar homeostasis. The main goals of this study were to investigate the impact of ACLY on proliferation, cell death, and chemosensitivity to bortezomib. To address this hypothesis, we tried to develop stable ACLY knockout HMCLs and to inhibit ACLY using the chemical inhibitor SB-204990. At this time, the role of ACLY in multiple myeloma is still unclear, so further investigation is necessary to elucidate whether ACLY would be a viable target in multiple myeloma treatment.

Through the Multiple Myeloma Research Foundation's CoMMpass database and the Broad Institute's Cancer Dependency Map, we identified ACLY as a potential target in myeloma. Patients who expressed high levels of *ACLY* transcript had worse progression free and overall survival. Additionally, more multiple myeloma cell lines were dependent on *ACLY* than cell lines from other cancer types. We also looked at the dependency of myeloma cells on other genes that code for proteins involved in fatty acid synthesis or cholesterogenesis. Myeloma cell lines were more dependent on *ACLY* than *SLC25A1* and *ACSS2*. From this we could conclude that ACLY is the primary source of cytosolic acetyl-CoA, not conversion of acetate or citrate transport. Additionally, if ACACA or FASN are not functional, myeloma cells could rely more on lipolysis and scavenging rather than de novo fatty acid synthesis. Almost all cell lines were dependent on the genes involved in the mevalonate pathway, indicating that almost all cells need cholesterol and protein prenylation. The only myeloma-specific dependency was *ACLY*.

From the cell lines in the DepMap analysis, we chose to continue this study using LP1 and OCI-My5 to try to make stable knockout cells because LP1 is not a dependent cell line, while OCI-My5 is dependent. During the process of generating knockout cells, we observed decreased expression of ACLY after one week of Cas9 induction with doxycycline in OCI-My5 and LP1. Flag was highly expressed in all samples except the control. In this system, Cas9 has a Flag tag, so it was expected that Cas9 was induced in the knockouts, but not in the control samples. We decided to continue with sgACLY2 and sgACLY5 because they seemed to be the only guide RNAs that decreased expression in both OCI-My5 and LP1. We single cell cloned these bulk cells and checked their expression levels after 4 weeks. The transduced OCI-My5 cells did not grow out. This result could possibly confirm the DepMap CRISPR screen, that OCI-My5 is dependent on ACLY for survival. After LP1 sgACLY2 and LP1 sgACLY5 single cell clones grew out, we observed decreased expression of ACLY in four cell lines, but the qRT-PCR and sequencing of genomic DNA did not confirm that ACLY was successfully knocked out. One explanation for the decrease in protein but not mRNA could be that any change to the DNA could still be transcribed and the ACLY TaqMan probe used in the qRT-PCR recognized and bound to a portion of the transcript that was not edited. Therefore, mRNA expression would appear unchanged. In the future, we could design and optimize more specific primers for qRT-PCR analysis rather than using the master mix and conduct the qRT-PCR on the bulk cells before single cell cloning. One possible explanation for the initial decrease in protein but no change to genomic DNA could be that the cell lines we developed were not actually single cell clones. LP1 cells tend to clump, so it is possible that cells stuck together when setting up the single cell cloning; knockout cells could have initially grown out, but the unedited cells could have outcompeted the knockout cells over multiple passages. When setting up single cell cloning

using LP1 in the future, we could dilute the cells more, but in a smaller volume to try to break

up the cells that adhere to each other. We could also check the expression of ACLY weekly to

ensure that their phenotype is consistent after repeated passaging.

We would like to try creating stable knockout cells again using the same method as

described. A recent publication used the sgRNA 5′-GAGCATACTTGAACCGATTC-3′ in a

lentiCRISPRv2 plasmid to knock out ACLY in colon cancer cells.[76] This sgRNA is the same as

our sgACLY4, one of the constructs that did not initially produce knockouts, but this should be

repeated. We could obtain OCI-My7 and JJN3 cells and try to knock out ACLY in these cell

lines in addition to LP1. The CERES scores for OCI-My7 (-0.2885) and JJN3 (-0.4539) are both

higher than LP1 (-0.4927), so we might have more success knocking out ACLY in these cell

lines. Additionally, we should first characterize the protein, mRNA and genomic DNA of the

bulk infected cells prior to single cell cloning. Then, if single cell clones grow out, we can

continue with functional assays.

To determine if the ACLY inhibitor SB-204990 functioned as a selective inhibitor in

myeloma cells, we decided the most direct way to measure ACLY function would be by

quantifying the intracellular concentration of acetyl-CoA. We used a fluorometric assay that

compared our samples to a standard curve to interpolate the concentration of acetyl-CoA in our

samples. Previous literature has shown that SB-204990 treatment results in decreased lipogenesis

in a dose dependent manner[81,85,92] so we expected acetyl-CoA concentrations to decrease in a

dose dependent manner, however this is not what was observed. Acetyl-CoA concentrations did

not change significantly. One explanation could be that after the drug was reconstituted in

DMSO, it was frozen at -20°C, and the drug could have degraded during freeze-thaw. The drug

should be prepared immediately prior to use to avoid this problem. Additionally, we should

optimize the amount of sample loaded; too little sample, too much sample, and unequal quantities would all result in inaccurate measures of acetyl-CoA in this assay. Another initial challenge to this assay was that some enzymes are added in small quantities in a step-wise manner, which may result in inaccurate readings if the enzymes are not mixed thoroughly. For example, 10µL of CoA quencher is added and after 5 minutes 2µL of quench remover is added. If this step is not reacted to completion, the free CoA would react to form NADH and generate more fluorescence. This could be fixed by adjusting the assay volume and diluting the enzymes to add amounts that will mix more thoroughly.

To investigate the impact of ACLY on proliferation and cell death, we treated cells with SB-204990, counted cells using FIT-C counting beads, and measured cell death using Annexin V-FITC and propidium iodide staining, all by flow cytometry. Previous literature has shown that SB-204990 delays the time of cells entering the cell cycle, reduces cell proliferation, and induces apoptosis.[58,79,93,94] We found that myeloma cells treated with 100µM SB-204990 had a lower cell count than cells treated at lower doses. We cannot draw conclusions on the impact of SB-204990 on cell death because we were not able to reproduce cell death data between trials. Cells treated with 100µM SB-204990 may have induced more apoptosis than at lower doses, but at this stage we cannot conclude this. This experiment should be repeated over the course of six days, set up in multiple replicates, and extra care should be taken to make sure that media is relatively fresh, cells have been thawed recently, and all cells have a relatively similar starting viability to obtain more reliable results

Combination treatment of bortezomib and SB-204990 also resulted in inconsistent data. In figure 18, although cell death appeared to increase upon addition of SB-204990 to bortezomib, the results in figure 18a showed that SB-204990 killed ~20% more than the results in figure 18b,

despite that these experiments were set up using the same methods. Additionally, at higher doses of bortezomib, there was no difference between cell death from combination treatment versus the bortezomib as a single agent. When repeating this assay, we should decrease the doses of bortezomib on MM.1s and OCI-My5. Because the IC_{50} of bortezomib is between 1nM and 10nM in these cell lines, it would be more probative to dose cells in that range to see a clear separation in response between the combination and corresponding single agent conditions.

At this point, we have yet to find the IC_{50} of SB-204990 in myeloma cells and it has not been previously published, so moving forward it is of the utmost importance to determine if the drug functions in myeloma cells before we can draw conclusions on how the drug functions in myeloma cells. To do this, we need to optimize the acetyl-CoA fluorometric assay for our myeloma cells. One optimization experiment we could do is a dose response using different volumes of lysis buffer to concentrate or dilute the samples and quantify the concentration of proteins prior to deproteinizing the samples. This would allow us to determine the optimal volume of lysis buffer to properly control the loading of the sample into the assay and the concentration of the sample that produces the clearest readout. After the assay is optimized, we can the dose response experiment to determine if SB-204990 effectively inhibits ACLY in myeloma cells and what concentrations of the drug to use in subsequent experiments.

We should also repeat the proliferation and cell death assays for the combination treatments of bortezomib and SB-204990, and single agent treatments of bortezomib alone and SB-204990 alone. We used the combination treatment of SB-204990 with bortezomib because our initial hypothesis was that decreased cytosolic acetyl-CoA may destabilize the endoplasmic reticulum and secretory pathways by decreased fatty acid synthesis. Because myeloma cells secrete copious amounts of proteins, they are particularly susceptible to accumulating misfolded

proteins that would cause ER stress if not degraded.[95] However, if myeloma cells are

differentially dependent on *ACLY*, but not on fatty acid synthesis or the mevalonate pathway, this

indicates that myeloma cells could use cytosolic acetyl-CoA for protein acetylation. One line of

treatment that is FDA approved for multiple myeloma is the pan-HDAC inhibitor panobinostat in

combination with bortezomib.[96] Bortezomib inhibits proteasomal degradation of ubiquitinated

proteins. Alternatively, ubiquitinated proteins can form a complex with HDAC6 and dynein and

are carried on microtubules to the microtubule organizing center to form aggresomes, which are

cleared by aggrephagy.[97] Panobinostat disrupts the binding of HDAC6 to this complex and the

hyperacetylation of microtubules, thus inhibiting aggrephagy, resulting in the accumulation of

misfolded proteins and ER stress, eliciting the unfolded protein response, a cellular mechanism

to reestablish ER homeostasis but triggers apoptosis if ER stress continues.[96] Interestingly, the

only post-translational modification made to α-tubulin is acetylation on lysine 40 in the luminal

face of the microtubule. This acetylation is required to form the reticulated lattice of acetylated

