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The Fanconi Anemia (FA/BRCA DNA damage repair pathway is reglated by NF-[kappa]B and mediates drug resistance in multiple myeloma

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The Fanconi Anemia (FA)/BRCA DNA Damage Repair Pathway is Regulated by NF-κB and Mediates Drug Resistance in Multiple Myeloma

by

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DEDICATION

I wish to dedicate this dissertation to my family. To my parents for their guidance and unconditional support and encouragement; and to Isaiah, for helping me to keep sight of what is truly important.

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AML	Acute myeloid leukemia
ANOVA	Analysis of variance
A-T	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia mutated and rad3-related
BER	Base excision repair
BCNU	Bis-chloronitrosourea
BMS	Bristol Myers Squibb
BrdU	Bromodeoxyuridine
CAM-DR	Cell adhesion-mediated drug resistance
CENP-E	Centromere-associated protein E
ChIP	Chromatin immunoprecipitation
CRAB	Hypercalcemia, renal insufficiency, anemia and bone lesions
DASH	Diffusion apoptosis slide halo
DEB	Diepoxybutane
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DSB	Double strand break
EMSA	Electrophoretic mobility shift assay
FA	Fanconi anemia
FAAP	Fanconi anemia-associated protein
FANC	Fanconi anemia complementation group
FGFR3	Fibroblast growth factor receptor 3
FN	Fibronectin
GSTP1	Glutathione S-transferase P1
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HSC	Hematopoietic stem cell
Hsp	Heat shock protein
HR	Homologous recombination
ICL	Interstrand crosslink
IκB	Inhibitor of NF-κB
IKK	IκB kinase
L-PAM	L-phenylalanine mustard
LR	L-PAM resistant
LRP	Lung resistance-related protein
M	Molar
MDR	Multi-drug resistance

MDR1	Multidrug resistance protein 1
MGUS	Monoclonal gammopathy of undetermined significance
mRNA	Messenger RNA
miRNA	Micro RNA
ml	Milliliter
MM	Multiple myeloma
MRD	Minimal residual disease
MRN	Mre11, Rad50, and NBS1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBS1	Nijmegen breakage syndrome 1
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa B
ng	Nanogram
NHEJ	Nonhomologous end joining
P-gp	P-glycoprotein
PARP	Poly(ADP-ribose) polymerase
PI	Propidium iodide
q-RT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
RPA	Replication protein A
SILAC	Stable isotopic labeling of amino acids in cell culture
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TLS	Translesion synthesis
TMZ	Temozolomide
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
UBE2T	Ubiquitin E2 ligase T
USP1	Ubiquitin-specific protease 1

THE FANCONI ANEMIA (FA)/DNA DAMAGE REPAIR PATHWAY IS REGULATED BY NF-κB AND MEDIATES DRUG RESISTANCE IN MULTIPLE MYELOMA

DANIELLE N. YARDE

ABSTRACT

The Fanconi Anemia (FA)/BRCA DNA damage repair pathway plays a critical role in the cellular response to stress induced by DNA alkylating agents and greatly influences drug response in cancer treatment. We recently reported that FA/BRCA DNA damage repair pathway genes are overexpressed and causative for resistance in multiple myeloma (MM) cell lines selected for resistance to melphalan. We hypothesized that the FA/BRCA DNA damage repair pathway mediates response and resistance to chemotherapeutic agents used to treat multiple myeloma and other cancers, and targeting this pathway is vital to overcoming drug resistance. In this dissertation, we show that FA/BRCA pathway genes are collectively overexpressed in MM, prostate, and ovarian cancer cell lines selected for resistance to melphalan and cisplatin, respectively. Interestingly, cells selected for resistance to topoisomerase II inhibitors selectively overexpress only FANCF. We also show that FA/BRCA pathway expression can be inhibited by the proteasome inhibitor bortezomib. FA/BRCA pathway mRNA expression was inhibited by bortezomib in myeloma cell lines and patient samples. FANCD2 gene and protein expression are downregulated by bortezomib, and remain attenuated in the face of melphalan treatment. Melphalan-induced FANCD2 foci formation was also

inhibited by bortezomib, and this drug enhanced melphalan-induced DNA damage, likely via inhibition of FA-mediated DNA damage repair. Next, we analyzed regulation of the FA/BRCA pathway. We demonstrate that NF-κB, specifically the RelB/p50 subunits, transcriptionally regulates members of the FA/BRCA pathway, and inhibition of these subunits by siRNA, BMS-345541, and bortezomib reduces FA/BRCA pathway expression. Furthermore, knocking down RelB and p50 simultaneously attenuates FANCD2 protein expression and results in diminished DNA repair and enhanced sensitivity to melphalan. Importantly, melphalan resistance was restored when FANCD2 was re-expressed in these cells. We also show that bortezomib regulates FANCD2 protein expression directly, by inhibiting FANCD2 synthesis. Finally, we demonstrate that low-dose bortezomib arrests cells in G0/G1 and also overcomes the S-phase arrest induced by melphalan, likely via inhibition of ATR.

Overall, our findings provide evidence for targeting the FA/BRCA pathway, either directly or indirectly, via inhibition of NF-κB or ATR, to enhance chemotherapeutic response and reverse drug resistance in multiple myeloma and other cancers.

INTRODUCTION

Cancer is defined as a malignant growth caused by uncontrolled cell division and lack of programmed cell death. In 2000, Hanahan and Weinberg identified six hallmarks of cancer, and suggested that these alterations within the cell dictate the origination of a malignancy (1). These six characteristics are: "self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis" (1). Hematologic malignancies exhibit many, if not all, of these traits, and understanding the mechanisms of tumorigenesis and drug resistance is crucial to both diagnosing and finding a cure for these diseases.

Multiple myeloma is an incurable malignancy arising from a clonal population of plasma cells (2). Although response to initial chemotherapy is typical, patients relapse due to the emergence of drug resistance. Our lab and many others have focused extensively on the mechanisms of drug resistance in myeloma. We previously reported that the FA/BRCA DNA damage repair pathway is associated with and causative for resistance to the DNA damaging agent melphalan (3-4). This dissertation analyzes the transcriptional regulation of the FA/BRCA pathway and highlights the important role that this pathway plays in response and resistance to chemotherapeutic agents commonly used in the clinic to treat myeloma.

Hematologic Malignancies

The formation of new blood cells is termed hematopoiesis. Pluripotent hematopoietic stem cells (HSCs) can give rise to both lymphoid and myeloid cells. Lymphoid progenitors, in turn, give rise to B and T cell lymphocytes, whereas myeloid and erythroid progenitors are responsible for the generation of macrophages, granulocytes and erythrocytes. Hematopoiesis is a dynamic process involving numerous growth factors, cytokines, and microenvironmental stimuli. Importantly, errors involving any of these homeostatic regulators can have detrimental effects, causing malignancies of these different cell lineages.

Hematologic malignancies account for 9.5% of all cancers diagnosed in the United States (leukemia-lymphoma.org). These diseases can be divided into two broad categories, based on whether they are derived from lymphoid or myeloid cell lineages. Lymphomas, the most common of the hematopoietic malignancies, are tumors that arise from lymphocytes. Myeloma falls under the category of a lymphoma, as it is a malignancy of plasma cells. Leukemia comprises the second type of hematologic malignancy, and occurs when the blood-forming tissues, or leukocytes, grow uncontrollably.

Although profound advances have been made in the treatment of hematologic malignancies, many of these cancers remain incurable due to the effects of minimal residual disease (MRD) and the emergence of drug resistance. Certain cancer cells can elicit mechanisms, such as the evasion of apoptosis or the overexpression of drug resistance genes, to avoid cell death and persist in the patient even after many rounds of chemotherapy. Unfortunately, these patients relapse and eventually succumb to disease

due to these remaining, often initially undetectable, tumor cells. Some of these cancer cells are intrinsically resistant to chemotherapeutic agents, while others become resistant to drugs throughout the course of treatment.

Multiple Myeloma

According to the National Cancer Institute (http://www.cancer.gov), 20,580 new cases of multiple myeloma (MM) were diagnosed in the United States in 2009, and in the same year, 10,580 deaths were associated with this disease. MM comprises nearly 15% of all hematologic malignancies (5), and is associated with roughly 2% of all cancer deaths in the United States (6-7), ranking it the second most common hematologic malignancy in this country. The lifetime risk of someone in the United States developing myeloma is 1 in 161 (7), with an increased incidence in men versus women (a ratio of 1.4:1, respectively), and also in African Americans as compared to Caucasians (8). The median age of diagnosis of MM is 71 years (9-10), and these patients typically present with the symptoms of "fatigue, bone pain, and recurrent infections" (11).

Multiple myeloma is a neoplasm arising from a clonal population of mature B cells. Patients with MM have large numbers of clonal antibody-secreting plasma cells in their bone marrow (2). The diagnosis of this disease requires the bone marrow to be comprised of at least 10% plasma cells (11). In comparison, the percentage of plasma cells in a healthy individual's bone marrow is less than 1% (12). Other criteria for diagnosis include secretion of excessive monoclonal immunoglobulin (Ig; as detected in the urine or serum), and end-organ damage as characterized by "CRABI" (hyper***c***alcemia, ***r***enal insufficiency, ***a***nemia and ***b***one lesions, and increased ***i***nfection

rate) (8, 11-12). According to Nair *et. al*., the most common Ig isotype (M-component) found in MM patients is IgG, and prognostically, IgA and IgD are the least favorable (13). Also, the 20-30% of patients who present with renal failure are considered to have a poor prognosis (10, 14).

The disease of MM is often preceded by a premalignant condition called monoclonal gammopathy of undetermined significance (MGUS). Myeloma progresses in these patients, who have less than 10% clonal plasma cells in their bone marrow, less than 3 grams/deciliter of paraprotein, and no myeloma-related end-organ damage (CRABI), at a rate of about 1% annually (8, 11, 15). Once the disease progresses, myeloma can be subdivided into two categories: asymptomatic (smoldering) and symptomatic (active). Patients with asymptomatic myeloma display an increase in M-protein concentration when compared to MGUS patients, but do not have the end-organ damage required for the diagnosis of symptomatic myeloma (8, 16).

Progression from MGUS to myeloma is believed to coincide with cytogenetic changes (11), and newly diagnosed MM patients present with an average of seven chromosomal abnormalities (12). The most common translocation, seen in 50% of patients, involves the immunoglobulin heavy-chain locus (17). These translocations usually involve partnering with chromosomes that encode for oncogenes, such as *cyclin D1* and *D3,* fibroblast growth factor receptor 3 (*fgfr3*)/MMSET, *c-maf*, and *mafB* (18-20). Also, certain translocations and abnormalities are associated with more aggressive disease. For example, patients with the del(13) or del(17p) abnormalities have shorter time to progression as well as decreased overall survival when compared to patients without these chromosomal deletions (21-22). Recently, though, it was found that the

poor prognosis associated with del(13) and other high-risk translocations could be overcome in relapsed or refractory patients treated with the novel therapeutic agents lenalidomide and bortezomib (23).

Therapeutic options for MM patients have changed markedly in recent years. The gold standard for treatment for decades has been melphalan plus prednisone (24). However, new agents, such as immunomodulatory drugs (thalidomide and lenalidomide) and proteasome inhibitors (bortezomib and second-generation derivatives), have recently been approved for myeloma treatment and have shown promise in the clinic, both alone and in the setting of combination chemotherapy (2, 25-28). New therapeutic options have nearly doubled the median survival, with the greatest improvements in survival rates seen in younger patients (6, 29).

Unfortunately, even with the exciting new options available for the treatment of MM, the emergence of drug resistance remains the largest hurdle in curing this disease. Therefore, discovering therapeutic agents that target specific pathways aberrantly expressed in drug sensitive and drug resistant cells will be necessary to cure this disease.

Multiple Myeloma and Drug Resistance

A number of drug resistance mechanisms have been described in multiple myeloma. These mechanisms of resistance can be intrinsic, due to intercellular changes that confer drug resistance, or extrinsic, where growth factors and physical effectors in the tumor cell's microenvironment mediate resistance. Furthermore, following chemotherapy, many tumors also become cross-resistant to other agents as well, a phenomenon known as the multi-drug resistance (MDR) phenotype (30). MDR in

myeloma has been shown to be mediated by overexpression of the drug transporter proteins P-glycoprotein (P-gp), multidrug resistance protein 1 (MDR1), and lung resistance-related protein (LRP) (30-32).

Drug resistance in myeloma is also known to be mediated by alterations in apoptotic machinery within the cell. For example, overexpression of Bcl-2, an anti-apoptotic member of the Bcl-2 family of proteins, confers resistance to chemotherapeutic agents such as doxorubicin, dexamethasone, and bortezomib (33-34). Additionally, the anti-apoptotic proteins Mcl-1 and Bcl-X_L have also been shown to contribute to drug resistance (35-37).

Aside from changes in apoptotic proteins, intercellular changes in other pathways are also known to influence drug resistance in multiple myeloma. For example, myeloma cells selected for resistance to melphalan by chronically exposing these cells to low doses of the drug display reduced DNA interstrand crosslinks and enhanced glutathione levels (38). Importantly, inhibition of glutathione synthesis in these cells reversed drug resistance (38). Furthermore, microarray analysis of these same melphalan resistant myeloma cells (8226/LR5 cells) revealed changes in over 1400 genes when compared to the drug sensitive cells (4). Overexpression of members of the glutathione pathway, DNA damage repair pathways, and cholesterol synthesis pathway were observed in the melphalan resistant cells (4).

Drug resistance is also influenced by the tumor cell's microenvironment. Adhesion to physical factors in the environment causes a drug resistance phenotype known as cell adhesion-mediated drug resistance (CAM-DR) (39-40). Adhesion of tumor cells to fibronectin (FN) via β1 integrins causes a G1 cell cycle arrest due to

increased $p27^{kip1}$ expression (40), and also enhances degradation of the pro-apoptotic Bcl-2 protein bim (40). Adhesion to bone marrow stromal cells (BMSCs) also protects myeloma cells from melphalan-induced cell death, and this resistance has been linked to upregulation of the Notch-1 signaling pathway (41).

Finally, growth factors are known to influence drug resistance. For example, interleukin (IL)-6 and insulin-like growth factor I (IGF-1), two cytokines produced in the bone marrow microenvironment, confer resistance to chemotherapeutic agents by blocking apoptotic stimuli and activating the PI3K/AKT MAPK pathways (37). These growth factors are also known to regulate the activity of certain transcription factors, such as STAT3 and NF-κB, which have been reported to enhance drug resistance (42-44).

Multiple Myeloma and NF-κB

The transcription factor nuclear factor-κB (NF-κB) consists of two proteins, each containing an N-terminal Rel homology domain (45). NF-κB can be a hetero- or homodimer of the subunits RelA, RelB, c-rel, p50 and p52 (46). The NF-κB dimers remain inactive in the cytoplasm by association with one of three inhibitors of NF-κB (IκB), specifically IκBα, IκBβ, or IκBε, or with p105 or p100 (the precursors of p50 and p52, respectively) (47). Following stimulation, IκB subunits are phosphorylated at conserved serine residues by the IκB kinase (IKK) complex, comprised of IKKα, IKKβ, and IKKγ/NEMO (48-49). These phosphorylation events target the IκB subunits for proteasomal degradation, leading to the release of the NF-κB dimer and subsequent translocation into the nucleus (50). Once inside the nucleus, NF-κB initiates the transcription of its target genes. (Figure 1).

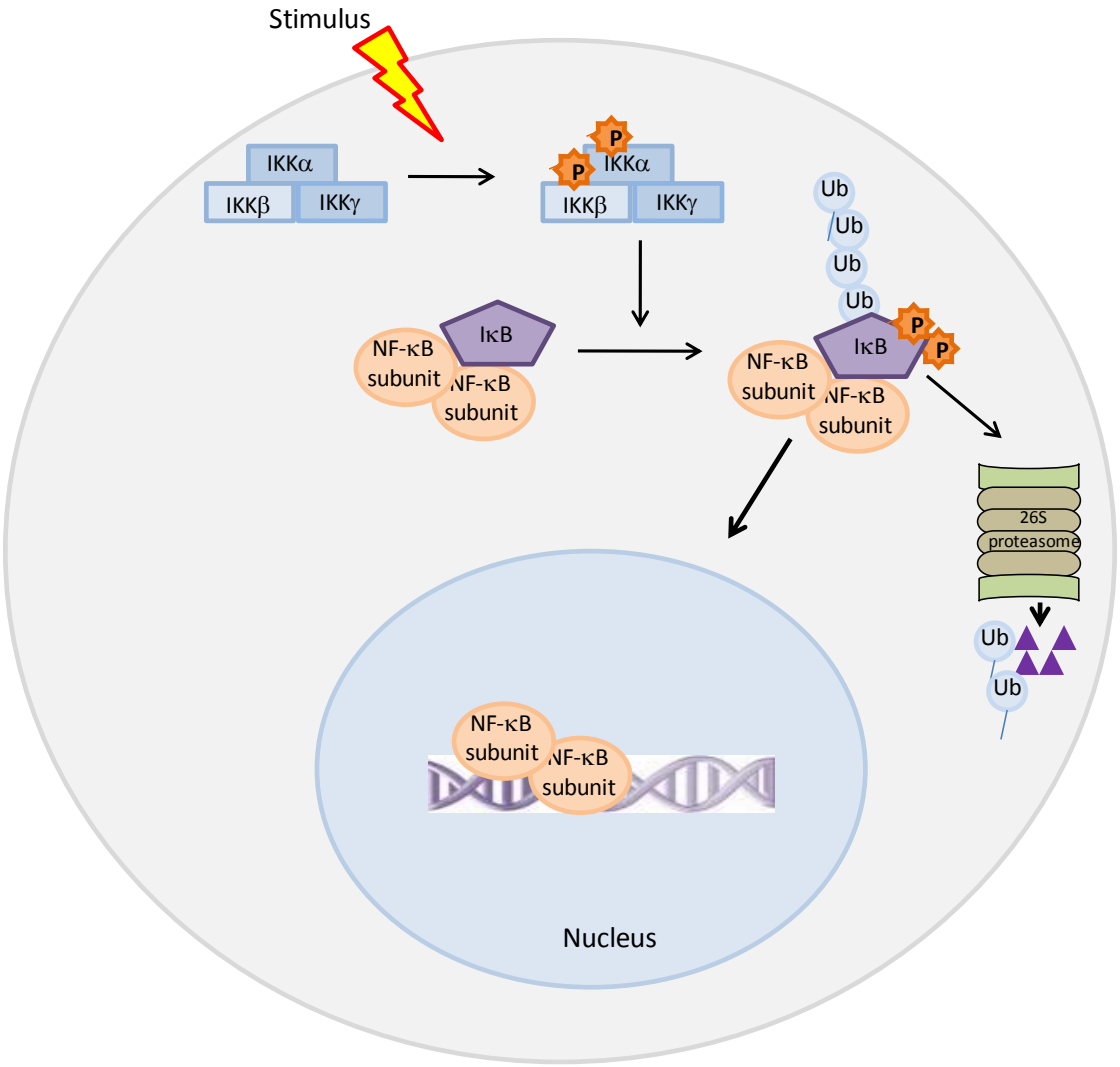


Figure 1. **The NF-κB pathway.** NF-κB is a homo- or hetero-dimer of the subuinits RelA, RelB, c-Rel, p50 and p52. IκB sequesters these proteins in the cytoplasm. Following stimulation, the IKK complex is activated and phosphorylates IkB, targeting it for proteasomal degradation. NF-κB is then free to translocate to the nucleus and activate transcription of its target genes.

NF-κB is known to be activated by one of three major pathways. The canonical pathway is typically induced in response to bacterial infection or to tumor necrosis factor (TNF)-α and other inflammatory cytokines, and is usually associated with the activation of the p50/RelA heterodimer (51). The second NF-κB activating pathway is termed the alternative, or noncanoncial, pathway. This pathway, associated with p52/RelB activation, is initiated by certain TNF receptor (TNFR) ligands, such as BAFF and lymphotoxin β (51). Finally, the atypical pathway provides a much slower and weaker NF-κB response when compared to activation of the other two pathways, and is induced in response to DNA damage and reactive oxygen species (ROS) (52).

NF-κB has been shown to regulate a plethora of genes involved in inflammation, proliferation, metastasis, angiogenesis, and apoptosis (53). As such, activation of this family of transcription factors is commonly observed in many different cancers (42, 54). For example, NF-κB has been reported to be constitutively activated in multiple myeloma (55). In two separate reports, Annunziata *et. al.*, and Keats *et. al.*, observed a number of NF-κB-activating gene mutations in myeloma cell lines as well as in primary myeloma patient samples (56-57). Furthermore, the RelB and p50 NF-κB subunits are upregulated following MM cell adhesion to the extracellular matrix protein fibronectin (FN) (58). This report provides a link between NF-κB and drug resistance, as adhesion to FN is known to cause a transient (*de novo*) drug resistance phenotype in these cells. Also highlighting the important role of the NF-κB pathway in drug resistance, microarray analysis of the FN-adhered cells revealed overexpression of 11 NF-κB-regulated genes (58). Finally, NF-κB has been found to be overexpressed in MM cell lines that have been selected for resistance to various chemotherapeutic agents (59), and NF-κB levels have

been found to be elevated in response to chemotherapy as well as in patient samples collected at the time of relapse (37, 60-62). Thus, inhibition of NF-κB, using agents such as the proteasome inhibitor bortezomib, is likely vital for the successful treatment of multiple myeloma.

The Proteasome and Proteasome Inhibition

Over 80% of proteins within the cell are degraded by the ubiquitin-proteasome system (63). Proteins tagged with ubiquitin have different functions, dependent upon the type of ubiquitination. Monoubiquitination, in which a single ubiquitin is attached to a single lysine residue, is important for such processes as endocytosis, DNA repair, and nuclear export of the protein (64-67). Proteins can also be multiubiquitinated, or monoubiquitinated on several lysines, and these proteins can be involved in endocytosis and nuclear export as well (68). Finally, proteins can also be polyubiquitinated. Proteins covalently bound to a polyubiquitin via lysine 63 characteristically signals DNA repair or endocytosis, whereas a protein linked to an ubiquitin chain via lysine 48 is typically targeted for proteasomal degradation (68).

Three enzymes are involved in the ubiquitin tagging of a protein. The E1 enzyme is known as the activating enzyme, as it utilizes ATP to activate the ubiquitin molecule by converting it to an E1-thiol-ester~ubiquitin moiety (69). The ubiquitin-conjugating E2 enzyme transfers the active ubiquitin from the E2 to the E3 enzyme. Finally, the E3 enzyme, which functions as a ligase, conjugates ubiquitin to the target protein (69). (Figure 2).

