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Ire1α Rnase-Dependent Lipid Homeostasis Promotes Survival In Myc-Transformed Cancers

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Ire1α Rnase-Dependent Lipid Homeostasis Promotes Survival In Myc-Transformed Cancers

Abstract
Myc family activation is a primary oncogenic event in many human cancers; however, these transcription factors are difficult to inhibit pharmacologically, suggesting that Myc-dependent downstream effectors may be more tractable therapeutic targets. Here, I show that Myc overexpression induces endoplasmic reticulum (ER) stress and engages the IRE1α-XBP1 pathway through multiple molecular mechanisms in a variety of c-Myc- and N-Myc-dependent cancers, e.g. Burkitt’s lymphoma and neuroblastoma. In particular, Myc-overexpressing cells require IRE1α-XBP1 signaling for sustained growth and survival in vitro and in vivo, dependent on elevated stearoyl-CoA-desaturase 1 (SCD1) activity. Pharmacological and genetic XBP1 inhibition induces Myc-dependent apoptosis, which is alleviated by exogenous unsaturated fatty acids. Of note, SCD1 inhibition phenocopies IRE1α RNase activity suppression both in vitro and in vivo. Furthermore, IRE1α inhibition enhances the cytotoxic effects of standard chemotherapy drugs used to treat c-Myc-overexpressing Burkitt’s lymphoma, suggesting that inhibiting the IRE1α-XBP1 pathway is a useful general strategy for treatment of Myc-driven cancers.

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IRE1α RNASE-DEPENDENT LIPID HOMEOSTASIS PROMOTES SURVIVAL IN MYC-TRANSFORMED CANCERS

Hong Xie

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2018

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IRE1α RNASE-DEPENDENT LIPID HOMEOSTASIS PROMOTES SURVIVAL IN MYC-TRANSFORMED CANCERS

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Hong Xie

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ABSTRACT

IRE1α RNASE-DEPENDENT LIPID HOMEOSTASIS PROMOTES SURVIVAL IN MYC-TRANSFORMED CANCERS

Hong Xie

M. Celeste Simon

Myc family activation is a primary oncogenic event in many human cancers; however, these transcription factors are difficult to inhibit pharmacologically, suggesting that Myc-dependent downstream effectors may be more tractable therapeutic targets. Here, I show that Myc overexpression induces endoplasmic reticulum (ER) stress and engages the IRE1α-XBP1 pathway through multiple molecular mechanisms in a variety of c-Myc- and N-Myc-dependent cancers, e.g. Burkitt’s lymphoma and neuroblastoma. In particular, Myc-overexpressing cells require IRE1α-XBP1 signaling for sustained growth and survival in vitro and in vivo, dependent on elevated stearoyl-CoA-desaturase 1 (SCD1) activity. Pharmacological and genetic XBP1 inhibition induces Myc-dependent apoptosis, which is alleviated by exogenous unsaturated fatty acids. Of note, SCD1 inhibition phenocopies IRE1α RNase activity suppression both in vitro and in vivo. Furthermore, IRE1α inhibition enhances the cytotoxic effects of standard chemotherapy drugs used to treat c-Myc-overexpressing Burkitt’s lymphoma, suggesting that inhibiting the IRE1α-XBP1 pathway is a useful general strategy for treatment of Myc-driven cancers.
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CHAPTER 1: Introduction

Introduction to Myc and its role in cancer

The Myc family of proto-oncogenes (*MYC, MYCN, MYCL*) encodes the c-Myc, N-Myc, and L-Myc basic helix-loop-helix leucine zipper (bHLHZ) transcription factors, respectively. Myc oncoproteins belong to a family of so-called “super-transcription factors” that potentially regulate the transcription of at least 15% of the entire genome (Dang et al., 2006). Upon activation, Myc recognizes CACGTG sequences termed “E boxes”, as a heterodimer paired with its binding partner Max, to regulate genes coordinating numerous cellular processes, including but not limited to proliferation, apoptosis, differentiation, self-renewal, and metabolism (Dang, 2013; 2012).

*Myc* is the only isoform expressed ubiquitously in a broad range of tissues and organs. In contrast, *MYCN* and *MYCL* are expressed in a more tissue-restricted manner, such as in the central nervous system and lung epithelium (Dang, 2012). However, it is generally believed that Myc family members can sometimes be functionally interchangeable (Malynn et al., 2000). Although *MYC* genes are normally induced by mitogenic stimulation and their activity tightly regulated under physiological conditions, oncogenic activation occurring via mutation, genomic amplification, and translocation leads to aberrant overexpression or stabilization of Myc proteins (Gabay et al., 2014).

*MYC* is the most frequently amplified oncogene in human cancers (Beroukhim et al., 2010). It was first discovered as the cellular homolog of the retroviral *v-Myc* oncogene identified from studies of oncogenic retroviruses (Bister and Jansen, 1986; Duesberg et al., 1977; Sheiness et al., 1978). Afterwards, chromosomal translocations that juxtapose *MYC* to immunoglobulin enhancers were discovered in Burkitt’s lymphoma (BL) (Dalla-Favera et al., 1982). Classic in *vitro* assays using normal primary rat embryo fibroblasts then documented c-Myc’s transforming activity in cooperation with activated RAS and the sufficiency of these two oncogenes to
transform normal cells (Land et al., 1983). In addition, multiple transgenic mouse models based on deregulated c-Myc expression have illustrated its transforming activity in vivo and support the notion that it is a human oncogene (Morton and Sansom, 2013).

**Targeting oncogenic c-Myc as a strategy for cancer treatment**

Dysregulation of Myc oncoproteins is a frequent event in a broad array of human cancers, including BL, breast cancer, neuroblastoma (NB), etc (Dang, 2012). The established role of c-Myc protein in tumor initiation and maintenance has made it an appealing target for cancer therapy (Gabay et al., 2014; Huang and Weiss, 2013; Schmitz et al., 2014). In the past two decades, numerous attempts have been made to develop strategies to directly or indirectly target c-Myc expression or activation (Brooks and Hurley, 2010; Delmore et al., 2011; Kiessling et al., 2007; Puissant et al., 2013). However, despite a confluence of detailed mechanistic insights and unmet medical needs, therapeutic strategies to directly manipulate c-Myc remain a historic challenge (McKeown and Bradner, 2014; Meyer and Penn, 2008). Therefore, essential steps involved in c-Myc deregulation have been exploited as new approaches to treat c-Myc-driven cancers. Examples include targeting c-Myc transcription with BET inhibitor JQ1 in multiple types of cancers (Delmore et al., 2011; Puissant et al., 2013), targeting MYC mRNA translation using pharmacological inhibition of the PI3K/AKT/mTOR pathway (Bjornsti and Houghton, 2004; Chapuis et al., 2010; Frost et al., 2004; Yu et al., 2001), targeting c-Myc stability through the ubiquitin-proteasome system (Popov et al., 2007; Tavana et al., 2016; Yada et al., 2004), or disrupting the Myc-Max complex (Annibali et al., 2014; Berg et al., 2002; Wang et al., 2007). These compounds appear to have significant therapeutic value for cancers with high levels of c-Myc activity, although some effects are c-Myc-independent. However, future advanced trials are needed to examine their efficiency and safety in humans.

**Oncogene activation and endoplasmic reticulum (ER) stress in cancer**
Cellular transformation into the malignant phenotype is characterized by hyperactivation of oncogenes leading to unregulated cell cycle progression and proliferation. In normal cells, metabolism is fine tuned to match oxygen, nutrient, and growth factor availability. However, in tumor cells, oncogenic transformation on one hand commits cancer cells to anabolic growth rates, but on the other hand generates metabolic stresses, which must be overcome to sustain survival and growth. The ER is the organelle in eukaryotic cells responsible for protein folding and transport, the disruption of which leads to accumulation of unfolded or misfolded proteins – a condition termed “ER stress”. In addition, ER is a principle site for membrane biogenesis and a major distribution “hub” for lipid trafficking to and from the cell (Jackson et al., 2016; Quon et al., 2018). In particular, increased ER protein load requires expansion of ER membranes via lipid synthesis, a process that is particularly important under ER stress. In addition, disrupted ER lipid composition also impairs protein-folding capacity. Therefore, the maintenance of ER functions by coordinating protein synthesis and lipid metabolism is necessary to support the high rates of anabolic metabolism required for tumor growth (Griffiths et al., 2013; Young et al., 2013).

A number of cell-intrinsic mechanisms contribute to cancer cell-specific induction of ER stress, such as the hyperactivation of mTORC1 due to deficiencies of the tumor suppressor tuberous sclerosis complex proteins (Tsc)-1 or Tsc2 (Saxton and Sabatini, 2017). Indeed, multiple studies demonstrated that heightened protein synthesis in mTORC1-activated cells induces ER stress (Clarke et al., 2014; Ozcan et al., 2008), which is further exacerbated by conditions of nutrient and O₂ deprivation characteristic of solid tumor microenvironments (Ackerman and Simon, 2014; Young et al., 2013). Another example is c-Myc activation. Activated c-Myc upregulates genes involved in ribosome biogenesis, resulting in substantial enhancement of protein translation and protein content (van Riggelen et al., 2010). This effect is required for c-Myc-mediated transformation, as haploinsufficiency of ribosomal gene L24 reduced protein synthesis rates and inhibited tumor progression in the Eμ-Myc model of B-cell lymphoma (Barna
et al., 2008). In addition, Hart et al. demonstrated that c-Myc activation increases ER protein load, therefore inducing ER stress (Hart et al., 2012).

**ER stress and the unfolded protein response (UPR) signaling**

Upon encountering ER stress, cells activate a series of complementary adaptive mechanisms to cope with protein folding alterations, which together are known as the unfolded protein response (UPR) (Hetz, 2012). In mammalian cells, the UPR has evolved into a complex network of signaling events, mediated by the activation of at least three major stress sensors: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase R (PKR) like endoplasmic reticulum kinase (PERK) (Hetz, 2012). Each stress sensor engages unique mechanisms to impact distinct transcription factors, initiating a specific molecular response (Malhotra and Kaufman, 2007; Ron and Walter, 2007). This process transduces information concerning the protein-folding status in the ER lumen to the nucleus and cytosol to buffer fluctuations in unfolded protein load. The outcome of UPR activation involves attenuation of protein synthesis, increased protein folding and elevated protein degradative pathways. If these adaptive pathways cannot resolve ER stress, cells enter apoptosis (Wang and Kaufman, 2014).

Interestingly, PERK promotes an autophagic program that sustains cell viability and promotes tumor growth in c-Myc-overexpressing cells (Hart et al., 2012; Nagy et al., 2013). In contrast, a role for the IRE1 pathway in c-Myc-overexpressing cells, and its potential utility as a therapeutic target for c-Myc-driven cancers, has not been investigated. IRE1 is the only ER stress sensor conserved from yeast to mammals, and the mammalian genome encodes two isoforms, IRE1α and IRE1β. IRE1α is more ubiquitously expressed, whereas IRE1β is restricted to the epithelium of the gastrointestinal tract (Chen and Brandizzi, 2013). IRE1 proteins have an ER-luminal sensor domain that recognizes unfolded proteins, as well as cytosolic kinase and endoribonuclease (RNase) domains that mediate responses through downstream effectors (See Figure 1A) (Chen and Brandizzi, 2013). Under conditions of ER stress, IRE1 is activated through
dimerization and autophosphorylation, and removes 26 nucleotides from unspliced X-Box Binding Protein 1 (XBP1u) mRNA to generate spliced XBP1 (XBP1s), producing a functional XBP1s transcription factor (Figure 1A) (Chen and Brandizzi, 2013). XBP1s in turn regulates the expression of numerous genes, the protein products of which operate ER-associated degradation (ERAD), the entry of proteins into the ER and protein folding, among other functions (Acosta-Alvear et al., 2007; Lee et al., 2003). XBP1s also modulates phospholipid synthesis, which is required for ER membrane expansion under ER stress (Hetz et al., 2011). In cancer settings, XBP1s has been demonstrated to promote tumor progression in triple-negative breast cancer (Chen et al., 2014) and multiple myeloma (Mimura et al., 2012), as well as chronic lymphocytic leukemia (Tang et al., 2014). Besides functioning through XBP1 splicing, the IRE1 RNase also selectively degrades ER-bound mRNAs to alleviate ER protein load, a process known as regulated inositol-requiring enzyme 1-dependent decay (RIDD) (Hollien et al., 2009).