α-tubulin. It has previously been shown that motor proteins have a stronger affinity for acetylated

microtubules.[98] We now propose that decreased supplies of cytosolic acetyl-CoA would result in

less acetylated α-tubulin, leading to decreased motor protein affinity, less efficient intracellular

trafficking, and thus less efficient formation of aggresomes and increased cellular stress. This

model is depicted in figure 19.
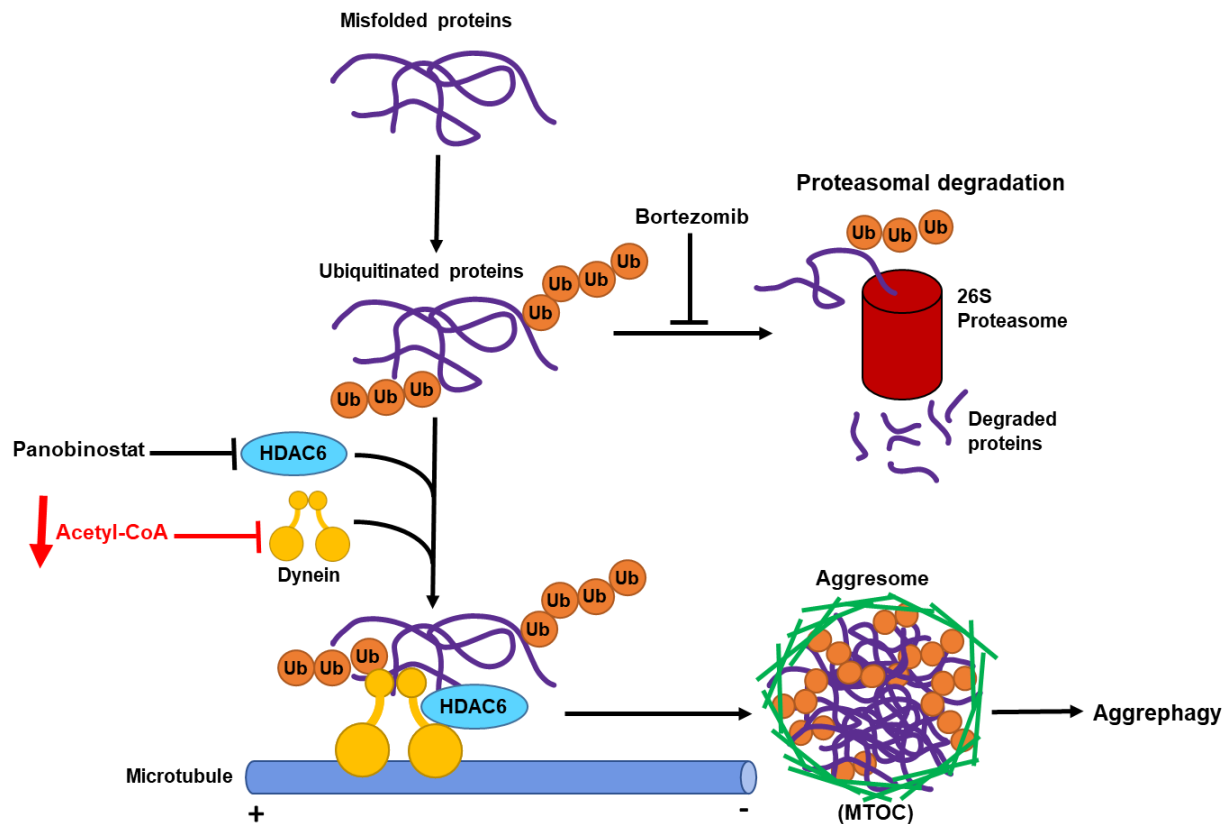
Figure 19 Model of mechanism of the proteasome inhibitor bortezomib combined with the pan-HDAC inhibitor panobinostat. Ub: ubiquitin, MTOC: microtubule organizing center.

Therefore, to more accurately understand the nature of the cell death, we should also evaluate the expression of ER stress induced proteins such as CHOP (CCAAT-enhancer-binding protein homologous protein) and eIF2-α phosphorylation. We could also evaluate expression of acetylated α-tubulin to determine if microtubule acetylation decreases. To investigate the impact of ACLY on ER structure, we could treat cells with an ACLY inhibitor and use immunofluorescence to visualize ER associated proteins such as calreticulin and proteins with KDEL motifs. If ACLY function is critical for maintaining ER structure, we would expect to see more diffuse fluorescence in cells treated with the inhibitor compared to untreated controls. To investigate the impact of ACLY on ER function, we could treat cells with an ACLY inhibitor and use an ELISA to quantify the monoclonal antibodies that are secreted. If ACLY function is

critical for maintaining secretion, we would expect to see less secretion of monoclonal antibodies in cells treated with the inhibitor compared to untreated controls. Decreased secretion would also be a potentially clinically relevant result because patients often develop kidney failure from filtering excessively secreted proteins by myeloma cells.

One major limitation to any of these proposed experiments is that this study would be carried out in myeloma cell lines. Myeloma cell lines are derived primarily from extramedullary disease, allowing them to survive in our flasks independent of the bone marrow microenvironment. This represents the most aggressive form of myeloma, so it does not directly represent the forms of disease we observe in patients. As secondary genetic features develop with disease progression, we do not know if myeloma cells in the earlier stages are dependent on ACLY. One experiment that could help elucidate this would be a dose response experiment using an ACLY inhibitor on patient samples taken from patients at different stages of disease. We could also determine expression of ACLY at different stages. This would help us to determine which cell lines would be best recapitulate different stages of disease. Because there are already chemical inhibitors of ACLY available, it could be clinically relevant to determine the function of ACLY in different stages of disease. If the myeloma cells are uniquely dependent on ACLY compared to other cells in the body, ACLY inhibitors may be a valuable tool in treatment of myeloma.

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Appendix I:

Protocols and Recipes

Protocol for Culturing Human Myeloma Cell Lines

Materials and Reagents:
1. Complete myeloma media (see recipe)
2. Frozen cells
3. T-25 flask
4. 15mL conical
5. Serological pipettes
6. Freeze media (see recipe)

Equipment:
1. 37°C water bath
2. Centrifuge
3. Aspirator
4. Cell culture hood

Protocol for thawing cells:
1. Pre-heat the complete myeloma media in a 37°C water bath.
2. Add 10mL complete myeloma media to a 15mL conical.
3. Remove vial of frozen cells from the -80°C freezer and warm cells in the 37°C water bath to thaw.
4. Transfer the thawed cells to the conical.
5. Centrifuge at 1100rpm for 5 minutes.
6. Aspirate supernatant.
7. Resuspend in 10mL fresh complete myeloma media.
8. Transfer to T25 flask.

Protocol for passaging cells:
1. Inside the sterile cell culture hood, transfer the cells in the flask to a conical.
2. Centrifuge at 1100rpm for 5 minutes.
3. Aspirate the supernatant.
4. Resuspend in 10mL fresh media.
5. Transfer back to a flask.

Protocol for freezing cells:
1. Take about 3-5 million cells in a 15mL conical and centrifuge them at 1100rpm for 5 minutes.
2. Aspirate supernatant.
3. Resuspend in 1mL freeze media and transfer to cryovial.
4. Store in -80°C freezer.

Protocol for Passaging HEK293T Cells

Materials and Reagents:
1. 293T complete media
2. Trypsin (Cellgro, Mediatech, Manassas, VA)
3. HEK293T cells
4. Sterile PBS
5. Serological pipettes
6. 50mL conical
7. T-75 flask

Equipment:
1. Cell culture hood
2. Aspirator
3. 37°C water bath

Protocol:
1. Pre-heat the HEK293T complete media and the trypsin in the 37°C water bath.
2. HEK293T cells are adherent, so gently tilt the flask to aspirate the media without disturbing the cells.
3. Wash with 5mL sterile PBS. Do not directly pipette the PBS onto the cells or they will dislodge. Instead pipette the PBS onto the walls of the flask and tilt the flask to gently wash the cells. Aspirate the PBS.
4. Add 3mL trypsin to the flask. Incubate for ~2 minutes until cells are dislodge in clumps, swirling the flask periodically.
5. Add 7mL of complete media to neutralize the trypsin. Pipette up and down to fully dislodge the cells and break up clumps.
6. Move cells to a 50mL conical. Centrifuge at 1100rpm for 5 minutes.
7. Aspirate the supernatant and resuspend in 25mL fresh media.
8. Transfer cells to a T-75 flask.

Protocol for Counting Cells

Materials and Reagents:
1. Cell culture
2. Eppendorf tubes
3. Trypan blue
4. 15mL conical
5. Hemocytometer
6. Ethanol
7. Kimwipe

Equipment:
1. Cell culture hood
2. Microscope

Protocol:
1. In the sterile cell culture hood, transfer cells from flask to a conical.
2. Add 25µL cells to an Eppendorf tube and add 75uL of trypan blue.
3. Pipette 10µL of the mixture onto a hemocytometer.
4. Count the clear and blue cells in the large 4x4 grids at the corners of the hemocytometer.
5. Clean the hemocytometer with ethanol and Kimwipe.

Protocol for Counting Cells with Beads

Materials and Reagents:
1. Cell culture
2. 15mL conical
3. 6mL round bottomed FACS tubes
4. FACS tube rack
5. AccuCheck Counting Beads (Thermo Fisher Scientific, #PCB100, Waltham, MA)

Equipment:
1. Cell culture hood
2. FACS analyzer

Protocol:
1. In the sterile cell culture hood, transfer cells from flask to a conical.
2. Add 100μL of cells to each FACS tube.
3. Thoroughly vortex AccuCheck counting beads prior to use. Mix 100μL AccuCheck counting beads in each tube of cells.
4. Vortex samples prior to putting them through the FACS analyzer.
5. Calculate the absolute cell count.