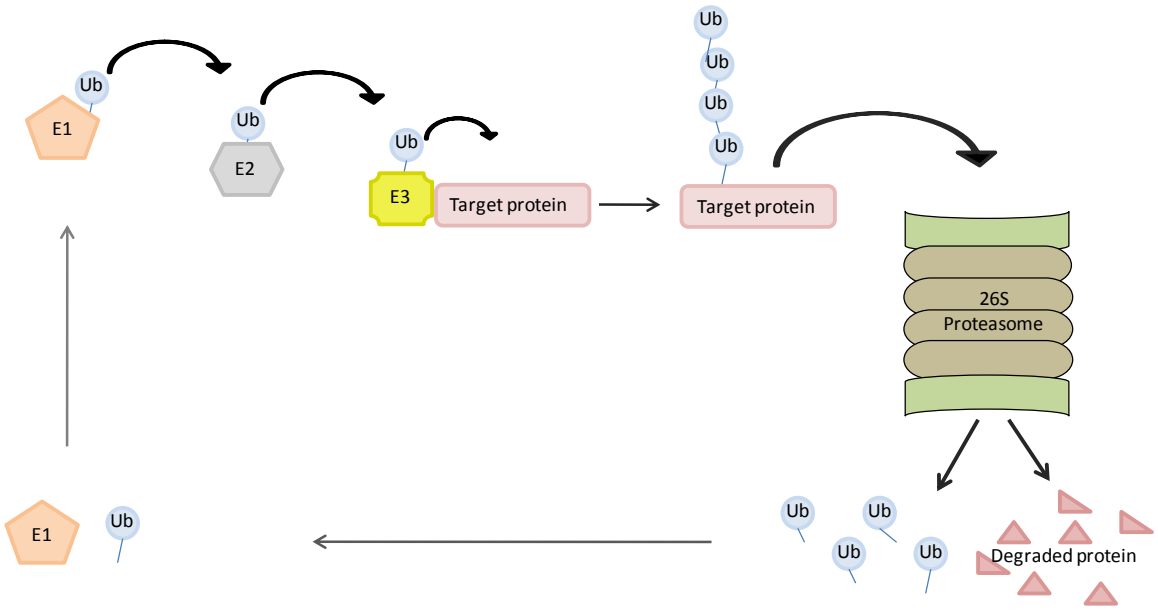


Figure 2. **Ubiquitination and the proteasome.** Polyubiquitination of proteins leads to their subsequent degradation by the proteasome. The E1 enzyme activates ubiquitin and transfers this molecule to the E2 conjugating enzyme. E2 then transfers the active ubiquitin to the E3 ligase, which in turn conjugates ubiquitin to the target protein. The E3 ligase also ligates ubiquitin molecules to one another, forming a polyubiquitin chain. Polyubiquitinated proteins are degraded by the 26S proteasome, which is comprised of the 19S regulatory caps (green) and the 20S core particle (brown), and the ubiquitin is recycled.

Once polyubiquitinated, most proteins are targeted for proteasomal degradation. There are approximately 30,000 proteasomes in a single cell, which are located in the cytosol as well as in the nucleus (70-71). The barrel-shaped 26S proteasome is formed by the 20S core particle and two 19S regulatory particles that cap each end (69). The 20S core particle is responsible for the catalytic activity of the proteasome, and contains two outer α rings plus two inner β rings (69). β1, β2, and β5 are responsible for the caspase-like, tryptic-like, and chymotryptic-like enzymatic activity of the 20S subunit (70). The 19S caps contain a base of six ATPase subunits and two non-ATPase subunits and a lid composed of 12 non-ATPase subunits (72). These caps recognize and unfold polyubiquitinated proteins, open the α rings of the 20S subunit, and cleave and recycle the polyubiquitin chains (72). (Figure 2).

Proteasomal degradation leads to the generation of polypeptides 3-23 amino acids in length, which are rapidly hydrolyzed by downstream proteases (69, 73). This controlled degradation of proteins is vital for the regulation of many cellular processes, including apoptosis, cell cycle progression, DNA repair and transcription (72). As mentioned above, NF-κB activation is dependent upon proteasomal degradation of its inhibitor (50). The proteasome is also responsible for the degradation of p53, the cyclin-dependent kinase inhibitors $p21^{cip1/waf1}$ and $p27^{kip1}$, and the proapoptotic protein bim (70, 74-75), and this degradation is necessary for cancer cell development and progression (76). Therefore, inhibiting the proteasome as a means to stabilize these and other proteins seems promising for the treatment of cancer.

Bortezomib (PS-341; Velcade), a dipeptidyl boronic acid, is a reversible inhibitor of the 26S proteasome (77). Bortezomib is an active, recently approved FDA agent, that

is being studied in combination with other drugs (26). Two independent labs have reported that bortezomib enhances melphalan response in myeloma cell lines, implicating NF-κB inhibition due to inhibited IκB degradation in this enhanced response (59, 78). Furthermore, bortezomib in combination with melphalan has shown encouraging activity in myeloma patients in a Phase I/II trial (25). Also, in a separate, multicenter Phase I/II study, the combination of bortezomib plus melphalan and prednisone was shown to be highly effective, even in patients with poor prognostic factors, and a Phase III trial is now ongoing (27, 79-80).

Although bortezomib shows great promise in the clinic for the treatment of MM, approximately 20% of newly diagnosed patients are inherently resistant to the drug, and all others will develop resistance over time (29). To this end, second-generation drugs such as carfilzomib and NPI-0052, which irreversibly inhibit the proteasome, are currently being tested in the clinic and have been shown to overcome boretezomib resistance (29, 81). Due to the complexity of drug resistance mechanisms, however, it is likely that the most durable responses will result from the development of rational drug combinations that capitalize on synergistic interactions.

DNA Damage

As mentioned above, bortezomib was only recently approved for the treatment of MM (77). Prior to bortezomib, the gold standard for treating myeloma for many years had been melphalan, and this cytotoxic agent remains the drug of choice for high dose chemotherapy (24). Melphalan, as well as an arsenal of other agents used in the clinic

today to treat cancers such as MM, targets tumor cells by damaging the DNA within these cells.

DNA within a cell can be damaged by endogenous means, such as by reactions that produce reactive oxygen and nitrogen species or by spontaneous hydrolysis reactions (82). Furthermore, DNA can also be damaged by exogenous forces such as ionizing radiation, ultraviolet light, and chemotherapeutic agents (82). In fact, it has been estimated that a single cell receives up to 10^5 lesions per day (83). Fortunately, cells are able to efficiently repair DNA via several different mechanisms as a means to maintain genomic integrity. Conversely, genomic instability caused by defective DNA damage repair is a hallmark of cancer, reinforcing the fact that DNA must be repaired properly in order to maintain cellular homeostasis.

A cell can respond to DNA damage using a number of mechanisms to repair the DNA, based on the type and extent of damage. Repair of damage involves activation of repair enzymes, numerous phosphorylation events, and recruitment of proteins to the site of damage (84). Base excision repair (BER), for example, removes oxidative lesions and other DNA modifications by removing the erroneous base and recruiting polymerase and ligase proteins to repair the damaged strand (85). DNA adducts and damage induced by ultraviolet rays, both of which distort the helix, is resolved by nucleotide excision repair (NER) (86). During this process, which involves greater than 30 proteins, the damaged nucleotide is removed and the complementary strand is used as a template for repair (87-88). Also, lesions can be bypassed by a system termed translesion synthesis (TLS), which is not an ideal method of repair as it induces error and thus is mutagenic (82).

DNA damage causing double strand breaks (DSBs) can induce a cell cycle checkpoint response leading to cell cycle arrest, and can also activate DNA damage repair or apoptotic pathways (84). If the damage can be reversed, the mechanism of repair is classically either nonhomologous end joining (NHEJ) or homologous recombination (HR) (89). NHEJ is typically initiated if the damage occurs when cells are in the G1 or early S phase of the cell cycle, whereas HR usually occurs during late S and G2 phases (88). As with other DNA damage repair pathways, NHEJ requires a large number of proteins to facilitate effective repair. Briefly, the heterodimer Ku70/Ku80 binds to the site of damage, which leads to the recruitment DNA-PK_{CS} and subsequent formation of a molecular bridge by the two strands (90). Overhangs are then filled in or removed by polymerases or nucleases, respectively, and a ligase, namely ligase IV/XRCC4, repairs the break (88). Due to the lack of a homologous strand as a template, NHEJ induces more errors than does HR.

Homologous recombination, a process integral for DSB repair as well as recovery of stalled replication forks, tends to be error-free as it uses an identical sister chromatid as a template to repair the damage (91-92). Once a DSB is induced, the histone variant H2AX is phosphorylated (a commonly used marker of DSBs), and the ATM kinase is activated and phosphorylates a number of proteins integral to DNA repair (93-94). Also, a complex containing Mre11, Rad50 and NBS1, known as the MRN complex, senses and binds to the site of damage (94). This complex resects the ends of the DNA, and the single-stranded DNA coated with RPA and Rad proteins then invades the homologous, undamaged DNA (94). Finally, DNA synthesis occurs using the homologous strand as the template (82).

The DNA damage repair pathways described above were explained singularly. It is important to note, however, that there is often much overlap between and among pathways when DNA damage takes place. For example, although NHEJ typically occurs in the G1 and early S phases, it has also been shown to be activated at different stages of the cell cycle as well (95). Also, inhibition of proteins known to be involved in the NER pathway led to ineffective DSB repair via NHEJ (88, 96). Finally, the efficient repair of DNA interstrand crosslinks has been shown to involve the NER, HR and TLS pathways (93).

DNA interstrand crosslinks (ICLs), which can be induced by chemotherapeutic agents such as melphalan and cis-platinum, cause some of the most detrimental damage to the DNA as they covalently link both strands, effectively inhibiting replication as well as transcription (97-98). DNA ICLs are first recognized and incised by NER proteins, such as ERCC1/XPF (99). This incision leads to a DSB, which is then resected and strand invasion occurs, ultimately leading the repair via HR (89). Throughout the process of ICL repair, numerous proteins are assembled at the damaged site, and the loading of these proteins onto the chromatin is coordinated by the Fanconi Anemia (FA)/BRCA DNA damage repair pathway (89, 93). This pathway and the critical role it plays in DNA damage repair is discussed in detail in a later section.

Based on the vital role that DNA damage repair mechanisms play in maintaining genomic stability, it is easy to see why toxically damaging the DNA of cancerous cells and inhibiting DNA repair pathways in these cells has proven to be an effective means of killing tumors. Conversely, it is understandable that defects in any of these repair

pathways can cause numerous problems, actually giving rise to cancer and other diseases that predispose to cancer.

Diseases of Defective DNA Damage Repair

Defects and mutations in DNA damage repair pathway genes are known to be causative for a number of diseases and, because of the genomic instability inherent in cells with ineffective repair mechanisms, these diseases are known to be associated with a high propensity for developing cancer. The cells of ataxia telangiectasia (A-T) patients, for example, do not express ATM, leading to impaired cell cycle checkpoints and genomic instability, typically resulting in a translocation between chromosomes 7 and 14 (100-101). A-T is characterized by such symptoms as progressive ataxia and impaired articulation, as well as a predisposition to lymphoma (101). As another example, patients with Li-Fraumeni syndrome develop a wide variety of tumors at an early age (101). This disease is caused by mutations in p53, a protein activated in response to DNA damage and involved in cell cycle regulation as well as in NER (101). Likewise, patients with Xeroderma pigmentosum (XP), caused by mutations in NER pathway genes, develop basal cell carcinoma at a greater than 1000-fold rate than that of the general population; and those with Nijmegen breakage syndrome (NBS), who have MRN complex defects, are predisposed to lymphoma (101-103).

Fanconi anemia (FA) was first described in 1927 and is reported to have a 1 in 300 carrier frequency, with a median age of survival of only 23 years (104). This disease, an autosomal recessive or X-linked disorder, is caused by defects in any of 13 known FA genes. As mentioned above, this pathway is integral in coordinating HR,

NER and TLS (93). FA is characterized by congenital malformations, pancytopenia, and hypopigmentation (105). This disease can also detrimentally affect the cardiac, renal and gastrointestinal systems (105). Furthermore, patients present with hematologic problems at a median age of seven years old, and 90% of these patients develop bone marrow failure by the age of 40 (104).

The cells of FA patients are hypersensitive to DNA damaging agents such as diepoxybutane (DEB) or mitomycin c, and chromosomal breakage analysis using these agents is used as a diagnostic tool for this disease (106). The chromosomal instability seen in FA patients predisposes them to cancer, with a median age of onset at just 14 years of age (105). Patients with the FA-D1 and –N subtypes are the most severely affected, with early onset of tumors and a "cumulative incidence probability of malignany" of 97% by a little over five years of age (107-108). Conversely, all FA patients who survive past 50 are of the subtype FA-A (108), suggesting that this is the least severe genotype. Finally, as a whole, over 50% of FA patients will develop AML by the age of 50, and they also have up to a 700-fold increased incidence of developing head and neck SCC when compared to the overall population (104, 109). The high propensity for cancer development in FA patients underscores the vital role that the FA/BRCA DNA damage repair pathway plays in maintaining genomic integrity.

The Fanconi Anemia (FA)/BRCA DNA Damage Repair Pathway

Members of the FA/BRCA DNA damage repair pathway have been identified using complementation studies in patients with Fanconi anemia. Thirteen FA complementation groups have been identified from these studies, and all 13 genes

(FANC A, B, C, D1, D2, E, F, G, I, J, L, M, and N) have since been cloned via complementation cloning, positional cloning or protein association (110-123). As mentioned above, this pathway has been shown to be important for DNA damage repair via HR, NER and TLS (93). A summary of each of these 13 genes is provided in Table 1, and a depiction of this pathway in relation to HR is portrayed in Figure 3.

Upstream FA Core Complex

Eight FA proteins (FANC A, B, C, E, F, G, L, and M) two Fanconi anemia associated proteins (FAAP24 and FAAP100), and Hes1 form a complex in the nucleus, termed the Fanconi anemia core complex (105, 124-127). The main function of the core complex is to monoubiquitinate FANCD2 and FANCI, and all components of this complex must be intact in order for the monoubiqutination/activation of FANCD2 and FANCI and consequent facilitation of DNA damage repair (99, 128). Furthermore, functions for some of these proteins beyond the realm of FA pathway activation have also been described.

FANCA interacts with FANCG and FANCL, and phosphorylation of FANCA is required for its accumulation in the nucleus (126, 129). FANCB and FANCL are also important for the nuclear accumulation of FANCA (126). ATR has been shown to phosphorylate FANCA and, outside of the FA core complex, this protein has also been found to be associated with the IKK signalsome as well as the SWI/SNF complex, the NER protein XPF, and the centromere –associated protein E (CENP-E) (105, 130-132). Finally, FANCA also associates with the chaperone protein Hsp90, and disruption of this

Gene	Year cloned	Location	Involvement in FA pathway
FANCA	1992	16q24.3	FA core complex subunit
FANCB	2004	Xp22.2	FA core complex subunit
FANCC	1996	9q22.3	FA core complex subunit
FANCD1/BRCA2	2004	13q12.3	Interacts with Rad51
FANCD2	2001	3p26	Monoubiquitinated by core complex
FANCE	2000	6p21.3	FA core complex subunit
FANCF	2000	11p15	FA core complex; flexible adaptor protein
FANCG	1998	9p13	FA core complex subunit
FANCI	2007	15q26.1	Monoubiquitinated by core complex
FANCJ/BRIP1	2005	17q22-24	Helicase
FANCL	2003	2p16.1	FA core complex subunit, E3 ligase
FANCM	2005	14q21.3	FA core complex, translocase, ATR activator
FANCN/PALB2	2007	16p12.1	Stabilizes BRCA2

Table 1. **Overview of FA pathway members.**

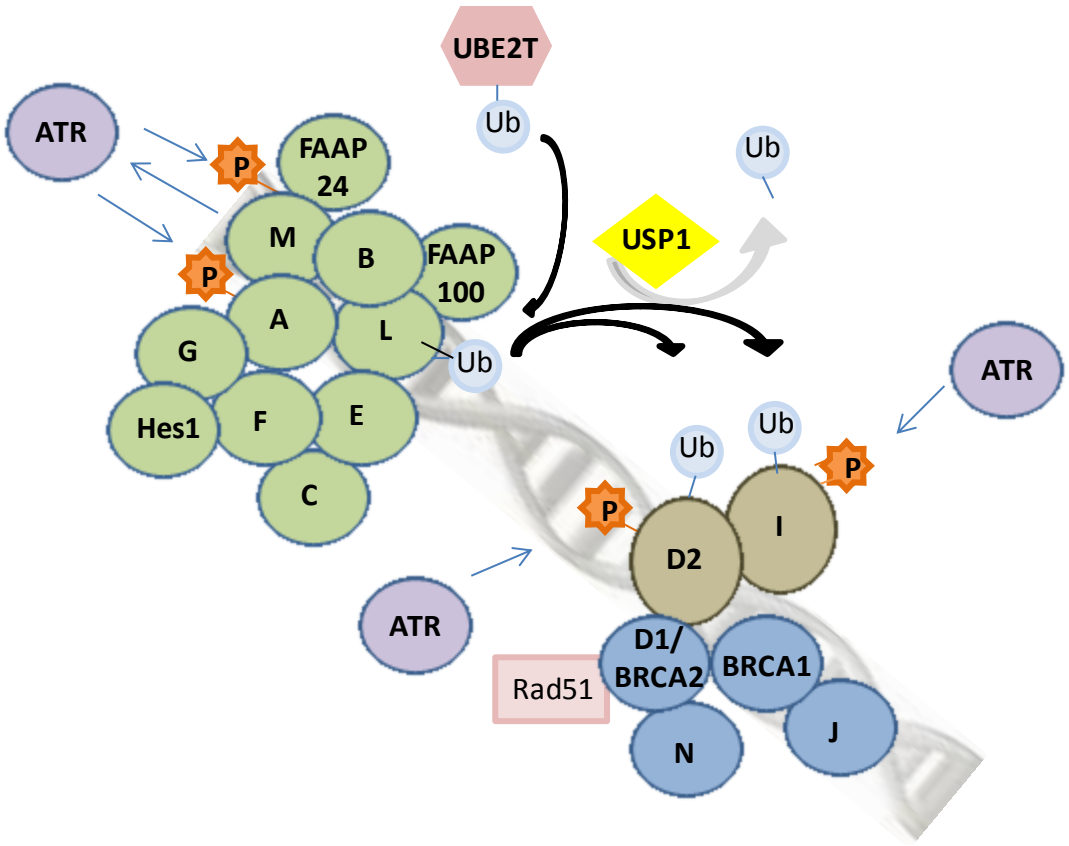


Figure 3. **The FABRCA DNA damage repair pathway.** Eight FA subunits, two Fanconi anemia-associated proteins, and Hes1 form the upstream FA core complex (in green). Intact core complex monoubiquitinates and thus activate FANCD2 and FANCI via the E3 ligase activity of FANCL. The ID complex (brown) in turn interacts with FA proteins downstream of the core complex (blue) to initiate DNA damage repair via homologous recombination. Phosphorylation of FANCA, FANCD2, FANCI, and FANCM by ATR is also required for activation of this pathway. UBE2T is the E2 enzyme involved in the monoubiquitination events, and USP1 functions as a negative regulator of this pathway by deubiquitinating FANCD2 and FANCI. FANCM loads the core complex on the chromatin and also activates ATR.

interaction results in proteasomal degradation of FANCA and export to the cytoplasm (133).

FANCB, which is the only X-linked FA gene, associates with FANCL in the core complex and, along with FANCG and FANCM, stabilizes the interaction between FANCL and FANCA (116, 126). The stability of both FANCB and FANCL is mediated by association with FAAP100, as the formation of a FANCB/FANCL/FAAP100 complex protects all three of these proteins for degradation (125).

FANCC is located primarily in the cytoplasm, but can be recruited to the nucleus via its binding partner, FANCE (134-136). Interestingly, the levels of FANCC mRNA are constant throughout the cell cycle, but FANCC protein is proteasomally degraded in a cell cycle-dependent manner (137). This protein has been linked to DSB repair mechanisms, as it is necessary for mitomycin c-induced MRN foci formation (138). Separate from its role in the FA core complex and MRN foci formation, FANCC is also necessary for maintenance of the G2/M checkpoint, is required for optimal STAT activation, has been found to interact with Hsp70, and can prevent apoptosis of hematopoietic cells by enhancing the enzymatic activity of glutathione S-transferase P1-1 (GSTP1) (139-142).

FANCC nuclear accumulation is regulated by FANCE, and the converse is also true (135). FANCE is also a partner of FANCD2, and is known to arbitrate the interaction between this protein and FANCC, as well as between FANCC and FANCF (143). FANCE and other core complex members must be functional to activate DNA damage repair via the FA/BRCA pathway. Interestingly, following DNA damage, phosphorylation of FANCE at threonine 346 and serine 374 by Chk1 leads to its

degradation (144). Therefore, phosphorylation of FANCE following DNA damage is postulated as one way in which the FA/BRCA pathway is negatively regulated (144).

The fifth subunit of the FA core complex is FANCF. Leveille *et. al.* identified this protein as a "flexible adaptor protein" (145). The N-terminal region of this protein stabilizes the interaction between FANCA and FANCG and between FANCC and E, and FANCG also binds directly to the C-terminal region of FANCF (145).

Phosphorylation of the FANCG protein by ATR is required for the direct interaction between FANCD2 and BRCA2, and FANCG also promotes formation of a complex between these two proteins and the Rad51 paralog XRCC3 (146). Interestingly, Wilson *et. al.* found that FANCG and XRCC3 were epistatic for mitomycin c sensitivity, providing another link to the FA/BRCA pathway and HR repair (146).

FANCL is one of the key mediators of FANCD2/I monoubiquitination. This protein, which binds to other FA core complex proteins via its WD40 repeats, is reported to have E3 ligase activity (115, 147). The PHD/RING finger motifs of FANCL allow it to recruit UBE2T, the E2 enzyme, to facilitate monoubiquitination (148).

Finally, FANCM is the most recently identified member of the FA core complex. This protein has an N-terminal helicase-ATPase domain and a C-terminal endonuclease domain (117). FANCM displays a high affinity for branched DNA structures and likely plays a role in replication fork remodeling (149). FANCM forms a stable complex with FAAP24 that binds to chromatin in response to DNA damage or during cell cycle progression, and recruits the core complex to the chromatin during S-phase (150-151). FANCM is phosphorylated by Plk1, ATM or ATR, and hyperphosphorylation and subsequent degradation of FANCM following replication releases the FA core complex

from the chromatin (151-152). In *Xenopus* egg extracts, Sobeck *et. al.* found that binding of xFANCM to the chromatin and subsequent phosphorylation of FANCM was dependent upon FANCD2 (151). Conversely, FANCM is required for FANCD2 monoubiquitination (153). Also, FANCM and FAAP24 interact with the checkpoint protein HCLK2 and regulate ATR signaling in a manner independent of the FA pathway (154).

ID Complex

Each of the upstream core components described above must be intact and functional in order for the activation/monoubiquitination of FANCD2 and FANCI to occur. These two proteins stabilize one another and are known as the ID complex (120). The ubiquitination of FANCI is important for the maintenance of ubiquitination on FANCD2, and vice versa (120). FANCI phosphorylation by ATR is also necessary for FANCD2 activation (155), and ATR-mediated phosphorylation of FANCD2 is necessary for DNA damage-induced foci formation (64).