Summary

The central goal of this study was to identify synthetic lethal targets in Myc-overexpressing cells as an alternative strategy for the treatment of Myc-dependent cancers. We demonstrated that the IRE1α-XBP1 pathway is engaged in both c-Myc- and N-Myc-driven cancers, and that c-Myc regulates this pathway through multiple molecular mechanisms: 1) directly activating ERN1, HSPA5, and XBP1 transcription, 2) stabilizing IRE1α protein, and 3) increasing ER protein load, thereby activating IRE1α RNase activity and promoting XBP1 splicing. Moreover, IRE1α-XBP1 signaling induces stearoyl-CoA desaturase (SCD) transcription, which generates unsaturated lipids required for ER membrane homeostasis. Pharmacological inhibition of IRE1α RNase activity or XBP1 depletion decreases growth and initiates apoptosis preferentially in Myc-overexpressing cells in vitro and in vivo, and is reversed by exogenous unsaturated lipids. Treatment with a SCD inhibitor phenocopies the effects of IRE1α suppression on in vivo tumor growth. Finally, a highly selective IRE1α inhibitor (B-I09) exhibits synergistic effects with standard
of care (e.g. Doxorubicin) to treat c-Myc-transformed BL. Taken together, these findings reveal an essential mechanism whereby oncogene-driven anabolic metabolism engages homeostatic stress responses to promote tumor growth. Importantly, the use of IRE1α inhibitors (like B-I09) could improve treatment of both c-Myc- (e.g. BL) and N-Myc- (e.g. neuroblastoma) driven malignancies.
CHAPTER 2 Materials and Methods

Cell Culture

Raji, Daudi, Ramos, EB-2, SK-N-AS, and BE2C cells were obtained from the American Type Culture Collection in 2016, and the Kelly cell line was obtained from Sigma Aldrich. MEC1, MEC2, and WaC3 cells were described previously (Kriss et al., 2012). 8498 cells were obtained from Dr. Alexander L. Kovalchuk (National Institute of Allergy and Infectious Disease, National Institute of Health, Rockville, MD, USA) and Dr. Herbert C. Morse III (National Institute of Allergy and Infectious Disease, National Institute of Health, Rockville, MD, USA). N-MycER SHEP cells were described previously (Ushmorov et al., 2008). Cells were cultured for a maximum of 6 weeks before thawing fresh, early passage cells, and routinely confirmed to be Mycoplasma negative. Raji, Daudi, Ramos, EB-2, MEC1, MEC2, WaC3, P493, 8498, N-MycER SHEP, and Kelly cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, and penicillin/streptomycin and cultured in 5% CO2 at 37 °C. SK-N-AS cells were cultured in DMEM supplemented with 15% FBS, 2mM glutamine, 1X non-essential amino acid (NEAA), and penicillin/streptomycin. BE2C cells were maintained in DMEM with 10% FBS, 4mM glutamine, and penicillin/streptomycin. During experimental conditions, FBS was decreased to 5%, with other components unchanged. For lipid deprivation experiments, lipid concentration in the culture medium was achieved through the dilution of complete FBS into Delipidized FBS (cat. 900-123, Gemini Bio-products) without changing other components. The P493 cells were derived from human peripheral blood B cells immortalized by an EBV genome that is complemented with an EBV nuclear antigen-estrogen receptor (EBNA2-ER) fusion protein and a tetracycline-repressible c-Myc transgene. With tetracycline (0.1µg/mL) and beta-estradiol (1µM), which activates EBNA2-ER, the cells proliferate with induction of endogenous c-Myc by EBNA2, achieving a "Low Myc" state that is equivalent to EBV-immortalized B lymphocytes. With tetracycline alone, c-Myc is suppressed and a "No Myc" state is achieved. In the absence of
tetracycline and beta-estradiol, ectopic c-Myc is induced in a "High Myc" tumorigenic state that resembles human BL. In N-MycER SHEP cells, N-MycER activation was performed by treatment with 4-Hydroxytamoxifen (4-OHT) at 200nM.

**Reagents**

B-I09 was described in a previous publication (Tang et al., 2014). Tetracycline (cat. 87128), beta-estradiol (cat. E8875), cycloheximide (cat. C7698), tunicamycin (cat. T7765), fatty acid free BSA (cat. A8806), OA (cat. O3008), POA (cat. P9417), sodium palmitate (cat. P9767), 4-OHT (cat. H7904), doxorubicin hydrochloride (cat. D1515) were purchased from Sigma Aldrich. JQ1 (cat. 4499) was from TOCRIS. 4µ8c (cat. 412512) was from Millipore. SCDi (cat. Cay10012562) and vincristine sulfate (cat. 11764) were purchased from Cayman. U-13C-glucose (cat. CLM-1396) was from Cambridge Isotope Laboratories.

**Plasmids, Virus production and Infection**

GIPZ non-silencing lentiviral shRNA control (Clone ID: RHS4346) and shRNA targeting XBP1 (Clone ID: V3LHS_387388) were purchased from Dharmacon. Inducible control shRNA (Forward: 5’-CCGGCCTAAGGTAAATGCTGCCTGTGGAGGCGACTTAACCTTAGGTTTTTG-3’, reverse: 5’-AATTCAAAAAACCTAAGGTAAATGCTGCCTGTGGAGGCGACTTAACCTTAGGTTTTTG-3’) or XBP1 shRNA (Forward: 5’-CCGGGACCCAGTCTAGTCTCTCAAACCTCAGGTGTTGAAGAACATGACTGGGTCTTTTTG-3’, reverse: 5’-AATTCAAAAAAGACCCAGTCTAGTCTCTCAAACCTCAGGTGTTGAAGAACATGACTGGGTCTTTTTG-3’) were cloned in pLKO-Tet-On lentiviral vector. To produce lentiviruses, 293T cells were co-transfected with the lentivirus expression vectors psPAX2 and pMD2.G using FuGene 6 transfection reagent (cat. E2691, Promega). Lentiviruses were collected 48 hours after
transfection. For N-MycER SHEP cells, the infection efficiency was >95% after 2 days infection, examined by GFP positive cells. For Kelly cells, viruses were used with 8µg/mL polybrene for infection and cells were selected with 0.75µg/mL puromycin for 5 days to establish stable cell lines.

**RNA Interference**

siRNA pools targeting human XBP1 (cat. L-009552), SCD (cat. L-005061), and non-targeting pool control (cat. D-001810) were from Dharmacon. For P493 cells, 5*10^6 cells were electroporated using an Amaxa Nucleofector with Program O-06, Nucleofactor kit V (cat. VCA-1003, Lonza) and 2µM siRNA for each reaction were used. For N-MycER SHEP cells, Lipofectamine RNAiMAX Reagent (cat. 13778, Invitrogen) was employed according to the manufacturer's instructions.

**Viability Assays**

Cell viability was determined using the FITC-Annexin V, PI Kit (cat. 556547) or APC-Annexin V (cat. 550475) for GFP-positive cells from BD Biosciences according to the manufacturer's instructions. Flow cytometry was performed using the BD Accuri C6 instrument or BD FACSCalibur flow cytometer, and double-negative cells were determined viable.

**Cell Growth Assay**

BL cell lines and CLL cells were seeded in 6-well plates and exposed to indicated treatments. Cells were counted at various time points using the Invitrogen Countess Automated Cell Counter (cat. C10281), as per the manufacturer's instructions. Control SHEP, 4-OHT SHEP, SK-N-AS, BE2C, and Kelly cells were seeded in 96-well plates and exposed to indicated treatments. At indicated time points, cell growth was analyzed using the WTS-1 reagent (cat. 11644807001, Roche), according to the manufacturer's instructions.

**Transmission Electron Microscopy**

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P493 cells with or without c-Myc overexpression were cultured at a concentration of $5 \times 10^5$ cells/mL. Cells were pelleted through centrifugation and washed with PBS once before fixation. Cells were then fixed with 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4, overnight at 4°C. After subsequent buffer washes, the sample were post-fixed in 2.0% osmium tetroxide for 1 hour at room temperature, and then washed again in buffer followed by distilled water. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in Embed812. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and ATM Advantage image capture software. At least 30 cells were analyzed from each group, and representative images are shown.

**RNA Extraction, Real-time Quantitative RT-PCR, and RT-PCR Analysis**

Total RNA was isolated using the RNeasy Mini Kit (cat. 74104, Qiagen), cDNA was synthesized using the High-Capacity RNA-to-cDNA Kit (cat. 4387406, ThermoFisher Scientific). qRT-PCR was performed on a ViiA7 Real-Time PCR system from Applied Biosystems. Predesigned Taqman primers were obtained from ThermoFisher Scientific for the following genes: TBP (HS00427620_M1), ACTB (HS01060665_G1), HSPA5 (HS00946084_G1), MYC (HS00153408_M1), MYCN (HS00232074_M1), XBP1t (HS00231936_M1), XBP1s (HS03929085_G1), LDHA (HS01378790_G1), ERN1 (HS00176385_M1), LXR (HS00172885_M1), FABP5 (HS02339437_G1), PPARA (HS00947536_M1), ACACA (HS01046047_M1), ACACB (HS01565914_m1), FABP6 (HS01031183_M1), PPARD (HS004187066_G1), ACAT1 (HS00608002_M1), CPT1A (HS00912671_M1), CPT1B (HS03046298_S1), SCD (HS01682761_M1), ACLY (HS00982738_M1), FASN (HS01005622_M1), ACSS2 (HS01122829_M1), DGAT1 (HS01017541_M1), DGAT2 (HS01045913_M1), PLIN2 (HS00605340_M1), PLIN3 (HS00998416_M1), HMGCS1 (HS00940429_M1), HMGCR (HS00168352_M1), ODC1 (HS00159739_M1), HERPUD1 (HS01124269_M1), DNAJB9 (HS01052402_M1), ATF3 (HS00231069_M1), and DDIT3.
VeriQuest Fast Probe qPCR Master Mix (cat. 75680) was purchased from Affymetrix. SYBR-green primers were utilized for mouse Actb (forward: 5'-AAATCTGGGACCACACCTTC-3', reverse: 5'-GGGGTGTTGAAGGTCTCAAA-3'), mouse Xbp1t (forward: 5'-GGCTGTCTGGCCCTAGAAGA-3', reverse: 5'-CTGTCAGAGTCCATGGGA-3'), mouse Xbp1s (forward: 5'-GGATCCGGAGCAGGTG-3', reverse: 5'-CTGTCAAATGACCCTCCCT-3'), human XBP1t (forward: 5'-GGCATCCTGGCTTGCCTCCA-3', reverse: 5'-GCCCGCTGAGGCTGTTCC-3'), human XBP1s (forward: 5'-CTGAGTCCGCAGTCCAGTGG-3', reverse: 5'-TCCAGTTGCTCAGAGGAGG-3'). SYBR Green PCR Master Mix (cat. 4309155) was purchased from ThermoFisher Scientific. RT-PCR assay for XBP1 splicing was described previously (Oslowski and Urano, 2011). Primers for XBP1 are: forward: 5'-CCTGGTTGCTGAAGAGGAGG-3', reverse: 5'-CCATGGGGAGATGTTCTGGAG-3'. Primers for internal control 18S rRNA are: forward: 5'-GGCCCTGTAATTGGAATTGGACT-3', reverse: 5'-CCAAGATCCAATCAGACCTT-3'.

**Western Blot Analysis**

Cells were lysed in 150mM NaCl, 10mM Tris PH7.6, 0.1% SDS, and 5mM EDTA containing Roche complete ultra protease/phosphatase inhibitor (cat. 05892791001). Nuclear fractionation was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (cat. 78833, ThermoFisher Scientific). Protein concentration was quantified with Pierce BCA Protein Assay Kit (cat. 23225, ThermoFisher Scientific). Isolated proteins were resolved by SDS-PAGE, and Western blot analysis was performed. IRE1α phosphorylation was monitored by Phos-tag SDS-PAGE as described previously (Yang et al., 2010). All primary antibodies were diluted in 1:1,000 in 5% w/v nonfat milk, unless otherwise noted. Blots were incubated with primary antibodies overnight at 4°C. XBP1s (cat. 619502, 1:500) antibody was from BioLegend. c-Myc (cat. ab32072), Actin (cat. ab3280), SCD1 (cat. ab19862) antibodies were purchased from Abcam. IRE1α (cat. 3294), BiP (cat.3177), PARP (cat. 9542), GAPDH (cat. 2118), total JNK (cat. 9252), phospho-threonine 183/185 JNK (cat. 9251), p62 (cat. 5114), LC3B (cat. 2775), N-Myc (cat.
9405), and HDAC1 (cat. 5356) antibodies were purchased from Cell Signaling Technology. DDRGK1 antibody (cat. HPA013373) was from Sigma Aldrich. HRD1 antibody (cat. NB100-2526) was from Novus. SEL1L antibody was generated in our laboratory. Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) followed by exposure to enhanced chemiluminescence substrate (cat. NEL103001EA, PerkinElmer) or SuperSignal West Femto Maximum Sensitivity Substrate (cat. 34095, ThermoFisher Scientific).

Chromatin Immunoprecipitation (ChIP) and ChIP-qPCR Assay

This assay was performed as described previously (Chen et al., 2014). qPCR using SYBR Green was performed per manufacturer’s protocol. The primers used for positive control ERdj4 are forward: 5’- GCAGCAACAAACAGTTTCCA-3’, reverse: 5’-GCACCCTAATCTCGTGTCGA-3’. Primers for negative control are forward: 5’-TTCAGGGGAAGAAAAACTTGGGA-3’, reverse: 5’-TCCGAAAACCCTGACTC-3’, which is located upstream of the SCD promoter. Primers for XBP1s binding site within the SCD promoter region are forward: 5’-AGAGGGAAACAGCAGATTGCG-3’, reverse: 5’-CTGTAAACTCCGGCTCGTCA-3’.

