IRE1α cofactor expression influences cell fate decisions in response to proteotoxic and non-proteotoxic stress

Abstract: ER stress activates multiple survival signaling pathways and subsequently confers resistance to chemotherapeutic agents. However, unresolved ER stress can lead to apoptosis and the transition from pro-survival to pro-apoptotic signaling in response to ER stress is poorly understood. Multiple myeloma (MM) is a plasma cell cancer that is highly dependent on ER function and responds heterogeneously to chemotherapy. This heterogeneity may be due to different contributions to survival signaling from the ER. IRE1α is a key mediator of the ER stress response and interacts with both pro-survival and pro-apoptotic cofactors. IRE1α activation can lead to survival or apoptosis depending on which cofactors are present. We hypothesize that MM IRE1α cofactor expression profile determines sensitivity to ER stress-induced apoptosis by affecting survival signaling. We further hypothesize that MM IRE1α cofactor expression patterns confer sensitivity or resistance to the non-proteotoxic chemotherapy melphalan by survival signaling. We will measure the sensitivity of 40 MM cell lines and 200 primary MMs to apoptosis in response to the ER stress inducer thapsigargin (Tg) and to melphalan. We will quantify pre-treatment protein expression levels of 10 IRE1α cofactors in each sample by microwestern array, and we will determine which IRE1α cofactors have expression patterns that correlate with apoptotic response. We will show that overexpression and siRNA knockdown of these cofactors modulates apoptosis in response to ER stress and melphalan treatment and that this effect is mediated through survival signaling downstream of IRE1α. These experiments will help determine how cell fate decisions are made in response to ER stress. This would also establish ER-derived survival signals as an important variable in determining cell fate in response to diverse stresses including chemotherapy.

<u>Hypothesis</u>: Differential expression of IRE1α cofactors determines the heterogeneity in ER stress thresholds required to switch from pro-survival to apoptotic signaling and influences apoptosis of multiple myeloma cells in response to melphalan chemotherapy.

Specific Aim 1: To test the hypothesis that the IRE1α cofactor expression pattern directly determines the threshold of thapsigargin-induced ER stress required to induce apoptosis in MM cells through modulation of survival signaling.

1a) Determine whether protein expression levels of IRE1α cofactors correlate with the apoptotic response to thapsigargin.

1b) Determine whether IRE1α cofactor expression directly affects the apoptotic response to thapsigargin.

1c) Determine whether thapsigargin-resistant cells have enhanced survival signaling through IRE1α cofactors.

Specific Aim 2: To test the hypothesis that IRE1 cofactor expression contributes to melphalan response in MM cells through ER-stress-mediated survival signaling.

2a) Determine whether protein expression levels of IRE1α cofactors correlate with the apoptotic response to melphalan.

2b) Determine whether IRE1α cofactor expression directly affects the apoptotic response to melphalan.

2c) Determine whether increased survival signaling through IRE1α cofactors mediates melphalan resistance.

Background and significance

Conditions that interfere with the function of endoplasmic reticulum (ER) or create an imbalance between the cellular demand for ER function and ER capacity are collectively called ER stress[27]. Upon ER stress, cells activate an evolutionarily conserved mechanism termed the unfolded protein response (UPR), which allows cells to cope with protein folding alterations and to restore homeostasis. When ER stress is not mitigated and homeostasis is not restored, the UPR triggers apoptosis. Thus, ER stress may result either in successful adaptation and survival or in cell death. The molecular mechanism underlying the cell's decision to switch from an adaptive response to apoptosis in response to ER stress is unknown.

IRE1α is an ER transmembrane protein that plays a central role in the UPR. As part of the UPR, IRE1α signaling affects multiple cell survival pathways involving p38, JNK, ERK, and NF-κB.[31-33] IRE1α activation alone is insufficient to selectively drive survival versus apoptosis. IRE1α cofactors, adaptors and inhibitors cooperate to activate, modulate and

Abbreviation	Full term	Definition
ER	Endoplasmic reticulum	The cellular organelle that processes proteins in the secretory pathway, among other functions.
MM	Multiple myeloma	A malignancy of antibody-secreting plasma cells. Most MMs secrete excessive immunoglobulins and depend highly on ER function for survival.
Tg	Thapsigargin	A drug that blocks Ca^{2+} uptake into ER by inhibiting SERCA pump. Causes ER stress by reducing the function of Ca^{2+}-dependent chaperones.
UPR	Unfolded protein response	The main ER stress response signaling pathway. Can induce adaptation to ER stress by increasing ER size, increasing chaperone proteins, and reducing ER protein input. Can also induce apoptosis if the cell is unable to compensate for the increased stress.
AT	Apoptotic threshold	The concentration of drug required to cause apoptosis in at least 75% of cultured cells.
hMCL	Human myeloma cell line	An immortalized cell line established from primary myeloma cells.
PMMC	Primary multiple myeloma cells	Malignant plasma cells isolated from a bone marrow aspirate of an MM patient.
MWA	Microwestern array	A high-throughput technique for quantifying protein levels and activation states.
kNN	k nearest neighbors classifier	A statistical classifier that designates samples to defined groups based on their similarity to samples that are known to be in a certain group.

Table 1. Abbreviations and definitions of common terms used in this proposal.