Protocol for PCR (Herculase II Fusion DNA Polymerase Kit)

Materials and Reagents:
1. Herculase II Fusion DNA Polymerase Kit (Agilent, Santa Clara, CA)
2. Forward primer (Integrated DNA Technologies, Coralville, IA)
3. Reverse primer (Integrated DNA Technologies, Coralville, IA)
4. Template DNA
5. Molecular grade dH_2O
6. 0.2mL PCR tubes
7. Cold block

Equipment:
1. Thermocycler

Protocol (adapted from manufacturer's instructions):
1. Keep all reagents in the cold block during use. In a PCR tube, combine the following components:

Component	Volume/reaction (µL)
Herculase buffer	10
dNTP (100mM)	0.5
Herc II pol	0.5
Forward primer (10µM)	1
Reverse primer (10µM)	1
Template DNA	2
Molecular grade	35
Total	**50**

2. Run on thermocycler:
   i)   95°C 3 min
   ii) 95°C 30 sec
   iii) 63.3°C 30 sec
   iv) 68°C 30 sec
   v)   4°C forever
   Repeat (iii) and (iv) for 40 cycles total

Protocol for PCR

Materials and Reagents:
1. 10x PCR buffer (see recipe)
2. Taq polymerase
3. dNTP (10µM)
4. MgCl$_2$ (25mM)
5. Forward primer (Integrated DNA Technologies, Coralville, IA)
6. Reverse primer (Integrated DNA Technologies, Coralville, IA)
7. Template DNA
8. Molecular grade water
9. 0.2mL PCR tubes
10. Cold block

Equipment:
1. Thermocycler

Protocol:
1. Keep all reagents in the cold block during use. In a PCR tube, combine the following components:

Component	Volume/reaction (µL)
MgCl$_2$ (25mM)	3
10x buffer	2.5
dNTP (10µM)	0.5
Taq polymerase	1
Forward primer (10µM)	0.5
Reverse primer (10µM)	0.5
Template DNA	3
Molecular grade water	14
Total	**25**

2. Run on thermocycler:
   i) 95°C 3 min
   ii) 95°C 30 sec
   iii) 60°C 30 sec
   iv) 68°C 30 sec
   v) 12°C forever
   Repeat (iii) and (iv) for 40 cycles total

Protocol for Casting and Running a 1% TAE Agarose Gel and DNA Purification

Materials and Reagents:
1. Agarose
2. 125mL flask
3. 50x Tris base, acetic acid and EDTA (TAE) (see recipe)
4. dH$_2$O
5. Saran wrap
6. Ethidium bromide
7. Purple 1kb DNA ladder (New England Biolabs, Ipswich, MA)
8. PCR product
9. 10x DNA gel loading dye (see recipe)
10. Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK)
11. Razor blade
12. Eppendorf tubes
13. Tris-HCl (pH 7.5)

Equipment:
1. Microwave
2. Fume hood
3. Gel caster (Bio-Rad, Hercules, CA)
4. Transparent gel tray (Bio-Rad, Hercules, CA)
5. 16 well gel comb (Bio-Rad, Hercules, CA)
6. Horizontal Gel Electrophoresis system (Bio-Rad, Hercules, CA)
7. PowerPac HC power supply (Bio-Rad, Hercules, CA)
8. UV light box

Protocol for casting gel:
1. Weigh 0.50 grams of agarose powder in a weight boat.
2. Transfer agarose powder to a 125mL flask.
3. Make 500mL 1xTAE by diluting 10mL 50xTAE in 490mL dH$_2$O.
4. Add 50mL 1xTAE to the flask containing the agarose powder.
5. Loosely cover flask with saran wrap.
6. Microwave flask for 2 minutes on power level 4, swirling the flask periodically.
7. Transfer the flask to the fume hood and allow to cool for 1 minute.
8. Add 10µL ethidium bromide to the flask. Swirl gently to mix.
9. Assemble the gel caster and transparent gel tray. Insert comb on one end of the mold. Make sure the mold is parallel to the bench.
10. Pour the agarose liquid into the transparent gel tray and allow to set.
11. Gently remove the comb, being sure not to tear any of the wells. Move the transparent gel tray to the gel electrophoresis system.
12. Fill the gel electrophoresis system with 1xTAE until the top of the gel is covered.

Protocol for running gel:
1. Prepare samples by adding 5µL of loading dye to 50µL of purified PCR product.

2. Load 6µL 100kb DNA ladder and 25µL PCR product.
3. Double check that the DNA is loaded on the negative end of the box and is running toward the positive end of the box. Run the gel at 120V until there is good separation of the ladder and the loading dye has run 80% of the way down the gel.
4. Stop the run and remove the gel from the running box. Being careful to wear protective eyewear and not exposing the skin, visualize the gel using a UV light box.

Protocol for DNA purification (adapted from manufacturer's instructions):
1. In the UV light box, using a razor blade, carefully cut out the bands of interest as close to the band as possible, being careful to minimize exposure of the skin to UV. Transfer the cut bands into Eppendorf tubes.
2. Add 300µL of Capture buffer type 3.
3. Mix by inverting the tube. Incubate at 60°C until the gel is completely dissolved.
4. Assemble the GFX Microspin Column and collection tube for each sample.
5. Transfer up to 800µL of the sample and capture buffer into the microspin column and collection tube.
6. Incubate the sample at room temperature for 2 minutes. Centrifuge at 16,000 x g for 30 seconds. Discard the flow-through.
7. Add 500µL of Wash buffer type 1 to the spin column.
8. Centrifuge at 16,000 x g for 30 seconds. Discard the flow-through.
9. Repeat steps 7 and 8.
10. Move the spin column to a fresh collection tube. Elute in 22.5µL Tris-HCl. Incubate sample at room temperature for 1 minute. Elute DNA by centrifuging at 16,000 x g.
11. Samples can be stored at -20°C.

Protocol for PCR Cleanup

Materials and Reagents:
1. GenElute PCR Clean-up Kit (Sigma-Aldrich, St. Louis, MO)
2. Tris HCl (pH 8)
3. PCR sample

Equipment:
1. Microcentrifuge

Protocol (adapted from manufacturer's instructions):
1. Assemble GenElute plasmid minispin column and collection tube. Add 500µL Column Preparation Solution to each spin column. Centrifuge at 12,000 x g for 1 minute. Discard the flow through.
2. Add 5:1 binding solution to PCR mix. Transfer to binding column. Centrifuge at maximum speed for 1 minute. Discard flow through.
3. Add 500µL diluted Wash Solution to the column. Centrifuge at maximum speed for 1 minute. Discard flow through.
4. Centrifuge at maximum speed for 2 minutes to dry the spin column. Discard flow through.
5. Transfer spin column to clean collection tube. Add 25µL Tris HCl (pH 8) directly to the center of the membrane. Incubate at room temperature for 1 minute. Centrifuge at maximum speed for 1 minute to elute.

Protocol for Restriction Enzyme Double Digestion

Materials and Reagents:
1. DNA
2. Cutsmart buffer (New England Biolabs, Ipswich, MA)
3. Calf intestinal phosphatase (CIP) (New England Biolabs, Ipswich, MA)
4. Xho1 (New England Biolabs, Ipswich, MA)
5. Nhe1 (New England Biolabs, Ipswich, MA)
6. Molecular grade dH_2O
7. 0.2mL PCR tubes
8. Cold block

Equipment:
1. 37°C water bath
2. Polyfoam floating rack

Protocol:
1. Keep all reagents in the cold block during use. In a PCR tube, combine the following components:

Component	Volume/reaction (µL)
DNA	20 (~1µg)
Cutsmart buffer	5
CIP	1
Xho1	0.5
Nhe1	0.5
Molecular grade	23
Total	**50**

2. Insert tubes into the floating rack and place in 37°C water bath for 1 hour or overnight.

Protocol for DNA Insert Ligation

Materials and Reagents:
1. Digested vector
2. Insert DNA
3. 10x T4 DNA ligase buffer (New England Biolabs, Ipswich, MA)
4. T4 DNA ligase (New England Biolabs, Ipswich, MA)
5. Molecular grade dH$_2$O
6. 0.2mL PCR tubes
7. Cold block

Equipment:
1. Thermocycler

Protocol:
1. Keep all reagents in the cold block during use. In a PCR tube, combine the following components:

Component	Volume/reaction
Digested vector	100ng
Insert DNA	30ng
10x T4 ligase buffer	2µL
T4 DNA ligase	1µL
Molecular grade	up to 20µL
Total	**20µL**

2. Run on the thermocycler at 22°C for 10min.

Protocol for Competent Cell Transformation (JM109)

Materials and Reagents:
1. Eppendorf tubes
2. Ice
3. Ligation mix
4. Digested plasmid
5. JM109 (Promega, Madison, WI)
6. SOC media (Cellgro, Mediatech, Manassas, VA)
7. 15mL conical
8. Ethanol

Equipment:
1. 42°C water bath
2. Timer
3. Shaking incubator
4. Polyfoam floating rack
5. LB/carbenicillin agar plates
6. Bacteria incubator
7. Bunsen burner
8. Cell spreader
9. Turn table

Protocol:
1. Pre-chill Eppendorf tubes on ice.
2. Add 3μL ligation mix to the tubes.
3. Thaw JM109 cells on ice for approximately 10 minutes.
4. Aliquot 50μL JM109 to each tube and put them on ice for 30 min. Gently flick tubes to mix periodically.
5. Heat shock the cells by putting the tubes in a floating rack in a 42°C water bath for exactly 20 seconds.
6. Put tubes back on ice for 2 minutes.
7. Add 450μL SOC media to each tube.
8. Put tubes into a 15mL conical and incubate at 37°C for 1 hour horizontally shaking at 200 rpm.
9. Pre-heat LB/carb agar plates in the 37°C bacteria incubator.
10. Plate 100μL competent cell and ligation product mixture on each plate.
11. Incubate plates face down at 37°C overnight.

Notes:
The competent cell mixture can be stored at 4°C and ligation mixture can be stored at -20°C.

Protocol for Overnight Bacteria Cultures

Materials and Reagents:
1. LB broth
2. 50mg/mL carbenicillin
3. 15mL Falcon tubes
4. P200 pipette tips

Equipment:
1. Shaking incubator
2. 37°C water bath

Protocol:
1. Warm LB broth to 37°C in the water bath.
2. In a Falcon tube, add 5mL LB broth and 10µL carbenicillin
3. Select a colony from a plate on a pipette tip and drop it into the Falcon tube with LB and carb.
4. Incubate in the shaking incubator overnight at 37°C horizontally shaking at 200rpm.