Analysis of Lipids by Fatty Acid Methyl Esterification (FAME)

Gas chromatography/mass spectrometry (GC/MS) analysis was used to examine total cellular fatty acids either with or without $^{13}$C enrichment. U-$^{13}$C-glucose was used to allow differentiation between de novo and non de novo produced lipids. For $^{13}$C enrichment studies, cells were cultured in RPMI 1640 media (cat. 11879020, ThermoFisher Scientific), and supplemented with 5% dialyzed FBS (cat. 100-108, Gemini Bio-products), with all unenriched glucose replaced with U-$^{13}$C-glucose. Cells were cultured to a level of $5\times10^5$/mL in T-75 flasks. Subsequently, they were collected by centrifugation and washed three times with ice cold PBS. The second PBS wash contained 1% fatty-acid-free albumin to remove residual lipids from the medium. After the last centrifugation, 1 mL of cold methanol was added prior to storage at -80 C.
A standard Bligh-Dyer chloroform extraction was used to recover both polar and non-polar lipids (BLIGH and DYER, 1959). Cells were initially sonicated in 2.7 ml of 75% methanol/25% water. Subsequently, chloroform and water were added to give a final mixture containing 38:31:31 methanol/water/chloroform. The mixture separated into two phases with the lipids in a chloroform-rich hydrophobic phase. After centrifugation, the bottom hydrophobic phase (~1.3 ml) was removed with a glass pipette. A second extraction with 0.7 ml chloroform was used to recover additional lipids from the methanol/water phase. The two hydrophobic fractions were combined in a single glass centrifuge tube and back extracted with 0.15 ml of de-ionized water. The hydrophobic fraction was dried under nitrogen in 30-ml thick-wall glass anaerobic tubes.

The dried lipid extracts were dissolved in 2 ml of a 4:1 methanol/toluene mixture that contained butylated hydroxytoluene (0.45 mM). Acetylchloride (14 mM) was added to produce catalytic H+ in situ for the methyl esterification reaction. The anaerobic tubes containing the reaction mixtures were capped with thick butyl rubber stoppers (Bellco) and heated at 100 °C for 1 hour. After cooling, the reaction mixtures were mixed with 0.56M aqueous sodium carbonate at a ratio of 2:5 to drive the fatty acid methyl esters into a hydrophobic phase that floated on top of the aqueous phase. The toluene was purified by centrifugation and analyzed with an Agilent 7890A GC/MS (7890A/5975C). Mass spectra were quantified with the MSD ChemStation software from Agilent. Isocor, written for the Python programming environment (www.python.org), was used to correct mass spectra for natural abundance contributions from $^{13}$C. Mean enrichment was calculated as: where: $f_i$ = fractional enrichment of the i-th carbon and n is the total number of carbons.

$$Mean\_enrichment = \frac{\sum_{i=1}^{n} f_i \cdot i}{\sum_{i=1}^{n} i}$$

**Cell Staining and Imaging**
P493 cells were cytospun onto the slides through CytoSep Dual Sample Chamber (cat. M967-20FW, Medline Industries, Inc.) in a 7620 CytoPro Cytocentrifuge Centrifuge (Wescor) at 212g for 7 minutes. For immunofluorescence, slides were incubated with 50mM ammonium chloride for 10 minutes, permeabilized with 0.25% Triton X-100 for 10 minutes, and blocked with 2% BSA for 30 minutes. Slides were then incubated with Ki-67 primary antibody (cat. 550609, BD Pharmingen) at 1:100. Secondary Alex Fluor 594 goat anti-mouse antibody (cat. R37121, ThermoFisher Scientific) was used at 1:200 for 1 hour at room temperature. Slides were mounted in Prolong Gold Antifade with DAPI (cat. P36935, ThermoFisher Scientific) before imaging. TUNEL staining was performed using ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (cat. S7111, Millipore), according to the manufacturer's instructions.

**Oleic acid, Palmitic acid, and Palmitoleic acid Treatment**

Oleic acid-BSA conjugation solution was purchased from Sigma Aldrich, in which oleic acid was dissolved in 10% BSA, the concentration of oleic acid is 3.33mM. For palmitic acid and palmitoleic acid, the powder was dissolved in 50% ethanol at 65°C until completely dissolved to achieve a 50mM stock, and then conjugated with BSA in 10% BSA solution to concentration of 3.33mM at 37°C for 1 hour. For the combination treatment, oleic acid and palmitic acid were added 1:1 to the medium.

**Xenograft Tumors**

For P493 High Myc xenografts and B-I09 treatment, 1.5×10^7 P493 High Myc cells were injected subcutaneously into the flank of 6-week-old female homozygous athymic nude mice (Strain code 490, Charles River Laboratories). When the tumor volumes reached approximately 150mm³, B-I09 was administered intraperitoneally at 50mg/kg on the first 5 days of each week for 2 weeks. For Ramos xenografts and SCDi treatment, 1×10^6 Ramos cells were introduced subcutaneously into the flanks of 10-week-old female homozygous C.B-17 SCID mice (Strain code 236, Charles River Laboratories). When tumor volumes reached approximately 50-150mm³,
SCDi was orally administered at 5mg/kg (dissolved in 0.5% methy cellulose) twice daily. For Kelly xenografts, $3 \times 10^6$ Kelly cells with tet-inducible SCR or tet-inducible shXBP1 construct were injected subcutaneously into the left or right flank of 5-week-old homozygous female BALB/c Nude mice (Strain code 194, Charles River Laboratories), respectively. When the tumor volumes reached approximately 50-150mm$^3$, dox diet (cat. S3888, Bio-Serv) was administered to the mice. Tumor volume was monitored by caliper measurements.

**Mice, Tumor Formation, and B Cell Purification**

Mice carrying the human MYC oncogene under the control of the Igλ regulatory elements (Kovalchuk et al., 2000) were crossed with wild-type C57BL/6J mice (Mouse strain 000664, The Jackson Laboratory), monitored for lymphoma development, and sacrificed when moribund (3-5 months old). Total RNA from superficial cervical lymphomas and mesenteric lymphomas from the same mouse was extracted for further analysis. For wild-type B cell purification, naïve B lymphocytes were purified from C57BL/6J mice spleens by magnetic depletion of CD43-positive cells (Miltenyi Biotech), according to manufacturer's instructions. LAP/MYC mice were described previously (Xiang et al., 2015). mRNA was extracted from 5 liver tumors and paired normal liver tissues from this mouse model for qRT-PCR analysis.

**Statistics**

For calculation of the Contribution Ratios to viability restoration corresponding to Figure 17E, Figure 18F, and Figure 19E. The Contribution Ratios of BSA were calculated using relative viability of B-I09+BSA to divide B-I09 treatment alone; while the Contribution Ratios of OA were calculated using relative viability of B-I09+OA to divide B-I09+BSA. For the correlation between HSPA5 expression and Myc signature (Figure 1D, and Table 1), raw data for 22 samples of interest in GSE2350 were downloaded from the GEO database. Multi-array average (RMA) was performed separately on the 9 and 13 samples, which were run on HG_U95A and HG_U95Av2, respectively. Resulting log2-transformed normalized intensities were quantile normalized across
all samples to lessen the platform-specific effects. Correlation of all genes across all samples was calculated against *HSPA5* (probeset ID 36614_at). *P*-value and FDR-corrected *P*-value were calculated for each gene. The set of genes with corrected *P*-values of ≤0.01 (106 genes) was overlapped with the set of genes described to be Myc-related in the Hallmark sets from MSigDB (Myc Union, 240 genes). A Fisher’s exact test was performed showing the overlap between these two lists and the full set of genes included in the array design. The overlap of 6 genes gave a *P*-value of 0.0364. Where necessary, data were statistically analyzed to generate mean ± standard deviation (SD). The levels of significance were determined using two tailed Student’s *t* test, two-way ANOVA with Bonferroni correction, or two tailed paired *t* test. Where appropriate, *P* values are provided in the figures or in the legends. *P*<0.05 was considered statistically significant.

**Study Approval**

All experiments involving the use of mice were performed following protocols approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.
CHAPTER 3 Results

ER stress and the IRE1α-XBP1 pathway are enhanced in human and mouse BL

Previous reports suggested a relationship between c-Myc expression and ER stress, although the details of this interaction remain unclear (Hart et al., 2012). We employed BL as a c-Myc-dependent disease model, in which MYC translocation into immunoglobulin loci leads to its constitutive transcriptional dysregulation and expression (Schmitz et al., 2014). Analysis of Oncomine data (www.oncomine.org) revealed that HSPA5 mRNA, which encodes the ER chaperone BiP, is elevated in BL cells relative to normal centroblasts (CB) (Figure 1B), a BL cell of origin. HSPA5 levels also correlated with MYC mRNA abundance and an established c-Myc signature (Figure 1C and 1D, and Table 1), suggesting that increased c-Myc engages ER stress response pathways in BL patients. We initially focused on the IRE1α arm of the UPR, whose regulatory and functional mechanisms have not been previously investigated in this setting. Gene expression profiles of two independent BL patient cohorts (Basso et al., 2005; Brune et al., 2008) revealed elevated XBP1s target mRNAs (So et al., 2012), relative to centroblasts (Figure 2A), consistent with increased IRE1α RNase activity and XBP1s accumulation. Interestingly, RIDD was not engaged, as indicated by the overexpression (rather than underexpression) of multiple RIDD targets in BL (Figure 2B). In addition, ratios of Xbp1s to total Xbp1 (Xbp1t) transcripts, an indicator of IRE1α RNase activity, were elevated in El/MYC murine BL lymphoma cells (Kovalchuk et al., 2000) and LAP/MYC murine hepatocellular carcinoma cells (Xiang et al., 2015), compared to their normal counterparts (Figure 3A and 3B), demonstrating that elevated c-Myc induces IRE1α RNase activity in multiple cancer types.
Figure 1: ER stress is enhanced in human BL.
A) Schematic model of the IRE1α-XBP1 pathway.
B) log2 median-centered intensity of HSPA5 in human BL (n=17) and CB (n=5, two-tailed Student t test). Microarray data were obtained from the Oncomine database. Whiskers denote the minimal to maximal values.
C) Correlation of MYC and HSPA5 in samples from (B). R^2 and P value were determined by a two-tailed Pearson correlation test.
D) Correlation between HSPA5 and an established Myc signature (see Methods for details).
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Table 1. Fisher's exact test to determine correlation between HSPA5 and Myc signature. Raw data for 22 samples of interest in GSE2350 were downloaded from the GEO database. RMA was performed separately on the 9 and 13 samples, which were run on HG_U95A and HG_U95Av2, respectively. Resulting log2-transformed normalized intensities were quantile normalized across all samples to lessen the platform-specific effects. Correlation of all genes across all samples was calculated against HSPA5 (probeset ID 36614_at). P-value and FDR-corrected P-value were calculated for each gene. The set of genes with corrected P-values of ≤0.01 (106 genes) was overlapped with the set of genes described to be Myc-related in the Hallmark sets from MSigDB (Myc Union, 240 genes). A Fisher's exact test was performed showing the overlap between these two lists and the full set of genes included in the array design. The overlap of 6 genes gave a P-value of 0.0364.
Figure 2. IRE1α-XBP1 signaling, but not RIDD is enhanced in human BL.
A. In two independent cohorts of BL patients and CB controls, heatmap shows the relative expression of genes directly regulated by XBP1s. Data were extracted from the Oncomine database. Expression signals are depicted using pseudo-coloring, in which expression for each gene is shown as high (red) or low (blue).
B. Comparison of RIDD targets between BL and CB. Data were obtained from the Oncomine database. \( P < 0.05 \) was regarded as significant (two tailed Student \( t \) test). Grey circles show genes without significant changes, read squares denote genes those are significantly upregulated in BL patients, while blue rhombuses represent significantly downregulated genes in BL.
Figure 3. IRE1α-XBP1 signaling is enhanced in murine BL and hepatocellular carcinoma.

A. qRT-PCR analysis of Xbp1s/Xbp1t ratios in mixed B lymphocytes isolated from 2 wild-type (WT) mouse spleens and tumor cells from superficial and mesenteric lymphomas in 7 Eλ/MYC mice. Actb was utilized as the endogenous control gene, and relative mRNA expression was determined by normalizing to expression in WT B lymphocytes. 3 technical triplicates were used in each sample.