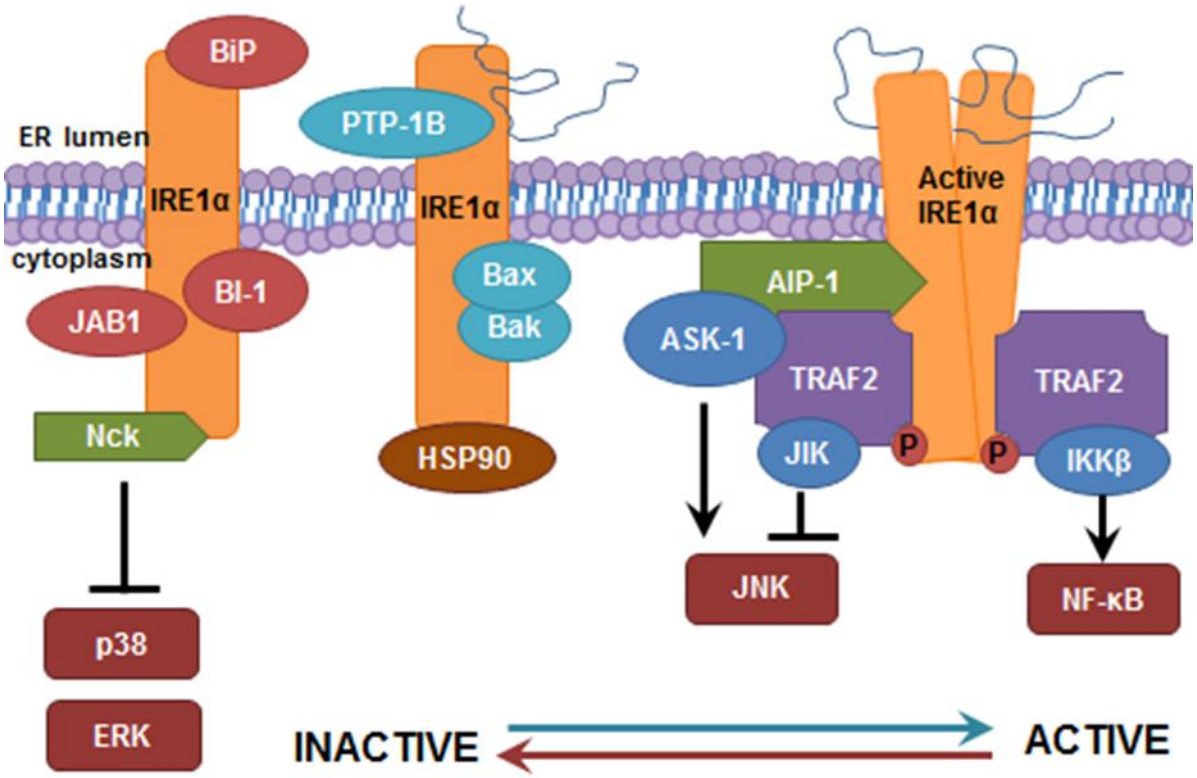

Figure 1. IRE1α cofactors modulate the oligomerization and activation of IRE1α, determining how survival and death signaling is modulated by ER stress. *Left*: Inactive IRE1α bound to inhibitory cofactors and anti-apoptotic adaptors. *Middle*: IRE1α bound to stabilizing and activating factors. *Right*: Active IRE1α promotes several downstream signaling pathways. We hypothesize that the expression pattern of these cofactors determines how the cell will respond to stress and how resistant the cell is to apoptosis.

fine-tune signaling downstream of IRE1α (Figure 1, Table 2). These cofactors influence pro-survival and pro-apoptotic signaling, and there is evidence that differential binding of IRE1α to its cofactors affects whether downstream signaling will lead to survival or cell death.[18,27] However, it is unclear how IRE1α balances pro-survival and pro-apoptotic signaling to decide between divergent cell fates and what role individual cofactors play in this decision.[27,28] We hypothesize that the expression levels of the various IRE1α cofactors in a given cell shapes the IRE1α stress response, determining the threshold of ER stress that is required to switch from a survival response to apoptosis. We expect that cells expressing high levels of pro-survival IRE1α binding factors and low levels of pro-apoptotic IRE1α cofactors require more ER stress to switch from survival to apoptosis, and vice versa.

Multiple myeloma (MM) is a malignancy of plasma cells, white blood cells whose role is to produce and secrete antibodies. Normal plasma cells are constitutively under ER stress

Protein	Role in IRE1α signaling	Antibody source	siRNA source	Expected effect
TRAF2	Adaptor, binds active IRE1α, activates JNK via ASK1, activates NF-κB via IKKβ[6,7,33]	pAb - CS	SC	Pro-apoptotic
NCK	Adaptor, binds inactive IRE1α, preventing ERK and p38 activation[9,32]	mAb - CS	SC	Anti-apoptotic
ASK1	Downstream of IRE1α-TRAF2. MAP3K that activates JNK[6,7]	pAb - CS	SC	Pro-apoptotic
BAX	Activates IRE1α, prevents IRE1α by BI-1[8]	mAb - CS	CS	Pro-apoptotic
BAK	Activates IRE1α, prevents IRE1α by BI-1[8]	mAb - CS	CS	Pro-apoptotic
PTP-1B	ER luminal tyrosine phosphatase, promotes IRE1α activation[13,45]	pAb - CS	CS	Pro-apoptotic
AIP1	Interacts directly with IRE1α, promotes JNK activation through TRAF2-ASK1[8,14]	mAb - CS	SC	Pro-apoptotic
BI-1	Inactivates IRE1α, inhibits IRE1α activation by Bax/Bak[15-17]	mAb - SC	SC	Anti-apoptotic
JIK	Binds active IRE1α and TRAF2, inhibiting JNK activation[18]	mAb - SC	SC	Anti-apoptotic
JAB1	Binds IRE1α in the absence of stress, inhibits IRE1α activation[18,35]	mAb - SC	SC	Anti-apoptotic

Table 2. IRE1α cofactors to be tested in our experiments. We expect that pro-apoptotic proteins will be highly expressed in drug-sensitive cells relative to drug-resistant cells. We also expect that overexpression of pro-apoptotic proteins will make a cell line more drug-sensitive, and that siRNA knockdown of pro-apoptotic proteins will make a cell line more drug-resistant. We expect the opposite effects for anti-apoptotic proteins. All antibodies are from rabbits. Antibody and siRNA sources: CS = Cell Signaling, SC = Santa Cruz.

due to elevated synthesis of secreted proteins, and the unfolded protein response is necessary for immunoglobulin (Ig) production.[40]Transforming events leading to MM affect proliferation and survival pathways, but leave secretory programs largely intact such that MM cells retain their Ig secretory capacity.[41] MM cells have an enlarged ER[42] and over 95% of MMs characteristically secrete large amounts of Ig, often leading to protein aggregation and organ damage.[43] Because MM cells exhibit this secretory phenotype, they are highly dependent on ER function for survival and have constitutively active ER stress response pathways.[37,38] These properties make MM a good model in which to study the effects of IRE1α signaling heterogeneity in cell fate decisions, as we expect that ER-derived signals will have a strong influence on cell fate in this highly secretory cancer.