Following monoubiquitination, the FANCD2/FANCI complex translocates to sites of damaged chromatin. Here, FANCD2 has been shown to interact with proteins such as BRCA1 and RAD51 in an S-phase-specific manner (156), BRCA2 and PCNA(157), and NBS1 (158). It is believed that FANCD2 and its interaction with these proteins initiates DNA repair via homologous recombination (128, 159). The FA/BRCA pathway has also been implicated in translesion synthesis repair (128, 160), and phosphorylation of FANCD2 by ATM/ATR has also been shown to initiate an inter-S-phase cell cycle checkpoint (64, 161). FANCD2 is deubiquitinated and thus inactivated

by the USP1 enzyme (162). Importantly, DNA crosslink repair occurs only after FANCD2 is deubiquitinated (163).

FA Proteins Downstream of ID

Finally, three FA proteins, FANCD1/BRCA2, FANCJ and FANCN, function downstream of the ID complex. Like FANCM, FANCJ also has helicase and ATPase activity (164). Sommers *et. al.* found that this ATP hydrolysis via FANCJ can destabilize protein/DNA complexes, FANCJ can control HR repair via inhibition of RAD51 strand exchange, and may also remove DNA structures that would inhibit efficient repair (165-166).

FANCN/PALB2, a breast cancer predisposition gene, interacts with and is essential for the stability of the tumor suppressor gene BRCA2 (167). FANCN also promotes the interaction between BRCA1 and BRCA2 (168). BRCA2 is important for loading RAD51 to the site of DNA damage, thus initiating HR repair (165).

In summary, the FA core complex is essential for the monoubiquitination of the ID complex members, and this activation event leads to interactions with numerous proteins and the initiation of DNA damage repair.

FA/BRCA Pathway and Drug Resistance

As described above, the FA/BRCA DNA damage repair pathway is complex and requires the coordinated efforts of numerous proteins for proper function. Defects in the FA/BRCA DNA damage repair pathway lead to genomic instability, which can ultimately give rise to cancers (105). Conversely, enhanced expression of this pathway

can lead to resistance of tumor cells to chemotherapeutic agents. Our lab was the first to show *overexpression* of FA/BRCA pathway genes (4). Gene expression profile analysis performed by our lab revealed that two FA/BRCA DNA damage repair genes (*fancf* and *rad51c*) were overexpressed in cells selected for resistance to melphalan when compared to the drug sensitive parent cell line (4). Further investigation of this pathway, using the more sensitive method of q-PCR, showed that many FA/BRCA pathway genes are overexpressed in two melphalan resistant myeloma cell lines when compared to the drug sensitive cell lines (3). Importantly, using siRNA techniques to knock-down FANCF, Chen *et. al*. demonstrated a causal relationship between levels of FANCF and melphalan response. Furthermore, overexpressing FANCF in the drug sensitive cell line conferred resistance to melphalan (3). Also, in a separate study using a different melphalan resistant MM cell line, Xiao *et. al.* found that resistance to melphalan could be reversed via inhibition of FANCD2 activation with the natural agent curcumin (169).

The FA/BRCA pathway has also been found to be important in drug resistance in other cancer types. Treatment of a FANCF-deficient ovarian cancer cell line with temozolomide (TMZ) or BCNU caused enhanced cytotoxicity in these cells when compared to the isogenic FANCF-corrected cell line (170). Also, FA-proficient glioma cell lines were determined to be more resistant to TMZ and BCNU when compared to a glioma cell line that was FA-deficient, and inhibition of the FA pathway increased drug sensitivity (170). Furthermore, the histone deacetylase (HDAC) inhibitor phenylbutyrate was found to enhance the sensitivity of head and neck cancer cells to cisplatin via inhibition of BRCA1 (171), and non-small-cell lung cancer cells were sensitized to

cisplatin following transfection with an adenovirus expressing a dominant-negative form of FANCA (172).

Interestingly, FA/BRCA pathway inactivation followed by re-activation has been reported in tumors when analyzing these cells before and after treatment. Alan D'Andrea's group, for example, reported that FANCF was methylated, and thus inactive, in ovarian tumors, and subsequently demethylated/activated upon acquisition of cisplatin resistance (173). Also, analysis of ovarian cancer cell lines and patient specimens revealed secondary mutations in BRCA2 following drug treatment, leading to restored BRCA2 function and cisplatin resistance (174-175). Overall, the FA/BRCA pathway has been linked to drug resistance in a variety of tumor types and thus should be considered an important target for overcoming drug resistance.

The FA/BRCA Pathway is Regulated by NF-κB and Mediates Drug Resistance in Multiple Myeloma

Due to the complexity of drug resistance and DNA damage repair mechanisms, it is likely that the most durable chemotherapeutic responses will result from the development of rational drug combinations that capitalize on synergistic interactions. **The main hypothesis of all work presented in this dissertation is that the FA/BRCA DNA damage repair pathway mediates resistance to chemotherapeutic agents used to treat multiple myeloma and other cancers, and targeting this pathway is vital to overcome drug resistance.**

As mentioned above, we recently reported that the FA/BRCA DNA damage repair pathway is causative for resistance to melphalan in MM cell lines (3-4). The goal

of Part I of this dissertation was to determine the extent of FA/BRCA pathway involvement in drug resistance, including both alkylating and non-alkylating agents, and in different tumor types. We analyzed FA/BRCA pathway mRNA expression in a prostate cancer model selected for resistance to melphalan and an ovarian cancer cell line selected for resistance to cisplatin. Substantiating the results that we have seen in the melphalan resistant MM cell lines, we show that both resistant cell lines also overexpress FA/BRCA pathway genes when compared to their respective isogenic drug sensitive parent cell line. Furthermore, we analyzed FA/BRCA pathway expression in 8226 cells selected for resistance to two topoisomerase II inhibitors, doxorubicin and mitoxantrone. Interestingly, we did not see global overexpression of FA/BRCA pathway genes. These drug resistant cells, however, were found to selectively overexpress FANCF.

We next wanted to determine if chemotherapeutic agents could be used to downregulate FA/BRCA pathway expression and thus reverse resistance. To this end, we analyzed FA/BRCA pathway expression following treatment with the proteasome inhibitor bortezomib. Bortezomib has been shown to enhance melphalan response both *in vitro* and *in vivo (28, 59, 78)*, but the mechanism by which this occurs is largely unknown. Also, Taniguchi's group has reported that proteasome function is necessary for FA/BRCA pathway activation (176). Based on these findings, we hypothesized that bortezomib enhances melphalan cytotoxicity via inhibition of the FA/BRCA pathway. The experiments performed in Part II of this dissertation test this hypothesis. We analyzed FA/BRCA pathway mRNA expression in MM cell lines as well as MM primary specimens following bortezomib treatment and found that bortezomib does indeed inhibit FA/BRCA pathway expression. We also found that bortezomib can inhibit FANCD2

mRNA and protein expression, even in the presence of melphalan, and it can also inhibit melphalan-induced FANCD2 foci formation. Using the alkaline comet assay, we also show that bortezomib enhances melphalan-induced ICL formation, likely through inhibition of DNA damage repair mediated by the FA/BRCA pathway.

The experiments in Parts III and IV of this dissertation further investigate the regulation of the FA/BRCA pathway. First, we hypothesized that NF-κB transcriptionally regulates members of the FA/BRCA pathway. This hypothesis is based on our knowledge that NF-κB activity is constitutively activated in MM cells and is further overexpressed in drug resistant cells as well as at time of relapse (56-59). Also, our lab previously reported that the DNA binding activity of the RelB and p50 subunits is enhanced in MM cells displaying a drug resistance phenotype via adhesion to fibronectin (58). Part III of this dissertation shows that NF-κB, specifically the RelB/p50 subunits, does indeed regulate FA/BRCA pathway transcription. Furthermore, inhibition of RelB/p50 reversed melphalan resistance, and this reversal was overcome by re-expressing FANCD2 in these cells.

Next, because we found that FA/BRCA pathway mRNA expression was attenuated by bortezomib, but not to the same extent as the inhibition of FANCD2 protein expression, we hypothesized that bortezomib has a direct effect on FANCD2 protein expression. Therefore, we analyzed FANCD2 protein stability via stable isotopic labeling of amino acids in cell culture (SILAC) and mass spectrometry. We also analyzed the effects of various miRNAs on the expression of FANCD2. Finally, we studied ATR activation in cells stimulated with bortezomib, as well as cell cycle regulation by bortezomib in these cells. We show that bortezomib inhibits FANCD2

synthesis and overcomes melphalan-induced S-phase arrest, likely via inhibiton of ATR activity.

Overall, the results presented in this dissertation show that drug response and resistance in MM, and possibly other cancers, is mediated by the FA/BRCA DNA damage repair pathway. Therefore, we believe that targeting the FA/BRCA pathway to inhibit DNA damage repair, either directly or via inhibition of NF-κB, is vital for reversing, or possibly circumventing, drug resistance in cancers such as multiple myeloma.

MATERIALS & METHODS

Cell Culture

The 8226 and U266 human multiple myeloma cell lines were obtained from the American Type Culture Collection (Manassas, VA), and the corresponding melphalan-resistant cells (i.e., 8226/LR5, 8226/Dox40, 8226/MR20 and U266/LR6) were generated in our laboratory as previously described (38, 177-179). All cell lines were maintained in RPMI 1640 (GIBCO, Carlsbad, CA) supplemented with 5% FBS (Omega Scientific, Tarzana, CA), 1% penicillin/streptomycin, and 100 mM L-glutamine (Invitrogen, Carlsbad, CA). The 8226/LR5 and U266/LR6 melphalan resistant cells were passaged weekly in medium containing 5 μM or 6 μM melphalan (Sigma-Aldrich, St. Louis, MO), respectively. The 8226/Dox40 cell line was maintained in 40 μM doxorubicin (Sigma-Aldrich), and the 8226/MR20 line was passaged weekly in 20 μM mitoxantrone (Sigma-Aldrich). All cell lines are routinely tested (every three months) for mycoplama contamination and kappa/lambda expression.

Cytotoxicity Assays

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis was used to compare levels of sensitivity to melphalan in the absence or presence of bortezomib (Millennium Pharmaceuticals, Cambridge, MA). 8226/S, 8226/LR6, U266/S and U266/LR6 cells were seeded at 20,000-25,000 cells/well in a 96-well plate. These

cells were pre-treated for 8 hours with $3x10^{-9}$ M bortezomib or vehicle control, followed by a 40 hour treatment with 2-fold serial dilutions of melphalan, ranging from $2x10^{-4}$ M to $6.3x10^{-6}$ M. Cells were maintained at 37°C, 5% CO_2 for a total of 48 hours before harvesting. 50 μl of MTT dye (2 mg/ml in PBS; Sigma-Aldrich) was added to each well and the cells were incubated at 37°C for an additional four hours. Plates were then centrifuged, media aspirated, and formazan was solubilized by adding 100 μl DMSO. Optical density was measured at 540 nm using a Dynex II plate reader (Dynex Technologies, Chantilly, VA). The control used to normalize the bortezomib plus melphalan samples was bortezomib treatment alone. Three independent experiments were performed. IC_{50} values were solved for using a probit model (PROC PROBIT in SAS software), and a paired t-test was then utilized to test for a significant difference between the IC_{50} values in the melphalan-treated versus combination bortezomib plus melphalan-treated groups.

MTT analysis was also used to analyze growth inhibition of the 8226/S and 8226/LR5 cell lines following treatment with BMS-345541 (Calbiochem, Gibbstown, NJ). Cells were seeded at 8,000-10,000 cells per well and treated with 1-5 μM BMS-345541 for a period of 96 hours. Following incubation with drug, cells were analyzed as described in the previous paragraph.

mRNA Isolation and qPCR Analysis

Quantitative RT-PCR was used to analyze FA/BRCA pathway mRNA expression in 8226 and U266 cells following drug treatment. Specifically, 8226/S and 8226/LR5 cells were treated with 10 nM bortezomib. Samples were collected and analyzed at 2, 4,

8, and 24 hours post-bortezomib. For the combination bortezomib plus melphalan studies, 8226/LR5 cells were pre-treated with 3 nM bortezomib for eight hours followed by 16 hours treatment with 25 μM melphalan. In a different study, 8226/S, 8226/LR5, U266/S and U266/LR6 cells were treated with 4 μM BMS-345541 and analyzed at 2, 4, 8, 12, 16 and 24 hours post-drug treatment.

Following drug treatment, total RNA was extracted using the RNeasy® Micro kit (Qiagen, Valencia, CA) and, subsequently, cDNA was synthesized using the SuperScript First Strand Synthesis kit (Invitrogen), according to the manufacturers' instructions. Quantitative RT-PCR was then carried out using either a customized microfluidic card (Applied Biosystems, Foster City, CA) or an Assay on Demand probe (Applied Biosystems), with the ABI 7900 Sequence Detection System (Applied Biosystems). Results were analyzed using the comparative CT method of analysis and SDS 2.1 software (Applied Biosystems). All samples were standardized to the endogenous control gene *gapdh*, and externally normalized to the corresponding vehicle control sample.

Initially, a customized microfluidic card was used to simultaneously analyze the expression of 11 FA/BRCA pathway-related genes, *brca1, brca2, fanca, fancc, fancd2, fance, fancf, fancg, fancl, rad51,* and *rad51c,* in 8226 cells treated with either 10 nM bortezomib or 4 μM BMS-345541. In order to incorporate recent additions to the FA/BRCA family, later studies were performed using a customized card that allowed for the analysis of 15 FA/BRCA-related genes, *brca1, brca2, fanca, fancb, fancc, fancd2, fance, fancf, fancg, fanci, fancj, fancl, fancm, fancn*, and *usp1*. This updated card was used to analyze the effect of BMS-345541 treatment on U266 cells. Finally, *fancd2* gene

expression was determined using the *fancd2* 20X Assay on Demand probe for the combination bortezomib plus melphalan studies.

The effect of BMS-345541 and bortezomib on FA/BRCA gene expression was statistically analyzed by determining whether relative change of gene expression deviated from 1 for each cell line (e.g., 8226/S, 8226/LR5, U266, and U266/LR6). A relative change less than 1 indicates downregulation of mRNA expression compared to the vehicle control. Since each gene showed a non-linear pattern of relative change over time (see Figures 2, 19 and 21), instead of using a linear slope for testing, a linear model with time as a categorical explanatory variable was used to test the null hypothesis of fold change=1 at each time point. Also, since multiple genes related to the FA/BRCA pathway were tested simultaneously, the p-value was adjusted based on the false discovery rate to control for simultaneous testing (180).

Western Blot Analysis

Immunoblot analysis was used to analyze FANCD2 and NF-κB family protein expression in 8226/S, 8226/LR5, U266/S and U266/LR6 cells following drug treatment. To analyze the effect of bortezomib on FANCD2 expression, 8226/LR5 and U266/LR6 cells were pre-treated with 3 nM bortezomib or vehicle control for eight hours and subsequently stimulated with melphalan or vehicle control for 16 hours (24 hours drug treatment total). Following stimulation with drug, cells were washed twice in ice cold PBS and cell extracts were prepared by resuspending in high-salt lysis buffer (50 mM Tris-HCl [pH 7.4], 1 M NaCl, 0.1% NP-40, 1 mM dithiothreitol) supplemented with 1 Complete, Mini protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis,

IN), 25 mM NaF, 2 mM sodium orthovanadate, and 0.1 M NaP_2O_4, per 10 mL lysis buffer. Samples were then sonicated, incubated on ice for 30 min, and lysates were quantified with Bio-Rad reagent (Bio-Rad, Hercules, CA). 25 μg lysate was separated using a 3% to 8% NuPage gel (Invitrogen) and transferred to Protran nitrocellulose membrane (Whatman, Dusseldorf, Germany). FANCD2 protein levels were examined using an α-FANCD2 antibody (1:5000; Novus Biologicals, Littleton, CO), which detects both non- and mono-ubiquitinated FANCD2. Equal loading was determined by probing for the housekeeping protein β-actin (1:10,000; Sigma). Membranes were visualized using Western Lightning Plus-ECL chemiluminescence substrate (Perkin-Elmer, Waltham, MA).

IKK phosphorylation was also examined in 8226/S and 8226/LR5 cells following stimulation with 25 and 50 μM melphalan, respectively, for 5-120 minutes. Proteins were lysed and analyzed as described above, and α-phospho-IKKα/IKKβ (1:100, Cell Signaling, Danvers, MA), α-IKKα (1:500; Cell Signaling), α-IKKβ (1250; Cell Signaling), and α-β-actin antibodies were used. The phospho-IKKα/IKKβ antibody specifically recognizes IKKα and IKKβ after phosphorylation at Ser-176/Ser-180 and Ser-177/Ser-181, respectively. Finally, following siRNA transfection of various NF-κB subunits, protein expression was analyzed using α-FANCD2, α-p65 (1:1500), α-p50 1:1000), α-c-Rel (1:200), α-RelB(1:500), and α-p52 (1:1000) (Santa Cruz Biotechnology) antibodies. TRAF2 (1:500; Imgenex, San Diego, CA) and $FLIP_L$ (1:200; BD Biosciences, San Jose, CA) antibodies were used as positive controls, and GAPDH (1:500; Cell Signaling) served as the negative control in these experiments.

Immunofluorescent Microscopy

Immunofluorescence techniques were used to analyze nuclear FANCD2 foci formation. Cells were treated with either vehicle control or bortezomib for 8 hours, followed by 5 hours treatment with melphalan. A cytospin was performed using 60,000 cells/slide, followed by fixation with 2% paraformaldehyde (Sigma-Aldrich). Slides were washed twice in PBS and cells were permeabilized using 0.3% Triton X-100 in PBS supplemented with 1% normal goat serum (NGS; Sigma). Following permeabilization, cells were blocked for 30 minutes with 5% NGS, 0.1% Triton X-100 in PBS. Slides were then stained with α-FANCD2 antibody (1:1000) for 2 hours in a humidified chamber. Next, samples were washed three times in PBS and incubated for one hour with Alexa Fluor 488 secondary antibody (1:1000; Molecular Probes, Invitrogen). Finally, samples were washed three times in PBS and a coverslip was mounted on each slide using VECTASHIELD Hardset Mounting Medium containing 4', 6'-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA). Fluorescence was detected using the Zeiss Axiovert Upright Fluorescent Microscope, and 100 cells per condition (control; melphalan; bortezomib; and bortezomib plus melphalan) were scored as having greater or less than 5 foci. Three independent experiments were performed and Tukey's method of statistical analysis was employed to compare differences between the four different groups.

Comet Assay

DNA damage was assessed using the Alkaline Comet Assay as previously described (4). 8226/LR5 cells were either pre-treated with 3 nM bortezomib or control

for 8 hours followed by 5 hours treatment with 25 μM melphalan. 8226/LR5 cells were also transfected with FANCD2 or control siRNA duplexes and, 48 h later, the cells were exposed to various amounts of 25 μM melphalan or vehicle control for 5 h. Following drug treatment, samples were irradiated at 9 Gy to induce single strand breaks (MARK I model 68A irradiator; J.L. Shepherd and Associates, San Fernando, CA). 5,000 cells were then washed once in PBS at 4°C, and 1% agarose was added to each sample. The agarose/cell suspension was loaded onto a frosted glass microscope slide and allowed to solidify. The slides were then placed in ice cold lysis buffer (2.5 M NaCl, 10 mM EDTA, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO, and 10 mM Tris; pH 10.0) for 1 hour at 4°C. Following lysis, slides were washed for 1 hour in alkaline wash buffer (0.03 M NaOH, 1 mM EDTA; pH 12.2). The samples were then electrophoresed for 20 minutes at 25 V and re-equilibrated with TBE. The DNA was stained with SYBR Green (1:10,000; Molecular Probes, Eugene OR) and slides were washed twice in TBE. 50 random images per slide were captured using fluorescent microscopy and quantified using Loats Associates comet analysis software (Loats Associates, Inc., Westminster, MD). The percent cross-linking was calculated using the equation: % cross-linking = 1 - [(value-mean control$_{\text{no IR}}$)/(mean control$_{\text{IR}}$ – mean control$_{\text{no IR}}$)]. The control used for the bortezomib plus melphalan studies was bortezomib treatment alone. A hierarchical linear model was fit, with condition (melphalan or melphalan plus bortezomib) as a fixed effect, and independent experiment as a random effect, to analyze the results of three independent experiments. (Confirmatory analyses were also performed without including the experiment effect). Mean percentage interstrand crosslinks (% ICLs) and 95% confidence intervals were generated. Analyses were performed in SAS, PROC MIXED.

To examine the effect of the loss of RelB/p50 on re-sensitization of 8226/LR5, cells were co-transfected with RelB and p50 siRNAs or siRNA control duplexes for 48 hours, followed by stimulation with 25-100 μM melphalan for five hours and irradiated and processed as described above. A linear regression was used to compare differences in melphalan-induced ICL formation in 8226/S and 8226/LR5 siRNA-transfected cells (i.e., LR5 RelB/p50 and LR5 siControl) relative to the 8226/LR5 wild-type cell line.

Patient Sample Plasma Cell Isolation and Purification

FA/BRCA pathway mRNA expression was analyzed in myeloma cells purified from patients enrolled in an Institutional Review Board approved clinical trial for patients with primary refractory myeloma, after obtaining informed patient consent. As per protocol, patients were treated with 1.3 mg/m^2 bortezomib on days 1, 4, 8, and 11, every three weeks for two cycles. Following completion of the bortezomib cycles, patients underwent high-dose melphalan treatment immediately followed by one dose of bortezomib as a conditioning regimen for tandem autologous peripheral blood stem cell transplants. Samples were collected and analyzed from three patients at the time of screening (baseline controls) and at 24 h after receiving the day 1 of cycle #1 bortezomib dose. Samples analyzed from a fourth patient were collected at screening, after 1 dose of bortezomib, after 2 cycles of bortezomib, three months post-transplant, and at relapse.

Plasma cells were isolated using a negative selection protocol, providing for >95% purity. Briefly, 1 mL of bone marrow aspirate was centrifuged in a Ficoll-Plaque Plus gradient (Amersham Biosciences, Piscataway, NJ), and a cytospin of the isolated cells was used to determine the initial purity of the plasma cell population. In parallel, 10

mL of the bone marrow sample was incubated with Millennium's Rosette Antibody Cocktail (Millennium Pharmaceuticals) for 20 minutes, followed by Ficoll extraction. To assess the efficiency of selection, a new cytospin was prepared from these cells. Once the myeloma cells were isolated, RNA was extracted, cDNA synthesized, and q-PCR analysis of FA/BRCA-related mRNA expression was performed as described above.

Promoter Region Analysis

Putative transcription factor binding sites on the promoter regions of *brca1, brca2, fanca, fancb, fancc, fancd2, fance, fancf, fancg, fanci, fancj, fancl, fancm, and fancn* (-3000 to +1 segments of the genomic sequences) were detected using a public version of 'P-Match' (http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi) and the database provided by TRANSFAC (http://www.gene-regulation.com/pub/databases.html). Matrix-scores (>0.990) were determined using 3 sets of optimized cut-off values.

Electrophoretic Mobility Shift Assays

NF-κB DNA binding activity was assessed utilizing the electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from 8226 and U266 cells, and EMSAs were carried out as described previously (ref). For gel shifts, nuclear extracts were incubated on ice with NF-κB-specific antibodies for 30 min prior to EMSA analysis.