B. mRNA was extracted from liver tumors and paired normal liver tissues from the LAP/MYC mouse model. MYC and Xbp1s/Xbp1t examined with qRT-PCR. Actb was used as an endogenous control gene. Relative mRNA expression was determined by normalizing to levels in each normal sample. Three technical triplicates were used in each sample.
c-Myc activation initiates ER stress and the IRE1α-XBP1 pathway through multiple mechanisms

To study the effect of c-Myc on ER stress responses, we employed human P493 B-cell lymphoma cells, in which c-Myc levels can be manipulated with tetracycline and beta-estradiol (Yustein et al., 2010) (annotated by "No Myc", "Low Myc", and "High Myc") (Figure 4A). Cell proliferation, cell size, total RNA content, and protein content increased proportionally with c-Myc abundance (Figure 4A-4C). Along with the canonical target gene LDHA, c-Myc induction correlated with elevated ERN1 (IRE1α), HSPA5 (BiP), XBP1t, XBP1s (XBP1s), and XBP1s/XBP1t (Figure 4D-4F), suggesting that c-Myc regulates multiple components of the IRE1α stress response pathway in these cells.

c-Myc appeared to induce the IRE1α-XBP1 pathway through a variety of mechanisms. First, re-stimulation of previously c-Myc-depleted P493 cells via tetracycline withdrawal revealed a time-dependent increase in LDHA, ERN1, HSPA5, XBP1t, and XBP1s mRNA (Figure 5A) and protein (Figure 5B), with maximal expression levels achieved by 24-48 hours. Importantly, IRE1α, BiP, XBP1s, and c-Myc protein levels in P493 High Myc cells were comparable to multiple bona fide BL cell lines: Raji, Daudi, Ramos, and EB-2 (Figure 5C), consistent with the notion that P493 High Myc cells are a faithful BL model (Yustein et al., 2010). Chromatin immunoprecipitation-sequencing (ChIP-seq) analysis of P493 cells confirmed c-Myc binding to E-box sequences in the ERN1, HSPA5, and XBP1 promoters, confirming that c-Myc activates their transcription directly (Figure 5D). Second, c-Myc regulates IRE1α RNase activity, as demonstrated by increased XBP1s/XBP1u ratios (Figure 4E) and phosphorylated IRE1α protein levels (Figure 4F) in c-Myc expressing cells. Consistent with this observation, XBP1s/XBP1t ratios were reduced upon c-Myc suppression, but only fully restored 48 hours post c-Myc induction (Figure 5A and 5B). These data likely reflect a delay between c-Myc-dependent target gene induction (<24 hours) and accumulation of sufficient misfolded/unfolded proteins to stimulate IRE1α RNase activity (between 24 hours and 48 hours). Third, utilizing a cycloheximide (CHX) chase assay, we
observed that IRE1α protein half-life was regulated by c-Myc (Figure 6A). IRE1α protein stability can be positively regulated by DDRGK domain-containing protein 1 (DDRGK1) through ufmylation modification (Liu et al., 2017). However, in contrast to a previous study, this may be DDRGK1 independent in BL, as DDRGK1 expression was negatively correlated with IRE1α stability in P493 cells (Figure 6B). In addition, Sun et al. demonstrated that IRE1α is a substrate of the SEL1L-HRD1 ERAD complex, and ERAD-mediated IRE1α degradation is attenuated by ER stress (Sun et al., 2015). However, no significant changes in SEL1L or HRD1 protein levels were observed in P493 cells with variable c-Myc expression (Figure 6C and 6D), suggesting that the exact mechanism of how c-Myc regulates IRE1α protein stability needs to be further investigated.

Treating c-Myc-overexpressing BL cell lines with the non-specific Myc inhibitor JQ1 (Figure 7A and 7B) or low dose CHX (Figure 7C) significantly reduced HSPA5 and XBP1s transcripts and proteins, as well as XBP1s/XBP1t ratios. Consistent with these biochemical findings, transmission electron microscopy revealed an irregular ER structure, with substantially expanded membranes and distended lumens in c-Myc-overexpressing P493 cells (Figure 7D). Taken together, our data are consistent with a model in which c-Myc engages the IRE1α-XBP1 stress pathway through multiple mechanisms (Figure 7E), by: 1) directly activating ERN1, HSPA5, and XBP1 transcription, 2) stabilizing IRE1α protein, and 3) increasing ER protein load, thereby activating IRE1α RNase activity and promoting XBP1 splicing (See Discussion).
Figure 4. c-Myc activation impacts ER stress and the IRE1α-XBP1 pathway.
A. Growth curve and immunoblot analysis for P493 cells with different levels of c-Myc expression (annotated as “No Myc”, “Low Myc”, and “High Myc”, n=3).

B. Left: P493 No Myc, Low Myc, and High Myc cells analyzed by flow cytometry to determine cell size using forward scatter (results are representatives of >3 independent experiments). Right: quantification of relative cell size (n=3, two-way ANOVA test with Bonferroni correction).

C. Total RNA (n=3) and protein (n=6) content of one million P493 No Myc, Low Myc, and High Myc cells determined by Nanodrop 1000 and BCA protein quantification, respectively (two-way ANOVA test with Bonferroni correction).

D. qRT-PCR analysis of LDHA, ERN1, HSPA5, XBP1l, XBP1s, and XBP1s/XBP1l ratios in P493 cells with different levels of c-Myc (n=3, two-way ANOVA test with Bonferroni correction).

E. RT-PCR analysis of XBP1 splicing in P493 cells, and quantification of band intensity of XBP1s/XBP1u, relative intensity was determined by normalizing to the ratio of No Myc cells.

F. Immunoblot analysis for IRE1α phosphorylation (phos-tag SDS-PAGE), BiP, and XBP1s in P493 cells. SE, short time exposure; LE, long time exposure. For qRT-PCR, ACTB was utilized as the endogenous control gene. *, P<0.05, **, P<0.01, ***, P<0.001.
Figure 5. c-Myc transcriptionally regulates ERN1, HSPA5, and XBP1.

A. In P493 cells, c-Myc was suppressed with tetracycline (0.1 µg/mL) for 24 hours, which was then withdrawn to re-express c-Myc. At indicated times, mRNA was collected for qRT-PCR analysis. Three technical triplicates were used in each sample, and results are representative of >3 independent experiments. ACTB was utilized as the endogenous control gene.

B. Corresponding RT-PCR analysis of XBP1 splicing and protein expressions of IRE1α, BiP, XBP1s, and c-Myc in the same experimental settings of (A).

C. Immunoblots comparing protein levels between P493 cells and 4 bona fide BL cell lines: Raji, Daudi, Ramos, and EB-2.

D. ChIP-seq analysis shows binding of c-Myc to the promoters of ERN1, HSPA5, and XBP1 in P493 cells, canonical (CACGTG) or non-canonical (CACCGG) E-boxes were found in the binding regions.
Figure 6. c-Myc regulates IRE1α protein stability.
A. Immunoblots of IRE1α decay in P493 cells with different c-Myc levels after treatment with 100µg/mL cycloheximide for the indicted times. The graph represents the quantification of IRE1α protein levels.
B. Immunoblots for DDRGK1 expression in P493 No Myc, Low Myc, and High Myc cells.
C. Immunoblots for SEL1L and HRD1 expression in P493 No Myc, Low Myc, and High Myc cells.
D. Protein expression of SEL1L and HRD1 in experiment of Figure 5B.
Figure 7. Inhibition of c-Myc and protein synthesis suppresses IRE1α-XBP1 pathway.

A. qRT-PCR analysis of Raji cells treated with 500nM JQ1 for 24 hours.
B. Immunoblot analysis of BL cell lines (Raji, Ramos, and Daudi) treated with 500nM JQ1 for 24 hours or 48 hours.
C. qRT-PCR analysis of Ramos cells treated with 0.1µg/mL CHX for 4 hours.
D. Comparison of ER structures using transmission electron microscopy in P493 cells with or without c-Myc overexpression. Scale bar, 500nm.
E. Schematic model of c-Myc regulating ER stress and the IRE1α-XBP1 pathway: 1) c-Myc directly activates ERN1, HSPA5, and XBP1 transcription, 2) c-Myc stabilizes IRE1α protein with unknown reasons, and 3) c-Myc increases ER protein load, thereby activating IRE1α RNase activity and promoting XBP1 splicing. For qRT-PCR, ACTB was utilized as the endogenous control gene. Results are representative of 3 independent experiments. *, P<0.05, **, P<0.01, ***, P<0.001.
Synthetic lethality between c-Myc overexpression and IRE1α RNase activity inhibition *in vitro* and *in vivo*

To investigate the effects of IRE1α inhibition in the context of c-Myc overexpression, we used the highly specific IRE1α RNase inhibitor B-I09 (Figure 8A), which was previously shown to mimic XBP1 deficiency and suppress chronic lymphocytic leukemia (CLL) progression *in vivo* by inducing apoptosis without causing systemic toxicity (Tang et al., 2014). B-I09 treatment resulted in a dose-dependent decrease in XBP1s protein in P493 High Myc cells treated with tunicamycin (Figure 8B), which blocks N-linked glycosylation and amplifies ER stress (Oslowski and Urano, 2011), without altering c-Myc levels (Figure 8B) or IRE1α phosphorylation (Figure 8C). Similarly, B-I09 treated P493 High Myc cells displayed a dose-dependent decrease in cell proliferation and viability (Figure 8D and 8E). Importantly, the effects of B-I09 were subtler in Low Myc and No Myc cells, especially at ≤10µM, as shown by Ki-67 and TUNEL staining (Figure 8D and 8E, Figure 9A-9C). It is noteworthy that the ability of B-I09 to specifically induce apoptosis in c-Myc-overexpressing cells was significantly higher than that of Doxorubicin (Figure 10), a traditional chemotherapeutic drug targeting highly proliferating cells, or that of JQ1 (Figure 10), which showed potent anti-tumor effects in multiple types of cancers with either c-Myc or N-Myc overexpression (Delmore et al., 2011; Puissant et al., 2013). We also evaluated three CLL cell lines (MEC1, MEC2, and WaC3) with variable c-Myc levels (Figure 11A). Although at base line WaC3 cells grow much more slowly than MEC1 and MEC2 CLL cells (Figure 11A), they were more sensitive to B-I09 treatment induced growth arrest and apoptosis, as indicated by Annexin V/PI staining and PARP cleavage assay (Figure 11B). These results decrease the possibility that variable cell proliferation rates between P493 High Myc, Low Myc, and No Myc cells are a confounding factor for the effects of B-I09, and confirm that c-Myc overexpression could be an important indicator for B-I09 usage in different types of cancers. Similar effects were observed using 4µ8c, a distinct (albeit less potent) IRE1α RNase inhibitor (Cross et al., 2012; Tang et al., 2014) (Figure 12A and 12B). Finally, B-I09 treatment inhibited P493 High Myc xenograft tumor
growth *in vivo*, with no obvious toxicity as indicated by the maintenance of mouse body weight during treatment (Figure 13A and 13B).
Figure 8. Synthetic lethality between c-Myc overexpression and IRE1α RNase inhibition with B-I09 in P493 cells.

A. Chemical structure of B-I09.

B. P493 High Myc cells treated with indicated concentrations of B-I09 for 24 hours; 5µg/mL tunicamycin was added 6 hours before harvesting. Immunoblots show the expression of IRE1α, XBP1s, and c-Myc.

C. Western blot shows IRE1α phosphorylation (phos-tag SDS-PAGE) of P493 High Myc cells treated with indicated concentrations of B-I09, in the presence of DMSO or 5µg/mL tunicamycin for 6 hours.
D. Before experimental treatment, Low Myc cells were generated by culturing with 0.1µg/mL tetracycline and 1µM beta-estradiol, while No Myc cells were generated by culturing with 0.1µg/mL tetracycline alone. After 48 hours, cells were treated with different concentrations of B-I09, and counted at indicated time points (n=3, two-way ANOVA test with Bonferroni correction). *, P<0.05; ***, P<0.001.

E. Representative contour plots of P493 High Myc, Low Myc, and No Myc cells treated with indicated concentrations of B-I09 for 48 hours and analyzed by Annexin V/PI staining.
Figure 9. 10µM B-I09 significantly induces cell proliferation arrest and apoptosis in P493 High Myc cells, with much subter effects in Low Myc and No Myc cells.

A. Immunoblot analysis of XBP1s and c-Myc in P493 High Myc cells treated with 10µM B-I09 for 48 hours.

B. Ki-67 staining representative images (left) and quantifications (right) upon 10µM B-I09 treatment for 48 hours.

C. TUNEL staining representative images (left) and quantifications (right) upon 10µM B-I09 treatment for 48 hours. For both Ki-67 and TUNEL staining, 5 fields per slide were quantified. Scale bar, 100µM. Two-way ANOVA with Bonferroni correction was used to determine significance. *, P<0.05, ***, P<0.001. n.s., not significant.
Figure 10. B-I09 has higher specificity to induce apoptosis in c-Myc overexpressing P493 cells than Doxorubicin and JQ1. P493 cells treated with indicated concentrations of B-I09, Doxorubicin, or JQ1 for 48 hours. Viability was examined and relative viability determined by normalizing to cells treated with Control or DMSO. Results are representative of 3 independent experiments. *, "High Myc" vs. "Low Myc"; #, "High Myc" vs. "No Myc". Two-way ANOVA with Bonferroni correction was used to determine significance. ***, $P<0.001$. ###, $P<0.001$. 
Figure 11. The effects of B-I09 in CLL cell lines are not dependent on higher cell proliferation rate.

A. Growth curve of CLL cell lines (MEC1, MEC2, and WaC3) (n=3); Immunoblots show expression of c-Myc and XBP1s of each.

B. CLL cells treated with 20µM B-I09 for 72 hours, relative cell growth (n=3) and viability were determined. Immunoblots show expression of PARP cleavage upon B-I09 treatment. Two-way ANOVA with Bonferroni correction was used to determine significance. ***, \( P<0.001 \).
Figure 12. 4µ8c exhibits similar effects as B-I09 in P493 cells, albeit less potent.

A. Cells cultured with different concentrations of 4µ8c, and counted at indicated times (n=3).
B. Cells treated with indicated concentrations of 4µ8c for 48 hours. Viability was examined and relative viability determined by normalizing to viability of cells treated with DMSO. Results are representative of 3 independent experiments. *, "High Myc" vs. "Low Myc"; #, "High Myc" vs. "No Myc". Two-way ANOVA with Bonferroni correction was used to determine significance. **, \( P<0.01 \); ***, \( P<0.001 \). ###, \( P<0.001 \).
Figure 13. Synthetic lethality between c-Myc overexpression and IRE1α RNase inhibition with B-I09 in vivo.

A. Growth of P493 High Myc subcutaneous tumors treated with vehicle or B-I09 (50mg/kg intraperitoneally, once per day, 5 days per week, 2 weeks). Relative tumor volume was determined by normalizing to volume when treatment was started (n=6 for Control, n=5 for B-I09, two tailed Student t test). *, P<0.05, **, P<0.01.