Most MM patients are treated at some point with the genotoxic chemotherapy melphalan.[44] The preferred therapy for MM is autologous stem cell transplant (ASCT), and the most common conditioning regimen for ASCT is high dose melphalan (200 mg/m^2).[44] For MM patients that are ineligible for ASCT due to age or comorbidities, front-line treatment is

combination chemotherapy that often includes melphalan (16 mg/m^2).[44] Clinical response to melphalan is variable, and there are currently no predictors of whether a patient will benefit from melphalan.[44]

Cells have different intrinsic tolerances to various types of stress and their initial state before the treatment likely determines how they will respond to therapy. The output of a cellular response involves integrating many signaling inputs, so a given signaling event may result in a very different outcome depending on the status of other signaling pathways. ER-mediated signaling can thus affect the ability of an unrelated stimulus, such as non-proteotoxic chemotherapy like melphalan, to induce survival or cell death. Because ER stress modulates core survival signaling pathways through IRE1α, IRE1α signaling affects a cell's intrinsic tolerance to non-proteotoxic chemotherapy.[39] Constitutive ER signaling can either confer stress sensitivity through apoptotic signaling or stress resistance through pro-survival signaling. Since IRE1α cofactor expression affects whether downstream signaling is mainly pro-survival or pro-apoptotic, we hypothesize that IRE1α cofactor expression underlies the heterogeneity observed in MM response to melphalan. Expression levels of IRE1α cofactors that affect survival or cell death signaling should contribute to resistance or sensitivity, respectively, to melphalan.

In this study we aim to show that different MM cell lines and patient samples differentially express IRE1α cofactors, and that these expression patterns directly determine the threshold of thapsigargin-induced ER stress required to induce apoptosis. We also aim to show that these IRE1α cofactor expression patterns modulate cell fate signals from the ER and thereby confer sensitivity or resistance to melphalan in MM.

Innovation

This proposed study would be the first to gain systems-level mechanistic understanding of IRE1α signaling and its role in determining cell fate in stress responses. Currently we know which molecular factors are involved in regulating IRE1α signaling, but we lack a comprehensive understanding of how these factors interact to produce a given

response to stimulus. This study can shed light on how the cell decides to live or die in response to ER stress. In general, this study can help us understand how cells choose between mutually exclusive outcomes.

Our study could represent a substantial paradigm shift in the field by demonstrating that IRE1α-mediated survival signaling can influence cell fate in response to various stressors, even stressors that do not directly involve the ER. This would show that by changing the cell's baseline survival signaling, cellular housekeeping processes are important variables that can affect the outcome of stress in any context. This could change the way we understand chemoresistance by identifying common clinical variables that affect response to chemotherapy independent of a drug's mode of action and therefore need to be considered in all chemotherapy regimens. This represents a move towards an integrated cell biological framework for understanding the cell's response to sudden stress.

This study has important implications for understanding heterogeneity between MM patients in response to melphalan and could lead to improved predictive tools for determining which patients are likely to benefit from chemotherapy. The knowledge we gain about ER stress response heterogeneity could be used to make clinical decisions about chemotherapy drug combinations or dosage adjustments. Understanding the heterogeneity in ER stress response could enable pre-identification of patients that have higher or lower thresholds needed to overcome ER-mediated survival signaling, and may require lower or higher dosing chemotherapy. By identifying variables that affect the cell's sensitivity to ER stress, this study could also be helpful in projecting which patients are likely to benefit from ER stress-inducing proteotoxic chemotherapies like bortezomib. The knowledge gained will help to tailor the treatment regimens to maximize efficacy and minimize toxicity for individual patients.

Specific Aim 1: To test the hypothesis that the IRE1α cofactor expression pattern directly determines the threshold of thapsigargin-induced ER stress required to induce apoptosis in MM cells through modulation of survival signaling.

***Rationale*:** Our goal of relating ER stress sensitivity to IRE1α cofactor levels requires us to measure apoptosis in response to ER stress. We will use Tg, a drug that inhibits ER Ca^{2+} reuptake by inactivating the SERCA pump[2], which reduces the activity of Ca^{2+}-dependent ER luminal chaperones and leads to ER stress,[12] as a proxy for ER stress. This will allow us to measure the amount of ER stress required to induce apoptosis in various MM cells. In order to relate these data to IRE1α cofactors, we will measure protein expression levels of IRE1α cofactors. Protein is a more accurate measure of cell conditions than mRNA, since proteins are the functional signaling units in most cellular pathways. Protein is even more accurate than mRNA under ER stress conditions, which promote selective mRNA translation and high mRNA degradation. The protein levels of each component will be evaluated by microwestern array (MWA), a multiplexed immunoblotting technology that uses ~200-fold less protein and antibody than traditional Western blots and has a significantly accelerated workflow.[10] This method allows us to feasibly quantify levels of 10 different proteins for each of our 40 cell lines and 200 patient samples.

Using statistical analysis, we will relate each sample's ER stress tolerance to its IRE1α cofactor expression profile, and we will determine which cofactors significantly correlate with ER stress tolerance across all samples. We will demonstrate the causal role of these cofactors in determining cell fate in response to ER stress by modifying their protein expression levels by siRNA knockdown and lentiviral overexpression; if these cofactors play a significant role in cell fate, then by changing their protein levels we should change cellular sensitivity to ER stress. We will then show that different sensitivity to ER stress reflects differences in pro-survival and pro-apoptotic signaling, as determined by phosphorylation of ERK, JNK, p38 and NF-κB. These measurements are also well-suited to MWA format, since they involve measuring several protein parameters across many samples.