Protocol for Bacteria Glycerol Stocks

Materials and Reagents:
1. Pure glycerol
2. dH_2O
3. overnight bacteria cell culture

Equipment:
1. -80°C freezer

Protocol:
1. Make 50mL 50% glycerol by adding 25mL pure glycerol to 25mL dH_2O. Mix well.
2. In a cryovial, add 500µL 50% glycerol and 500µL overnight bacteria culture.
3. Store at -80°C for later use.

Notes:
When using the glycerol stock to make more overnight cell cultures, do not thaw the entire vial. Keep the vial on ice and chip a small amount from the glycerol stock.

Protocol for Miniprep

Materials and Reagents:
1. GenElute HP Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO)
2. Overnight culture
3. Tris HCl (pH 8)

Equipment:
1. Microcentrifuge

Protocol (adapted from manufacturer's instructions):
1. Pellet 3mL of overnight culture.
2. Resuspend the cells in 200μL resuspension solution containing RNase A.
3. Add 200μL of lysis buffer. Invert to mix until mixture is clear and viscous.
    a. Do not allow lysis to exceed 5 minutes.
    b. Do not overload with cells or the cells will not lyse properly.
    c. Do not vortex.
4. Add 350μL Neutralization/binding buffer. Gently invert to mix. Centrifuge at maximum speed for 10 minutes to collect the precipitate.
5. Assemble the Miniprep Binding Column and microcentrifuge tube. Add 500μL Column Preparation Solution. Centrifuge at 12,000 x g for 1 minute. Discard flow through.
6. Transfer cell lysate to the column. Centrifuge at 12,000 x g for 1 minute. Discard flow through.
7. Add 500μL Wash Solution 1 to the column. Centrifuge at 12,000 x g for 1 minute. Discard flow through.
8. Add 750μL Wash Solution 2 to the column. Centrifuge at 12,000 x g for 1 minute. Discard flow through.
9. Centrifuge at 12,000 x g for 1 minute to dry membrane. Discard flow through.
10. Transfer column to a clean collection tube. Elute by adding 50μL Tris HCl directly to the spin column membrane. Incubate at room temperature for 1 minute. Centrifuge at 12,000 x g for 1 minute.

Protocol for Transfecting HEK293T

Materials and Reagents:
1. Dulbecco's Modified Eagle Media (Cellgro, Mediatech, Manassas, VA)
2. pCMV-dR8.2 dvpr (Weinberg Lab: Stewart et al RNA 2003 Apr;9(4):493-501. Obtained from Addgene, plasmid #8455, Cambridge, MA)
3. pCMV-VSV-G (Weinberg Lab: Stewart et al RNA 2003 Apr;9(4):493-501. Obtained from Addgene, plasmid #8454, Cambridge, MA)
4. Lipofectamine (Thermo Fisher Scientific, Waltham, MA)
5. Syringe (BD Biosciences, Franklin Lakes, NJ)
6. Acrodisc Syringe Filter 0.45µm Supor Membrane (Pall, Port Washington, NY)
7. HEK293T media (see recipe)
8. Eppendorf tubes
9. Miniprep DNA samples
10. HEK293T cells
11. Cell culture plates

Equipment:
1. Cell culture hood
2. 37°C cell incubator
3. -80°C freezer

Protocol:
1. Plate 500,000 HEK293T cells in 6mL 293T media one day before use.
2. Prepare tubes A and B for transfection:

   Tube A   **Tube B**
   1µg DNA   7µL lipofectamine
   1µg pCMV-dR8.2 dvpr   93µL plain DMEM
   100ng pCMV-VSV-G   *let stand at room temp. for 5minutes
   Plain DMEM up to 100µL
      a. pCMV-dR8.2 dvpr is a packaging plasmid
      b. pCMV-VSV-G is a pseudotyping plasmid
3. Mix tubes A and B and let stand at room temperature for 30 minutes.
4. Add the mixture to the plate of 293T cells dropwise while swirling the plate to evenly distribute the reagents. Incubate for overnight at 37°C.
5. Change the media to 3mL 293T media. Incubate 6 hours at 37°C.
6. After 6 hours, change the media to 1.5mL 293T media. Incubate overnight at 37°C.
7. The next day, collect the viral supernatant using a syringe and a 0.45µm syringe filter.
      a. HEK293T cells secrete lentivirus into the supernatant.
8. Freeze the supernatant in 1mL aliquots at -80°C.

Protocol for Lentiviral Transduction of Myeloma Cells

Materials and Reagents:
1. Polybrene (EMD Millipore, Burlington, MA)
2. Blasticidin (Thermo Fisher Scientific, Waltham, MA)
3. Doxycycline (Sigma-Aldrich, St. Louis, MO)
4. Complete myeloma media (see recipe)
5. Eppendorf tubes
6. Lentivirus
7. Myeloma cells that contain doxycycline inducible cas9 construct
8. 6-well plate
9. 24-well plate

Equipment:
1. 37°C water bath
2. 37°C cell incubator
3. Centrifuge

Protocol:
1. Pellet 500,000 myeloma cells (with doxycycline inducible cas9 construct) per viral condition in Eppendorf tubes.
2. Thaw virus in 37°C water bath.
3. Add 500μL virus and 0.4μL/mL polybrene to each pellet. Mix thoroughly. Transfer to a 24-well plate.
4. Centrifuge for 90 minutes at 37°C spinning at 2250rpm.
5. Change media to 1mL complete myeloma media. Put cells in a different well of the same 24-well plate. Incubate at 37°C overnight.
6. Transfer to 6-well plate. Add 2mL media and the appropriate amount of blasticidin.
   a. Need to do a dose curve of blasticidin to determine appropriate amount of blasticidin to add.
   b. Cells that received virus will be blasticidin resistant.
7. Grow cells at 37°C, adding media and blasticidin as needed as cells grow.
8. After cells grow out, add 1μg/mL doxycycline.

Protocol for Single Cell Cloning

Materials and Reagents:
1. Cells
2. 20% FBS complete myeloma media (see recipe)
3. 96-well plates
4. P200 multichannel pipette
5. Aluminum foil
6. 50mL sterile reservoirs

Equipment:
1. 37°C water bath
2. 37°C cell incubator
3. Cell culture hood

Protocol:
1. Pre-heat the 20% FBS complete myeloma media in the 37°C water bath.
2. Count cells.
3. Dilute cells to ~1.3 cells/mL media in 20% FBS complete myeloma media.
    a. Prepare enough for four 96-well plates.
    b. Each plate requires ~20mL.
4. Using a P200 multichannel pipette, aliquot 200µL per well.
5. Wrap plates in aluminum foil and label the top.
6. Incubate at 37°C for four weeks, checking around the two-week mark for colony growth.
    a. Colonies will often form as little clumps of cells along the edges of the well.
    b. Looking from the bottom of the plate is the easiest way to look for colonies.

Protocol for Preparing Whole Cell Lysates

Materials and Reagents:
1. Cells
2. Eppendorf tubes
3. Lysis buffer (see recipe)
4. RIPA buffer (see recipe)
5. Ice
6. 6x loading dye (see recipe)
7. 2-Mercaptoethanol

Equipment:
1. Microcentrifuge
2. Vortexer
3. 95°C hot plate

Protocol:
1. Count cells.
2. Collect a pellet of 2-10 million cells in Eppendorf tubes.
3. Add 50μL lysis buffer to each sample, vortex to break up the pellet.
4. Incubate samples on ice for 30 minutes.
5. Centrifuge samples for 10 minutes at 4°C, spinning at 15,000rpm.
6. Collect supernatant in a fresh Eppendorf tube.
7. Measure the protein concentration by the BCA protein assay (see protocol).
8. Take 30μg protein and add RIPA to 25μL.
9. Mix 50μL 6X loading dye and 3μL 2-ME.
10. Add 5μL loading dye and 2-ME to each lysate sample.
11. Boil samples at 95°C for 5 minutes.
12. Centrifuge at 16,000rpm for 3 minutes.

Protocol for Bicinchoninic Acid (BCA) Assay

Materials and Reagents:
1. Cuvettes
2. RIPA buffer (see recipe)
3. Whole cell lysates
4. 2mg/mL bovine serum albumin (BSA) (Thermo Fisher Scientific, Waltham, MA)
5. BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL)

Equipment:
1. 37°C incubator
2. Spectrophotometer

Protocol:
1. Grab enough cuvettes for 6 standards and unknown samples.
    a. It is helpful to keep these in an empty cuvette container.
2. Add 50μL RIPA buffer to each of the standard cuvettes. Add 48μL to the unknown samples.
3. Prepare standards of 50, 25, 12.5, 6.25, 3.125 and 0μg protein by serially diluting 50μL 2mg/mL BSA, skipping the 0μg standard tube.
4. Add 2μL whole cell lysate to the unknown sample cuvettes.
5. Mix 25mL reagent A and 500μL of reagent B (1:50 dilution)
6. Add 1mL A+B to each cuvette.
7. Incubate at 37°C for 30 minutes.
    a. Standards should be a gradient from light green to dark purple.
8. Using a spectrophotometer, measure absorbance at 562nm.
9. Interpolate the amount of protein in the unknown samples from the standard curve.