B. Body weight of mice bearing P493 High Myc xenografts treated with Control or B-I09.
B-I09 suppresses growth and induces apoptosis in human and mouse BL cells

c-Myc is overexpressed in all BL cells tested (Figure 5C), which also exhibited growth and survival defects upon B-I09 exposure, although their sensitivity to the drug varied (Figure 14A-14G). For example, B-I09 treated Ramos cells displayed a dose-dependent inhibition of XBP1 splicing, reduced proliferation and viability, without apparent alterations in c-Myc protein levels or IRE1α phosphorylation (Figure 14A-14C). The modest increase in IRE1α protein abundance (Figure 14A) was consistent with previous studies of XBP1 deletion in leukemic and hepatic cells, and may reflect a negative feedback mechanism (Lee et al., 2008; Tang et al., 2014), or changes in IRE1α protein stability. Importantly, the viability of B-I09-treated Ramos cells was rescued by treatment with CHX (Figure 14D), indicating that elevated protein synthesis and proteotoxicity at least partially contribute to IRE1α activation. We employed Ramos (Epstein-Barr viral negative (EBV-)) and Daudi (EBV positive (EBV+)) cells as representatives of two categories of human BL (EBV- and EBV+) (FRCPH et al., 2012) for further study (see below). Finally, elevated IRE1α and XBP1s expression, and increased sensitivity to B-I09, was observed in 8498 cells isolated from the Eκ/MYC mouse lymphoma model, as compared to wild type murine B-lymphocytes (Figure 14H). Collectively, these data suggest an essential protective role for IRE1α RNase activity downstream of elevated c-Myc in BL cells.
Figure 14. B-I09 suppresses growth and induces apoptosis in human and mouse BL cells.

A. (Left) Ramos cells were treated with indicated concentrations of B-I09 for 24 hours. Western blot shows the expressions of IRE1α, XBP1s, and c-Myc. (Right) Immunoblots show IRE1α phosphorylation (phos-tag SDS-PAGE) of Ramos cells treated with indicated concentrations of B-I09, in the presence of DMSO or 5µg/mL tunicamycin for 6 hours.

B. Ramos cells were treated with indicated concentrations of B-I09 for 72 hours, cell numbers were counted at indicated times (n=3).

C. Ramos cells were treated with indicated concentrations of B-I09 for 48 hours, cell viability was examined and relative cell viability was determined by normalizing to viability upon DMSO treatment. Immunoblots show PARP cleavage with 10µM B-I09 treatment for 48 hours.

D. Ramos cells pre-treated with CHX (0.5µg/mL) for 2 hours, and then cultured with DMSO, 5µM, or 10µM B-I09 for 48 hours. Cell viability was then examined and relative cell viability determined by normalizing to viability upon DMSO treatment, or DMSO+CHX treatment, respectively.

E. Raji cells were treated with B-I09 for the indicated times, cell number was counted (n=3) and cell viability examined.

F. Daudi cells were treated with B-I09 for the indicated times, cell number was counted (n=3) and cell viability examined.

G. EB-2 cells were treated with B-I09 for the indicated times, cell number was counted (n=3) and cell viability examined.

H. For B cells isolated from WT mouse spleens and 8498 cells from Eκ/MYC lymphoma tumor, protein expression of IRE1α, XBP1s, and c-Myc was compared, and their sensitivities to different B-I09 concentrations at 48 hours examined. For all viability assays, results are representative of 3 independent experiments; P values were determined by two-way ANOVA with Bonferroni correction. **, P<0.01; ***, P<0.001. n.s., not significant.
IRE1α RNase inhibition induces growth and viability defects by decreasing SCD1 accumulation

There are multiple mechanisms whereby IRE1α might regulate growth and survival in c-Myc-overexpressing cells. For example, the IRE1α cytoplasmic region also contains a kinase domain that phosphorylates and activates the c-Jun N-terminal kinase (JNK) pathway, inducing apoptosis (Urano et al., 2000). However, phospho-JNK protein levels were unaffected by B-I09 treatment (Figure 15A). Additionally, XBP1s can regulate the expression of BECN1, thereby inducing autophagy (Margariti et al., 2013; Tian et al., 2015), which confers a cytoprotective advantage in c-Myc-overexpressing mammalian (Hart et al., 2012) and Drosophila (Nagy et al., 2013) cells. However, autophagy (based on p62 and LC3-II abundance) was not suppressed by B-I09 treatment (Figure 15B), indicating no decline in autophagic flux in this setting.

Both c-Myc and the IRE1α-XBP1 pathway have been previously implicated in regulating lipid metabolism in normal and malignant tissues (Cubillos-Ruiz et al., 2015; Lee et al., 2008; McGehee et al., 2009; Piperi et al., 2016; So et al., 2012), suggesting that alterations in lipid homeostasis trigger growth and viability defects in IRE1α inhibited, c-Myc-overexpressing BL cells. To investigate this possibility, we quantified mRNA levels of 20 lipid metabolism genes in IRE1α inhibitor treated P493 cells, including those involved in synthesis, storage, and catabolism (Figure 15C). B-I09 treatment inhibited XBP1 splicing, without altering c-Myc activity (based on unchanged LDHA levels). Expression of lipid synthesis genes, e.g. HMGCR1, HMGCS1, ACLY, ACACA, FASN, and SCD was increased by c-Myc and inhibited by B-I09 (Figure 15C). De novo lipogenesis generates diverse free fatty acids from glucose and glutamine, which can be probed by supplying uniformly $^{13}$C-labeled glucose (U-$^{13}$C-glucose) and subsequent mass spectrometry analysis of saponified fatty acids (Kamphorst et al., 2011) (Figure 15D). Analysis at steady state labeling quantifies the unlabeled fraction (M+0) relative to labeled forms arising from lipogenesis. Consistent with mRNA expression, de novo lipogenesis was significantly higher in High Myc cells and suppressed by B-I09 treatment (Figure 15E).
**Figure 15.** B-I09 treatment affects lipid metabolism in P493 cells.

A. P493 High Myc cells treated with 10μM B-I09 for 24 hours and 48 hours. Phospho-JNK and total JNK protein levels analyzed.

B. P493 High Myc, Low Myc, and No Myc cells treated with 10μM B-I09 for 48 hours. Autophagy markers including p62 and LC3 analyzed by Immunoblots.

C. Heatmap shows relative expression of lipid metabolism genes in P493 cells with different c-Myc levels upon 10μM B-I09 treatment for 48 hours (n=3). Expression signals are

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**Table:**

<table>
<thead>
<tr>
<th>Lipid Metabolism Genes</th>
<th>P493 High Myc</th>
<th>P493 Low Myc</th>
<th>P493 No Myc</th>
<th>B-I09 (10μM)</th>
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<tr>
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<tr>
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<td>-</td>
<td>+</td>
</tr>
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<td>GAPDH</td>
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**Diagram:**

- **C16:0:**
  - High Myc: 0.8, 0.6, 0.4, 0.2, 0.0
  - No Myc: 0.8, 0.6, 0.4, 0.2, 0.0

- **C18:0:**
  - High Myc: 0.8, 0.6, 0.4, 0.2, 0.0
  - No Myc: 0.8, 0.6, 0.4, 0.2, 0.0

- **C18:1:**
  - High Myc: 0.8, 0.6, 0.4, 0.2, 0.0
  - No Myc: 0.8, 0.6, 0.4, 0.2, 0.0

**Legend:**

- M+0: Black
- M+2: Red
- M+4: Orange
- M+6: Yellow
- M+8: Purple
- M+10: Magenta
- M+12: Cyan
- M+14: Green
- M+16: Blue
- M+18: Pink

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**Diagrams:**

- **Diagram A:** Shows the results of B-I09 treatment on Phospho-JNK and total JNK protein levels in P493 High Myc cells.
- **Diagram B:** Illustrates the effects of B-I09 on autophagy markers p62 and LC3 in P493 cells with different Myc levels.
- **Diagram C:** Displays a heatmap of lipid metabolism gene expression in P493 cells with varying Myc levels after B-I09 treatment.
- **Diagram D:** Depicts the metabolic pathway of glucose and fatty acid metabolism, highlighting the role of B-I09 in lipid metabolism.
- **Diagram E:** Demonstrates the labeled/total ratio of different fatty acids in P493 cells with different Myc levels and B-I09 treatment.
depicted using pseudo-coloring, in which expression for each gene is shown as high (red) or low (blue).

D. Schematic model of U-$^{13}$C-glucose tracing and fatty acid labeling.

E. U-$^{13}$C-glucose tracing and fatty acid labeling in P493 High Myc and No Myc cells with 10µM B-I09 treatment for 24 hours (n=3). Labeled/Total ratios were calculated for palmitate (C16:0), stearate (C:18:0), and Oleate (C18:1).
We focused on SCD for several reasons: 1) SCD mRNA abundance was the most altered across different conditions among all genes tested (Figure 15C); 2) SCD encodes the rate-limiting enzyme in monounsaturated fatty acid formation. Furthermore, increased levels of monounsaturated fatty acids (e.g. C18:1 oleic acid) are a hallmark of human c-Myc driven lymphomas, based on comprehensive lipid profiling (Eberlin et al., 2014); and 3) previous work demonstrated that synthesizing or scavenging unsaturated lipids is critical to maintain cell viability in multiple transformed cell types, especially encountering elevated protein synthesis (Kamphorst et al., 2013; Young et al., 2013). Consistent with mRNA expression, decreased SCD1 protein accumulation upon IRE1α RNase inhibition was confirmed in multiple cell lines (Figure 16A-16C). More importantly, U-13C-glucose labeling enables us to determine SCD1 activity by calculating mean enrichment of labeled C18:1/C18:0 (Figure 15D), and we found that SCD1 enzymatic activity was dramatically inhibited by B-I09 effects on SCD1 abundance (Figure 16D).

ChIP analysis demonstrated XBP1s binding to the SCD proximal promoter, regulated by both tunicamycin (utilized to increase XBP1s expression) and B-I09 (Figure 16E), consistent with previous observations in mouse liver cells (Lee et al., 2008). XBP1 knockdown resulted in decreased levels of SCD1 protein (Figure 16F). SCD is a known c-Myc transcriptional target (Zeller et al., 2003) (Figure 15C); however, c-Myc protein levels were only modestly changed, if at all, in response to IRE1α RNase inhibition (Figure 16A-16C), suggesting that decreased SCD1 was at least partly c-Myc-independent and XBP1s-dependent. In summary, we identified SCD as a transcriptional target downstream of IRE1α-XBP1 signaling in BL.
Figure 16. B-I09 treatment decreases SCD1 expression, and SCD (encoding SCD1) is transcriptionally regulated by XBP1s.

A. Immunoblot analysis for P493 High Myc, Low Myc, and No Myc cells treated with DMSO or 10µM B-I09 for 48 hours. XBP1s, c-Myc, and SCD1 expression examined.

B. Immunoblot analysis for P493 High Myc, Low Myc, and No Myc cells treated with DMSO or 20µM 4µ8c for 48 hours. XBP1s, c-Myc, and SCD1 expression examined.

C. CLL cell lines treated with 20µM B-I09 for 48 hours; c-Myc and SCD1 expression monitored.

D. Mean enrichment of C18:1/C18:0 was calculated in P493 High Myc cells from (Figure 15E) (n=3, two tailed Student t test).
E. ChIP-qPCR assay performed using anti-XBP1s antibody to detect enriched gene promoter fragments in 3 conditions: Control (Ct); Tunicamycin (5µg/mL) treatment for 6 hours (Tm); and Tunicamycin+B-109 (10µM) treatment for 6 hours (Tm+B-I09). IgG was used as mock ChIP control. ERdj4 serves as a positive control for XBP1s binding. Values represent relative increase of real-time PCR signals compared to the signal of IgG ChIP under Control condition. 3 technical triplicates are presented.

F. P493 High Myc cells transfected with non-targeting (NT) siRNA or siRNA targeting XBP1 (siXBP1) for 48 hours, XBP1s and SCD1 expression levels were examined.
To test the critical role of SCD1 in IRE1α-inhibited, c-Myc-overexpressing conditions, P493 High Myc cells were cultured with the monounsaturated fatty acids oleic acid (OA, C18:1) or palmitoleic acid (POA, C16:1), which are the enzymatic products of SCD1. OA partly rescued cell proliferation (Figure 17A) and both OA and POA essentially restored cell viability without affecting the ability of B-I09 to reduce XBP1s and SCD1 expression (Figure 17B and 17C). However, a combination of OA and saturated palmitic acid (Palm, C16:0) did not rescue viability as effectively as OA alone, even though exposure to Palm itself was not toxic (Figure 17D). These results suggest that unsaturated fatty acids are critical to maintain the viability of B-I09-treated cells.

Because long-chain fatty acids are relatively insoluble in aqueous solutions, they were conjugated to lipid free-bovine serum albumin (BSA) before use. Surprisingly, control BSA partially rescued cell growth and viability in the absence of exogenous OA (Figure 17A, 17B, and 17D). This raised the possibility that BSA might enter B-I09 treated cells through macropinocytosis (Commissso et al., 2013; Palm et al., 2015), and contribute to viability by supplying free amino acids, although free BSA could rescue viability by scavenging lipids in the medium (Francis, 2010). To test this, cells were cultured in medium with reduced lipid concentration (see Methods). Treatment with B-I09 significantly reduced cell viability in lipid-limited conditions, and whereas exogenous BSA alone rescued the viability of multiple B-I09 treated cell lines in replete medium, it had no significant effect on the survival of lipid-limited cells. In contrast, addition of exogenous BSA-conjugated OA fully rescued viability in lipid-limited cells, suggesting that BSA functioned primarily as a fatty acid shuttle in these experiments (Figure 17E).