Samples: Human multiple myeloma cell lines (hMCLs, n=40) will be obtained from Dr Bernard Klein (Centre de Recherches en Cancérologie Nantes/Angers, Université Montpellier, France) and would be maintained as previously described.[1] These hMCLs

recapitulate the molecular diversity of MM in patients.[1] Further, to increase the clinical significance of our results, we will test primary multiple myeloma cells (PMMCs, n=200) from pre-treatment human MM patients. Bone marrow aspirates will be obtained from pre-treatment human MM patients after written informed consent at University of Chicago Medical Center. Live mononuclear cells will be isolated from BM aspirates by Histopaque® density gradient centrifugation[4,5] (Sigma Aldrich) and CD138+ cells (PMMCs[4,5]) will be enriched from this fraction with anti-CD138 immunomagnetic MicroBeads® (Miltenyi Biotec).[4,5] An aliquot of PMMCs will be retained for protein expression profiling and the remaining sample would be maintained in culture for dose-response assays.[1,3] While PMMCs can be maintained in culture, they often lose viability over the course of several weeks. To ensure maximum cell viability and relevance of results, the dose-response assays would be performed within a week of isolating PMMCs.[1]

Power analysis: To detect a correlation between one of the ten protein expression levels and drug sensitivity (p-value = 0.05, power = 0.9), conservatively assuming that several variables are co-dependent due to regulation by the same biological processes (variance inflation factor 5) and that protein expression levels have a relatively low dynamic range (SD = 0.5), we need to recruit 170 MM patients.[46] To ensure our analysis has sufficient power if we fail to obtain useable PMMCs from some patients, we intend to recruit 200 newly-diagnosed MM patients.

Aim 1a: *Determine whether protein expression levels of IRE1α cofactors correlate with the apoptotic response to thapsigargin. (Figure 2.)*

We will measure the apoptotic threshold of MM cells in response to Tg. We define the apoptotic threshold (AT) as the concentration of drug required to cause apoptosis in at least 75% of cultured cells. AT is a metric for sensitivity of cells to the drug and cells with lower AT are more sensitive to the drug than the cells that have relatively higher AT. To determine the apoptotic threshold, cultured cells (1×10^5 cells/mL)[1] will be treated with varying concentrations of the ER stress inducer thapsigargin (Tg). Tg is reported to have

maximal effect on ER Ca^{2+} levels in the range of 50-100 nM,[2] so we will test Tg concentrations of (0, 10, 20, 30,…,100) nM. In response to Tg, annexin V staining can be seen within 24h,[12] so we will assay cell death and apoptosis after 24h Tg treatment. Cell death and apoptosis will be assessed using LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen) and Annexin V staining (Invitrogen) respectively. The percentage of dead and apoptotic cells will be quantified by flow cytometry (BD FACSCanto®). Subsequently, we will plot the percentage of apoptotic cells versus the Tg concentration independently for each of the samples to generate separate dose-response curves for each of the hMCLs and PMMCs. From the resulting plot, AT would be determined as the concentration of Tg at which at least 75% of the cultured cells undergo apoptosis.

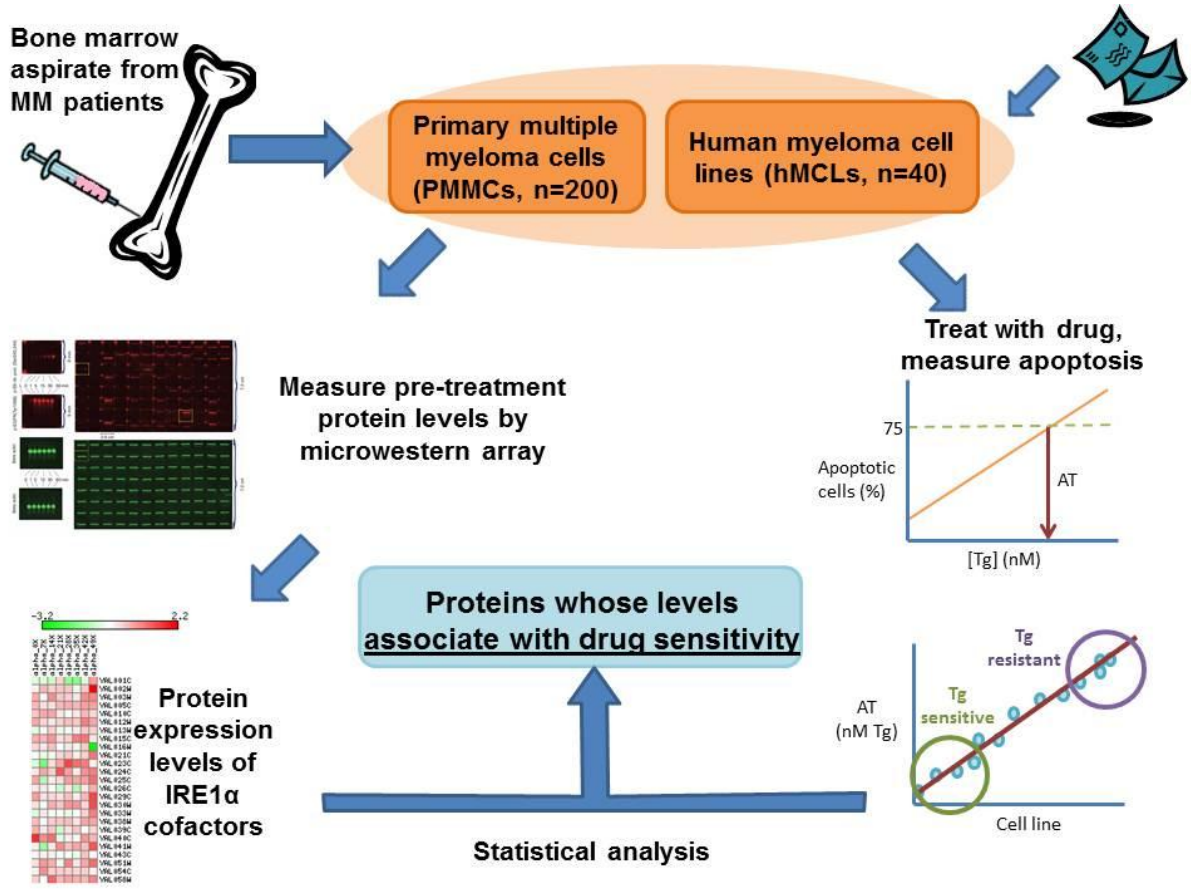

Figure 2. Workflow for identifying proteins whose levels associate with Tg sensitivity. We will purify PMMCs from 200 MM bone marrow aspirates. We will measure the apoptotic threshold of hMCLs and PMMCs in response to Tg. We will quantify pre-treatment protein expression levels of IRE1α cofactors by microwestern array. We will correlate protein levels with Tg sensitivity to determine which proteins are differentially expressed between Tg-sensitive and Tg-resistant MM cells.