NF-κB DNA binding activity was measured using a probe containing the well-described sequence of the κB enhancer, 5'-TAGTTGAGGGGACTTTCCCAG-3'. When

indicated, nuclear extracts were incubated with the following oligonucleotides containing FANCD2-specific consensus NF-κB binding sites: 5'-TTCAGACAGGGGCTCTCCCATTGCAA-3' (Probe I); 5'-TTTCCCCAGGAAACCCCAATTTGCAA-3' (Probe II); 5'-TTAATATACTAAAAAACCCTGAATAA-3' (Probe III); and, 5'-TTTGAAGTGGGGCTTCCCAGACTGAA-3' (Probe IV). As a control for loading, NF-1 DNA binding activity was measured using a probe derived from the adenovirus-2 origin of replication (5'-CTTATTTTGGATTGAAGCCAATAT-3'). Klenow (exo-) (New England Biolabs, Ipswich, MA) was added to the samples in the presence of [α^{32}P]ATP for 30 minutes at 37°C. A gel filtration column (Roche, Basel, Switzerland) was used to remove unincorporated nucleotide, and 20-40 kcpm of probe was incubated with 5 μg protein extract plus 1μg poly dI:dC for 20 minutes at room temperature. Protein-DNA complexes were resolved on a 5% nondenaturating polyacrylamide gel and detected by autoradiography.

Combination Index Analysis

As a means to determine if bortezomib and BMS-345541 exact their cytotoxic effects by altering the same pathway (ie – NF-κB), the dose-effect relationship between these two drugs was analyzed in the 8226/LR5 and U266/LR6 cell lines. Cells were seeded at 15,000 cells per well and drugs were added both alone and in combination, simultaneously for a total of 72 hours. The concentration of bortezomib added to the 8226/LR5 cell line varied from 3-10.5 nM, and from 2-5.3 nM in the U266/LR6 cell line. BMS-345541 was added to the 8226/LR5 and U266/LR6 cells at a range of

concentrations from 500-1750 nM and 1500-4000 nM, respectively. The drugs were added at a constant molar ratio (1:167 for the 8226/LR5 cell line and 1:750 for the U266/LR6 cells). Following 72 hours incubation with drug, MTT dye was added and samples were processed as described above. Percent survival was determined relative to vehicle control-treated samples. The dose-effect relationship was analyzed using CalcuSyn software (Biosoft, Ferguson, MO), and is based on the Chou-Talalay multiple drug effect equation (181). A combination index value equal to 1 indicates that the two drugs are additive, >1 indicates synergism, and <1 denotes antagonism. Three independent experiments were performed.

Transfection of siRNA, plasmids, and miRNA

8226 cells were seeded in complete medium at a concentration of 2×10^5 cells/ml. After 24 hours, $4\text{-}5 \times 10^6$ cells/sample were resuspended in 200 μl of cytomix buffer (containing 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM Hepes, 2 mM EGTA, 5 mM $MgCl_2$, 2 mM ATP, and 5 mM glutathione [pH 7.6]), mixed with the indicated 'ON-TARGET plus' siRNA duplexes (Dharmacon, Lafayette, CO) at a final concentration of 67 nM, and electroporated at 140 V/975 μF using the Bio-Rad GenePulser XCell (Bio-Rad, Hercules, CA). A similar transfection protocol was used for the overexpression of untagged, full-length FANCD2 using pIRES-neo-FANCD2 (exon 44 variant; 20 μg) plasmid DNA.

The following siRNA sequences were used (target mRNAs in parentheses): 5'-UGGUUUACAUGUCGACUAA-3', 5'-UGGUUUACAUGUUGUGUGA-3', 5'-UGGUUUACAUGUUUUCUGA-3', and 5'-UGGUUUACAUGUUUUCCUA-3'

(siControl, Non-Targeting pool); 5'-CGUACGCGGAAUACUUCGA -3' (Luciferase); 5'-GUGCCAGGAUCACGUAGAAUU-3' (c-Rel); 5'-CGAACAGCCUUGCAUCUAGUU-3' (p52); 5'-GGAUUGAGGAGAAACGUAAUU-3' (p65); 5'-CUGCGGAUUUGCCGAAUUAUU-3' (RelB); 5'-GGAGACAUCCUUCCGCAAAUU-3' (p50); and, 5'-UGGAUAAGUUGUCGUCUAU-3', 5'-CAACAUACCUCGACUCAUU-3', 5'-GGAUUUACCUGUGAUAAUA-3', 5'-GGAGAUUGAUGGUCUACUA-3' (FANCD2 Targeting pool). Transfected cells were typically used in experiments 24-48 hours post-transfection.

The effects of hsa-miR-16, has-miR-23a, and hsa-miR-27 on FANCD2 expression were also analyzed in the 8226/LR5 cell line. The same transfection protocol as described above was used to transfect cells with pre- or anti-miRNAs at a concentration of 50 nM. Samples were collected at 24, 48 and 72 hours post-transfection.

Chromatin Immunoprecipitation Assays

8226/S and 8226/LR5 cells were cross-linked in formaldehyde (20×10^6 cells/sample), lysed in SDS-rich buffer, and the resulting nuclear fractions were sonicated until the average chromatin size reached 200-1000 bp (~2-4, 10 sec pulses/sample). The cell extracts were pre-cleared with heat-inactivated, protein A-coated Staphylococcus aureus cells (PANSORBIN® cells, Calbiochem), and were immunoprecipitated with normal rabbit IgG or α-NF-κB/Rel specific antibodies. After three washes, DNA/protein cross-links were reversed at 65°C; and, a close-to-pure fraction of DNA was obtained with a ChIP-IT mini-column (Active Motif) and analyzed by quantitative PCR.

The ChIP DNA was subjected to quantitative RT-PCR using the following primer pairs: 5'-AATGAATGGGCAGCCGTTA-3' and 5'-TAGCCTCGCTCCACCTGACT-3' (GAPDH, control samples); 5'-GGGTCTGCAGGAGATCAACTAAGAAA-3' and 5'-GTGCCTGGCCCTATGCTGTAACTA-3' (FANCD2, STATx); and, 5'-GAGCCAAGAGGTACCCTGATAAAGTC-3' and 5'-CAGCTTTGGTTTAATACCTGTCAGAATT-3' (FANCD2, NF-κB).

Diffusion Apoptosis Slide Halo (DASH) Assay

Apoptosis was analyzed using the DASH Assay kit (Trevigen, Helgerman, CT), which detects DNA fragmentation. This assay captures and detects damage-induced low molecular-weight DNA fragments as diffuse halos in an agarose microgel. After the indicated siRNA transfections, the cells were treated with 25-100 μM melphalan or vehicle control for 24 hours, then washed with PBS and embedded in low melting agarose. The embedded cells were lysed for 10 minutes under alkaline conditions, and DNA was precipitated and visualized with SYBR Green fluorogenic dye. Fifty images were randomly captured per slide, and the logarithmic radius of each nucleoid was calculated using the tail-length parameter of the Loats Associates comet analysis software.

Analysis of variance (ANOVA) was employed to test whether nuclear DNA diffusion after melphalan treatment was different among all experimental groups. The Tukey's Honest Significance Difference method was used to adjust P-values for multiple comparisons (182). To examine the relationship between the nuclear DNA diffusion and ICL, Pearson correlation was used to study their association (183).

Stable Isotopic Labeling of Amino Acids in Cell Culture

FANCD2 protein half-life and doubling time was analyzed via stable isotopic labeling of amino acids in cell culture (SILAC). 8226/LR5 cells were cultured for six doublings (approximately seven days) in "heavy media" (RPMI 1640 deficient in lysine, arginine, glutamine, and phenol red, and supplemented with 5% dialyzed FBS, 1% penicillin/streptomycin, 100 mM L-glutamine, [U-$^{13}C_6$]-L-Lysine HCl and [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine). Following isotopic labeling, cells were washed twice in PBS and re-suspended in "light media" (RPMI 1640 routinely used for cell culture). Cells were divided into two flasks, 3 nM bortezomib or vehicle control was immediately added, and samples were harvested at 0, 4, 8, 12, 24 and 36 hours. Labeling with heavy media followed by a chase with light media allows for the simultaneous analysis of FANCD2 degradation (decrease in isotopically labeled FANCD2) and FANCD2 synthesis (incorporation of light/normal amino acids).

Samples were harvested in high salt lysis buffer (50 mM Tris-HCl pH7.4, 0.1% NP-40, 1M NaCl, protease inhibitor cocktail (Roche), 25 mM NaF, 2 mM Na_3VO_4, and 0.1 M Na_2HPO_4), sonicated, and centrifuged at 14,000 rpm for 10 minutes. Aliquots of the cell lysate (equivalent to 300,000 cells) were denatured by boiling in loading buffer and separated by 4-12 % Criterion XT Bis-Tris gels for 80 minutes at 150 V. The gel was stained with colloidal Coomassie Brilliant Blue G-250 (Bio-Rad). Bands from the mass of interest were excised and destained with 50% MeOH and 50 mM ammonium bicarbonate. Samples were then reduced with 2 mM tris (2-carboxyethyl) phosphine, alkylated with 20 mM iodoacetamide, and digested with trypsin (Promega, Madison, WI)

overnight at 37ºC. Following digestion, peptides were extracted and concentrated under vacuum centrifugation.

Using a Proxeon nanoLC system, the sample (~150,000 cells) was loaded onto a trap column (300 µm x 5 mm), washed for 20 min, then eluted onto a C18 PepMap 100 column (LC packings/Dionex, Sunnyvale, CA) with 75 µm inner diameter, 3 µm particle size, and 100 Å pore size. Peptides were eluted on a 35 minute gradient from 5% B to 50% B over 35 minutes at a flow rate of 300 nl/min. The solvent system was composed of aqueous 2% acetonitrile with 0.1% formic acid (A) and aqueous 90% acetonitrile with 0.1% formic acid (B). Peptide precursors were selected in the first quadrupole with a mass window of 0.2 (Q1); fragments were filtered at a resolution of 0.7 (Q3). The scan width was set to 0.002, and 15 milliseconds of acquisition time was used per transition. Collision energy values were calculated using the equation: $CE = (0.034 * m/z) + 3.314$ V, based on the mass-to-charge ratio of the doubly charged peptide precursor.

Peak areas were identified and calculated using MRMer, which has been implemented as a pipeline in GenePattern (184). The raw data files from the instrument are converted into mzxml format; then, the LC-MRM peaks are extracted and visualized for data evaluation. Resulting files were then compared using Post MRMer, developed in house for data visualization and evaluation, as well as comparison of sample groups. MRM was performed on a Finnigan TSQ Quantum triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA). The internal standard DAFFGNPR was added at a concentration of 20 fmole/injection to the samples after in-gel digestion and was used as a normalization constant.

BrdU/PI Cell Cycle Analysis

8226/LR5 cells were pre-treated with 3 nM bortezomib for eight hours and subsequently treated with 25 μM melphalan. $1x10^6$ cells were incubated with 30 μg/ml bromodeoxyuridine (BrdU) for 30 minutes after 8, 24, 36, and 48 hours drug treatment. Samples were then washed once in cold PBS and fixed overnight at 4°C with 4 ml cold PBS plus 6 ml cold 100% EtOH. Next, samples were centrifuged and 4 ml Pepsin solution (0.04% pepsin, 0.1% HCl) was added. Samples incubated while shaking for 1 hour at 37°C. The DNA was then denatured by the addition of 2 N HCl for 30 minutes at 37°C, followed by washing once with 0.1 M sodium borate to neutralize the acid. Samples were then washed once in PBTB (0.5% Tween-20, 0.5% BSA in PBS), and incubated in the dark for one hour with 10 μl FITC-conjugated anti-BrdU in 200 μl PBTB. Next, samples were centrifuged and incubated with 200 μl PBTB containing 10 μg/ml propidium iodide and 0.25 mg RNase A for 30 minutes at 37°C. Finally, samples were centrifuged, re-suspended in 400 μl PBS, and analyzed using a FACScan flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

RESULTS

Part I:

Fanconi Anemia/BRCA Pathway Expression in Drug Resistant Cell Lines

Our lab was the first to report that members of the FA/BRCA DNA damage repair pathway are overexpressed in melphalan resistant myeloma cell lines (3-4). Furthermore, inhibition of FANCF in the melphalan resistant 8226/LR5 cell line partially reversed resistance in these cells, and FANCF overexpression in the drug sensitive cells conferred resistance to melphalan (3). Furthermore, members of the FA/BRCA pathway have been shown to mediate sensitivity to cisplatin, BCNU, and TMZ in different tumor types as well (170, 172-175). Based on these reports, we hypothesized that overexpression of FA/BRCA pathway members is a common mechanism by which tumor cells acquire resistance to chemotherapeutic agents. This section of the dissertation summarizes our findings regarding FA/BRCA pathway mRNA expression in isogenic drug sensitive and drug resistant tumor cell lines. We found that cells selected for resistance to the alkylating agents melphalan and cisplatin overexpress FA/BRCA pathway genes, whereas cells selected for resistance to topoisomerase II inhibitors specifically overexpress FANCF.

FA/BRCA Pathway mRNA is Overexpressed in Cells Selected for Resistance to Melphalan and Cisplatin

The DU-145/M4.5 prostate cancer cell line was selected for resistance to melphalan by chronically treating the parental DU-145 cell line with low doses of melphalan for a period of three years (185). The DU-145/M4.5 cells are approximately 27-fold resistant to melphalan when compared to the drug sensitive parental cells (185). Quantitative-RT-PCR analysis of FA/BRCA pathway mRNA expression was performed in these cell lines using a customized microfluidic card to analyze expression of 11 FA/BRCA pathway related genes (*brca1, brca2, fanca, fancc, fancd2, fance, fancf, fancg, fancl, rad51 and rad51c*). Similar to our published results showing overexpression of FA/BRCA pathway genes in melphalan resistant myeloma cell lines (3-4), the DU-145/M4.5 melphalan resistant prostate cancer cell line shows enhanced FA/BRCA pathway expression when compared to the drug sensitive parent cell line (Figure 4A). Specifically, nine of 11 genes analyzed (*brca1, brca2, fanca, fancc, fancd2, fance, fancg, rad51 and rad51c*) show at least two-fold enhanced expression in the melphalan resistant versus melphalan sensitive cells.

We next analyzed FA/BRCA pathway mRNA expression in cells selected for resistance to cisplatin, another alkylating agent. The C13* ovarian cancer cell line was selected for resistance to cisplatin (186). This cell line is approximately 15-fold resistant to cisplatin when compared to the drug sensitive OV2008 cell line, and is also cross-resistant to melphalan (186). Analysis of FA/BRCA pathway mRNA expression revealed overexpression of many of these genes, similar to the results seen in cells selected for resistance to melphalan (Figure 4B). Based on these results, we conclude that selecting tumor cells for resistance to alkylating agents typically results in overexpression of

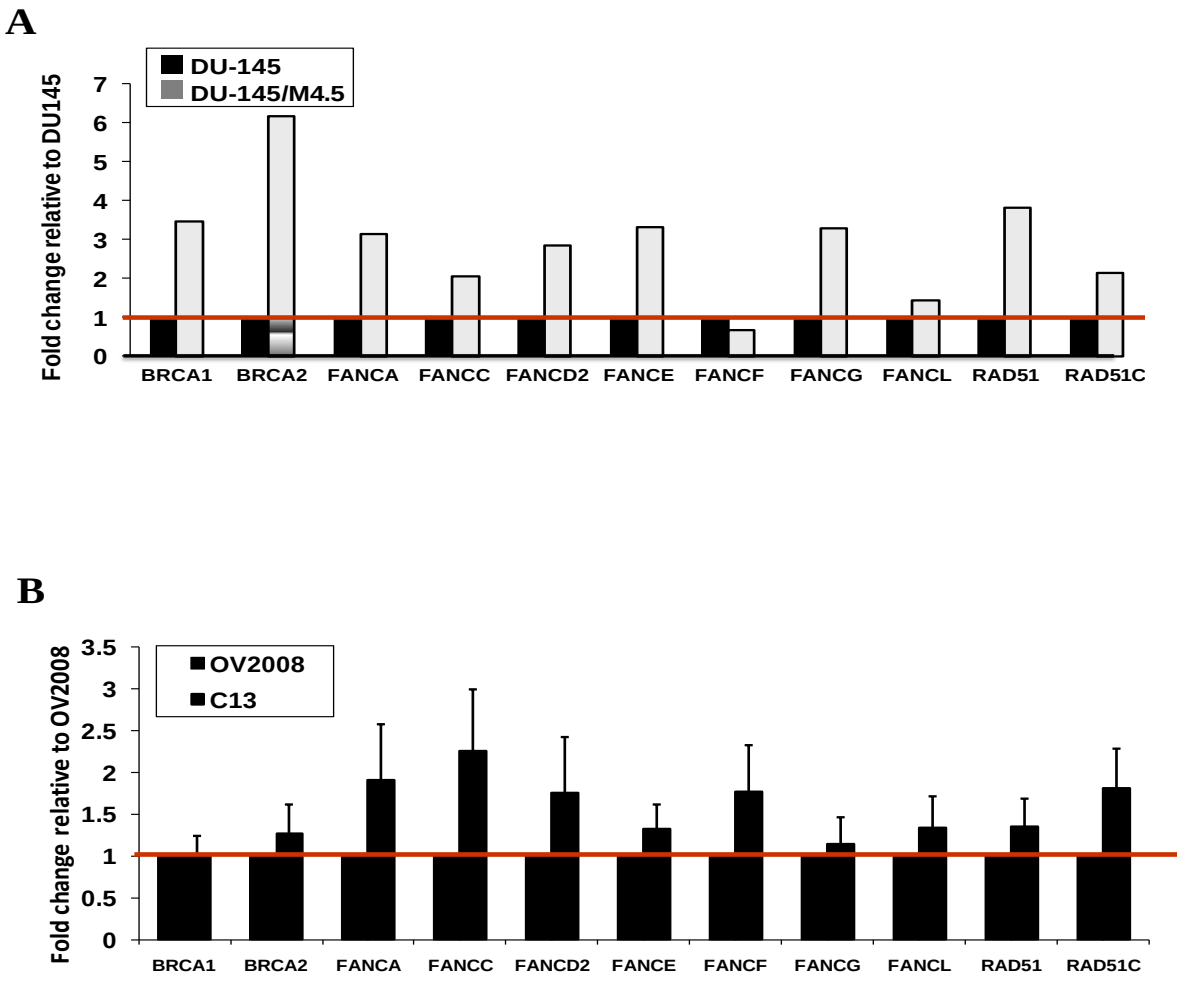


Figure 4. **Melphalan and cisplatin resistant cells overexpress FA/BRCA pathway genes.** mRNA levels of FA/BRCA pathway genes were analyzed in melphalan and cisplatin resistant cell lines using a customized microfluidic card and q-PCR analysis. (A) The DU-145/M4.5 prostate cancer cell line is ~27-fold resistant to melphalan when compared to the drug sensitive DU-145 parental cell line. n=1. (B) C13* cells are ~15–fold resistant to cisplatin when compared to the drug sensitive OV2008 parental cell line. n=3.

members of the FA/BRCA pathway, and targeting this pathway is likely vital for overcoming drug resistance.

FANCF is Specifically Overexpressed in Myeloma Cells Selected for Resistance to Topoisomerase II Inhibitors

We next analyzed FA/BRCA pathway mRNA expression in myeloma cells selected for resistance to doxorubicin and mitoxantrone. These two chemotherapeutic agents are classified as topoisomerase II inhibitors, which function by inhibiting this enzyme, resulting in single and double strand breaks. The 8226/Dox40 cell line was selected for resistance to doxorubicin via chronic stimulation with increasing doses of this drug for a period of 10 months, and these cells are approximately 10-fold resistant to doxorubicin when compared to the drug sensitive 8226/S parent cell line (178). Unlike cells selected for resistance to alkylating agents, the doxorubicin resistant myeloma cells do not show global enhanced expression of FA/BRCA pathway genes (Figure 5A). These cells do, however, specifically overexpress FANCF.

FA/BRCA pathway mRNA expression was next analyzed in the mitoxantrone resistant 8226/MR20 cell line. When compared to the drug sensitive parent cell line, these cells are close to 40-fold resistant to mitoxantrone. Interestingly, similar to the results seen with the 8226/Dox40 cells, the 8226/MR20 cells show enhanced expression of FANCF (Figure 5B).

Collectively, the results presented in this section of the dissertation show enhanced mRNA expression of many FA/BRCA pathway members in tumor cells

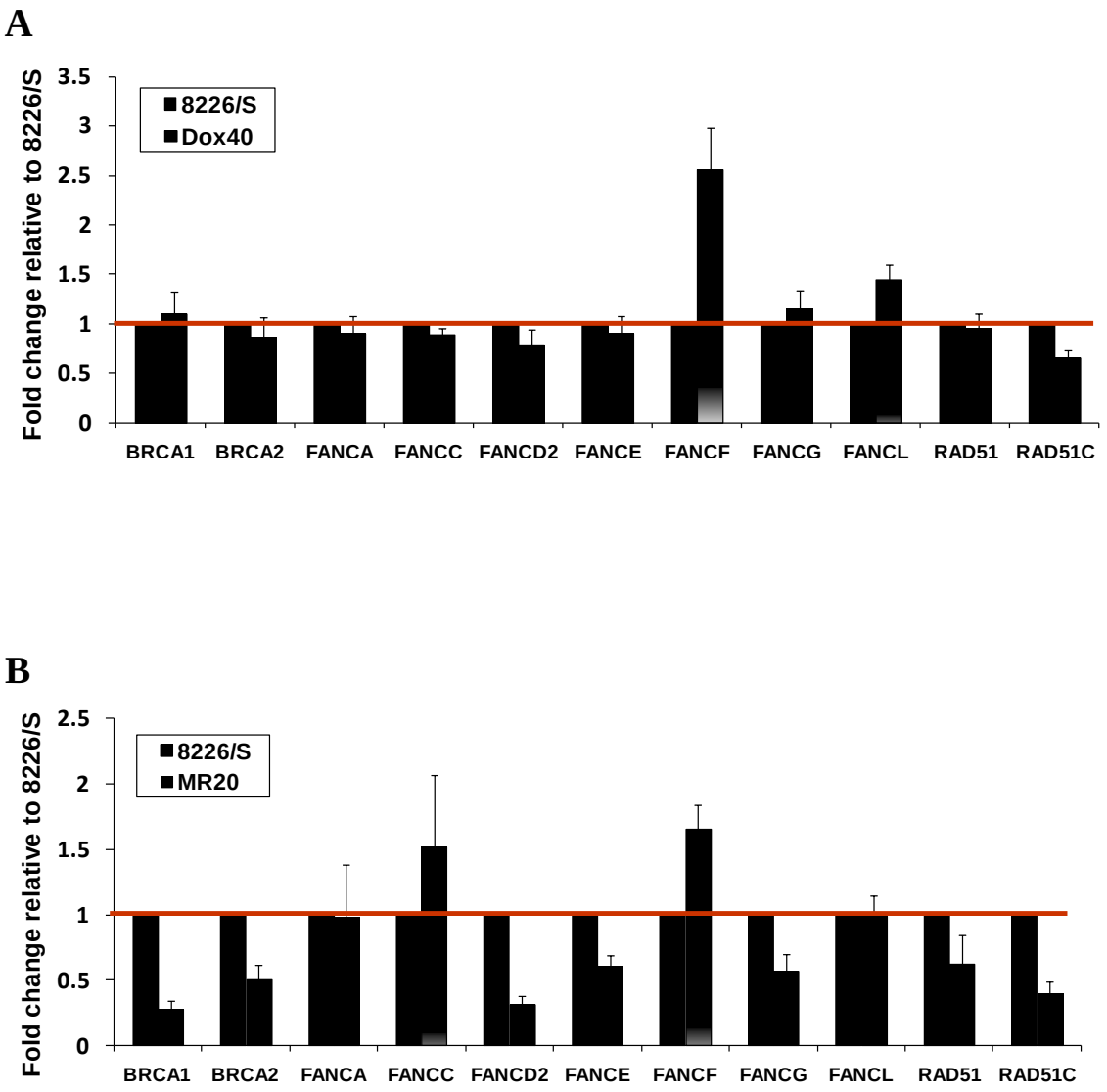


Figure 5. **Doxorubicin and mitoxantrone resistant myeloma cells overexpress FANCF.** mRNA levels of FA/BRCA pathway genes were analyzed in doxorubicin and mitoxantrone resistant myeloma cell lines using a customized microfluidic card and q-PCR analysis. (A) The 8226/Dox40 cell line is ~10-fold resistant to doxorubicin when compared to the drug sensitive 8226/S parental cell line. n=3. (B) 8226/MR20 cells are ~37–fold resistant to mitoxantrone when compared to the drug sensitive 8226/S parental cell line. n=3.

selected for resistance to melphalan or cisplatin, whereas cells selected for resistance to topoisomerase II inhibitors specifically overexpress FANCF.