Protocol for Western Blotting

Materials and Reagents:
1. Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad, Hercules, CA)
2. Precision Plus Protein Standards Dual Color (Bio-Rad, Hercules, CA)
3. Blotting paper (VWR, Radnor, PA)
4. Nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK)
5. ATP-citrate lyase antibody (Cell Signaling Technology, #4332, Danvers, MA)
6. Anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Dallas, TX)
7. Antibody dilution buffer (see recipe)
8. 5% milk (see recipe)
9. 1x Tris buffered saline (TBS) w/ TWEEN (TBS-T) (see recipe)
10. ECL1 (see recipe)
11. ECL2 (see recipe)
12. 10x Tris glycine SDS (TGS) buffer (see recipe)
13. Gel transfer buffer (see recipe)
14. dH_2O
15. Prepared samples

Equipment:
1. PowerPac HC power supply (Bio-Rad, Hercules, CA)
2. Buffer dam (Bio-Rad, Hercules, CA)
3. Buffer tank and lid (Bio-Rad, Hercules, CA)
4. Gel holder cassette and foam pads (Bio-Rad, Hercules, CA)
5. Vertical electrophoresis electrode assembly (Bio-Rad, Hercules, CA)
6. Transfer electrode assembly (Bio-Rad, Hercules, CA)
7. Autoradiography cassette (Fisher Scientific, Pittsburgh, PA)
8. Amersham hyperfilm ECL (GE Healthcare, Buckinghamshire, UK)
9. Ice pack
10. Magnetic stir plate
11. Casserole dish
12. Ice bucket
13. Rocker
14. Saran wrap
15. Developer

Protocol for running SDS-PAGE:
1. Make 1xTGS buffer by diluting 80mL 10xTGS in 720mL dH_2O.
2. Assemble the gel electrophoresis apparatus.
    a. Insert the pre-cast gel in the vertical electrophoresis electrode assembly all the way to the bottom, then scoot the gel up until the top edge of the gel is butted up against the rubber seal of the inner chamber.
    b. If only running one gel, use a buffer dam on the other side of the inner chamber.
3. Fill inner chamber with 1xTGS. Wait two minutes to see if the chamber leaks to the buffer tank.
4. Fill the buffer tank with remaining buffer.

5. Load 6µL of the protein standard ladder and between 10-20µL samples.
    a. Load 20µL sample if using >30µL well gel
    b. Load 10µL sample if using 15µL well gel
    c. Load the same amount of each sample in the gel
    d. Don't forget to load a control sample.
6. Run gel for ~50 minutes at 150V or until the desired amount of separation is reached.

Protocol for gel transfer:
1. Pour half of transfer buffer into a casserole dish and the other half into the buffer tank. Place transfer electrode assembly in buffer tank with the black side facing the front.
2. Break open the pre-cast gel and trim it if necessary.
3. Pre-soak two sheets of blotting paper, nitrocellulose membrane, and foam pads in the transfer buffer just prior to use.
4. Arrange the cassette for transfer in the following order:
    a. Black side of cassette facing down
    b. Foam pad
    c. Blotting paper
    d. Gel
    e. Nitrocellulose membrane
    f. Blotting paper
    g. Foam pad
    h. Clear side of cassette facing up
    i. Close the cassette
5. Load the cassette in the transfer electrode assembly with a magnetic stir bar and an ice pack.
    a. The black side of the cassette should face the black side of the transfer electrode assembly.
    b. If running two transfers in the same tank, make sure that both cassettes are facing the same direction—always black to black
6. Place the buffer tank in an ice bucket. Pour in the rest of the transfer buffer from the casserole dish. Close the buffer tank. Pack the system on ice. Stir at 350rpm.
7. Transfer a total of 600mA.
    a. 400mA for 1.5 hours
    b. 300mA for 2 hours
    c. 200mA for 3 hours

Protocol for blocking:
1. After the gel transfer, block the membrane in 5% milk on the rocker for 1 hour at room temperature.
    a. Block longer at 4°C
2. Rinse the blot in 1x TBS-T.
3. Incubate blot in primary antibody overnight.
    a. Prepare primary antibody according to the manufacturer's directions.
    b. 1:1000 dilution of rabbit anti-ACLY antibody in 10mL antibody dilution buffer
4. Wash three times with 1x TBS-T for 5 minutes each.
5. Incubate blot in secondary antibody for 1 hour.

      a.   1:5000 dilution of anti-rabbit IgG-HRP in 5% milk.
6. Wash three times with 1x TBS-T for 5 minutes each.

Protocol for visualizing blot with ECL:
1. Mix 1mL of ECL1 and 1mL of ECL2 for each nitrocellulose membrane. Directly pipette onto membrane.
2. Wrap the membrane in saran wrap. Let it sit in the dark for 1 minute. Arrange membrane in film cassette in the top left corner.
3. In the developing room, expose the film to the blot. Remove the film from the cassette. Place in developer.
   a. Flip film three times to fit four exposures onto one sheet of film.
   b. The room needs to be dark while developing and before the developer beeps.
   c. Wait until the developer beeps to load another sheet of film or to turn on lights.

Protocol for Extracting RNA from Cultured Cells

Materials and Reagents:
1. RNeasy Mini Kit (Qiagen, Hilden, Germany)
2. QIAshredder (Qiagen, Hilden, Germany)
3. 70% ethanol
4. Tris HCl (pH 8)
5. Cultured cells
6. Eppendorf tubes

Equipment:
1. Microcentrifuge

Protocol (adapted from manufacturer's instructions):
1. Pellet a maximum of 1 billion cells. Add 350µL RLT buffer. Transfer the sample to a QIAshredder. Centrifuge at maximum speed for 3 minutes.
2. Add 350µL 70% ethanol to the lysate. Mix well.
3. Assemble RNeasy mini spin column and collection tube. Transfer up to 700µL sample to the spin column. Centrifuge at 8,000 x g for 30 seconds. Discard flow through.
4. Add 700µL RW1 buffer. Centrifuge at 8,000 x g for 30 seconds. Discard flow through.
5. Add 500µL RPE buffer. Centrifuge at 8,000 x g for 30 minutes. Discard flow through.
6. Add 500µL RPE buffer. Centrifuge at 8,000 x g for 2 minutes. Discard flow through.
7. Transfer spin column to a fresh collection tube. Centrifuge at maximum speed for 1 minute to dry the membrane.
8. Transfer spin column to a fresh collection tube. Add 30µL Tris HCl directly to the RNeasy mini spin column membrane. Incubate at room temperature for 1 minute. Centrifuge at 8,000 x g for 1 minute to elute.

Protocol for Reverse Transcription: Make cDNA from RNA

Materials and Reagents:
1. High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA)
2. RNase inhibitor (Life Technologies, Carlsbad, CA)
3. Molecular grade water
4. RNA sample
5. 0.2mL PCR tubes
6. Cold block

Equipment:
1. Thermocycler

Protocol:
1. Normalize RNA to 100ng/µL.
2. Keep all components on the cold block while in use. In a 0.2mL PCR tube, prepare reverse transcription reaction with the following components:

Component	Volume per reaction (µL)
10x RT buffer	2
25x dNTP mix (100mM)	0.8
10x RT random primers	2
Reverse transcriptase	1
RNase inhibitors	1
Molecular grade water	3.2
RNA (100ng/µL)	10
Total volume	**20**

3. Run on thermocycler:
   i) 25°C 10 min
   ii) 37°C 2 hours
   iii) 85°C 5 min
   iv) 4°C forever

Protocol for qRT-PCR

Materials and Reagents:
1. TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, MA)
2. 20x TaqMan GAPDH probe (Thermo Fisher Scientific, Waltham, MA)
3. 20x TaqMan probe (gene of interest) (Thermo Fisher Scientific, Waltham, MA)
4. Template cDNA
5. Molecular grade water
6. Cold block

Equipment:
1. CFX96 Real-Time System (Bio-Rad, Hercules, CA)

Protocol:
1. Dilute cDNA 1:10.
2. Keep all reagents in the cold block during use. For each reaction, combine the following components:

Component	Volume/reaction (µL)
TaqMan Gene Expression Master Mix	12.5
20x TaqMan probe	1.2
Molecular grade water	1.3
cDNA	10
Total	**25**

   a. In addition to running a probe for the gene of interest, GAPDH control is needed for each sample.
3. Run on real time system:
   i) 50°C 20 seconds
   ii) 95°C 10 minutes
   iii) 95°C 15 seconds
   iv) 50°C 1 minute
   v) 12°C forever
   Repeat (iii) and (iv) for 40 cycles total

Protocol for Purification of DNA from Cultured Cells

Materials and Reagents:
1. DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany)
2. PBS
3. Tris HCl (pH 8)
4. Ethanol
5. Cultured cells
6. Eppendorf tubes

Equipment:
1. Microcentrifuge

Protocol (adapted from manufacturer's instructions):
1. Pellet a maximum of 5 million cells. Resuspend in 200µL PBS. Add 20µL proteinase K.
2. Add 200µL buffer AL (without added ethanol). Vortex and incubate at 56°C for 10 minutes.
3. Add 200µL pure ethanol to the sample. Vortex.
4. Assemble DNeasy mini spin column and collection tube. Transfer sample to the spin column. Add 500µL buffer AW1. Centrifuge at 6,000 x g for 1 minute. Discard flow through and change to clean collection tube.
5. Add 500µL buffer AW2. Centrifuge at 20,000 x g for 3 minutes. Discard flow through and change to clean collection tube.
   a. Do not let the spin column touch the flow through. This will result in carryover of ethanol.
6. Add 200µL Tris HCl directly to the DNeasy mini spin column membrane. Incubate at room temperature for 1 minute. Centrifuge at 6,000 x g for 1 minute to elute.

Protocol for Topo TA Cloning and Competent Cell Transformation

Materials and Reagents:
1. Eppendorf tubes
2. Ice
3. 2% X-Gal (see recipe)
4. Purified PCR product
5. Molecular grade water
6. TOPO TA Cloning Kit for Sequencing (Thermo Fisher Scientific, Waltham, MA)
7. OneShot TOP10 Electrocompetent E.coli (Thermo Fisher Scientific, Waltham, MA)
8. SOC media ((Cellgro, Mediatech, Manassas, VA)
9. 15mL conical
10. Ethanol

Equipment:
1. 42°C water bath
2. Timer
3. Shaking incubator
4. Polyfoam floating rack
5. LB/carbenicillin plates
6. Bacteria incubator
7. Bunsen burner
8. Cell spreader
9. Turn table

Protocol:
1. Spread 40μL of 40mg/mL X-gal onto each LB/carb plate (2 plates per reaction). Incubate plates at 37°C.
2. In an Eppendorf tube, combine the following components:

Component	Volume/reaction (μL)
Purified PCR product	3
Salt solution	1
Molecular grade water	2
TOPO vector	1
Total	**6**

3. Incubate the reaction for 15 minutes at room temperature.
4. Thaw TOP10 OneShot chemically competent *E. coli* on ice for 10 minutes.
5. Add 5μL of the ligation mixture to 25μL TOP10 cells and put them on ice for 30 min.
6. Heat shock the cells in a floating rack in a 42°C water bath for 30 seconds.
7. Put tubes back on ice for 2 minutes.
8. Add 250μL room temperature SOC media to each tube.
9. Put tubes in 15mL conical. Incubate at 37°C for 1 hour shaking at 200 rpm.
10. Plate 100μL onto one plate and 200μL mixture onto other plate.
11. Incubate plates face down at 37°C overnight.
12. Pick ~10 white or light blue colonies for further analysis and Prepare overnight cultures for the selected colonies.