We will quantify protein expression levels of IRE1α cofactors in MM cells. Each hMCL and PMMC will be analyzed for pre-treatment protein expression levels of IRE1α cofactors (Table 1). The protein levels of each component will be evaluated by microwestern array (MWA); MWAs will be performed as described previously.[10] β-actin levels will be used to control for array printing biases and also to normalize samples to a standard scale of protein expression, as described previously.[10]

We will determine which IRE1α cofactors are differentially expressed in Tg-sensitive versus Tg-resistant MM cells. Using our AT data, we will group samples into Tg-sensitive (AT < 0.5 SD below the mean) and Tg-resistant (AT > 0.5 SD above the mean) classes. To determine if Tg sensitive and Tg resistant cells express significantly different IRE1α cofactors, we will use a k nearest neighbors (kNN) classifier.[21] To determine which IRE1α cofactors are most highly correlated with drug sensitivity, we will use weighted voting class determination to obtain a ranked list of proteins based on the strength of their association with drug sensitivity.[19] All statistical analysis will be performed using the Bioconductor package in R.[20] For our weighted voting and kNN classifiers, we will split our hMCL and PMMC data into a training set and a validation set (n=20 hMCL, n=100 PMMC, determined randomly). On the training set we will vary input parameters (e.g. *k* in kNN) and compare the accuracy of the resulting classifiers using leave-one-out bootstrapping.[22] We will use the input parameters that produce the highest accuracy to validate our models on the test set.[22] To determine how each protein's expression varies relative to AT, we will correlate expression and AT as continuous variables and perform regressions. We will observe the scatterplot of these data to determine whether the relation is linear[19]. If the relation is linear, we will perform linear regressions[19]; if it appears nonlinear we will allow for nonlinear components in our regression and experiment with curve fits until we achieve an $r^2 > .90$.[19] All statistical analysis will be performed using the Bioconductor package in R.[20]

Aim 1b: *Determine whether IRE1α cofactor expression directly affects the apoptotic response to thapsigargin.*

From aim 1a, we will have a ranked list of proteins whose expression correlates with Tg-induced apoptosis. We will take any protein with significantly correlated expression and demonstrate that changing its expression level in an MM cell line directly affects the AT of that cell line. We will change expression levels by forced overexpression and siRNA knockdown (Figure 3). We will test modulation of these cofactors in the 3 most Tg-sensitive and the 3 most Tg-resistant hMCLs, as determined in aim 1a.

For *siRNA knockdown of individual IRE1α cofactors*, we will plate 1 x 10^5 cells/mL and allow them to grow for 24h, then transfect with 0.25μg siRNA duplex in serum-free medium for 6h.[23] As controls, we will treat the cells with no siRNA and with scrambled siRNA. After 6h, serum will be reintroduced and cells will incubate for an additional 24h. An aliquot of cells will be used to measure knockdown efficiency by western blot against the target cofactor; the rest of the cells will be treated with [Tg] determined to be that cell line's AT in aim 1a and apoptosis will be quantified as described in aim 1a. We expect that knockdown of a pro-apoptotic cofactor will reduce Tg sensitivity and observed apoptosis, and that knockdown of an anti-apoptotic cofactor will increase Tg sensitivity and observed apoptosis.

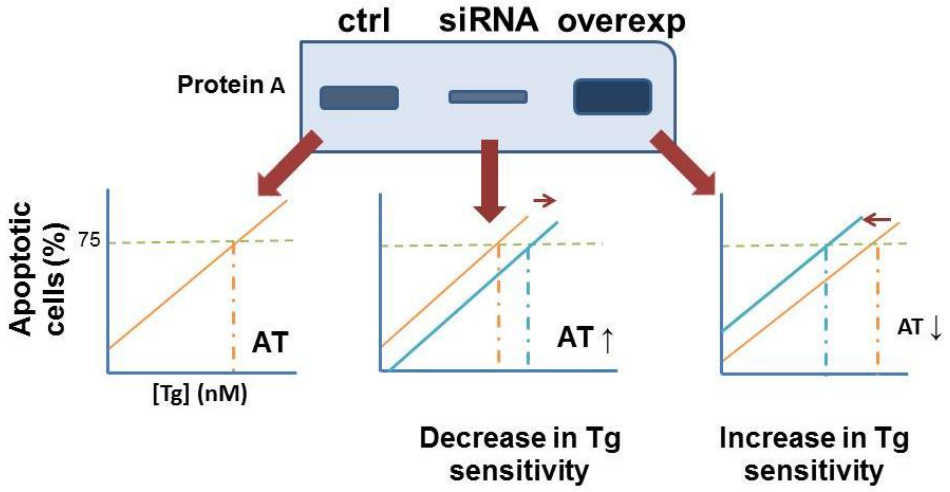

Figure 3. IRE1α cofactor levels will be modified by siRNA knockdown and lentiviral overexpression. We expect that changing the level of a cofactor will also change the observed apopototic threshold in response to stress. In this example, Protein A is a pro-apoptotic cofactor of IRE1α; knocking down A increases cell survival and increases the AT, while overexpressing A reduces cell viability and decreases the AT.

For overexpression of IRE1α cofactors in human MM cell lines, we will use lentiviral constructs developed for each of the cofactors to be tested. Lentiviral transfection will be done using Lenti-Smart kit (Invitrogen) and protein overexpression would be confirmed by a parallel western blot, with mock transfected cells as the controls. Cells overexpressing the cofactor of interest will be treated with [Tg] determined to be that cell line's AT in aim 1a and apoptosis will be quantified as described above. We expect that overexpression of a pro-apoptotic cofactor will increase Tg sensitivity and observed apoptosis, and that overexpression of an anti-apoptotic cofactor will reduce Tg sensitivity and observed apoptosis.