Part II:

Bortezomib Enhances Melphalan Response in Multiple Myeloma Cell Lines and Patient Samples

Although there are many options available today for the treatment of multiple myeloma (MM) that did not exist even ten years ago, this disease remains incurable, mainly due to the emergence of drug resistance following chemotherapy. Our laboratory previously reported that members of the FA/BRCA pathway are overexpressed in melphalan resistant cell lines, and causative for this resistance (3-4). Specifically, using siRNA techniques to inhibit FANCF expression in the melphalan resistant 8226/LR5 cell line partially reversed resistance (3). Conversely, overexpression of FANCF in the drug sensitive 8226/S cell line conferred resistance to melphalan (3). Based on these results, we hypothesized that bortezomib enhances melphalan response by inhibiting FA/BRCA pathway expression.

Current MM treatment regimens include combining different agents to circumvent or overcome drug resistance. Others have reported that the proteasome inhibitor bortezomib enhances melphalan response *in vitro* (59, 78), as well as in clinical trials (25, 27-28, 79). However, the mechanism by which this enhanced response occurs remains unclear. Based on our previous reports that the FA/BRCA pathway is involved in melphalan resistance, we hypothesized that bortezomib enhances melphalan response by inhibiting expression and function of the FA/BRCA DNA damage repair pathway.

This hypothesis is further substantiated by a recent report, which found that proteasome function is required for FA/BRCA pathway activation (176). In this portion of the dissertation, we analyze the role of the FA/BRCA pathway in enhanced melphalan response by bortezomib. We show that bortezomib inhibits mRNA expression of many FA/BRCA pathway members. We also demonstrate that FANCD2 mRNA and protein expression is inhibited, even in the presence of melphalan, and that bortezomib inhibits melphalan-induced FANCD2 foci formation. Bortezomib also enhanced DNA damage induced by melphalan treatment, likely via inhibition of FANCD2 expression. Importantly, we also show that bortezomib inhibits FA/BRCA pathway expression in MM patient specimens. Combined, these results show that bortezomib enhances melphalan response via inhibition of the FA/BRCA pathway, and provide a target for overcoming or even circumventing drug resistance in multiple myeloma and possibly other cancers.

Bortezomib Enhances Melphalan Response in Myeloma Cell Lines

To test our hypothesis that bortezomib enhances melphalan response via inhibition of the FA/BRCA pathway, we first needed to confirm the work of others that bortezomib does indeed enhance melphalan cytotoxicity. Since we believed that bortezomib enhances melphalan response by inhibiting this pathway, we first treated cells with bortezomib, followed by exposure to melphalan. Using MTT assays, we pre-treated two melphalan sensitive cell lines (8226/S and U266/S), and two melphalan resistant cell lines (8226/LR5 and U266/LR6), with a non-toxic dose (3nM) of bortezomib for eight hours, followed by treatment with varying doses of melphalan for 40 hours (48 hours

total drug treatment). The results show that pre-treatment with bortezomib enhances sensitivity to melphalan when compared to melphalan treatment alone (Figure 6). In all four cell lines, statistical analysis of the IC_{50} values generated from three independent experiments revealed a statistically significant reduction in the IC_{50} values of cells pre-treated with bortezomib versus cells exposed only to melphalan. These results confirm the work of others and show that pre-treatment with a non-toxic dose of bortezomib enhances melphalan response.

Bortezomib Downregulates FA/BRCA Pathway mRNA Expression

We next wanted to determine if bortezomib inhibits FA/BRCA pathway expression and function. To this end, we analyzed FA/BRCA pathway gene expression in the 8226/S and 8226/LR5 cell lines following treatment with 10 nM bortezomib. Samples were collected at 2, 4, 8, and 24 hours, and mRNA expression was analyzed using a customized microfluidic card (which allowed for simultaneous analysis of eight FA genes, *fancd1/ brca2, fanca, fancc, fancd2, fance, fancf, fancg, fancl,*and three DNA damage response genes, *brca1, rad51 and rad51c*) and quantitative RT-PCR. Bortezomib decreased expression of certain FA/BRCA pathway members in both cell lines in as little as 2 hours, with maximal effect seen at 24 hours (Figure 7). Specifically, bortezomib significantly reduced expression of *brca2, fancc, fancd2, fance, fancf, fancg, fancl,* and *rad51c* in the drug sensitive 8226/S cell line at 24 hours (Table 2). In the 8226/LR5 melphalan resistant cell line, *fancd2, fance, fancf and fancg* were reduced significantly following 24 hours treatment with melphalan (Table 2). Importantly, the downregulation of mRNA expression seen following treatment with bortezomib is not

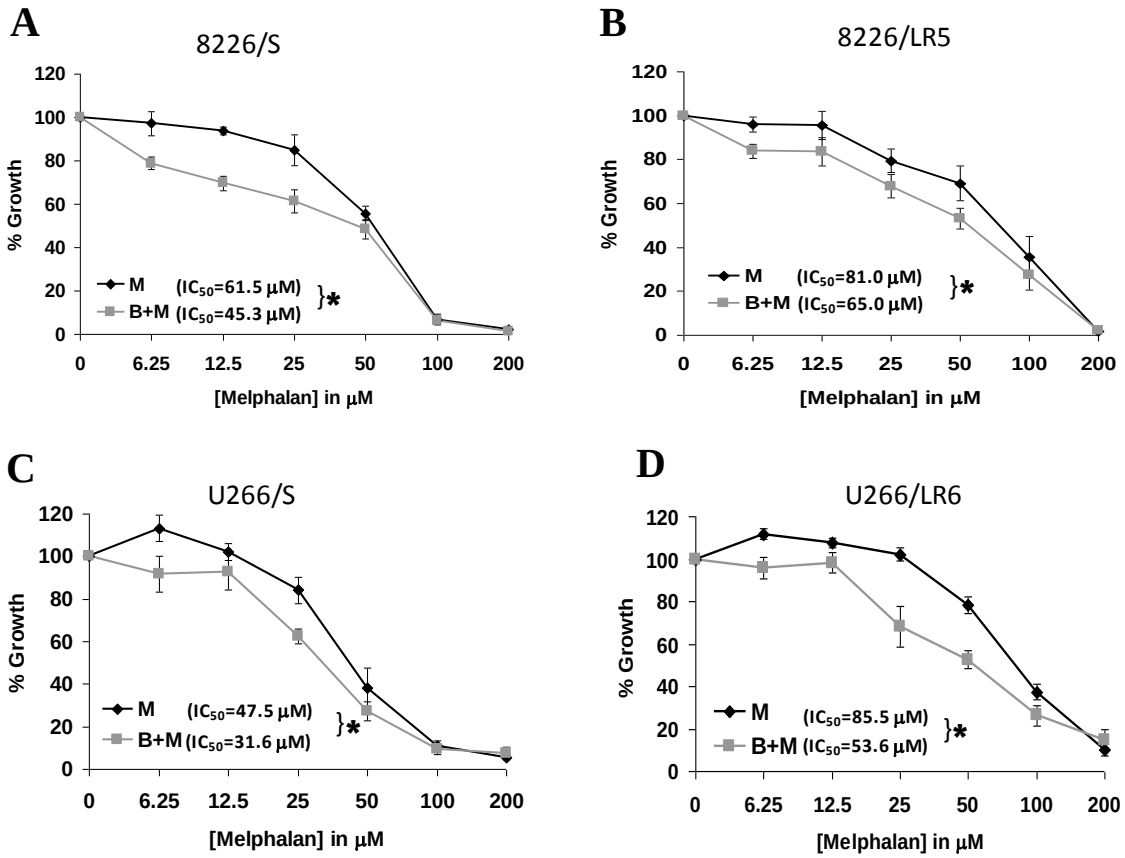


Figure 6. **Low-dose bortezomib enhances melphalan response in melphalan sensitive and melphalan resistant myeloma cell lines.** Melphalan sensitivity was studied in melphalan sensitive and resistant myeloma cell lines in the absence or presence of a non-toxic (3 nM) dose of bortezomib. Percent growth is shown following exposure to melphalan alone or bortezomib plus melphalan treatment, in the (A) 8226/S melphalan sensitive and (B) 8226/LR5 melphalan resistant cell lines, as well as in the (C) U266/S and (D) U266/LR6 cell line pairs. The control used for the bortezomib plus melphalan studies was bortezomib treatment alone. At least 3 independent experiments were performed, and the mean of these experiments and SEM are depicted in the growth curve. Mean IC_{50} values are also shown. *Statistical significance was determined using a paired t-test, and $p \leq 0.02$. Bortezomib enhanced melphalan activity at statistically significant levels in all four cell lines.

due to a global inhibition of transcription, as expression of genes not related to the FA/BRCA pathway (*b2m, ipo8* and *tfrc*) were unaffected by bortezomib treatment (Figure 8). These results suggest that bortezomib may function in part by inhibiting the FA/BRCA pathway.

Bortezomib Inhibits FANCD2 mRNA, Protein Expression, and Foci Formation, Even in the Presence of Melphalan

Of the FA/BRCA pathway-related genes that did change following bortezomib treatment, FANCD2 mRNA expression was most consistently and dramatically decreased in both the drug sensitive 8226/S and drug resistant 8226/LR5 cell lines. To further characterize the effect of bortezomib on melphalan response, FANCD2 mRNA and protein expression was analyzed in the drug resistant 8226/LR5 and U266/LR6 cell lines. Cells were pre-treated with either vehicle control or 3 nM bortezomib for eight hours and subsequently exposed to 25 μM melphalan for 16 hours. Results showed that melphalan treatment alone did not affect FANCD2 mRNA expression in the 8226/LR5 cell line whereas bortezomib treatment, both alone and in combination with melphalan, decreased expression of FANCD2 (Figure 9A). Analysis of the U266/LR6 cell line revealed that melphalan treatment alone enhanced expression of FANCD2, and, comparable to results seen in the 8226/LR5 cell line, bortezomib treatment inhibited FANCD2 gene expression (Figure 9B). Importantly, decreased FANCD2 mRNA expression seen with low-dose bortezomib was maintained even in the presence of melphalan in both cell lines.

FANCD2 protein expression was also analyzed in the 8226/LR5 and U266/LR6 cell lines following the same method of treatment with bortezomib, melphalan, or the

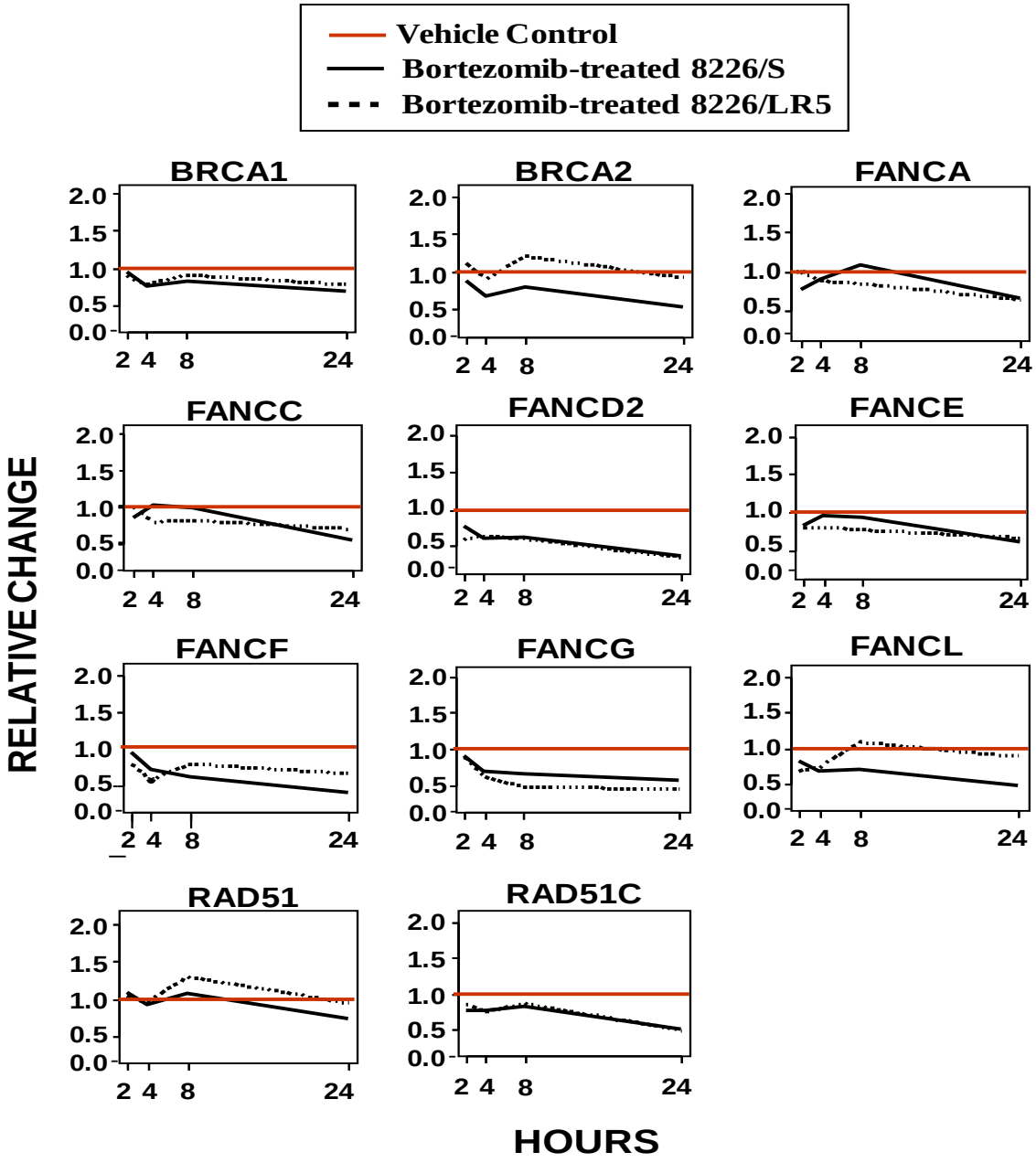


Figure 7. **Bortezomib downregulates FA/BRCA pathway mRNA expression in melphalan sensitive and resistant myeloma cell lines**. 8226/S and 8226/LR5 cells were treated with 10 nM bortezomib and harvested at indicated times. FA/BRCA gene expression was determined in quadruplicate samples by qPCR using a customized microfluidic card. Results depict fold change normalized to vehicle control samples. Statistical analysis is shown in Table 2. Three independent experiments were performed.

A 8226/S

Time (h)	2	4	8	24
BRCA1	0.7612	0.1484	0.2899	0.0853
BRCA2	0.2677	0.0152	0.0836	0.0028
FANCA	0.1521	0.5206	0.6368	0.0644
FANCC	0.0853	0.8026	0.8499	0.0010
FANCD2	0.1003	0.0153	0.0179	0.0019
FANCE	0.0152	0.5130	0.2899	0.0006
FANCF	0.6905	0.0853	0.0310	0.0053
FANCG	0.1140	0.0018	0.0010	0.0006
FANCL	0.0958	0.0113	0.0153	0.0010
RAD51	0.6368	0.5206	0.6407	0.0777
RAD51C	0.1102	0.1085	0.2268	0.0062

B 8226/LR5

Time (h)	2	4	8	24
BRCA1	0.4833	0.1285	0.5249	0.1285
BRCA2	0.7798	0.7964	0.5891	0.8040
FANCA	0.9920	0.5249	0.3552	0.0582
FANCC	0.9588	0.2723	0.3010	0.1110
FANCD2	0.0100	0.0166	0.0111	0.0014
FANCE	0.1285	0.1285	0.0926	0.0217
FANCF	0.0590	0.0036	0.0589	0.0078
FANCG	0.3010	0.0059	0.0014	0.0014
FANCL	0.1918	0.2959	0.7484	0.6246
RAD51	0.8462	0.8040	0.1843	0.7798
RAD51C	0.5891	0.3126	0.6188	0.0590

Table 2. **Statistical analysis of FA/BRCA gene expression following bortezomib treatment in 8226 cells.** To test the effect of bortezomib on FA/BRCA gene expression in 8226/S (A) and 8226/LR5 (B) cells, we examined whether fold change of gene expression deviated away from 1. Since each gene showed a non-linear pattern of fold change over time (see Figure 2), a linear model with time as a categorical explanatory variable was used to test the null hypothesis of fold change=1 at each time point for each gene in each cell line. Since we tested 11 FA/BRCA related genes, we adjusted for p value based on the false discovery rate to control for simultaneously testing (Benjamini, 1995).

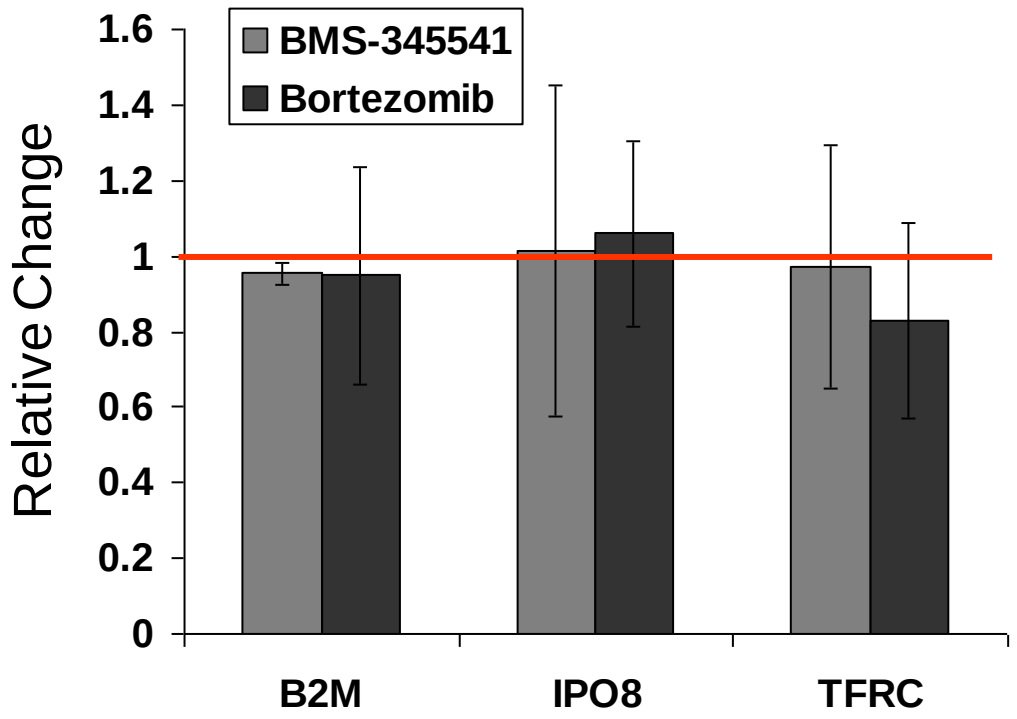


Figure 8. **Gene expression levels of B2M, IPO8 and TFRC remain unchanged in 8226/S cells treated with BMS-345541 and bortezomib**. 8226/S cells were treated with BMS-345541 or bortezomib as described in *Figures 7 and 24*. Relative changes in gene expression were obtained by internally standardizing against GAPDH and externally standardizing against the control, equal to 1. Results depict the mean relative change of three independent experiments and SEM. No significant difference in B2M, IPO8 or TFRC gene expression was seen following treatment with BMS-345541 or bortezomib.

combination (Figure 10). The antibody against FANCD2 recognizes both non-ubiquitinated (short, lower band) and monoubiquitinated, activated (long, upper band) FANCD2. Demonstration of the monoubiquitinated form of FANCD2 is evidence for the presence and activation of the upstream FA complex (187). In this study, we found that in both cell lines melphalan increased FANCD2 monoubiquitination/activation. By comparison, bortezomib attenuated total FANCD2 protein levels, resulting in a reduced amount of monoubiquitinated protein in the cells when compared to basal monoubiquitinated FANCD2 levels. Furthermore, bortezomib effectively reduced FANCD2 levels, in the presence or absence of melphalan, compared to melphalan alone. These results show that bortezomib impedes FA/BRCA pathway activation by inhibiting FANCD2 mRNA and protein expression.