Protocol for Annexin V-FITC/Propidium Iodide Apoptosis Assay

Materials and Reagents:
1. Propidium iodide (Sigma-Aldrich, St. Louis, MO)
2. Annexin V-FITC (BioVision, Palo Alto, CA)
3. 6mL round bottomed FACS tubes
4. FACS tube rack
5. 1x phosphate buffered saline (PBS)
6. 1x Annexin buffer (see recipe)
7. Samples

Equipment:
1. Centrifuge
2. FACS analyzer

Protocol:
1. Transfer samples to 6mL round bottomed FACS tubes.
2. Add 3mL PBS to each sample.
3. Centrifuge at 1100rpm for 5 minutes. Discard the supernatant.
4. Add the following components to each sample:

Component	Volume per sample (µL)
1x Annexin buffer	100
Propidium iodide (1mg/mL)	1
Annexin V-FITC (0.15 mg/mL)	0.25

5. Let samples sit in the dark for 15 minutes.
6. Add 300µL of 1x Annexin buffer to each sample. Samples are ready to be analyzed.

Protocol for PicoProbe Acetyl-CoA Fluorometric Assay (adapted from manufacturer's instructions)

Materials and Reagents:
1. PicoProbe Acetyl-CoA Fluorometric Assay Kit (Biovision, #K317, Palo Alto, CA)
2. Deproteinizing Sample Preparation Kit (Biovision, #K808, Palo Alto, CA)
3. Acetyl-CoA assay lysis buffer (see recipe)
4. dH$_2$O
5. Cultured Cells
6. Eppendorf tubes
7. 1x PBS (see recipe)
8. Cold block
9. 96-well plates

Equipment:
1. Microcentrifuge
2. Sonicator
3. Fluorescence plate reader

Protocol for preparing reagents:
1. PicoProbe: already in DMSO. Warm to room temperature when ready to use. Store at -20°C.
2. Substrate mix: dissolve with 220µL assay buffer. Store at -20°C and use within 2 months
3. Quench remover: dissolve in 220µL dH$_2$O and KEEP ON ICE while in use. Store at -20°C.
4. Acetyl-CoA standard: dissolve in 100µL dH$_2$O to get a 10mM (10nmol/µL) Acetyl CoA standard solution. KEEP ON ICE while in use. Store at -20°C.

Protocol for preparing acetyl-CoA standard curve:
1. 0-1nmol range
    a. Take 10µL of the acetyl-CoA standard and add 990µL dH$_2$O to dilute 100x to 0.1mM (100pmol/µL)
    b. Take 10µL of the 0.1mM standard and add 400µL dH$_2$O to dilute 5x to 0.2mM
    c. Add 0, 10, 20, 30, 40, 50µL into a series of wells in a 96 well plate
    d. Adjust final volumes to 50µL per well with dH$_2$O to generate 0, 200, 400, 600, 800, 1000pmol per well acetyl-CoA standards
2. 0-100pmol range
    a. Take 10µL of acetyl-CoA standard and add 990µL dH$_2$O to dilute 100x to 0.1mM (100pmol/µL)
    b. Take 10µL of 0.1mM standard and add 490µL dH$_2$O to dilute 50x to 2µM (2pmol/µL)
    c. Add 0, 10, 20, 30, 40, 50µL into a series of wells on a 96 well plate
    d. Adjust final volumes to 50µL per well with dH$_2$O to generate 0, 20, 40, 60, 80, 100pmol per well acetyl-CoA standards

Protocol for preparing cultured cells:
1. Make sure all reagents are thawed and kept on ice.
2. Collect cultured cells in an Eppendorf tube and wash with ice cold PBS
3. Add 100μL acetyl-CoA assay lysis buffer to cells in an Eppendorf tube
4. Incubate on ice for 5 minutes
5. Sonicate 4-5 times each on cold block
6. Spin for 10 minutes on maximum speed at 4°C and transfer supernatant to a new tube
7. Quantify the amount of protein in each sample.
8. Take 100μL of sample and mix with 20μL of ice cold PCA in an Eppendorf tube
9. Vortex and place on ice for 5 minutes. Centrifuge at 13000 rpm for 2 minutes
10. Transfer 96μL of the supernatant to a fresh tube
11. Note: at this point the samples may be frozen at -70°C for up to a month
12. Neutralize the sample by adding 4μL of ice cold neutralization solution
13. Place on ice for 5 minutes
14. Spin samples at 13000 rpm for 15 minutes
15. Collect the supernatant
16. Note: the deproteinized samples have been diluted to 80% of the original concentration. Quantitation results should be divided by 0.8 to correct measured values back to the original sample concentrations

Protocol for preparing assay:
1. Add 10μL of the sample into duplicate wells of a 96 well plate. One will serve as the sample and the other will serve as the background
2. Bring the volumes of each well to 50μL with assay buffer
3. Add 10μL of CoA Quencher to each standard, sample, and background well
4. Incubate for 5 minutes at room temperature
5. Add 2μL of Quench Remover
6. Mix and incubate for 5 minutes at room temperature
7. Add buffer, substrate mix, conversion enzyme, and PicoProbe in the amounts specified.

Component	0-1nmol	Background
Buffer	40μL	41μL
Substrate Mix	2μL	2μL
Conversion Enzyme	1μL	---
Enzyme Mix	5μL	5μL
PicoProbe	2μL	2μL

Component	0-100pmol	Background
Buffer	41.8μL	42.8μL
Substrate Mix	2μL	2μL
Conversion Enzyme	1μL	---
Enzyme Mix	5μL	5μL
PicoProbe	0.2μL	0.2μL

8. Incubate for 10 minutes at 37°C.
9. Measure fluorescence using Ex/Em=535/587nm with a plate reader

Calculations:

1. Correct background by subtracting the value of the 0 acetyl-CoA standard from all readings
2. Determine background values for each sample tested and correct acetyl-CoA values for this background
3. Plot the standard curve
4. Calculate the amount of acetyl-CoA in each reading using the standard curve
5. **C=Ay/Sv**   (pmol/µL; or nmol/mL; or µM)
   Ay is the amount of Acetyl CoA (pmol) in your sample from the standard curve
   Sv is the sample volume (10µL) added to the sample well
   Acetyl CoA mw: 809.6g/mol
6. Adjust final concentration for the dilution in the deproteinization step

Recipes

<u>1x TBS-T (2L)</u>
200mL 10x TBS
1800mL dH$_2$O
2mL TWEEN20

<u>1x Annexin buffer</u>
50mL 10x Annexin buffer
450mL dH$_2$O

<u>2% X-Gal</u>
200mg X-Gal powder (Sigma Aldrich, St. Louis, MO)
10mL N,N-Dimethylformamide

<u>5% milk</u>
25g milk powder
500mL 1x TBS-T

<u>6x Laemmli buffer</u>
1M Tris base (pH 6.8)
1g SDS
10mL glycerol
0.1g bromophenol blue
33.7mL dH$_2$O

<u>10x Annexin buffer</u>
100mM HEPES (pH 7.4, NaOH)
1.4M NaCl
25mM CaCl$_2$

<u>10x DNA gel loading dye</u>
50% glycerol
0.25% xylene cyanide

<u>10x PCR buffer</u>
100mM Tris (pH 8.0)
500mM KCl
15mM MgCl$_2$
<u>10x TBS (for 5L)</u>
400g NaCl
10g KCl
150g Tris base

10x TGS (for 10L)
303g Tris base
1442g glycine
100g SDS
pH 8.3
~60mL HCl

20% FBS complete myeloma media
RPMI-1640 medium
20% heat inactivated FBS
1% HEPES buffer
100U/mL penicillin/streptomycin
2mM L-glutamine
(all from Cellgro, Mediatech, Manassas, VA)

50x TAE (for 500mL)
121g Tris Base
50mL EDTA (0.5M)
28.55mL Glacial acetic acid
pH 8.0
~20-25mL HCl

Acetyl-CoA assay lysis buffer
20mM Tris Base
150mM NaCl
1mM EDTA
1mM EGTA
1% Triton X-100
2.5mM sodium pyrophosphate*
1mM β-glycerolphosphate*
1mM sodium orthovanadate*
1µg/mL leupeptin*
1mM PMSF*
*Add just prior to use

Antibody dilution buffer
5% BSA
0.1% sodium azide
in TBS-T

Complete myeloma media
RPMI-1640 medium
10% heat inactivated FBS
1% HEPES buffer
100U/mL penicillin/streptomycin
2mM L-glutamine (all from Cellgro, Mediatech, Manassas, VA)

ECL1
200µL luminol (250mM)
88µL p-coumeric acid (90mM)
1mL Tris base (2M, pH 8.5)
18.71mL dH$_2$O

ECL2
12µL 30% hydrogen peroxide
1mL Tris base (2M, pH 8.5)
19mL dH$_2$O

Freeze media
90% Fetal bovine serum (FBS)
10% DMSO

Gel transfer buffer
100mL 10x TG
200mL methanol
700mL dH$_2$O

HEK293T complete media
500mL Dulbecco's Modified Eagle Media (DMEM)
10% Heat-inactivated fetal bovine serum (FBS)
100 U/mL penicillin/streptomycin
2mM L-glutamine
1% non-essential amino acids
1mM sodium pyruvate
1% HEPES buffer
(all from Cellgro, Mediatech, Manassas, VA)