Aim 1c: *Determine whether thapsigargin-resistant cells have enhanced survival signaling through IRE1α cofactors.*

IRE1α cofactors may regulate apoptosis through downstream survival signaling. Activation of a protein and its subsequent downstream signalling is quantified by the ratio of intensity of bands in western blot for phosphorylated protein to the total protein. For the three most Tg-resistant and the three most Tg-sensitive cell lines, we will measure pre-treatment ratios of p-ERK1/2 to total ERK1/2, p-NF-κB to total NF-κB, p-Akt to total Akt, p-JNK to total JNK, and p-p38 to total p38 by Western blot using pan-specific and phospho-specific antibodies for each protein (Cell Signaling). We expect that Tg-resistant will have high pre-treatment levels of active pro-survival factors including p-ERK, p-NF-κB and p-Akt and low levels of active pro-apoptotic factors like p-JNK and p-p38 compared to Tg sensitive cells. On similar lines, Tg-sensitive cells will have lower activity of pro-survival signals and higher activity of pro-apoptotic signals compared to Tg resistant cells.

To show that IRE1α cofactors directly affect the levels of downstream survival signaling, we will measure these activation ratios in cells with siRNA knockdown or forced overexpression of a given cofactor. Cells will be transfected as described in aim 1b and post-transfection activation levels of ERK, NF-κB, Akt, JNK and p38 will be measured by Western blot. We expect that overexpression of a pro-apoptotic cofactor will decrease survival signals

and increase apoptotic signals, and that knockdown of a pro-apoptotic cofactor will increase survival signals and decrease apoptotic signals. We expect the opposite phenotype from overexpression or knockdown of an anti-apoptotic cofactor.

Pitfalls and alternatives

Pitfall #1: We are not able to obtain any distinct classification of hMCLs and PMMCs into resistant and sensitive cells.

Reason #1 Our measurement of apoptotic threshold does not account for cell growth, which in some assays could skew our estimates of apoptotic cells.

Alternative: Tg affects cell viability independent of proliferation rate, and proliferation does not significantly affect the observed percentage of Tg-induced apoptotic cells detected by annexin V staining.[12] If, despite this, it seems necessary to control for proliferation, we will count cells at the time of flow cytometry and normalize our observations to the initial number of cells plated.

Reason #2: The dynamic range of Tg may be too small to capture significant variability in AT in MM cells. Tg-induced apoptosis may not be entirely due to ER stress but may also involve mitochondrial permeabilization due to increased cytosolic [Ca^{2+}].[11]

Alternative: Switch to a different drug such as tunicamycin (Tm), which has a broader dynamic range. In addition, Tm is an ER-stress inducing drug that does not affect cytosolic Ca^{2+} levels and would help to check for any mitochondrial dependent and UPR-independent apoptosis that might be confounding our results. However, because Tm inhibits glycosylation reactions that are necessary for the function of many proteins, it may have broad effects on cell signaling and simply confound our results in a different way from Tg. Tg is well characterized relative to Tm and widely used for specific induction of ER stress,[29] so despite its limitations we will perform our initial experiments with Tg.

Reason #3: The definition of apoptotic threshold (AT) used is not optimum for highlighting the variability in sensitivity to ER stress.

Alternative: Revise the AT definition to the concentration of drug required to cause apoptosis in at least x% of cultured cells. Based on the data we obtain from our experiments, x can be a higher or a lower value than 75. We will adjust the value of x to maximize the range of observed AT in response to Tg.

Pitfall #2: There is no correlation between IRE1α protein expression and the clusters of resistant and sensitive MM cells.

This seems highly unlikely as we have included many major components of the ER stress response which have been shown to influence susceptibility to ER stress-induced apoptosis.[5-18] However, it is still a plausible outcome and would suggest that other factors play more dominant roles in determining cell fate in response to ER stress.

Reason #1: Rather than only expression, post translational modifications of cofactors or cofactor interaction with IRE1α influence the observed AT.

Alternative: To see if IRE1α cofactor PTMs correlate with Tg sensitivity, we will use antibodies against modified forms of these cofactors (Cell Signaling; Santa Cruz). To see if physical interactions between IRE1α and cofactors correlate with Tg sensitivity, we will perform co-immunoprecipitation by pulling down IRE1α and then measure levels of eluted cofactors by MWA as described above. Statistical analysis of these results will be performed as described in aim 1.

Pitfall #3: Modulation of expression of individual protein levels may not be sufficient to change apoptosis phenotypes.

Reason #1: Modulation of individual protein levels may not be sufficient to change apoptosis phenotypes, and modulation of two or more co-factors is required to change the phenotype. A future direction would be to identify the minimum IRE1α cofactor set whose modulation is sufficient to alter apoptosis phenotype and to develop techniques to modulate that expression pattern.

Specific Aim 2: To test the hypothesis that IRE1 cofactor expression contributes to melphalan resistance through ER-stress-mediated survival signaling.

***Rationale*:** In this aim, we are asking a parallel question to aim 1: whereas in aim 1 we examine the relationship of IRE1α cofactor expression to cell fate in response to ER stress, here we examine the relationship between these cofactors and cell fate in response to ER-independent stress. We want to evaluate cell fate effects in these two contexts by consistent criteria so we can compare results between our aims, so we will use the same analysis pipeline in both aims. A benefit of using the same analysis pipeline is that cells only need to be prepared once from each patient, since responses to Tg and melphalan can be tested in parallel on a given sample; in addition, both aims can use the same cofactor expression profile data for untreated cells, obviating the need to measure baseline protein levels twice. The experiments proposed in aim 1 can be generalized to evaluate any stress rather than only ER stress, and by simply replacing Tg treatment with melphalan treatment we can obtain the necessary data for assessing the role of IRE1α cofactors in melphalan response.