To further determine the effects of bortezomib on FANCD2 activation and function, immunofluorescent microscopy techniques were used to analyze FANCD2 DNA repair foci formation, a hallmark of FA pathway activation. 8226/LR5 cells were treated with vehicle control or bortezomib for 8 hours, followed by a five hour treatment with 50 μM melphalan (Figure 11). FANCD2 foci formation, as measured by percentage of cells with greater than five foci, was found to be enhanced following melphalan but not bortezomib treatment. However, melphalan-induced FANCD2 foci formation was inhibited when cells were first treated with 3 nM bortezomib. Tukey's method of statistical analysis confirmed a difference in foci formation between control and melphalan alone; melphalan alone versus bortezomib alone; and melphalan alone versus the combination of melphalan and bortezomib. In contrast, no difference in foci formation was seen between the control, bortezomib alone, and the combination of

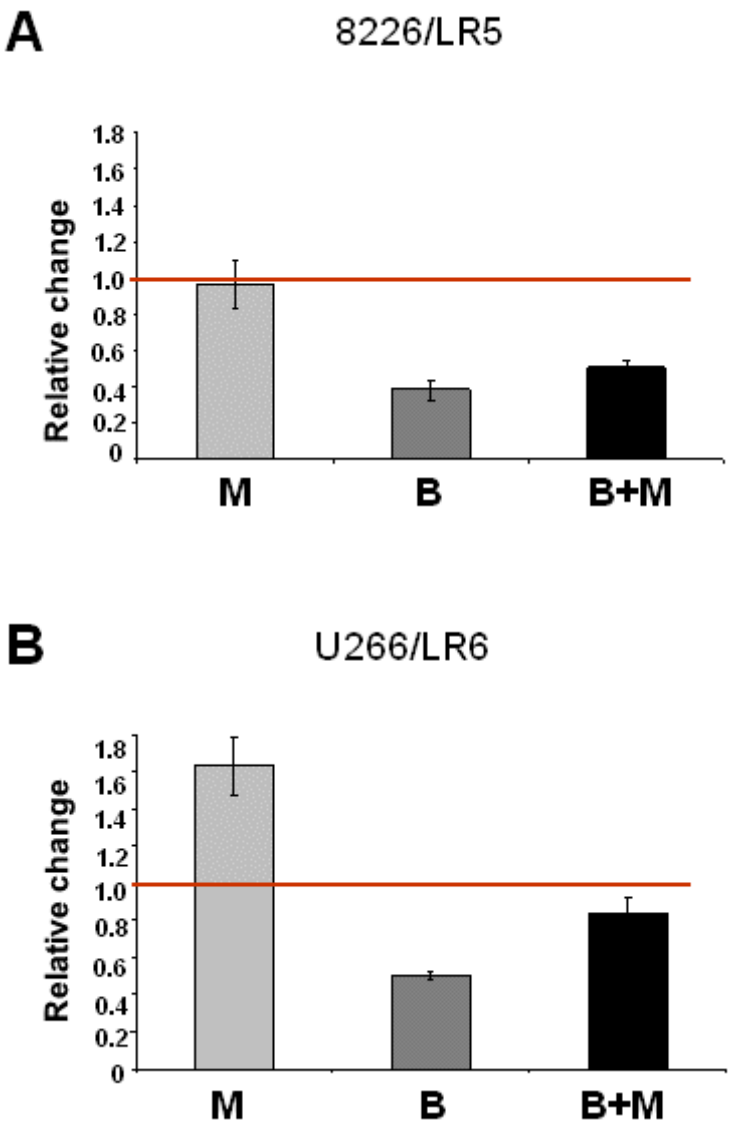


Figure 9. **Bortezomib inhibits FANCD2 mRNA expression, even in the presence of melphalan.** 8226/LR5 (A) and U266/LR6 (B) cells were pre-treated with 3 nM bortezomib followed by 16 hours treatment with melphalan. Fold changes were obtained by internally standardizing against GAPDH and externally standardizing against the control, equal to 1 (noted by the solid line). Three independent experiments were performed. Data shown are mean value and SEM. *P<0.05.

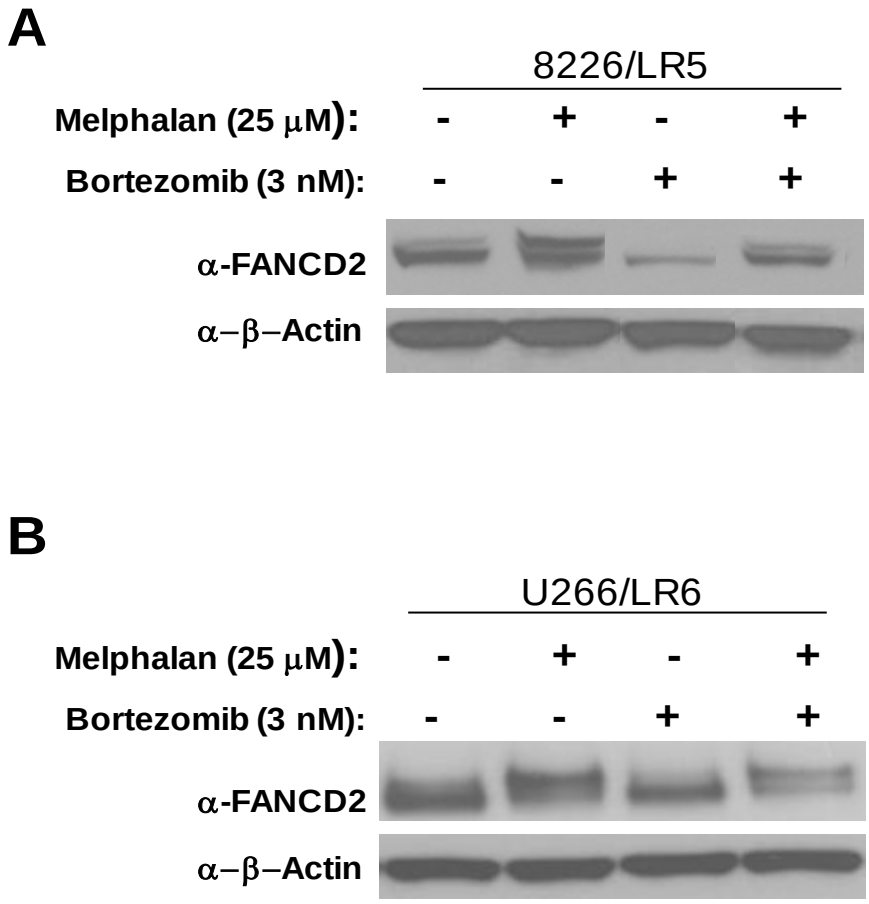


Figure 10. **Bortezomib inhibits FANCD2 protein expression.** Immunoblot analysis of 8226/LR5 (A) and U266/LR6 (B) cells were pre-treated with 3 nM bortezomib followed by 16 hours treatment with melphalan. Two distinct bands are seen: the upper, monoubiquitinated band and the lower, non-ubiquitinated band. A representative blot of three independent experiments is shown.

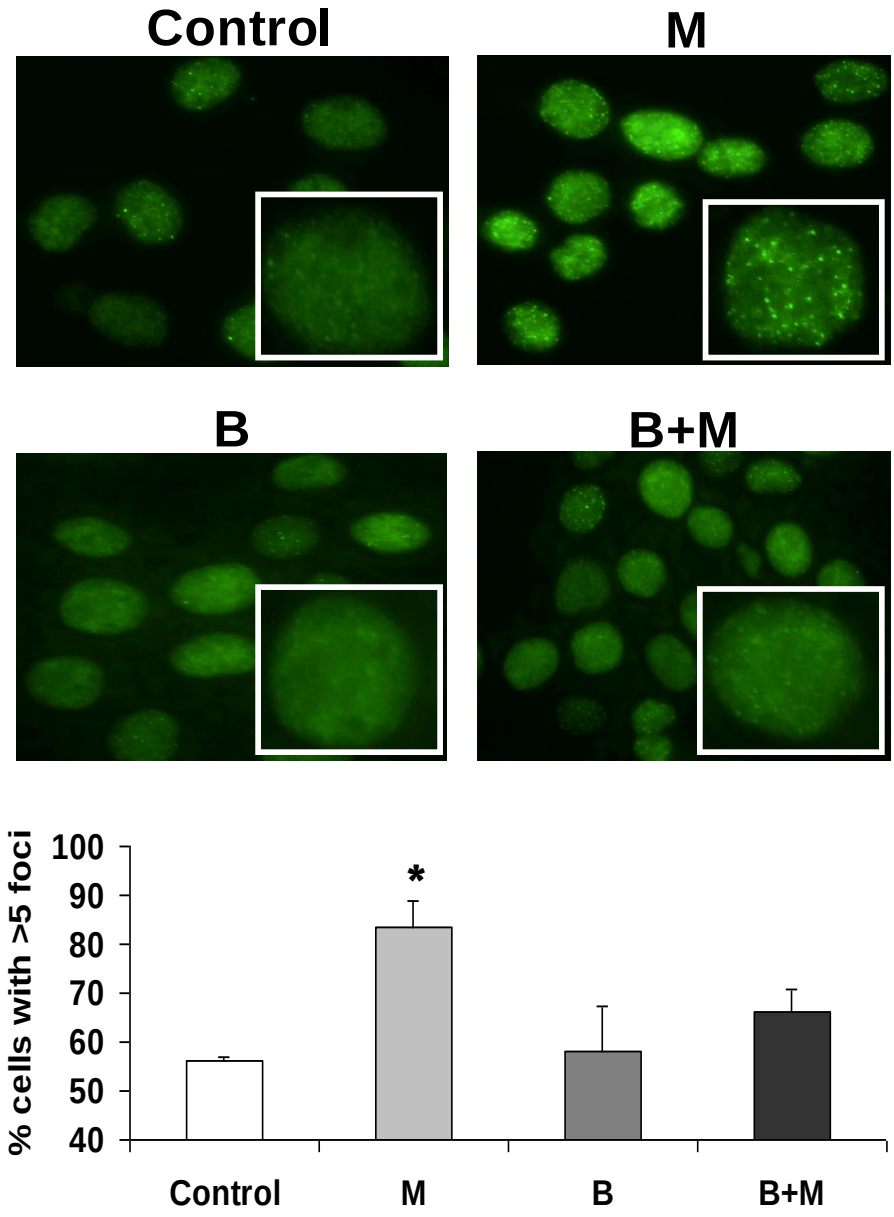


Figure 11. **Bortezomib inhibits melphalan-induced FANCD2 foci formation.** Immunofluorescent microscopy was used to detect FANCD2 foci formation. Melphalan-induced FANCD2 foci formation was inhibited when cells were pre-treated with bortezomib. Inserts demonstrate the foci typically observed for single cells in respective treatment groups. Three independent experiments were performed. *Tukey's method of statistical analysis showed a significant difference between melphalan alone and control; melphalan alone and bortezomib alone; and melphalan alone and the combination of bortezomib plus melphalan. No difference was seen between control, bortezomib alone, and bortezomib plus melphalan.

bortezomib plus melphalan. Based on these results, we conclude that a non-toxic dose of bortezomib inhibits FANCD2 gene expression, FANCD2 protein levels, and FANCD2 foci formation, even in the presence of melphalan.

Previous reports have shown that FANCD2 monoubiquitination and foci formation is S-phase specific (156). Furthermore, others have reported that bortezomib can reduce the percentage of cells in S-phase (188-189). Therefore, we next wanted to determine if the reduction of FANCD2 protein expression and foci formation following bortezomib treatment was due in part to a lesser number of cells in the S-phase of the cell cycle. In these studies, 8226/LR5 cells were pre-treated with bortezomib for eight hours and subsequently exposed to melphalan for 16 hours. Using BrdU incorporation and PI staining, we observed that 3 nM bortezomib treatment does indeed decrease the percentage of cells in S-phase, even in the presence of melphalan (Figure 12). These results may in part explain the decrease in total FANCD2 protein expression as well as FANCD2 foci formation following bortezomib treatment.

Bortezomib Enhances Melphalan-Induced DNA Damage Via Inhibition of FANCD2

Our lab previously reported that DNA interstrand cross-links (ICLs) induced by treatment with melphalan were reduced in melphalan resistant cell lines (8226/LR5 and U266/LR6) when compared to their respective drug sensitive parent cell lines (8226/S and U266/S) (3). Moreover, DNA damage repair mediated by the FA pathway, as indicated by ICL removal, was enhanced in the melphalan resistant cell lines (3-4). Based on our previous published results and our present observation that bortezomib reduces FANCD2 levels and foci formation, we hypothesized that the amount of DNA damage,

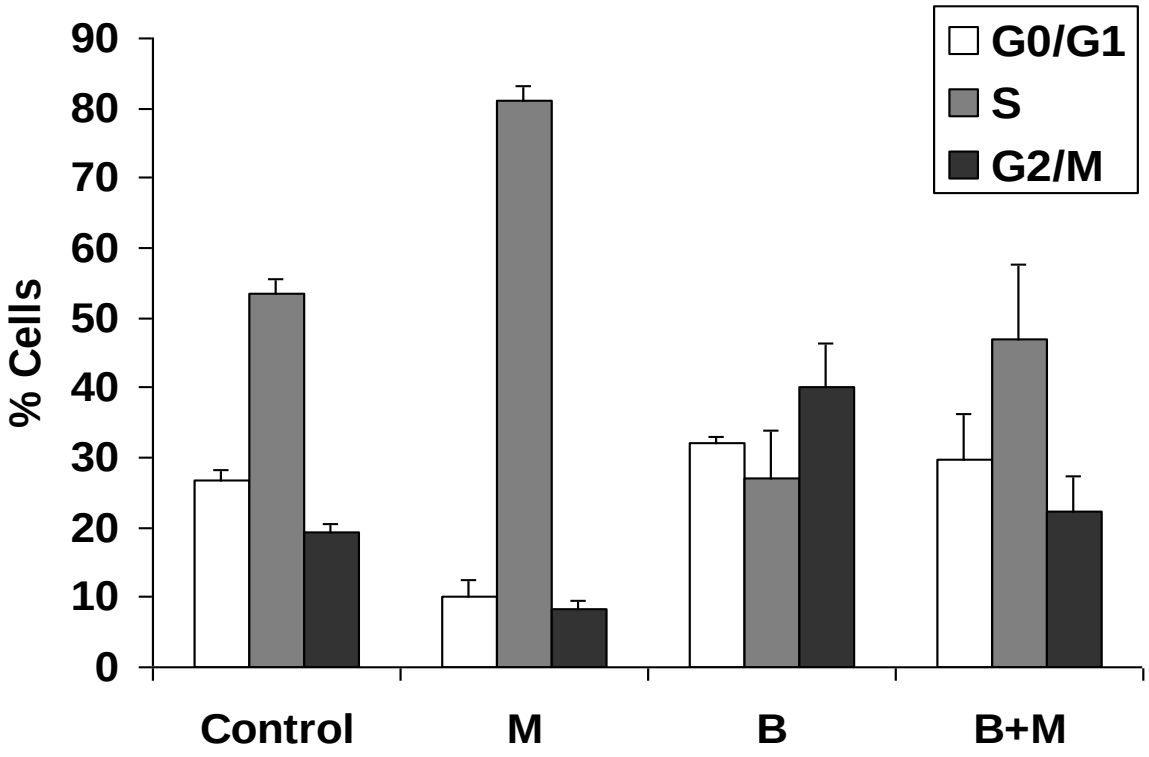


Figure 12. Bortezomib treatment decreases the proportion of cells in S-phase. 8226/LR5 cells were pre-treated with 3 nM bortezomib followed by treatment with 25 mM melphalan for 16 hours. To determine the effect of bortezomib on cell cycle, cells were then incubated with BrdU/PI for 30 minutes, fixed, and analyzed using flow cytometric techniques. The mean percentage of cells in each phase of the cell cycle and SEM are depicted, based upon three independent experiments.

as measured by ICLs, would be greater in cells treated with the combination of bortezomib plus melphalan as compared to cells treated with melphalan alone. Using the alkaline comet assay, DNA damage was measured in 8226/LR5 cells following treatment with 3 nM bortezomib alone, 25 μM melphalan alone, or the combination (Figure 13). Bortezomib treatment alone did not induce DNA damage when compared to control cells (Figure 14). Melphalan treatment induced DNA damage and DNA interstrand cross-links at levels similar to those previously reported by our lab (3). As predicted, when cells were treated with the combination of bortezomib plus melphalan, DNA damage was enhanced when compared to cells treated with melphalan alone (Figure 13A).

Because bortezomib by itself did not induce DNA damage, the increase in damage seen when cells are pre-treated with bortezomib followed by melphalan treatment (as compared to melphalan treatment alone) is likely due to reduced DNA damage repair. To determine if inhibition of the FA/BRCA pathway, and in particular FANCD2, could result in enhanced DNA damage, we used siRNA to specifically inhibit expression of FANCD2. 8226/LR5 cells were transfected with either a control siRNA (siControl) or siRNA targeting FANCD2 (siFANCD2). Following transfection, cells were treated with 25 μM melphalan for 5 hours, and alkaline comet assays were performed. As shown in Figure 13B, siRNA-mediated inhibition of FANCD2 increased the percentage of melphalan-induced ICLs when compared to the control cells. Interestingly, similar differences in percentage of ICL formation are observed when comparing bortezomib plus melphalan or siFANCD2 plus melphalan to their respective controls. This observation underscores the role of FANCD2 inhibition in the ability of bortezomib to enhance melphalan-induced DNA damage. Taken together, these results

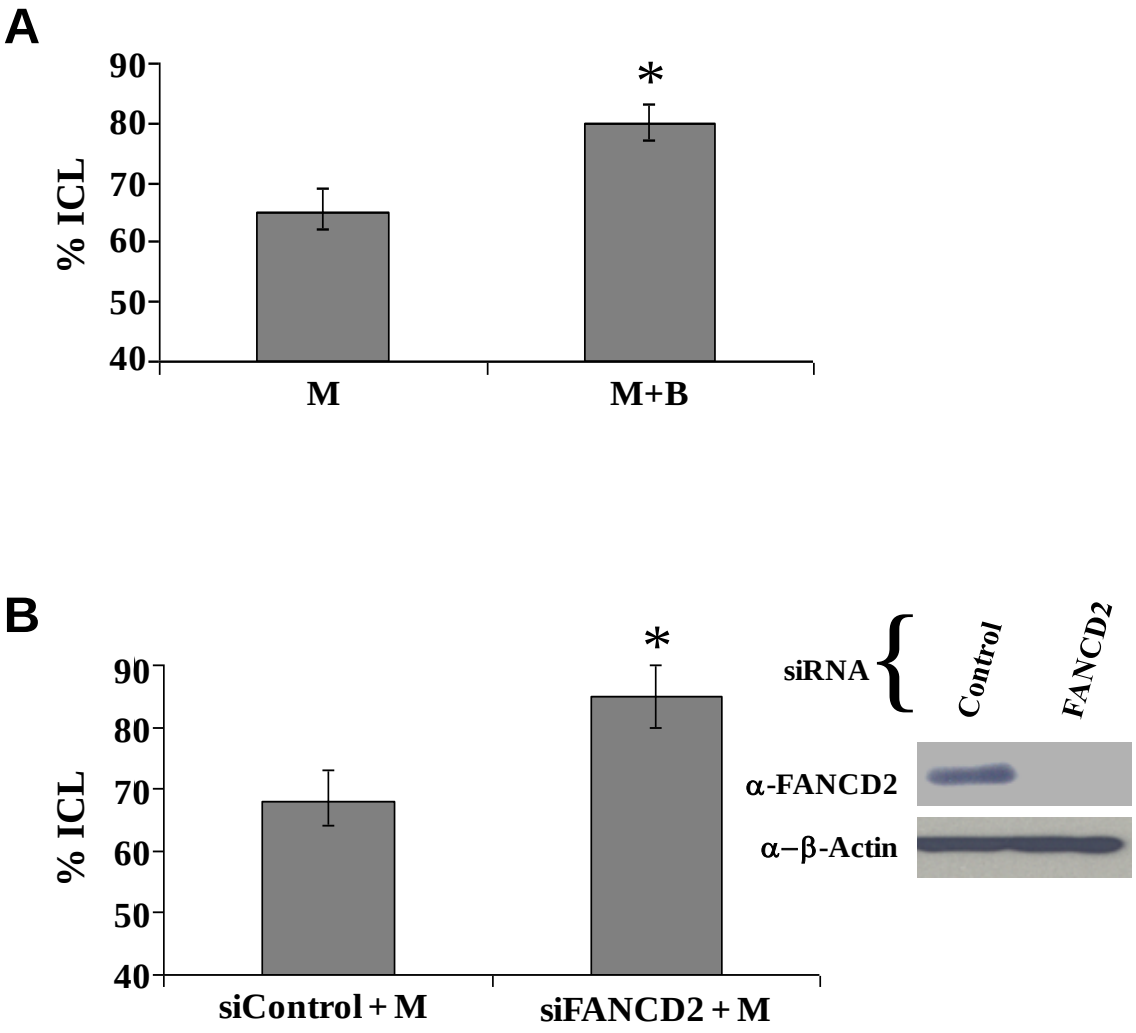


Figure 13. **Bortezomib enhances melphalan-induced DNA damage.** The alkaline comet assay was used to assess DNA damage. (A) 8226/LR5 cells were pre-treated with borteozmib for eight hours followed by five hours treatment with 25 mM melphalan. (B) FANCD2 expression was inhibited in 8226/LR5 cells using siRNA techniques. These cells were then treated with 25 mM melphalan for five hours and DNA damage was assessed. Three independent experiments were performed. A significant increase (*$p<0.0001$) in the percentage of interstrand crosslinks (% ICLs) was seen in cells treated with combination bortezomib plus melphalan when compared to melphalan treatment alone, and when comparing siControl+M to siFANCD2+M.

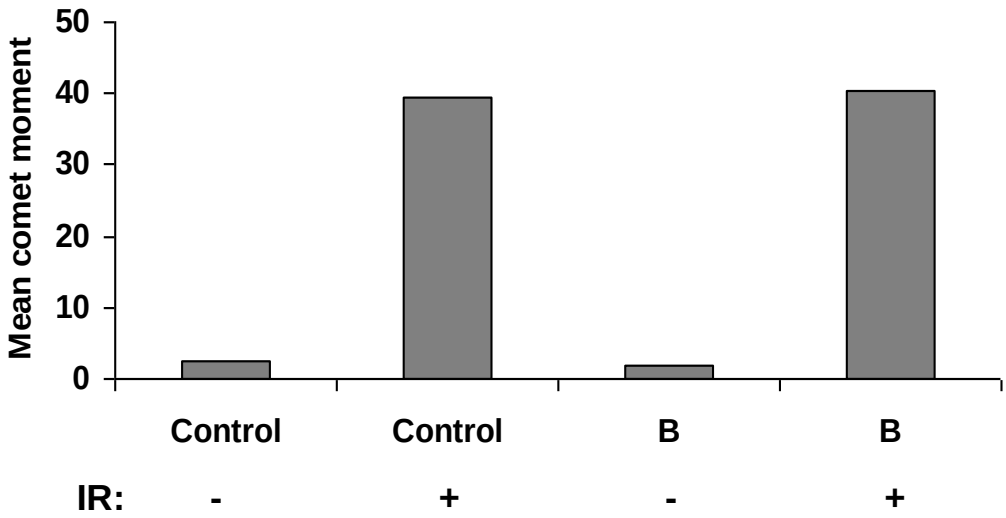


Figure 14. **Low-dose bortezomib does not induce DNA damage.** Comet moment analysis of 8226/LR5 cells treated with bortezomib revealed that low-dose (3 nM) bortezomib does not induce DNA damage when compared to control-treated samples. Three independent experiments were performed.

indicate that bortezomib enhances melphalan-induced DNA damage, leading ultimately to enhanced melphalan cytotoxicity, likely by targeting FANCD2 and the FA/BRCA pathway.