A requirement for SCD1 function in c-Myc-overexpressing P493 cells was further validated using a commercially available SCD1 inhibitor (SCDi), which phenocopied B-I09 treatment by inducing growth arrest and cell death (Figure 17F and 17G). However, apoptosis was only induced after 72 hours treatment (Figure 17G), suggesting other mechanisms might also exist.
Figure 17. B-I09 treatment results in phenotypes dependent on SCD1 loss in P493 cells.

A. Cell growth of P493 High Myc cells treated with 10µM B-I09, and rescued with BSA control or OA (n=3).
B. Relative viability of P493 High Myc cells treated with 10µM B-I09, and rescued with BSA control, OA, or POA for 48 hours.
C. Immunoblots of experiment from Figure 17A.
D. P493 High Myc cells treated with DMSO or 20µM B-I09 for 48 hours, with OA, or Palm, or the combination (1:1 ratio), cell viability examined, and relative viability calculated by normalizing to DMSO treatment group.
E. P493 High Myc cells cultured in media with normal lipid concentration (100%) or delipidated condition (10%), treated with 10µM B-I09, and rescued with BSA or OA for 48 hours. Contribution ratios of viability restoration of BSA and OA in 100% or 10% lipid media were calculated (see Methods for details).

F. P493 cells treated with 0.5µM SCDi for 72 hours, cell growth were determined (n=3).

G. P493 cells treated with 0.5µM SCDi for 72 hours, viability were examined. For viability assays, results are representative of 3 independent experiments. \( P \) values were determined by two-way ANOVA with Bonferroni correction, if not specified elsewhere. ***, \( P<0.001 \).
The regulation of SCD1 by IRE1α-XBP1 signaling and its role in maintaining cell growth and survival were further validated in bona fide BL cell lines (Figure 18A-18G and Figure 19A-19F). Importantly, SCD expression was increased in BL cells relative to centroblasts (Figure 20A). Therefore, we evaluated the therapeutic potential of SCDi for BL growth in vivo. SCDi administration significantly decreased tumor growth and tumor weight (Figure 20B). However, this was also accompanied by a slight weight loss during treatment (Figure 20C), as observed in a previous study (Mason et al., 2012). Taken together, IRE1α RNase inhibition resulted in BL cell phenotypes, dependent on SCD1 loss. In addition, targeting SCD1 phenocopied B-I09 to decrease in vivo tumor growth. However, based on the toxicity of SCDi, IRE1α RNase inhibition may be a safer therapeutic strategy for BL patients.
Figure 18. B-I09 treatment results in phenotypes dependent on SCD1 loss in Ramos cells.

A. Immunoblot analysis of Ramos cells treated with indicated concentrations of B-I09 for 48 hours.

B. Cell growth of Ramos cells treated with 20µM B-I09, and rescued with BSA control or OA (n=3).

C. Relative viability of Ramos cells treated with 10µM B-I09, and rescued with BSA control, OA, or POA for 48 hours.

D. Immunoblots of experiment from Figure 18B.
E. Ramos cells treated with DMSO or 20µM B-I09 for 48 hours, with OA, or Palm, or the combination (1:1 ratio), cell viability examined, and relative viability calculated by normalizing to DMSO treatment group.

F. Ramos cells cultured in media with normal lipid concentration (100%) or delipidated condition (25%), treated with 10µM B-I09, and rescued with BSA or OA for 48 hours. Contribution ratios of viability restoration of BSA and OA in 100% or 25% lipid media were calculated (see Methods for details).

G. Relative viability of Ramos cells treated with 0.5µM SCDi, and rescued with BSA control or OA for 48 hours. For viability assays, results are representative of 3 independent experiments. P values were determined by two-way ANOVA with Bonferroni correction, if not specified elsewhere. **, P<0.01; *** P<0.001; n.s., not significant.
Figure 19. B-I09 treatment results in phenotypes dependent on SCD1 loss in Daudi cells.
A. Immunoblot analysis of Daudi cells treated with 10µM B-I09 for 48 hours.
B. Cell growth of Daudi cells treated with 10µM B-I09, and rescued with BSA control or OA (n=3).
C. Relative viability of Daudi cells treated with 10µM B-I09, and rescued with BSA control, OA, or POA for 48 hours.
D. Immunoblots of experiment from Figure 19B.
E. Daudi cells treated with DMSO or 10µM B-I09 for 48 hours, with OA, or Palm, or the combination (1:1 ratio), cell viability examined, and relative viability calculated by normalizing to DMSO treatment group.
F. Daudi cells cultured in media with normal lipid concentration (100%) or delipidated condition (25%), treated with 10µM B-I09, and rescued with BSA or OA for 48 hours. Contribution ratios of viability restoration of BSA and OA in 100% or 25% lipid media were calculated (see Methods for details).

G. Relative viability of Daudi cells treated with 0.5µM SCDi, and rescued with BSA control or OA for 48 hours. For viability assays, results are representative of 3 independent experiments. P values were determined by two-way ANOVA with Bonferroni correction, if not specified elsewhere. **, P<0.01; ***, P<0.001; n.s., not significant.
Figure 20. SCDi treatment decreases Ramos xenograft growth in vivo.

A. Normalized reads of SCD in human BL and CB from healthy donors. Microarray data were obtained from the Oncomine database. Whiskers denote the minimal to maximal values. $P$ values were determined by two-tailed Student $t$ test.

B. Tumor growth and weight of xenografted Ramos tumors treated with Control or SCDi (5mg/kg, orally twice daily). $P$ values were determined by two-tailed Student $t$ test. **, $P<0.01$, ***, $P<0.001$.

C. Body weight of mice bearing Ramos xenografts treated with Control or SCDi.
N-Myc overexpressing cells also engage the IRE1α-XBP1-SCD1 pathway to maintain viability

To determine if these findings extend to other Myc family members, we employed the “N-MycER” SHEP neuroblastoma cell line in which N-Myc activity is induced by tamoxifen treatment (Figure 21A). N-Myc activation via 4-hydroxytamoxifen (4-OHT) increased XBP1 splicing (Figure 21B and 21C) and XBP1s protein abundance (Figure 21D), indicating N-Myc also engages the IRE1α-XBP1 pathway. Whereas B-I09 had only modest effects on the proliferation of untreated “Control SHEP” cells (N-Myc negative), it robustly inhibited proliferation and induced apoptosis in 4-OHT treated “N-Myc SHEP” cells (N-Myc positive), in both a dose- and time-dependent manner (Figure 22A-22D). Furthermore, this decrease in cell viability was largely restored by CHX treatment (Figure 22E). Mechanistically, B-I09 treatment resulted in decreased SCD1 (Figure 22F), and all phenotypes were partly or totally reversed by OA (Figure 22G and 22H). Similarly, N-Myc SHEP cells were more sensitive to SCDi treatment or SCD knockdown with siRNA than controls (Figure 22I and 22J). To confirm B-I09 IRE1α target specificity, cells were treated with scrambled shRNA (shSCR) or shRNA targeting XBP1 (shXBP1) (Figure 23A). Like B-I09 treatment, XBP1 depletion with shXBP1 decreased SCD1 protein levels and induced apoptosis in N-Myc SHEP cells, while Control SHEP were largely resistant (Figure 23A and 23B).

MYCN amplification is found in ~25% of neuroblastoma (NB) cases, and remains the best-characterized genetic marker of high risk disease (Huang and Weiss, 2013). We compared three NB cell lines with or without MYCN amplification: SK-N-AS (no MYCN amplification), BE2C (MYCN amplification), and Kelly (MYCN amplification): cells with MYCN amplification expressed higher levels of HSPA5 and XBP1s, and exhibited enhanced sensitivity to B-I09 (Figure 24A and 24B). Importantly, XBP1 knockdown in Kelly cells dramatically impaired tumor growth and tumor weight in vivo (Figure 24C-24E), without affecting overall mouse body weight (Figure 24F). In summary, like c-Myc, N-Myc overexpressing cells also engage the IRE1α-XBP1-SCD1 pathway...
to maintain cell proliferation and viability, and targeting this axis could be a potential therapeutic strategy for N-Myc overexpressing cancers, e.g. NB.
Figure 21. N-Myc overexpression also engages the IRE1α-XBP1 pathway.

A. (Upper) SHEP N-MycER cells were treated with 4-OHT (200nM) for indicated times to activate N-Myc nuclear translocation. N-MycER expression was examined in the nuclear fragment. (Lower) N-Myc target ODC1 examined with qRT-PCR upon N-Myc activation (n=3).

B. XBP1s/XBP1t ratios examined with qRT-PCR upon N-Myc activation (n=3).

C. XBP1 splicing analyzed by RT-PCR upon N-Myc activation, and relative band intensity was determined by normalizing to Control cells at 24-hour time point.

D. XBP1s protein accumulation determined by Immunoblots upon N-Myc activation.
Figure 22. IRE1α-XBP1-SCD1 pathway is critical to maintain cell proliferation and viability in N-Myc overexpressing cells.

A. SHEP cells cultured in vehicle (Control) or 4-OHT containing media for 48 hours before treatment with DMSO or B-I09. WST-1 assay was used to examine cell growth. Relative absorbance was determined by normalizing to absorbance at time 0 hour (n=6).
B. Control and 4-OHT SHEP cells were exposed to 100, 50, 25, 12.5, 6.25, 3.125, 1.5625µM B-I09 for 72 hours, IC_{50} was then determined (n=3, P value was determined by two-tailed Student t test).

C. Representative contour plots of Control and 4-OHT SHEP cells treated with 30µM B-I09 for 96 hours.

D. Control and 4-OHT SHEP cells treated with indicated concentrations of B-I09 for different times, viability was then measured. Relative viability was determined by normalizing to viability of cells with DMSO treatment.

E. SHEP cells pre-treated with CHX (0.5µg/mL) for 2 hours, and then cultured with DMSO or 30µM B-I09 for 72 hours. Relative viability was determined by normalizing to viability upon DMSO treatment, or DMSO+CHX treatment, respectively.

F. Immunoblots analysis for Control and 4-OHT SHEP cells with B-I09 treatment for 72 hours.

G. Control or N-Myc SHEP cells treated with DMSO or 30µM B-I09, rescued with BSA or OA for 72 hours (n=6). Absorbance was measured using WST-1 reagents. *, comparison of B-I09 and DMSO treatment. #, comparison of B-I09+BSA or B-I09+OA and B-I09 treatment.

H. Control or N-Myc SHEP cells treated with DMSO or B-I09, and rescued with BSA or OA for 72 hours. Viability was examined and relative viability was determined by normalizing to viability upon DMSO treatment. *, comparison of B-I09 and DMSO treatment. #, comparison of B-I09+BSA or B-I09+OA and B-I09 treatment.

I. Control or N-Myc SHEP cells treated with 0.5µM SCDi for indicated times, viability was examined. Relative viability was determined by normalizing to viability of cells with DMSO treatment.

J. Control or N-Myc SHEP cells treated with siNT or siSCD for 72 hours, and viability was determined. Relative viability was determined by normalizing to viability of cells with siNT treatment. For viability assays, results are representative of 3 independent experiments. P values were determined by two-way ANOVA with Bonferroni correction, if not specified elsewhere. *, P<0.05, ***, P<0.001. ###, P<0.001. n.s., not significant.
Figure 23. XBP1 depletion results in apoptosis in N-Myc overexpressing SHEP cells.

A. SHEP cells infected with lentivirus containing shSCR or shXBP1 constructs for 48 hours, and vehicle or 4-OHT subsequently added. After 72 hours, cells were for Immunoblots analysis of XBP1s and SCD1.

B. SHEP cells infected with lentivirus containing shSCR or shXBP1 constructs for 48 hours, and vehicle or 4-OHT subsequently added. After 72 hours, cells were imaged and harvested for viability assays. For viability assays, results are representative of 3 independent experiments. P values were determined by two-way ANOVA with Bonferroni correction. ***, P<0.001. n.s., not significant.
Figure 24. XBP1 depletion sensitizes N-Myc overexpressing neuroblastoma cells to growth inhibition in vitro and in vivo.

A. qRT-PCR comparing 3 neuroblastoma cell lines SK-N-AS, BE2C, and Kelly in terms of expressions of MYCN, ODC1, HSPA5, and XBP1s.

B. Three neuroblastoma cell lines were tested for IC50 of B-I09 treatment for 72 hours.

C. Kelly cells with tetracycline inducible shSCR or shXBP1 constructs injected subcutaneously into the left or right flanks of mice, respectively. When tumor sizes reached 50-150mm3, doxycycline chow was used to knockdown XBP1. Tumor volume was monitored every 3 days; tumor weight was measured upon harvesting (two tailed paired t test).

D. Bulk tumors are shown: 2 tumors from the same mouse are shown together; left, Tet-shSCR; right, Tet-shXBP1.
E. Validation of *XBP1* knockdown in Kelly xenograft tumors by qRT-PCR (two-tailed paired Student *t* test).