Lysis buffer
500µL RIPA buffer
5µL PMSF (100mM)
5µL protease inhibitor cocktail

RIPA buffer
150mM NaCl
1% NP-40
0.5% Na-deoxycholate
0.1% SDS
50mM Tris base
pH 7.4
~2mL HCl

Appendix II:

Primers and Plasmids

	sgRNA sequence	Forward primer	Reverse primer	Exon	Specificity score
1	TTCCACGACGTTTGATCAGC	GTTCCACGACGTTTGATCAGCG TTTTAGAGCTAGAAATAGCAA	GCTGATCAAACGTCGTGGAA CGGTGTTTCGTCCTTTCC	3	96
2	GACCAGCTGATCAAACGTCG	GACCAGCTGATCAAACGTCGGT TTTAGAGCTAGAAATAGCAA	CGACGTTTGATCAGCTGGTC GGTGTTTCGTCCTTTCC	3	94
3	CGACGTTTGATCAGCTGGTC	GCGACGTTTGATCAGCTGGTCG TTTTAGAGCTAGAAATAGCAA	GACCAGCTGATCAAACGTCG CGGTGTTTCGTCCTTTCC	3	93
4	GAGCATACTTGAACCGATTC	GAGCATACTTGAACCGATTCGT TTTAGAGCTAGAAATAGCAA	GAATCGGTTCAAGTATGCTC GGTGTTTCGTCCTTTCC	2	97
5	AGAATCGGTTCAAGTATGCT	GAGAATCGGTTCAAGTATGCTG TTTTAGAGCTAGAAATAGCAA	AGCATACTTGAACCGATTCT CGGTGTTTCGTCCTTTCC	2	93
6	GAGAGCAATTCGAGATTACC	GAGAGCAATTCGAGATTACCGT TTTTAGAGCTAGAAATAGCAA	GGTAATCTCGAATTGCTCTCG GTGTTTCGTCCTTTCC	11	96

F_c: AAACTCGAGTGTACAAAAAGCAGGCTTTAAAG

R1: Rc(GN_19)GGTGTTTCGTCCTTTCC

F1: GN_19GTTTTAGAGCTAGAAATAGCAA

R_c: AAAGCTAGCTAATGCCAACTTTGTACAAGAAAGCTG

Note: Rc is the reverse complement of the guide sequence.
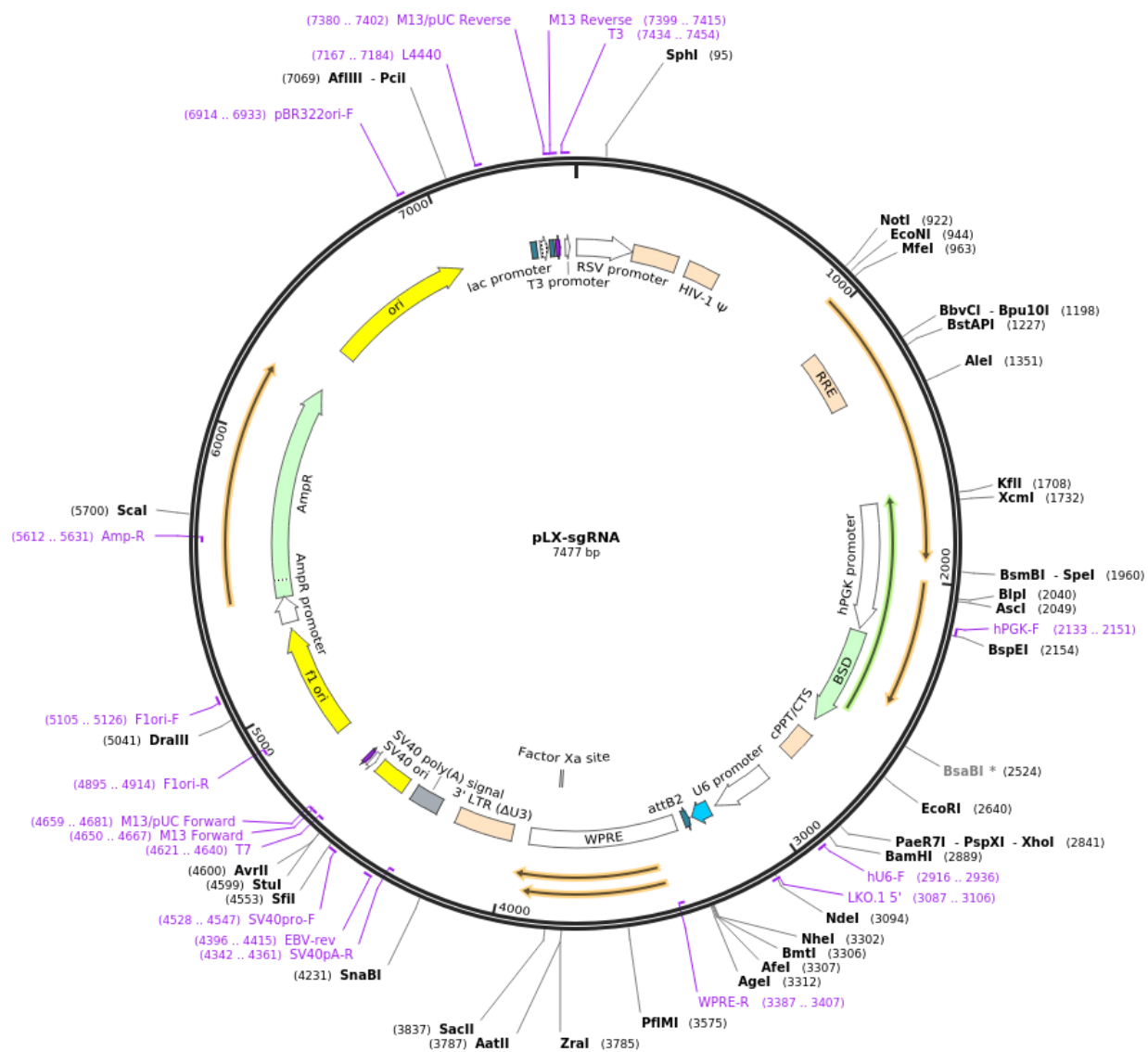
Sequence:

Red: Xho1 recognition site
Orange: U6 promoter
Green: gRNA scaffold
Blue: Nhe1 recognition site

5'

tgcaagcttaatgtagtcttatgcaatactcttgtagtcttgcaacatggtaacg
atgagttagcaacatgccttacaaggagagaaaaagcaccgtgcatgccgattgg