Bortezomib Reduces FA/BRCA Pathway Gene Expression in Patient Specimens

To extend the results obtained *in vitro* to a more clinically relevant model, we analyzed FA/BRCA pathway mRNA expression in purified plasma cells from myeloma patients prior to and after treatment with bortezomib. As seen in Figure 15A, a single dose of bortezomib reduced FA/BRCA mRNA expression when compared to baseline (screening) levels in three patients. Plasma cells were analyzed from a fourth patient prior to therapy, following one dose of bortezomib, two cycles of bortezomib, three months after high dose melphalan and stem cell transplant, and at time of relapse (Figure 15B). A moderate reduction in FA/BRCA pathway mRNA expression was seen in this patient's plasma cells following one dose and two cycles of bortezomib. Certain FA/BRCA genes were highly overexpressed, however, at three months post-high dose melphalan (as compared to screening levels; Figure 15B). This patient clinically relapsed only five months after completion of therapy, and FA/BRCA pathway mRNA expression levels at this time were similar to levels at the time of screening. Importantly, these preliminary results fully support our hypothesis that bortezomib treatment leads to the inhibition of FA/BRCA gene expression in MM patients.

Clinical response in five patients involved in the bortezomib study was compared to modulation of FA/BRCA pathway mRNA expression in these patients following treatment with bortezomib. One patient who showed no response, based on the Bladé

multiple myeloma response criteria and as indicated by the levels of IgG in the blood, also showed an increase in FA/BRCA pathway mRNA expression levels following one dose as well as after two cycles of bortezomib (Figure 16A). Comparatively, one patient displayed a partial response and another showed complete response, and FA/BRCA gene expression was reduced in both of these patients following one dose of bortezomib treatment (Figure 16 B and C). Finally, mRNA expression levels of two patients were analyzed at the time of relapse and compared to levels post-transplant. As seen in Figure 16D, FA/BRCA pathway expression levels showed a general trend of overexpression at the time of relapse. Importantly, these results support our hypothesis that effective bortezomib treatment leads to the inhibition of FA/BRCA mRNA expression in MM patients.

Collectively, the results presented in this section of the dissertation show that bortezomib potentiates melphalan activity by inhibiting FA/BRCA pathway gene expression, reducing FANCD2 protein expression and foci formation, and enhancing DNA damage likely via inhibition of DNA damage repair. Importantly, these results appear to translate to the clinic, as bortezomib was also shown to modulate FA/BRCA pathway mRNA expression in multiple myeloma patient specimens.

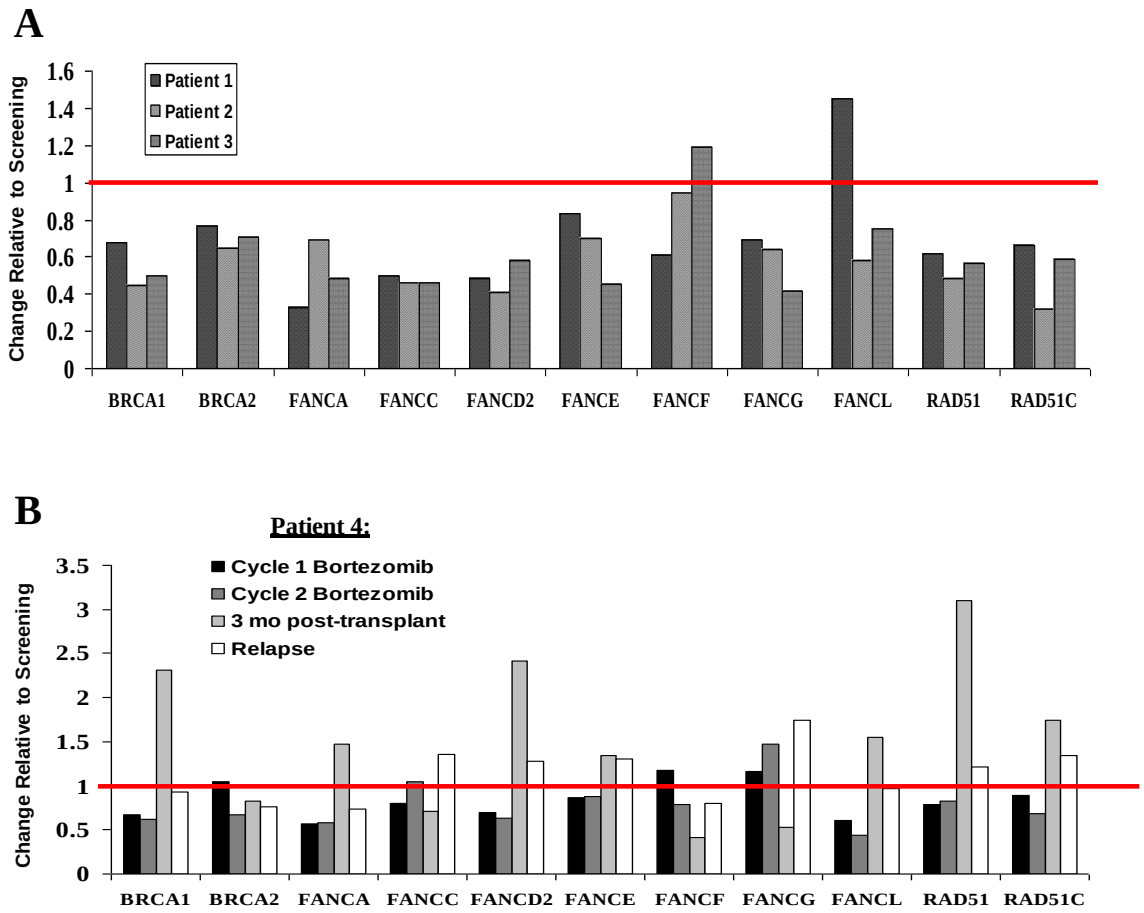
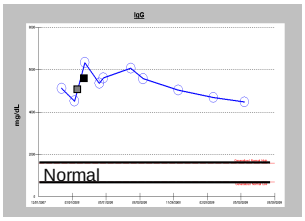
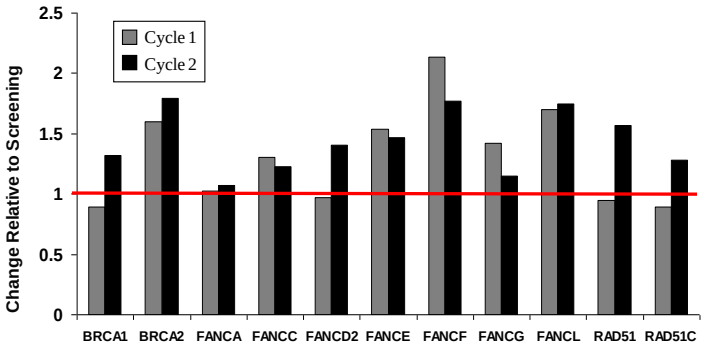


Figure 15. **Bortezomib modulates FA/BRCA pathway mRNA expression in myeloma patient specimens.** (A) Bone marrow aspirates were collected in three patients prior to treatment with bortezomib (screening) and 24 hours post-bortezomib. Plasma cells were isolated via negative selection, with >95% purity, and FA/BRCA pathway gene expression was determined using a customized microfluidic card and qPCR analysis. Fold changes were obtained by internally standardizing against GAPDH and externally standardizing against the screening sample, equal to 1 (noted by the solid red line). (B) Aspirates from one patient were collected at screening, after one dose of bortezomib, following two cycles of bortezomib, 3 months post-transplant, and at time of relapse. FA/BRCA pathway gene expression was analyzed as described in *panel A*.

A

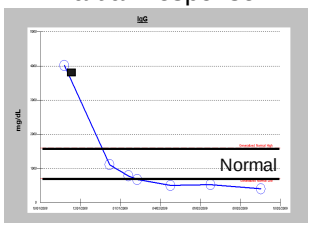
No Response

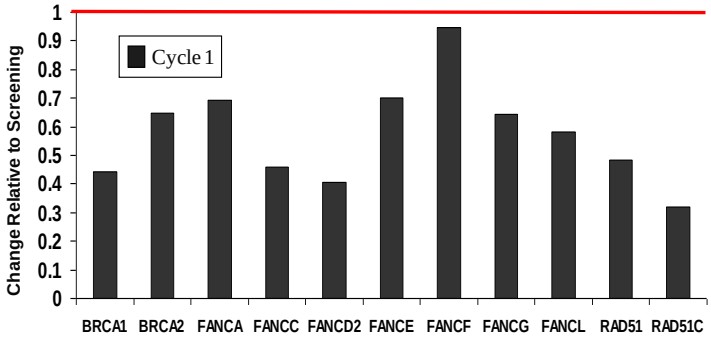




B

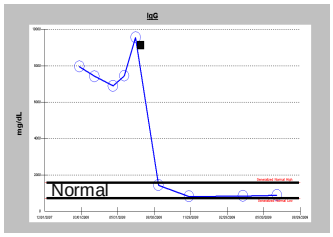
Partial Response

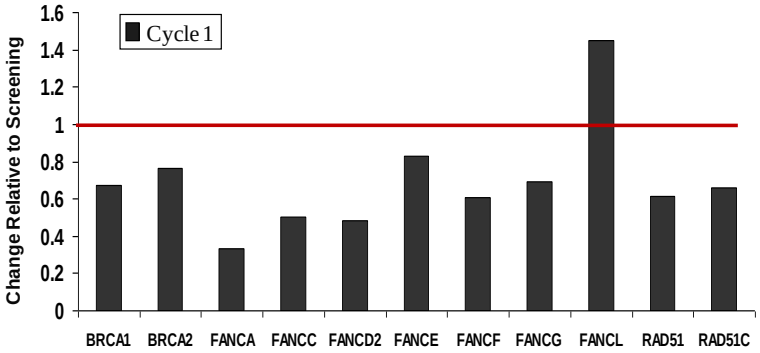




C

Complete Response





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D

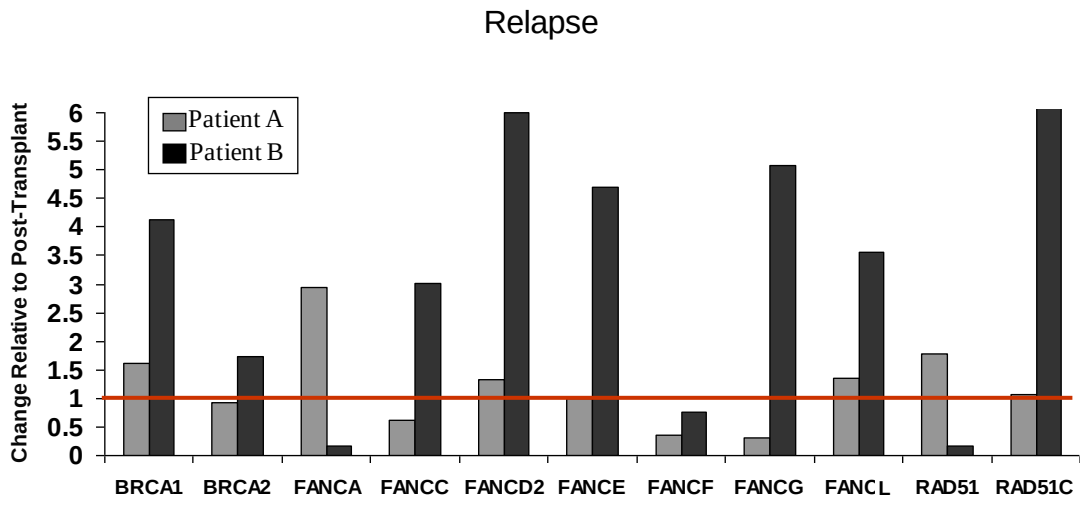


Figure 16. **Analysis of FA/BRCA pathway mRNA expression in samples taken from bortezomib-treated myeloma patients.** Following treatment with bortezomib, FA/BRCA pathway mRNA expression was analyzed in a patient who showed (A) no response, (B) partial response, and (C) complete response to treatment. Response was assessed based on the Bladé Criteria of response. Fold changes were obtained by internally standardizing against GAPDH and externally standardizing against the screening sample, equal to 1 (noted by the solid red line). (D) Aspirates from two patients were collected at time of relapse and externally standardized against corresponding samples taken three months post-transplant.

Part III:

NF-κB Transcriptionally Regulates the FA/BRCA Pathway

Our lab previously reported that many FA/BRCA pathway-related genes are overexpressed in cells selected for resistance to melphalan (3-4), and others have also implicated this pathway in the acquisition of drug resistance (170, 173). Furthermore, treatment with bortezomib was shown to inhibit expression of many of these genes in multiple myeloma cell lines as well as in patient specimens (190), suggesting that these genes may be co-regulated. This portion of the dissertation analyzes the transcriptional regulation of members of the FA/BRCA DNA damage repair pathway. Since NF-κB is constitutively activated in MM (47, 191), and since bortezomib is a known inhibitor of NF-κB (59, 78), we hypothesized that NF-κB transcriptionally regulates FA/BRCA pathway family members. Analysis of the promoter regions of the FA/BRCA family members revealed a number of putative NF-κB binding sites, and BMS-345541, a specific inhibitor of NF-κB, was able to significantly reduce expression of the FA/BRCA pathway-related genes analyzed. Using EMSAs, siRNA experiments, and ChIP analysis, we determined that two NF-κB subunits, RelB and p50, can bind to the promotor region of FANCD2. We also found that inhibiting RelB/p50 caused a reduction in FANCD2 protein levels and sensitized cells to melphalan. Importantly, re-introducing FANCD2 in these cells once again conferred resistance. Taken together, these results indicate that NF-κB, specifically the RelB and p50 subunits, trancriptionally regulate members of the FA/BRCA pathway.

Analysis of Promoter Regions of FA/BRCA Pathway Members Reveals Putative NF-κB Binding Sites

As a means to determine possible transcriptional regulators of members of the FA/BRCA pathway, the promoter regions of the 13 FA family members (*fanca, fancb, fancc, fancd1/brca2, fancd2, fance, fancf, fancg, fanci, fancj, fancl, fancm,* and *fancn*) and *brca2* were analyzed. Putative transcription factor binding sites within the -3000/+1 segments of the genomic sequences were identified using a public version of P-Match and the database provided by TRANSFAC. As seen in Figure 17, the promoter regions of 11 of the 14 genes analyzed contained putative NF-κB binding sites. Interestingly, this analysis revealed four putative NF-κB binding sites on the promoter region of *fancd2*.

Low-Dose Bortezomib Inhibits NF-κB DNA Binding Activity

Using higher doses of bortezomib, others have reported that bortezomib inhibits NF-κB activity (59, 78). We hypothesized that NF-κB transcriptionally regulates members of the FA/BRCA pathway, and so treatment with bortezomib reduces FA/BRCA pathway mRNA and protein expression via inhibition of NF-κB DNA binding activity. In our system, we wanted to analyze NF-κB DNA binding activity in the 8226/LR5 cell line following treatment with low-dose bortezomib or the combination of bortezomib plus melphalan. Using electrophoretic mobility shift assays (EMSAs), it was revealed that exposure to 3 nM bortezomib for 24 hours reduced NF-κB DNA binding activity, whereas treatment with melphalan enhanced this activity (Figure 18). Interestingly, and correlating with our FANCD2 Western blot data, NF-κB activity

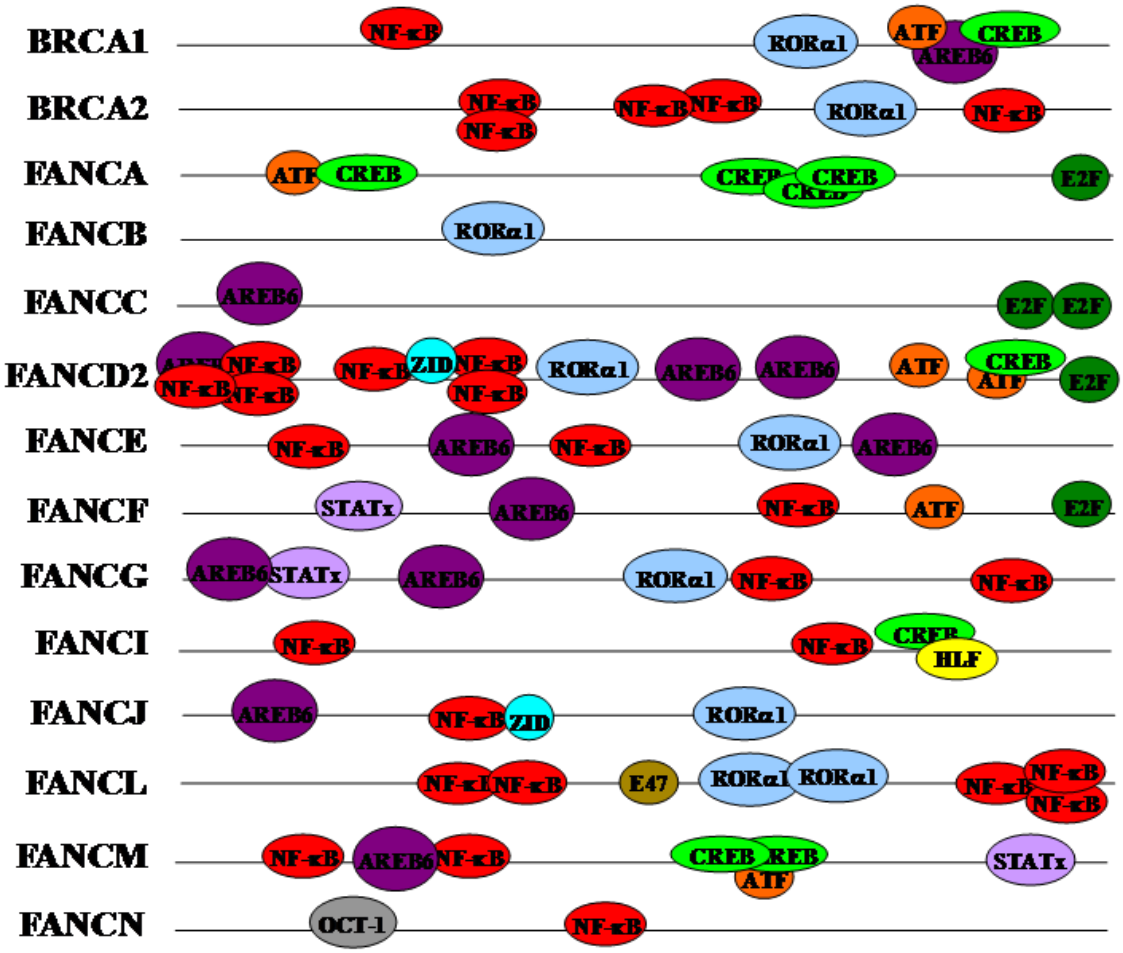


Figure 17. **Schematic view of putative transcription factor (TF)-binding sites in FA/BRCA promoter regions.** A public version of 'P-Match' (http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi) and the database provided by TRANSFAC (http://www.gene-regulation.com/pub/databases.html) were used to identify putative TF-binding sites within -3000/+1 segments of FA/BRCA genomic sequences. Using 3 sets of optimized cut-off values, matrix-scores (>0.990) were determined.

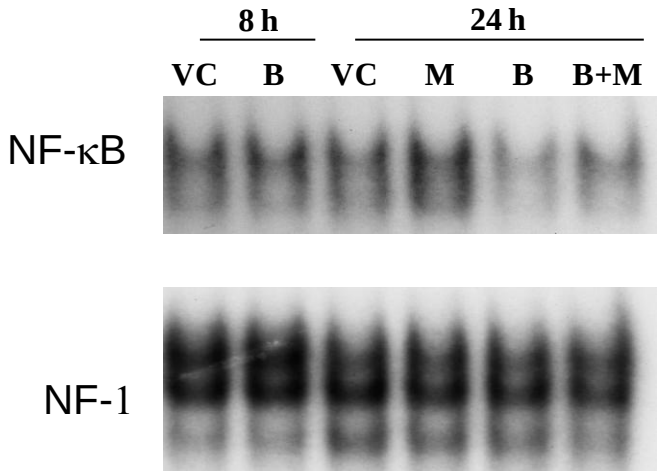


Figure 18. **Bortezomib inhibits NF-κB DNA binding activity.** 8226/LR5 cells were pre-treated with 3 nM bortezomib or vehicle control for eight hours followed by 16 hours treatment with melphalan or control. Samples were collected following eight and 24 hours treatment with bortezomib and subjected to an electrophoretic mobility shift assay (EMSA) to analyze NF-κB DNA binding activity. NF-1 was used as a loading control. A film representative of three independent experiments is shown.

was attenuated in cells pre-treated with bortezomib and subsequently exposed to melphalan, when compared to cells exposed only to melphalan as well as to control cells.

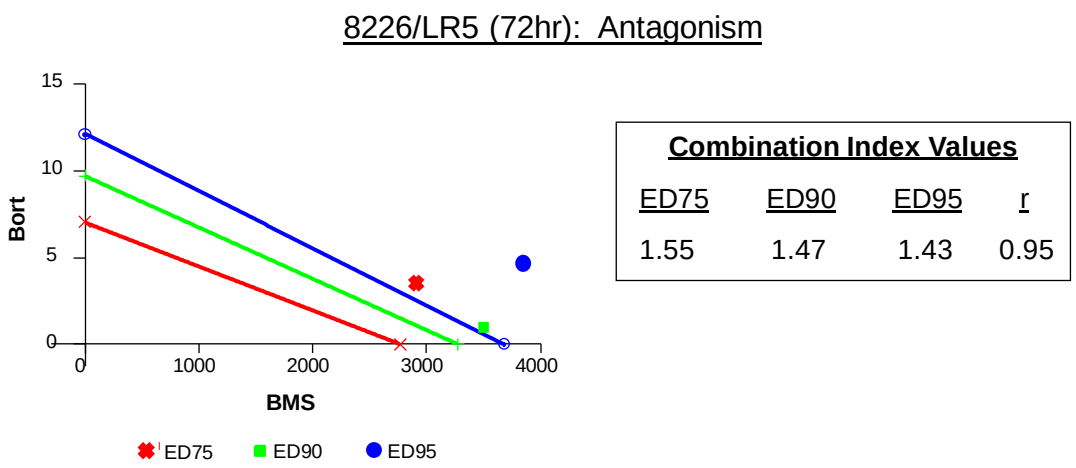
Bortezomib and BMS-345541, a Specific Inhibitor of NF-κB, are Antagonistic

We next wanted to determine if bortezomib was exacting its cytotoxic effects via inhibition of NF-κB. To this end, combination index analysis was performed using bortezomib and BMS-345541. BMS-345541 is a selective inhibitor of the IKK complex and a putative anti-tumor agent (192). If bortezomib is functioning through the NF-κB pathway, then combining it with a specific inhibitor of this same pathway should result in antagonism. 8226/LR5 and U266/LR6 cells were treated simultaneously with varying doses of bortezomib and BMS-345541 at a constant molar ratio for a total of 72 hours. Analysis of the dose-effect relationship between these two compounds, using the Chou-Talalay multiple drug effect equation (181), revealed that these compounds are antagonistic in their cytotoxic effects (Figure 19), suggesting that the effects of both agents are driven by inhibition of the NF-κB pathway.