F. Body weight of mice bearing Kelly xenograft tumors (n=10). For qRT-PCR, *TBP* and *ACTB* were used as endogenous control genes, 3 technical triplicates were used in each sample, and data are representative of 3 independent experiments. *, *P*<0.05, **, *P*<0.01, ***, *P*<0.001. n.s., not significant.
B-I09 enhanced *in vitro* cytotoxicity of BL chemotherapeutic drugs

Given the c-Myc-dependent toxicity of B-I09 observed in BL cell lines, we explored whether B-I09 treatment would improve standard therapies currently used to treat BL clinically. Apoptosis triggered in BL cells by either doxorubicin or vincristine treatment was further enhanced by B-I09. Combination indices (CIs) showed additive or synergistic effects with doxorubicin and vincristine in Daudi and Ramos cells (Figure 25A-25C, Table 2), and the effects of B-I09 depend on SCD1 activity, as SCD1 downregulation was maintained with combinational treatment and OA partially rescued cell viability (Figure 25D and 25E). These results suggest that B-I09 and other IRE1α inhibitors could be used to treat a variety of Myc-driven malignancies, including c-Myc overexpressing BL and N-Myc overexpressing neuroblastoma, to improve standard of care.
Figure 25. B-I09 combination indices (CI) with Doxorubicin and Vincristine.

A. Daudi cells treated with Doxorubicin plus B-I09 for 48 hours.
B. Ramos cells treated with Vincristine plus B-I09 for 48 hours.
C. Daudi cells treated with Vincristine plus B-I09 for 48 hours. Relative viability was determined by normalizing to viability upon DMSO treatment.
D. Western blot examining SCD1 expression after treatment with 10µM B-I09 or 0.5µM Vincristine alone, or the combination of both in Daudi and Ramos cells after 48 hours.
E. Daudi or Ramos cells treated with 10µM B-I09 or 0.5µM Vincristine alone, or the combination of both, rescued with OA for 48 hours, viability was determined. Data are representative of 3 independent experiments.
### B-I09 combination Indices (CI) with Doxorubicin in Daudi

<table>
<thead>
<tr>
<th>Doxorubicin (nM)</th>
<th>B-I09 (µM)</th>
<th>CI value</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>5</td>
<td>0.842</td>
</tr>
<tr>
<td>12.5</td>
<td>5</td>
<td>0.798</td>
</tr>
<tr>
<td>25.0</td>
<td>5</td>
<td>0.758</td>
</tr>
<tr>
<td>6.25</td>
<td>10</td>
<td>0.866</td>
</tr>
<tr>
<td>12.5</td>
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<td>0.853</td>
</tr>
<tr>
<td>25.0</td>
<td>10</td>
<td>0.835</td>
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</table>

### B-I09 combination Indices (CI) with Vincristine in Daudi

<table>
<thead>
<tr>
<th>Vincristine (nM)</th>
<th>B-I09 (µM)</th>
<th>CI value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.796</td>
</tr>
<tr>
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<td>5</td>
<td>0.625</td>
</tr>
<tr>
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<td>5</td>
<td>0.700</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0.520</td>
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</table>

### B-I09 combination Indices (CI) with Vincristine in Ramos

<table>
<thead>
<tr>
<th>Vincristine (nM)</th>
<th>B-I09 (µM)</th>
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<tr>
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<tr>
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<tr>
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<td>0.547</td>
</tr>
<tr>
<td>0.7</td>
<td>20</td>
<td>0.437</td>
</tr>
</tbody>
</table>

Table 2. B-I09 combination indices (CI) with Doxorubicin and Vincristine in Daudi and Ramos cells. CI was calculated using CompuSyn software. CI<0.9 indicates synergistic effects, 0.9<CI<1.1 indicates additive effects, and CI>1.1 indicates antagonistic effects.
Discussion

Myc overexpression drives tumor growth and progression by altering the expression of thousands of target genes that regulate myriad cellular processes (Dang, 2012). Although Myc-transformed tumors are highly dependent on sustained Myc transcriptional activity, directly inhibiting Myc as a therapeutic approach has proven technically difficult and largely unsuccessful (McKeown and Bradner, 2014). Alternative approaches, such as inhibition of Myc-mediated downstream effectors, need to be investigated. Paradoxically, Myc is known to activate both proliferation and apoptosis, depending on the cellular context (Dang, 2012; Hoffman and Liebermann, 2008). However, the mechanisms that cancer cells use to escape Myc-induced apoptosis remain poorly understood. In this study, we found Myc activated the pro-survival IRE1α-XBP1 pathway in a broad spectrum of human and murine cancers, including BL, CLL, neuroblastoma, and hepatocellular carcinoma. In addition, our findings indicate that Myc-transformed cancer cells rely on this pathway to sustain cell proliferation and viability. We further identify SCD1 as a critical IRE1α-XBP1 effector required for maintenance of ER homeostasis and prevention of Myc-mediated cytotoxic ER stress.

Disruption of ER-dependent protein folding and transport results in the accumulation of misfolded proteins and consequent activation of ER stress responses. Specifically, cells initiate at least one of three major UPR signaling pathways (PERK, IRE1, ATF6) that collectively attenuate protein synthesis, increase protein folding and elevate protein degradation to sustain cell survival. If these responses do not restore ER homeostasis, persistent UPR signaling can ultimately trigger apoptosis (Hetz, 2012). The UPR has recently been appreciated as a central player in tumor development, making it an appealing target in both solid and hematological malignancies (Clarke et al., 2014; Urra et al., 2016). However, several fundamental questions need to be addressed to rationally target the UPR and improve patient outcomes. For example, what constitute cell-autonomous drivers of UPR in different types of cancer and how they function to integrate stress management must be elucidated. In addition, how to identify patients most likely to respond to
UPR inhibition remains unclear. In the current study, we determined that both c-Myc and N-Myc overexpression activates the IRE1α-XBP1 pathway through multiple molecular mechanisms. More importantly, this signaling pathway induces SCD1 as a critical downstream effector that generates unsaturated lipids to maintain ER membrane homeostasis in the face of Myc-dependent proteotoxicity. Consistent with our results, an accompanying report (Zhao et al., 2018) utilizing a triple negative breast cancer model also demonstrates c-Myc regulation of IRE1α-XBP1 signaling, and that cells with higher c-Myc expression are more sensitive to pharmacological IRE1α inhibition and genetic XBP1 depletion. Taken together, we identified IRE1α-XBP1 signaling as a critical survival pathway downstream of Myc activation in multiple cancers; therefore, Myc activation might be utilized to predict responses to IRE1α RNase inhibitor treatment in patients with CLL, NB, and breast cancers.

Previous studies clearly show that XBP1s regulates ER-associated degradation, protein entry into the ER, and protein folding (Acosta-Alvear et al., 2007; Lee et al., 2003). However, to maintain ER homeostasis in cells with oncogene-driven protein synthesis, ER lipid membrane biogenesis must also be regulated to accommodate elevated protein load. For example, our previous studies revealed an essential role for unsaturated lipids in maintaining ER homeostasis and viability in cells with constitutive mTORC1 activity (Young et al., 2013). The relationship between the UPR and ER lipid homeostasis is underscored by data demonstrating mutant IRE1α and PERK proteins lacking the ability to sense unfolded proteins retain their responsiveness to increased lipid saturation (Volmer et al., 2013), and that SCD1 inhibition initiates ER stress in multiple conditions (Liu et al., 2011; Roemeling et al., 2013). In this study, we extend the model by demonstrating that IRE1α-XBP1 signaling itself regulates SCD1 expression (Figure 26). In the case of Myc-transformed cancer cells, this feedback loop is essential for cell proliferation and viability. Interestingly, the accompanying study describes c-Myc/XBP1s complex formation in the nucleus; it's possible they coordinately transcriptionally regulate SCD. Carroll et al. found that oncogenic Myc requires the Myc superfamily member MondoA for tumorigenesis (Carroll et al., 2015), and decreased lipid biosynthesis plays an important role in MondoA deficient cell death.
In our study, we also found IRE1α RNase inhibition decreased de novo lipogenesis, yet SCD1 loss does not fully explain cellular responses to IRE1α inhibition. Therefore, it’s possible that decreased lipid biosynthesis also contributes to the observed phenotypes. Future studies will investigate how Myc, MondoA, and XBP1s coordinate with each other to regulate lipid metabolism. Our results also extend an emerging theme in which oncogenic transformation simultaneously induces anabolic metabolism to increase proliferation, along with homeostatic pathways that maintain cell viability. These include lipid and protein scavenging in RAS-transformed tumors (Commisso et al., 2013; Kamphorst et al., 2013), autophagy downstream of c-Myc overexpression (Hart et al., 2012), and lipid storage downstream of HIF2α activation (Qiu et al., 2015).

The role of the IRE1α-XBP1 pathway in human cancers may be more general, as Genovese et al. recently demonstrated that pancreatic ductal adenocarcinoma cells exhibiting a Myc gene expression signature undergo an anabolic switch that increases protein metabolism and adaptive activation of IRE1α-mediated survival pathways (Genovese et al., 2017). Increased XBP1s levels are frequently associated with human multiple myeloma (Mimura et al., 2012) and disruption of XBP1 splicing by inhibiting IRE1α may be a promising therapeutic option in this and other malignancies. Finally, XBP1s promotes triple-negative breast cancer progression via HIF1α (Chen et al., 2014), suggesting that different oncogenic pathways may engage distinct downstream UPR responses, and therefore harbor non-overlapping vulnerabilities to specific inhibitors.

Our findings support the use of IRE1α RNase inhibitors as an approach to target multiple tumors. BL, characterized by MYC translocation and dysregulation, is a highly aggressive malignancy, which clinically presents as the most common pediatric cancer in specific geographic locations, such as equatorial Africa, Brazil, and Papua New Guinea. Although intensive chemotherapy can achieve long-term survival, these non-targeted agents are unsafe in older patients due to immune suppression and cannot be efficiently deployed in less developed regions.
because of the need for extensive supportive care. Thus, targeted treatment strategies with fewer side effects are urgently needed. We provide a proof of principle of combining novel IRE1α inhibitors with chemotherapy drugs to decrease cytotoxicity and improve survival. In addition, MYCN amplification is a major prognostic factor in neuroblastoma (Huang and Weiss, 2013); targeting IRE1α underlying N-Myc overexpression is thus a promising strategy to treat a second fatal pediatric disease, where standard of care is extremely difficult and lengthy, and imposes significant toxicities.
Figure 26. Proposed model illustrating the regulation of ER stress and the IRE1α-XBP1 pathway by c-Myc and N-Myc, and the protective role of an IRE1α-XBP1-SCD1 axis to counterbalance anabolic metabolism mediated by Myc overexpression. c-Myc induces ER stress through multiple molecular mechanism, including transcriptional regulation of ERN1, HSPA5, and XBP1; stabilization of IRE1α protein; and upregulation of ER protein load, thereby activating IRE1α RNase activity and promoting XBP1 splicing. In turn, XBP1s directly regulates SCD1 transcription, which is required to maintain cellular lipid metabolism homeostasis. And this feedback loop is essential for proliferation and viability in cells that have high either c-Myc or N-Myc activity.
CHAPTER 4 Conclusions

The therapeutic potential of targeting ER stress-associated machinery

Cancer cells continuously divide and proliferate, which can be externally challenged by restricted supplies of nutrients and oxygen, while internally stressed by oncogenic activities. Therefore, maintaining cellular homeostasis is a key for cancer cells to survive and grow. The ER is the principal intracellular organelle responsible for protein and lipid synthesis. Disruption of ER homeostasis by biochemical, physiological, and pathological stimuli results in ER stress, which must be overcome to survive. In response to cellular stress, a well-established signaling cascade, the UPR, is activated. This intricate mechanism is an important means of reestablishing cellular homeostasis and alleviating the inciting stress. At the same time, most normal cells are not subjected to stress, and the UPR pathways remain largely inactive in these cells. Therefore, the importance of the UPR in the maintenance of malignancy has inspired great interest in exploring the therapeutic potential of targeting UPR components.

Cancer therapeutic approaches using ER stress-associated machinery can be divided into two categories: 1) increasing misfolded proteins in ER to overload protein folding machinery, therefore inducing more severe ER stress and cell death; 2) inhibiting UPR adaptive and pro-survival pathways, leading to failure of homeostasis restoration. For the first approach, several targets have been identified. For example, misfolded proteins are recognized by molecular chaperons and lectin-like proteins in the ERAD pathway and subsequently degraded as a part of an ER quality control mechanism (Ruggiano et al., 2014). As a main effector for ERAD, proteasome activity inhibition has been intensively studied for the treatment of cancer, such as Bortezomib, which has been approved for clinical use against multiple myeloma (Kouroukis et al., 2014). Secondly, the heat shock protein 90 (HSP90) chaperone machinery is a key regulator of proteostasis under both physiological and stress conditions in eukaryotic cells, and upregulated in multiple types of cancers (Miyata et al., 2013). Consequently, HSP90 represents an ideal target.
for the development of new anti-cancer agents (Schopf et al., 2017). Indeed, a previous study showed that HSP90 inhibitors 17-AAG and radicicol induce apoptosis in myeloma plasma cells (Davenport et al., 2007). Furthermore, Mbofung et al. recently showed that inhibition of HSP90 with ganetespib enhances T-cell-mediated killing of patient-derived human melanoma cells by their autologous T cells in vitro and potentiates responses to anti-CTLA4 and anti-PD1 therapy in vivo (Mbofung et al., 2017). This is due to the upregulation of interferon response genes, which are essential for enhanced killing ability of T cells, suggesting that HSP90 inhibitors have immunomodulatory features (Mbofung et al., 2017). Finally, protein folding chaperon BiP acts as a survival factor in solid tumors and cancer cells, and its expression is correlated with metastasis or late stages of tumor progression (Casas, 2017; Jamora et al., 1996; Pyrko et al., 2007). Down-regulation of BiP by siRNA leads to decreased glioma cell growth, and lowers resistance of glioma cells to temozolomide. At the same time, BiP depletion sensitizes glioma cells to 5-fluorouracil and CPT-11, suggesting that targeting BiP with conventional agents might represent a novel approach to eliminate residual tumor cells after surgery and increase the effectiveness of malignant glioma chemotherapy (Pyrko et al., 2007).