Aim 2a) *Determine whether protein expression levels of IRE1α cofactors correlate with sensitivity or resistance to melphalan.*

We will measure the apoptotic response of MM cells to melphalan treatment. To determine the apoptotic response, cultured cells (1×10^5 cells/mL)[1] will be treated with varying concentrations of melphalan. Multiple myeloma cell lines have heterogeneous responses to melphalan, with effective cell killing concentrations varying from 400nM-150μM.[26] We will therefore treat with doses of (0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64,128, 256 μM), a scheme which gives us effective coverage over the range reported in the literature. Melphalan-induced cell death can be seen within 48-72h,[26] so we will assay cell death and apoptosis after 48 and 72h melphalan treatment. Cell death and apoptosis will be assessed as described in aim 1a. These data will be used to generate dose-response curves and determine the AT of each cell line in response to melphalan treatment.

We will utilize protein expression levels of IRE1α cofactors in MM cells, obtained in aim 1a. The same protein expression data set will be used for aims 1a and 2a, since these pre-treatment expression data are unaffected by drug treatment.

We will determine which IRE1α cofactors are differentially expressed in melphalan-sensitive versus melphalan-resistant MM cells. Using our AT data, we will group samples into melphalan-sensitive (AT < 0.5 SD below the mean) and melphalan-resistant (AT > 0.5 SD above the mean) classes. To determine if melphalan-sensitive and melphalan-resistant cells express significantly different IRE1α cofactors, we will use a kNN classifier.[21] To determine how each protein's expression varies relative to AT, we will correlate expression and apoptotic response as continuous variables and perform regressions[19] as described in aim 1a.. To determine which IRE1α cofactors are most highly correlated with melphalan sensitivity, we will use weighted voting class determination as described in aim 1.

Aim 2b) *Determine whether IRE1α cofactor expression directly affects the apoptotic response to melphalan.*

From aim 2a, we will have a ranked list of proteins whose expression correlates with sensitivity and resistance to melphalan-induced apoptosis. In this sub-aim, we will take any protein with significantly correlated expression and demonstrate that changing its expression level directly affects the apoptotic response of MM cells. We will change expression levels by forced overexpression and siRNA knockdown. We will test modulation of these cofactors in the 3 most melphalan-sensitive and the 3 most melphalan-resistant hMCLs, as described in aim 1b.

We expect that overexpression of a pro-apoptotic cofactor will increase melphalan sensitivity and observed apoptosis, and that overexpression of an anti-apoptotic cofactor will reduce melphalan sensitivity and observed apoptosis. These analyses will specifically identify novel molecular targets that influence sensitivity to DNA alkylation treatment through the ER stress signaling cascade and can be studied further in an attempt to sensitize MM cells to chemotherapy.

Aim 2c) *Determine whether increased survival signaling through IRE1α cofactors mediates melphalan resistance.*

In this sub aim we will show that IRE1 α cofactor expression pattern confers sensitivity or resistance due to activation of specific downstream signaling pathways. For the 3 most melphalan-resistant and 3 most melphalan-sensitive hMCLs, we will measure pre-treatment ratios of active pro-survival and pro-apoptotic factors as described in aim 1c. We expect that melphalan-resistant cells will have high pre-treatment levels of active pro-survival factors and low levels of active pro-apoptotic factors, whereas melphalan-sensitive cells will have lower activity of pro-survival signals and higher activity of pro-apoptotic signals.

To show that IRE1α cofactors directly affect the levels of downstream survival signaling, we will measure these activation ratios in cells with siRNA knockdown or forced overexpression of a given cofactor. Cells will be transfected as described in aim 1b and activation levels of ERK, NF-κB, JNK and p38 will be measured by Western blot. We expect that overexpression of a pro-apoptotic cofactor will decrease survival signals and increase apoptotic signals, and that knockdown of a pro-apoptotic cofactor will increase survival signals and decrease apoptotic signals. We expect the opposite phenotype from overexpression or knockdown of an anti-apoptotic cofactor.

Pitfall: ER stress mediated signaling may not be the sole factor conferring resistance to melphalan in MM cells. As a result, modulation of this pathway alone may not be successful in sensitizing these cells to chemotherapy. Nonetheless, in light of the fact that MM cells are especially resistant to treatment and that a unique quality of MM cells is the presence of chronic ER stress, we hypothesize that targeting the ER stress pathway through specific modulation of IRE1α signaling will sensitize cells to melphalan, and will be highly specific to MM cells compared to normal cells, given the chronic activation of the ER stress pathway in these cells.