tggaagtaaggtggtacgatcgtgccttattaggaaggcaacagacgggtctgac
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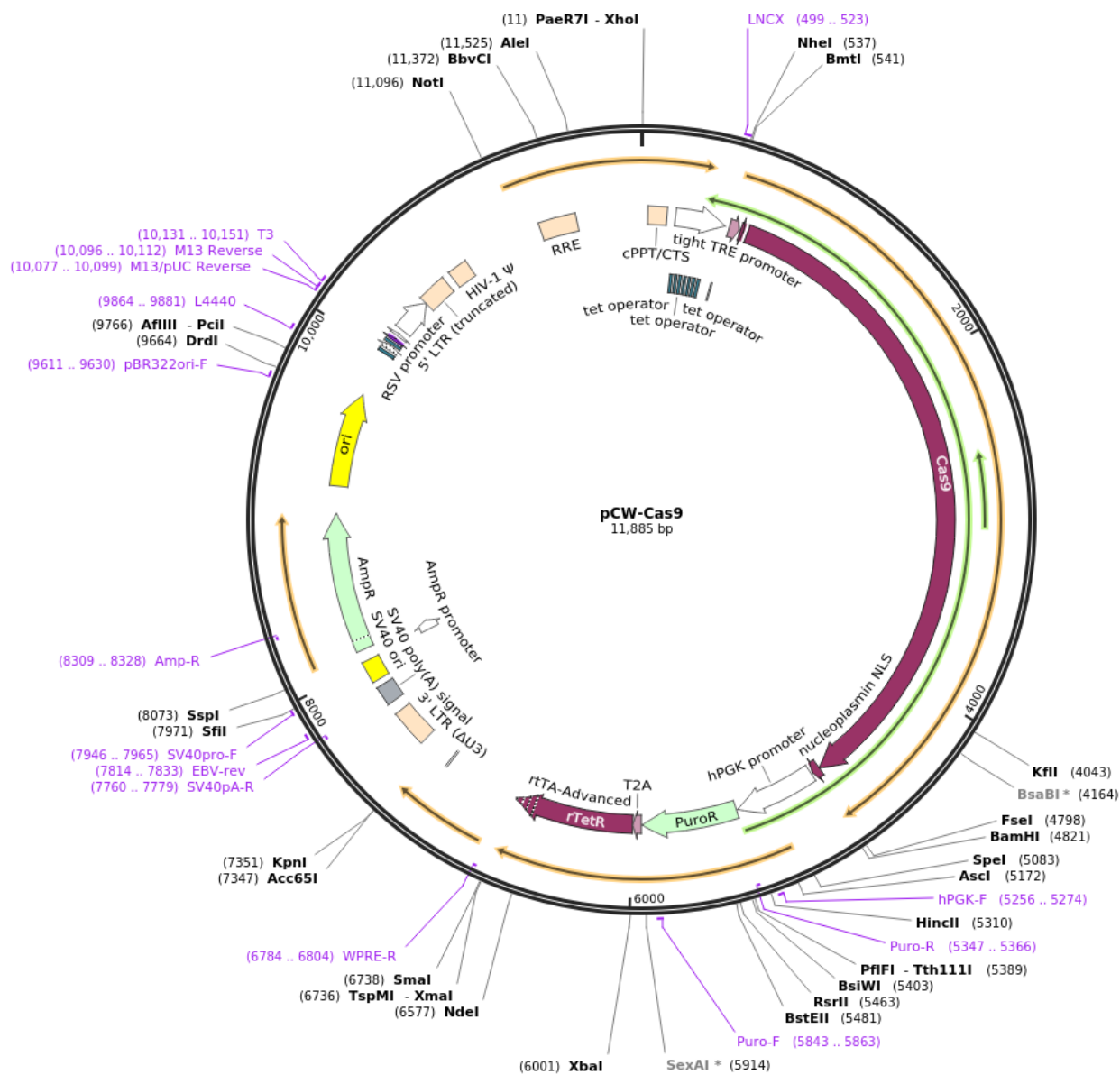
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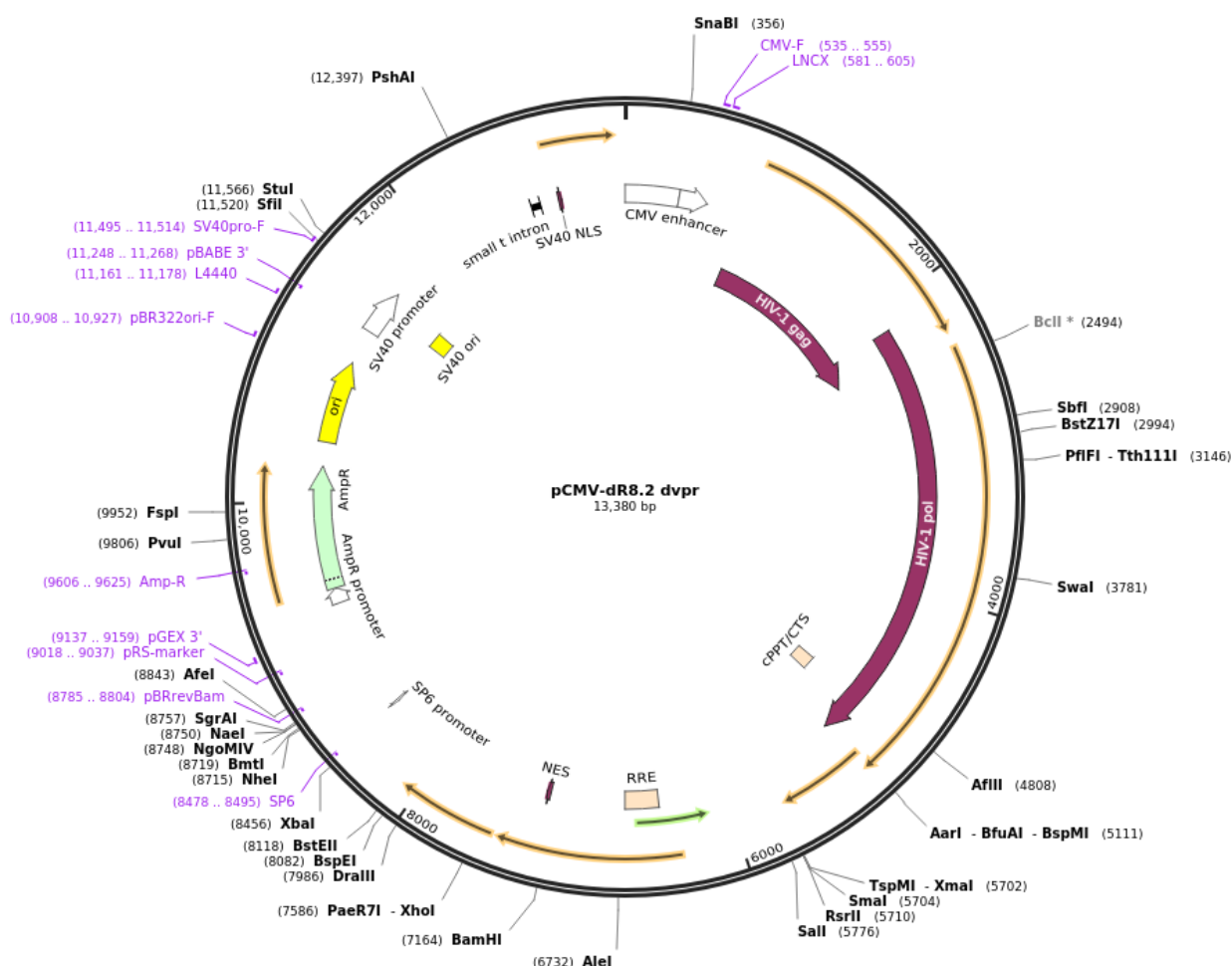
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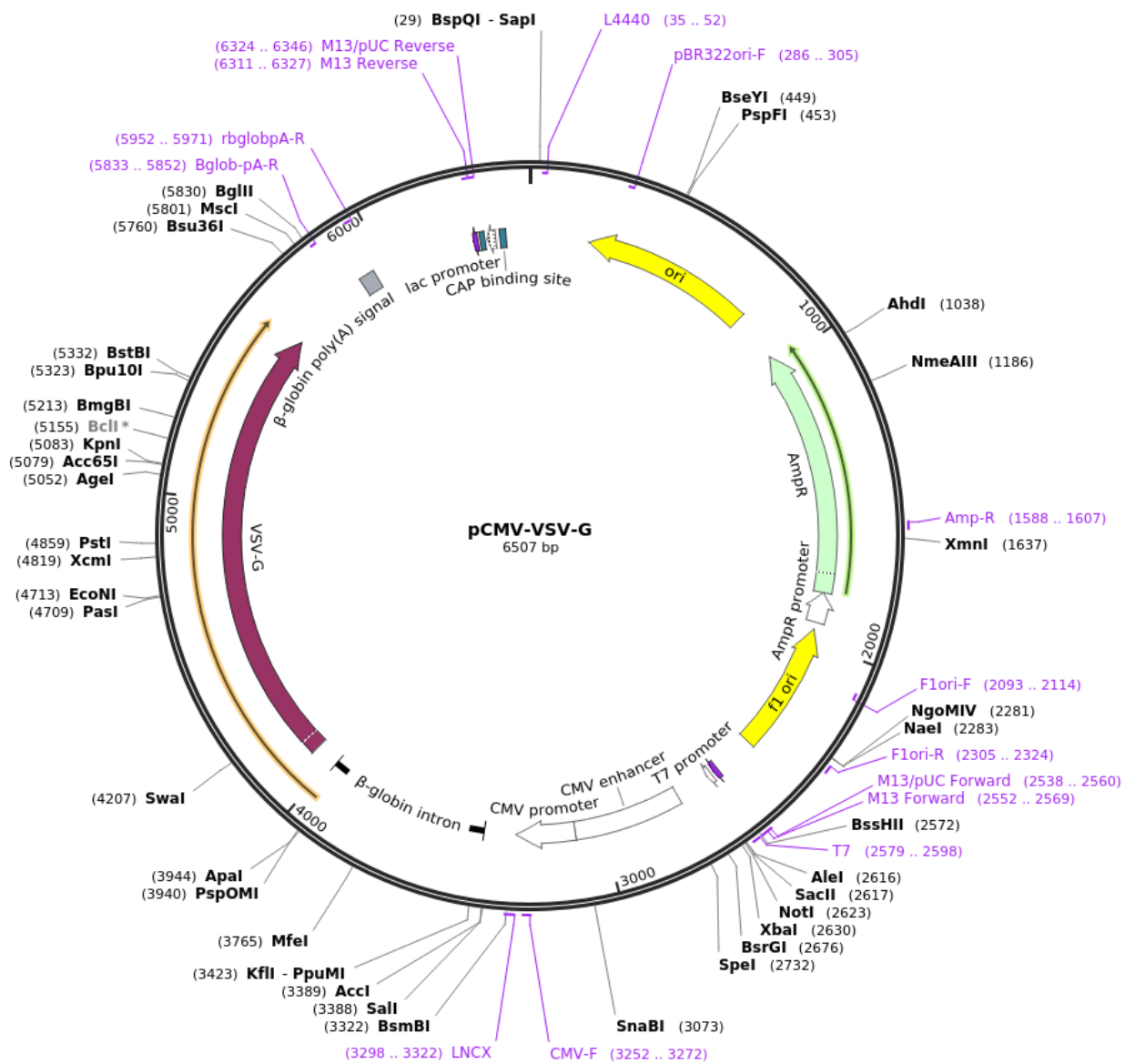atctacatagaaagtttctgctcctattatgggttctttctctaactggtaccat

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(29) **BspQI - SapI**

(6324 .. 6346) M13/pUC Reverse
(6311 .. 6327) M13 Reverse

L4440 (35 .. 52)

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(5833 .. 5852) Bglob-pA-R

(5830) **BglII**
(5801) **MscI**
(5760) **Bsu36I**

ori

lac promoter
CAP binding site

β-globin poly(A) signal

AhdI (1038)

NmeAIII (1186)

(5332) **BstBI**
(5323) **Bpu10I**

(5213) **BmgBI**
(5155) **BclI ***
(5083) **KpnI**
(5079) **Acc65I**
(5052) **AgeI**

AmpR

VSV-G

(4859) **PstI**
(4819) **XcmI**

Amp-R (1588 .. 1607)
XmnI (1637)

(4713) **EcoNI**
(4709) **PasI**

AmpR promoter

f1 ori

(4207) **SwaI**

F1ori-F (2093 .. 2114)
NgoMIV (2281)
NaeI (2283)

F1ori-R (2305 .. 2324)

M13/pUC Forward (2538 .. 2560)
M13 Forward (2552 .. 2569)

β-globin intron

CMV enhancer
CMV promoter

T7 promoter

BssHII (2572)

T7 (2579 .. 2598)

AleI (2616)
SacII (2617)
NotI (2623)
XbaI (2630)
BsrGI (2676)
SpeI (2732)

(3944) **ApaI**
(3940) **PspOMI**

(3765) **MfeI**

(3423) **KflI - PpuMI**
(3389) **AccI**
(3388) **SalI**
(3322) **BsmBI**

(3298 .. 3322) LNCX

CMV-F (3252 .. 3272)

SnaBI (3073)

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