Another strategy is to inhibit UPR pathways. As discussed in Chapter 1, three principle branches of the UPR have been identified. These operate in parallel and use unique mechanisms of signal transduction. Each branch is defined by a class of transmembrane ER-resident signaling components: IRE1, PERK, and ATF6. The IRE1 branch is the most conserved and sole branch of the UPR in lower eukaryotes, and evolution later added the PERK and ATF6 branches to metazoan cells. The functions of these pathways are to attenuate protein synthesis, increase protein folding, and elevate protein degradative pathways. Therefore, targeting these molecules becomes a potential strategy for the treatment of multiple types of cancer. For instance, Hart et al. showed that c-Myc and N-Myc activate the PERK/eIF2α/ATF4 arm of the UPR, and inhibition of PERK significantly reduced c-Myc-induced autophagy, colony formation, and tumor formation (Dey et al., 2013; Hart et al., 2012). In addition to enabling cell survival, PERK-ATF4 signaling
also triggers multiple steps in the metastatic cascade (Bu and Diehl, 2016), including angiogenesis (Blais et al., 2006), migration (Nagelkerke et al., 2013), survival (Dey et al., 2015), and colonization at secondary organ sites (Bobrovnikova-Marjon et al., 2010). PERK is also required for the metastatic dissemination of cancer cells that have undergone an epithelial-to-mesenchymal transition (EMT) (Feng et al., 2014). Given its critical role in driving tumor growth and metastatic progression, PERK has been a focus of drug discovery programs for cancer, which have identified several small-molecule inhibitors of this kinase. For example, GSK2656157 is an ATP-competitive inhibitor of PERK enzyme activity with a nanomolar level of IC$_{50}$, and its administration results in a dose-dependent slowing of the growth in multiple human tumor xenografts in mice (Atkins et al., 2013; Axten et al., 2013). Altered amino acid metabolism, decreased blood vessel density, and reduced vascular perfusion are potential mechanisms for the observed antitumor effect (Atkins et al., 2013; Axten et al., 2013). At the same time, PERK inhibition strongly reduced the ability of EMT cells to form tumor-spheres and migrate in trans-well assays, and pretreatment of metastatic cancer cells with PERK inhibitor results in significantly diminished metastatic capacity (Feng et al., 2014), suggesting that disruption of the PERK pathway significantly compromises the malignant phenotype of EMT. However, while PERK inhibitors decrease cancer cell growth and reduce metastatic spread, they also cause rapid onset of pancreatic atrophy, precluding their further consideration of clinical development (Atkins et al., 2013). In terms of ATF6, a previous study showed that knockdown of ATF6 is sufficient to enhance radiation induced cell death in glioblastoma cells, suggesting ATF6 as a potential therapeutic target to enhance the efficacy of radiation therapy (Dadey et al., 2016). In addition, ATF6 transcriptionally induces the expression of cancerous inhibitor of protein phosphatase 2A (CIP2A) and contributes to the prognosis of colon cancer (Liu et al., 2018). Furthermore, a novel selective ATF6 inhibitor, melatonin, induces human hepatoma cell apoptosis through COX-2 downregulation (Bu et al., 2017).

IRE1α-XBP1 signaling might be the best studied UPR pathway for future treatment of
cancer, which plays an indispensable role in tumor growth, metastatic progression, and chemoresistance (Shajahan et al., 2009). XBP1 expression and activation correlates with clinical outcome in breast cancer (Chen et al., 2014; Davies et al., 2008), and angiogenesis in pancreatic adenocarcinomas (Romero-Ramirez et al., 2009): XBP1 depletion reduces blood vessel formation, and expression of XBP1s restores angiogenesis in IRE1α dominant-negative expressing cells. In mouse models of glioblastoma, IRE1α is required for upregulation of pro-inflammatory cytokines and angiogenic factors, which contributes to tumor growth, angiogenesis, and invasiveness (Auf et al., 2010; Drogat et al., 2007). XBP1 is also critical in the development of plasma cells and is overexpressed in multiple myelomas (MMs). Several studies demonstrate that the IRE1α-XBP1 pathway is involved in the pathogenesis of this disease, and blockage of XBP1 splicing by IRE1α inhibition is a promising therapeutic option in MM (Mimura et al., 2012). Our study here, accompanied by a companion paper by Zhao, et al. (Zhao et al., 2018), establish the pro-survival functions of the IRE1α-XBP1 pathway in Myc-overexpressing cancers, including Burkitt’s lymphoma, neuroblastoma, and triple-negative breast cancer. These findings provide the evidence of inhibiting this pathway for the treatment of these types of cancer, which all together further expand the scenarios of using IRE1α inhibitors as a means of cancer treatment.

**Future directions**

**How do c-Myc and XBP1s co-operatively regulate gene expression?**

In Chapter 3, we show that SCD is a direct transcriptional target of XBP1s through ChIP-qPCR analysis (Figure 16E). At the same time, the c-Myc target gene database and multiple previous studies establish that c-Myc also regulates SCD transcription by binding to the E-box of its promoter region (Wu et al., 2017; Zeller et al., 2003). This raises the possibility that c-Myc and XBP1s co-regulate the expression of SCD. Furthermore, ChIP-seq and motif analysis of XBP1s in MDA-MB-231 cells revealed statistically significant enrichment of both HIF1α and XBP1s DNA binding motifs, which is “CACGT”. Since the E-box sequence is “CACGTG”, it is reasonable to
speculate that c-Myc is a co-regulator of XBP1s in the nucleus. Therefore, it would be interesting to investigate interactions between c-Myc and XBP1s, as well as the genes co-regulated by these two proteins, to provide more insights into defining their functions in tumor initiation and maintenance, and assist the development of therapeutic strategies to target these pathways.

**Combining B-I09 with traditional chemotherapy for the treatment of BL?**

BL is a highly aggressive non-Hodgkin’s lymphoma (NHL) that typically arises from germinal center B-lymphocytes. It is subdivided into three variants: endemic (eBL), sporadic (sBL), and immunodeficiency-associated (Jaffe, 2009). eBL clinically presents as the most common pediatric cancer in specific geographic locations, e.g. equatorial Africa, Brazil, and Papua New Guine (Orem et al., 2007). In these areas, the annual BL incidence has been estimated at 40-50 per million children younger than 18 years old, comprising approximately half of all childhood cancers and up to 90% of lymphoma diagnose (Orem et al., 2007). There is a low background incidence of sBL worldwide: In high-income countries, NHL accounts for ~7% of cancers in children younger than 20 years, in which ~40% are BL cases; in Western Europe and America, sBL accounts for 1-2% of adult lymphoma (Blum, 2004). In addition, BL is the second most common neoplasm in patients with HIV infection (Corti, 2016), which is a severe complication leading to poor outcomes. In developed countries, with intensive chemotherapy (hyper-CVAD: hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone alternating with methotrexate and cytarabine), the 5-year survival rate of BL has increased to be >90% in children (Uzunova and Burke, 2016). However, these non-targeted reagents are unsafe in older patients due to immune suppression and cannot be efficiently deployed in less developed regions because of the need for extensive supportive care. Therefore, targeted treatment strategies with fewer side effects are thus urgently needed.

In our study, we demonstrate that IRE1α RNase inhibitors (like B-I09) exhibit antitumor effects in BL cell lines *in vitro* and *in vivo*, without showing significant side effects. Furthermore,
we show that B-I09 exhibits synergistic effects with Doxorubicin and Vincristine to induce apoptosis in both Ramos and Daudi BL cell lines (Figure 25 and Table 2), and B-I09 maintains its ability to inhibit SCD1 expression in both cases. Doxorubicin is a chemotherapy medication used to treat multiple types of cancers, such as breast cancer, bladder cancer, lymphoma, and leukemia. It has antimitotic and cytotoxic activity through a number of proposed mechanisms of action: it forms complexes with DNA by intercalation between base pairs, and inhibits topoisomerase II activity by stabilizing the DNA-topoisomerase II complex, preventing the religation portion of the ligation-religation reaction that topoisomerase II catalyzes. Vincristine is an antitumor vinca alkaloid isolated from *Vinca rosea*, which is indicated for the treatment of acute leukemia, malignant lymphoma, Hodgkin’s disease, and acute panmyelosis. The antitumor activity of Vincristine is thought to be due primarily to inhibition at metaphase through its interaction with tubulin. Like other vinca alkaloids, Vincristine may also interfere with: 1) amino acid, cyclic AMP, and glutathione metabolism, 2) calmodulin-dependent Ca2+-transport ATPase activity, 3) cellular respiration, and 4) nucleic acid and lipid biosynthesis. Even though we have a detailed understanding of the mechanisms of B-I09, Doxorubicin, and Vincristine resulting in antitumor effects, further investigation should be performed to study mechanisms of their synergy. In addition, these outcomes should be confirmed *in vivo*, ideally with the *Ea/MYC* BL mouse model, which will potentially reshape the current standard of care to further increase efficacy and decrease toxicity.

**Targeting the IRE1α-XBP1 pathway in cancer-associated myeloid cells**

A function for the IRE1α-XBP1 pathway in cells of the immune system has been well established (Bettigole and Glimcher, 2015): for instance, Toll-like receptor (TLR) 2 and 4 specifically activates this axis, which is required for optimal and sustained production of proinflammatory cytokines in macrophages, and XBP1 deficiency results in a much greater bacterial burden in mice infected with TLR2-activating human intracellular pathogen *Francisella tularensis*, suggesting a critical role of XBP1 in mammalian host defenses (Martinon et al., 2010).
Neutrophils infiltrating acute lung injury lesions exhibit XBP1 hyperactivation compared with lung-resident neutrophils in naïve mice. In this setting, XBP1 was needed for optimal neutrophil granule release, whereas dampening XBP1 expression in neutrophils substantially alleviates acute lung injury (Hu et al., 2015). Furthermore, IRE1α-XBP1 signaling is required for the optimal differentiation of plasma cell (Reimold et al., 2001), eosinophil (Bettigole et al., 2015), and some dendritic cell populations (Iwakoshi et al., 2007).

More recently, IRE1α-XBP1 pathway functions in cancer-associated myeloid cells have been investigated. In ovarian cancer (OvCa), Cubillo-Ruiz et al. showed that dendritic cells in the OvCa microenvironment exhibit marked upregulation of ER stress response markers and robust XBP1 activation when compared to cells from naïve hosts, and this is induced by byproducts of lipid peroxidation, such as the unsaturated aldehyde 4-hydroxy-trans-2-nonenal (4-HNE) (Cubillos-Ruiz et al., 2015). XBP1 activation induces a triglyceride biosynthetic program in dendritic cells leading to abnormal lipid accumulation and subsequent inhibition of the capacity of these cells to support antitumor T cells. Accordingly, dendritic cell-specific XBP1 deletion restores their immunostimulatory activity in situ and extends host survival (Cubillos-Ruiz et al., 2015). Tumor-associated macrophages play critical roles during disease progression by promoting angiogenesis, cancer cell proliferation, invasion, and metastasis (Ostuni et al., 2015). Cysteine cathepsin proteases, produced by macrophages and cancer cells, modulate these processes. Interestingly, IL-4 synergizes with IL-6 or IL-10 to trigger IRE1α-XBP1 activation in macrophages through STAT6 and STAT3, a process that promoted cathepsin secretion (Yan et al., 2016), and pharmacological inhibition of IRE1α blocks cathepsin secretion and blunts macrophage-mediated cancer invasion (Yan et al., 2016). Tumor cells evade immune control by creating hostile microenvironments that perturb T cell metabolism and effector function (Chang et al., 2015; Scharping et al., 2016). A recent study shows that malignant ascites fluid obtained from patients with OvCa inhibit T cell glucose uptake and cause N-linked protein glycosylation defects, which therefore triggers IRE1α-XBP1 activation (Song et al., 2018). Consequently, XBP1 regulates the
abundance of glutamine carriers, thus limiting the influx of glutamine necessary to sustain mitochondrial respiration in T cells under glucose deprived conditions. Accordingly, mice bearing OvCa and lacking XBP1 in T cells demonstrate superior anti-tumor immunity, delayed malignant progression and increased overall survival (Song et al., 2018). These studies demonstrate a critical function of IRE1α-XBP1 signaling in the tumor immune microenvironment, and targeting this pathway seems to have a high potential to modulate tumor immunity, which inhibits tumor growth and progression. Since this pathway is also essential in cancer cells, it would be interesting to see how its manipulation could be applied in the clinics as a single agent or as a combinatorial treatment with immune check-point inhibitors.


The ATF6 pathway of the ER stress response contributes to enhanced viability in glioblastoma. Oncotarget 7, 2080–2092.


Gabay, M., Li, Y., and Felsher, D.W. (2014). MYC activation is a hallmark of cancer initiation and