REFERENCES

1. Moreaux, J. *et al.* A high-risk signature for patients with multiple myeloma established from the molecular classification of human myeloma cell lines. *Haematologica* **96**, 574–582 (2011).
2. Geiszt, M. *et al.* Thapsigargin inhibits Ca^{2+} entry into human neutrophil granulocytes. *Biochem J* **305**, 525-528 (1995).
3. Baughn, LB. *et al.* CDK2 phosphorylation of Smad2 disrupts TGF-beta transcriptional regulation in resistant primary bone marrow myeloma cells. *J Immunol* **182**, 1810-1817.
4. Zhan, F. *et al.* Gene Expression Profiling of Human Plasma Cell Differentiation and Classification of Multiple Myeloma Based on Similarities to Distinct Stages of Late-Stage B-Cell Development. *Blood* **101**, 1128–1140 (2003).
5. Ge, F. *et al.* Phosphoproteomic analysis of primary human multiple myeloma cells. *Journal of Proteomics* **73**, 1381–1390 (2010).
6. Nishitoh, H. *et al.* ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* **16**, 1345–1355.
7. Urano, F. *et al.* Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287**, 664–666 (2000).
8. Hetz, C. *et al*. Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1α. *Science* **312**, 572–576 (2006).
9. Hu, P. *et al*. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1α-mediated NF-kappaB activation and down-regulation of TRAF2 expression. *Mol. Cell. Biol.* **26**, 3071–3084 (2006).
10. Ciaccio, M. F. *et al*. Systems analysis of EGF receptor signaling dynamics with microwestern arrays. *Nature Methods* **7**, 148–155 (2010).
11. Kass, G. E. & Orrenius, S. Calcium signaling and cytotoxicity. *Environ. Health Perspect.* **107** Suppl 1, 25–35 (1999).
12. Denmeade, S. R. & Isaacs, J. T. The SERCA pump as a therapeutic target: making a ‘smart bomb’ for prostate cancer. *Cancer Biol. Ther.* **4**, 14–22 (2005).
13. Gu, F. *et al*. Protein-tyrosine phosphatase 1B potentiates IRE1 signaling during endoplasmic reticulum stress. *J. Biol. Chem.* **279**, 49689–49693 (2004).
14. Luo, D. *et al*. AIP1 is critical in transducing IRE1-mediated endoplasmic reticulum stress response. *J. Biol. Chem.* **283**, 11905–11912 (2008).
15. Lisbona, F. *et al.* BAX inhibitor-1 is a negative regulator of the ER stress sensor IRE1αlpha. *Mol. Cell* **33**, 679–691 (2009).
16. Bailly-Maitre, B. *et al.* Cytoprotective gene bi-1 is required for intrinsic protection from endoplasmic reticulum stress and ischemia-reperfusion injury. *Proc. Natl. Acad. Sci. USA* **103**, 2809–2814 (2006).
17. Chae, H.J. *et al.* Evolutionarily conserved cytoprotection provided by Bax Inhibitor-1 homologs from animals, plants, and yeast. *Gene* **323**, 101–113 (2003).
18. Hetz, C. & Glimcher, L. H. Fine-tuning of the unfolded protein response: Assembling the IRE1αlpha interactome. *Mol*. *Cell* **35**, 551–561 (2009).

19. Hill, T. & Lewicki, P. STATISTICS: Methods and Applications. StatSoft, Tulsa, OK. (2007).
20. Gentleman, R. *et al.* Bioinformatics and Computational Biology Solutions Using R and Bioconductor. *Statistics for Biology and Health*, Springer Science (2005).
21. Staunton, J.E. *et al*. Chemosensitivity prediction by transcriptional profiling. *Proc Natl Acad Sci U S A* **98**, 10787–10792 (2001).
22. Ruderfer, D. M. *et al.* Using Expression and Genotype to Predict Drug Response in Yeast. *PLoS ONE* **4**, e6907 (2009).
23. Santa Cruz Biotech siRNA protocol
24. Invitrogen Lipofectamine protocol
25. Börset, M. *et al.* TNF and IL-6 are potent growth factors for OH-2, a novel human myeloma cell line. *Eur. J. Haematol.* **53**, 31 (1994).
26. Hjertner, Ö. *et al.* Comparison of the effects of 2-chlorodeoxyadenosine and melphalan on myeloma cell lines. *Leukemia Research* **20**, 155-160 (1996).
27. Hetz, C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat. Rev. Mol. Cell Biol.* **13**, 89–102 (2012).
28. Ma, Y. & Hendershot, L. M. The role of the unfolded protein response in tumour development: friend or foe? *Nature Reviews Cancer* **4**, 966-977 (2004).
29. Corazzari, M. *et al.* Targeting homeostatic mechanisms of endoplasmic reticulum stress to increase susceptibility of cancer cells to fenretinide-induced apoptosis: the role of stress proteins ERdj5 and ERp57. *Br. J. Cancer* **96**, 1062–1071 (2007).
30. Nishitoh, H. *et al*. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* 16, 1345-1355 (2002).
31. Urano, F*. et al*. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287**, 664-666 (2000).
32. Nguyen, D. T. et al. Nck-dependent activation of extracellular signal-regulated kinase-1 and regulation of cell survival during endoplasmic reticulum stress. Molecular biology of the cell 15, 4248-4260, doi:10.1091/mbc.E03-11-0851 (2004).
33. Hu, P, *et al*. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1αlpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. *Molecular and cellular biology* **26**, 3071-3084 (2006).
34. Yoneda, T. et al. Activation of caspase-12, an endoplastic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. The Journal of biological chemistry 276, 13935-13940, doi:10.1074/jbc.M010677200 (2001).
35. Oono, K. et al. JAB1 participates in unfolded protein responses by association and dissociation with IRE1. *Neurochemistry international* **45**, 765-772 (2004).
36. Szegezdi, E. *et al.* Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO reports* **7**, 880-885 (2006).
37. Lin, J. H. et al. IRE1 signaling affects cell fate during the unfolded protein response. *Science* **318**, 944-949 (2007).

38. Ling, S. C. et al. Response of myeloma to the proteasome inhibitor bortezomib is correlated with the unfolded protein response regulator XBP-1. *Haematologica* **97**, 64-72 (2012).

39. Jiang, C.C. *et al.* Human melanoma cells under endoplasmic reticulum stress acquire resistance to microtubule-targeting drugs through XBP-1-mediated activation of Akt, *Neoplasia*, **11** 436-447 (2009).

40. Ma, Y., Shimizu, Y., Mann, M. J., Jin, Y. & Hendershot, L. M. Plasma cell differentiation initiates a limited ER stress response by specifically suppressing the PERK-dependent branch of the unfolded protein response. *Cell Stress Chaperones* **15**, 281–293 (2010).

41. Shapiro-Shelef, M. & Calame, K. Plasma cell differentiation and multiple myeloma. *Curr. Opin. Immunol.* **16**, 226–234 (2004).

42. Karpas, A. *et al.* Studies of four new human myeloma cell lines. *Leuk. Lymphoma* **46**, 101–112 (2005).

43. Dispenzieri, A. *et al.* International Myeloma Working Group guidelines for serum-free light chain analysis in multiple myeloma and related disorders. *Leukemia* **23**, 215 (2008).

44. Rajkumar, S. V. Treatment of multiple myeloma. *Nature Reviews Clinical Oncology* **8**, 479 (2011).

45. Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A. & Neel, B. G. The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell* **68**, 545–560 (1992).

46. Lenth, R. V. Java Applets for Power and Sample Size [Computer software]. Retrieved April 15, 2012, from http://www.stat.uiowa.edu/~rlenth/Power. (2006-9).