Basal NF-κB DNA Binding Activity is Enhanced in Melphalan Resistant Cells

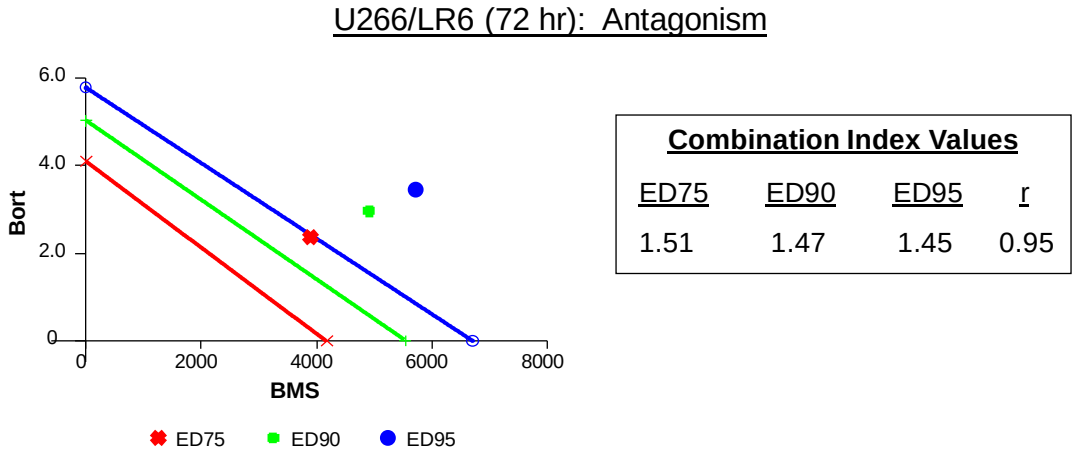
Our lab previously reported that melphalan resistant myeloma cells overexpress FA/BRCA pathway genes (3-4). Therefore, we hypothesized that, if NF-κB transcriptionally regulates members of this pathway, drug resistant multiple myeloma cells will display enhanced basal levels of NF-κB activity when compared to the drug sensitive parental cell lines. Using EMSAs to measure DNA-

A



Combination Index Values			
ED75	ED90	ED95	r
1.55	1.47	1.43	0.95

B



Combination Index Values			
ED75	ED90	ED95	r
1.51	1.47	1.45	0.95

Figure 19. **Bortezomib and BMS-345541 are antagonistic.** (A) 8226/LR5 and (B) U266/LR6 melphalan resistant myeloma cells were treated with bortezomib and BMS-345541 for 72 hours and combination index analysis was performed. Three independent experiments were performed, and a representative is shown. The results show that the two drugs act antagonistically in these cell lines, suggesting that both exact their cytotoxic effects through the same (NF-κB) pathway.

bound NF-κB complexes, we found that the melphalan resistant 8226/LR5 cells exhibit a higher degree of basal NF-κB DNA-binding activity relative to the drug sensitive 8226/S parental cells (Figure 20A).

We next analyzed NF-κB DNA binding activity in the 8226/S and 8226/LR5 cell lines following melphalan exposure. We previously reported that interstrand cross-link (ICL) accumulation in drug sensitive 8226/S cells is approximately 2-fold higher than that of melphalan-resistant 8226/LR5 cells (3, 38). Therefore, to assess directly the effect of acute melphalan exposure on NF-κB activation, we treated 8226/S and 8226/LR5 cells with 25 μM and 50 μM melphalan, respectively, as a means to induce a similar amount of ICLs. As shown in Figure 20A, 8226/LR5 cells also exhibit enhanced NF-κB DNA binding activity following melphalan exposure when compared to melphalan-treated 8226/S cells. In both cell lines, melphalan stimulation elicited a peak of NF-κB activity after 30 minutes, followed by a noticeable decrease in activity at 2 hours post-treatment. These findings indicate that acute exposure to melphalan further increases the magnitude, but fails to accelerate the rate, of NF-κB activation in drug resistant 8226/LR5 cells relative to drug sensitive cells.

Next, cellular extracts were immunoblotted with a phospho-specific IKKα/β antibody. The results showed that IKKα was constitutively phosphorylated in 8226/LR5 cells, but not in 8226/S cells (Figure 20B). Interestingly, after one hour treatment with melphalan, IKKα phosphorylation was markedly reduced in 8226/LR5 cells, and no IKKα activation was ever evident in 8226/S cells. Furthermore, we were unable to detect any basal or melphalan-induced IKKβ phosphorylation in either cell line. These results indicate that chronic exposure of myeloma cells to melphalan engages the specific

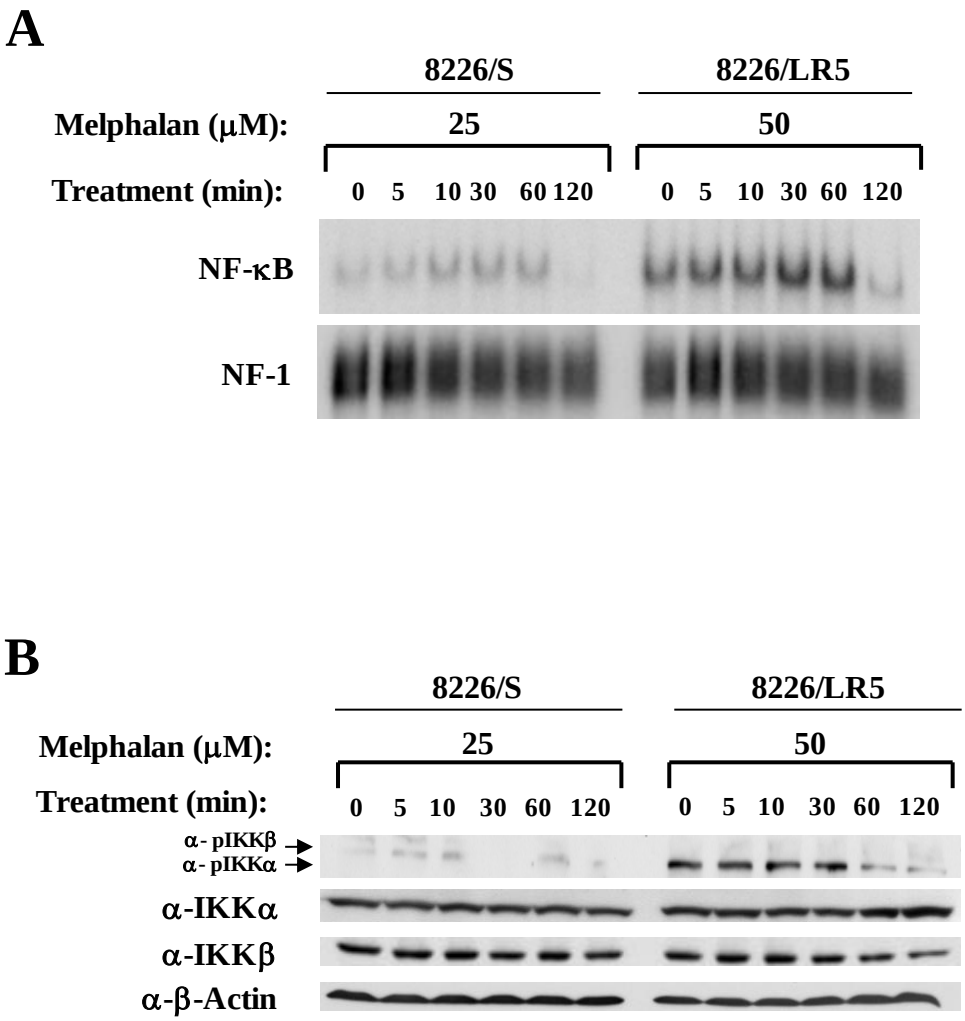


Figure 20. **Basal NF-κB DNA binding activity is enhanced in melphalan-resistant 8226 cells.** (A) Nuclear extracts were isolated from melphalan-treated 8226 cells at the indicated times and analyzed for NF-κB DNA binding activity by EMSA using a canonical κB site as a probe. Binding to a NF-1 probe was used as a loading control. (B) 8226/S and 8226/LR5 cells were treated with 25 μM and 50 μM melphalan, respectively, to induce comparable amounts of ICLs between sensitive and resistant cell lines. Cells were harvested at the indicated times post-treatment and IKK phosphorylation was determined with a α-phospho-IKKα/IKKβ specific antibody. Cellular extracts were then immunoblotted sequentially with α-IKKα, α-IκBα, α-IκBβ, and α-β-Actin antibodies. Representative blots from three independent experiments are shown.

phosphorylation of IKKα leading to a concomitant increase in basal NF-κB DNA-binding activity.

RelB and p50 Subunits are Responsible for Enhanced FANCD2-Specific NF-κB DNA Binding Activity in 8226/LR5 Cells

To further explore the possibility that NF-κB is a transcriptional regulator of the FA/BRCA pathway, we analyzed interactions between NF-κB and the promoter region of FANCD2. Using FANCD2-specific NF-κB binding sites as probes (denoted as I, II, III, and IV in Figure 21), nuclear extracts from 8226 cells were analyzed for NF-κB DNA-binding activity. The results revealed that NF-κB DNA-binding activity could only be detected with probe IV (Figure 21). Also, in support of our previous experiments that NF-κB activity is enhanced in drug resistant cells, extracts from 8226/LR5 cells exhibited a substantial increase in FANCD2-bound NF-κB complexes relative to the 8226/S cells (Figure 21).

We next performed gel shift analyses to determine which specific NF-κB subunits are required for this enhanced DNA binding activity. Incubation of cell extracts with α-p50 or α-RelB antibodies resulted in a complete loss of probe IV-specific electrophoretic signals in both the 8226/S and the 8226/LR5 cells (Figure 22A). Next, we examined the effects of siRNA inhibition of the diffrent NF-κB subunits on the binding activity of NF-κB toward FANCD2-probe IV. In correlation with the gel shift assays, knockdown of RelB or p50 reduced FANCD2-specific NF-κB activity in the 8226/LR5 cells (Figure

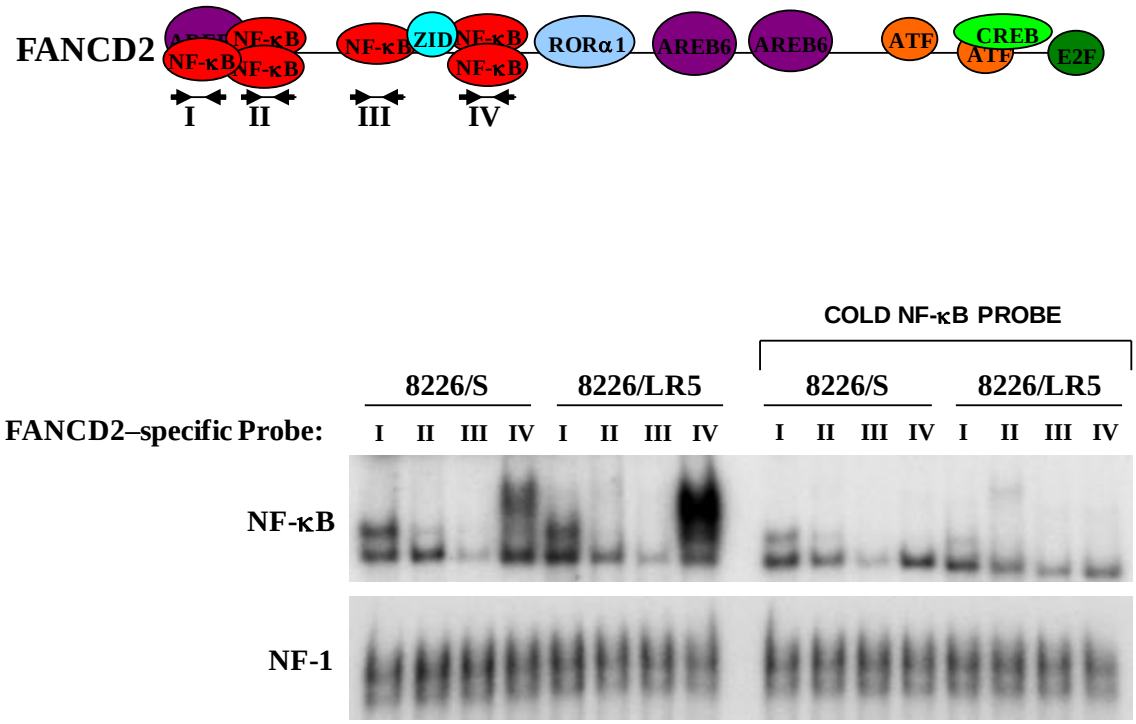
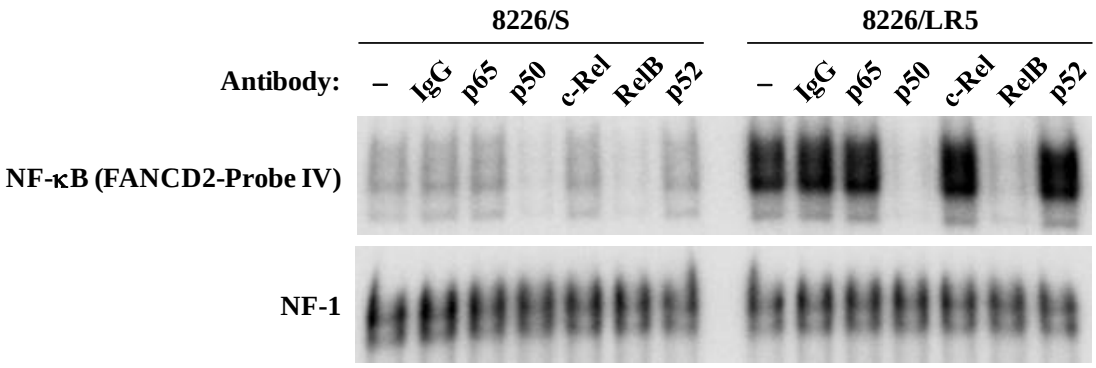


Figure 21. **NF-κB DNA binding activity is enhanced in the 8226/LR5 cell line using a FANCD2-specific probe.** Nuclear extracts from 8226/S and 8226/LR5 cells were analyzed for NF-κB DNA binding activity by EMSA using four FANCD2-specific NF-κB probes (I, II, III, and IV). The specificity of each binding reaction was demonstrated by competition with a 100-fold excess of unlabeled (cold) NF-κB canonical probe.
22B). Furthermore, direct immunoblotting of the cellular extracts used for the EMSA revealed a decrease in FANCD2 protein levels in RelB- and p50-depleted cells. A representative of three independent experiments is shown.

A



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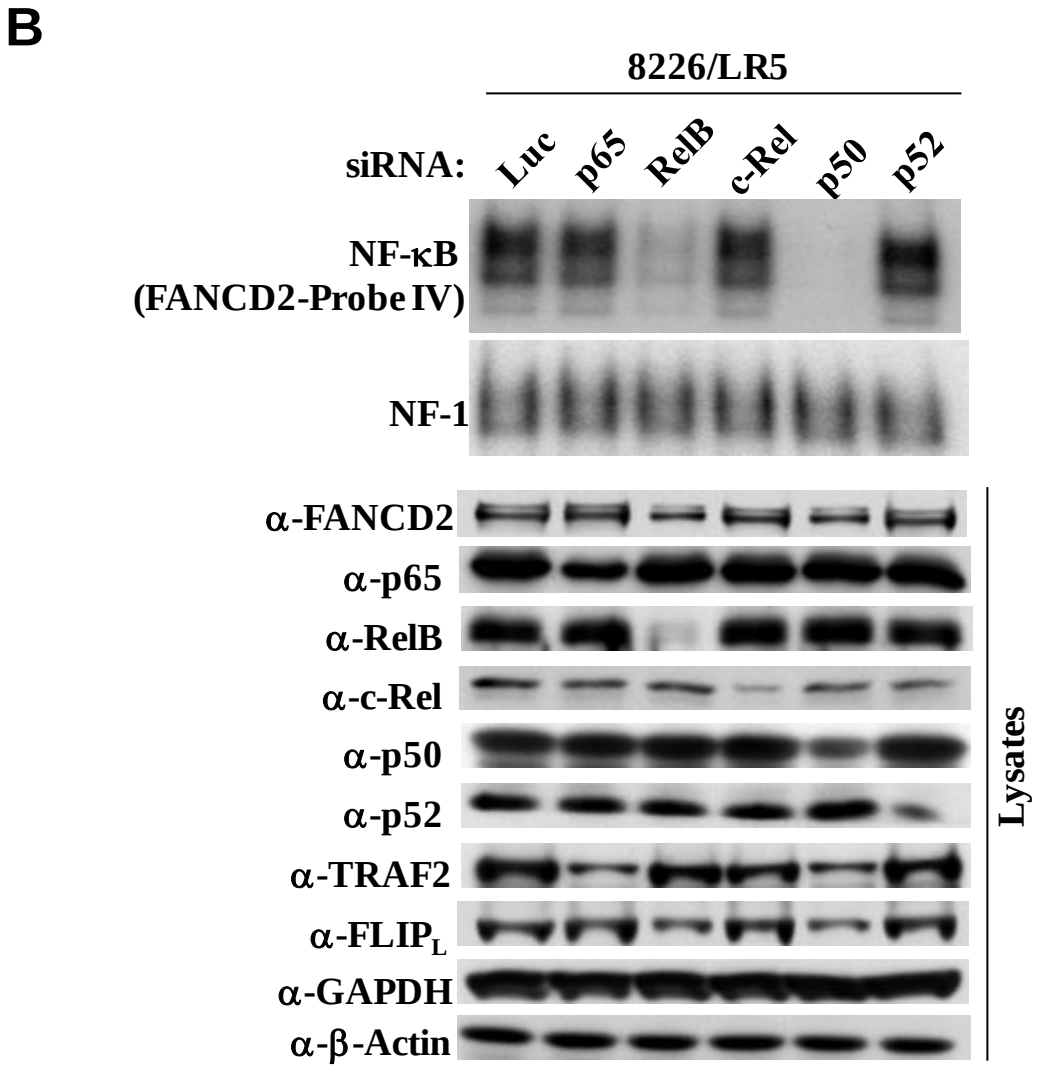

Figure 22. **RelB and p50 subunits are responsible for enhanced FANCD2-specific NF-κB DNA binding activity in the 8226/LR5 cell line**. (A) Nuclear extracts from 8226/S and 8226/LR5 cells were incubated with the indicated antibodies for 30 min prior to EMSA analysis. Binding to FANCD2-Probe IV was used as a measure of NF-κB activity on FANCD2 promoter region. (B) 8226/LR5 cells were transfected with the indicated siRNAs and examined by EMSA using FANCD2-Probe IV. Binding to the NF-1 probe was used as a loading control. The bottom panel shows expression levels of FANCD2, p65, RelB, c-Rel, p50, p52, and β-Actin at 48 h post-transfection.

Using the 8226/S and 8226/LR5 cells, we next performed chromatin immunoprecipitation assays to probe for binding between NF-κB subunits and the newly described NF-κB-binding site on the FANCD2 promoter (region IV, Figure 23). Quantitative RT-PCR analysis of α-NF-κB immunoprecipitates with FANCD2-specific primers revealed that 8226/LR5 cells exhibited a higher degree of coimmunoprecipitation of region IV with RelB, p52, and p50, when compared to 8226/S cells (Figure 23). In contrast, the association of region IV of the FANCD2 promoter with p65 and c-Rel was found to be comparable in both cell lines.

Based on the results presented above, we conclude that the increase in FANCD2-specific NF-κB activity observed in melphalan-resistant cells is due to the enhanced binding of RelB and p50 to the FANCD2 promoter in these cells.

BMS-345541 Downregulates FA/BRCA Pathway mRNA Expression in Melphalan Sensitive and Resistant Myeloma Cells

To examine the effect of loss of NF-κB function on FA/BRCA gene expression, we treated 8226 and U266 cells with 4 μM BMS-345541 and monitored the levels of mRNA transcripts over time, using a customized microfluidic card similar to the one used in the bortezomib studies. Following BMS-345541 treatment, expression of 9 of 11 genes analyzed in the 8226/S and all 11 genes in the 8226/LR5 cell lines significantly decreased in a time-dependent fashion during the first 4-8 hours post-treatment (Figure 24; Table 3). However, after 8 hours, exposure to BMS-345541 resulted in a gradual recovery in the expression of *brca1, fancc, fancl,* and *rad51* to baseline levels in both cell lines. Finally, FANCD2 protein expression in the 8226/S and 8226/LR5 cell lines was

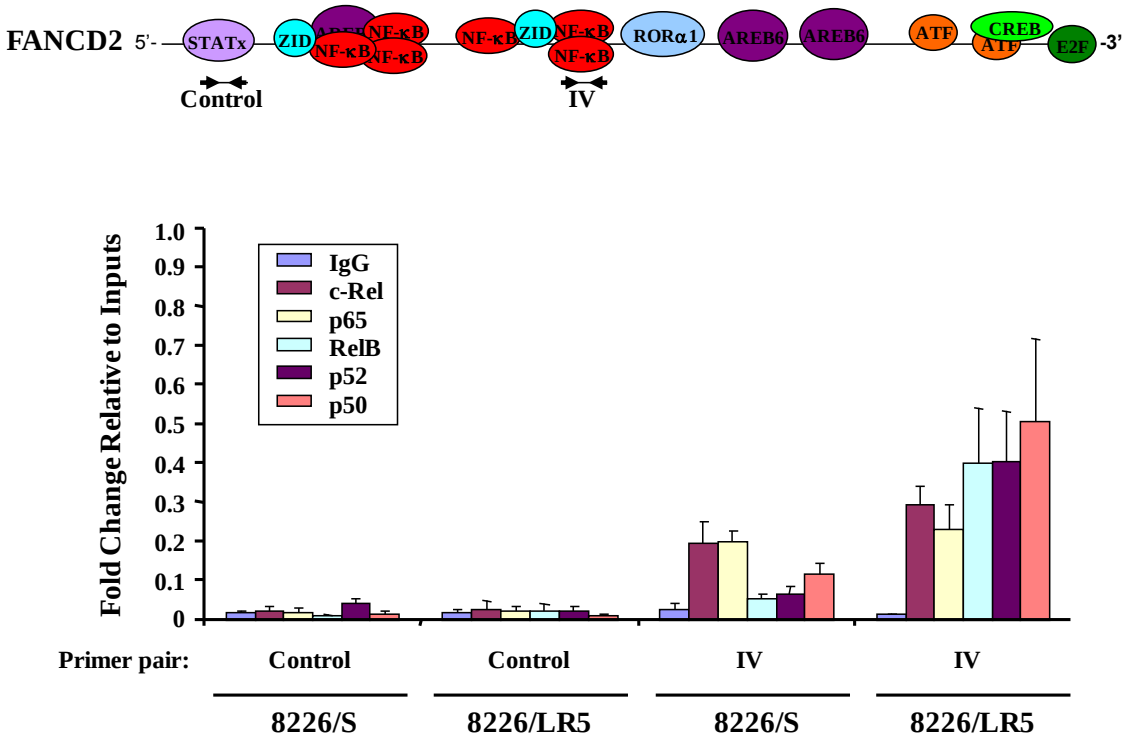

Figure 23. **NF-κB subunit binding to the promotor region of FANCD2.** Binding of NF-κB to the FANCD2 promoter is enhanced in 8226 melphalan-resistant cells as determined by chromatin immunoprecipitation analysis. DNA samples were subjected to qPCR using GAPDH and FANCD2-specific primer pairs, schematically represented as Control and IV. Results were obtained from three independent trials, and fold changes in gene expression were normalized to input samples.

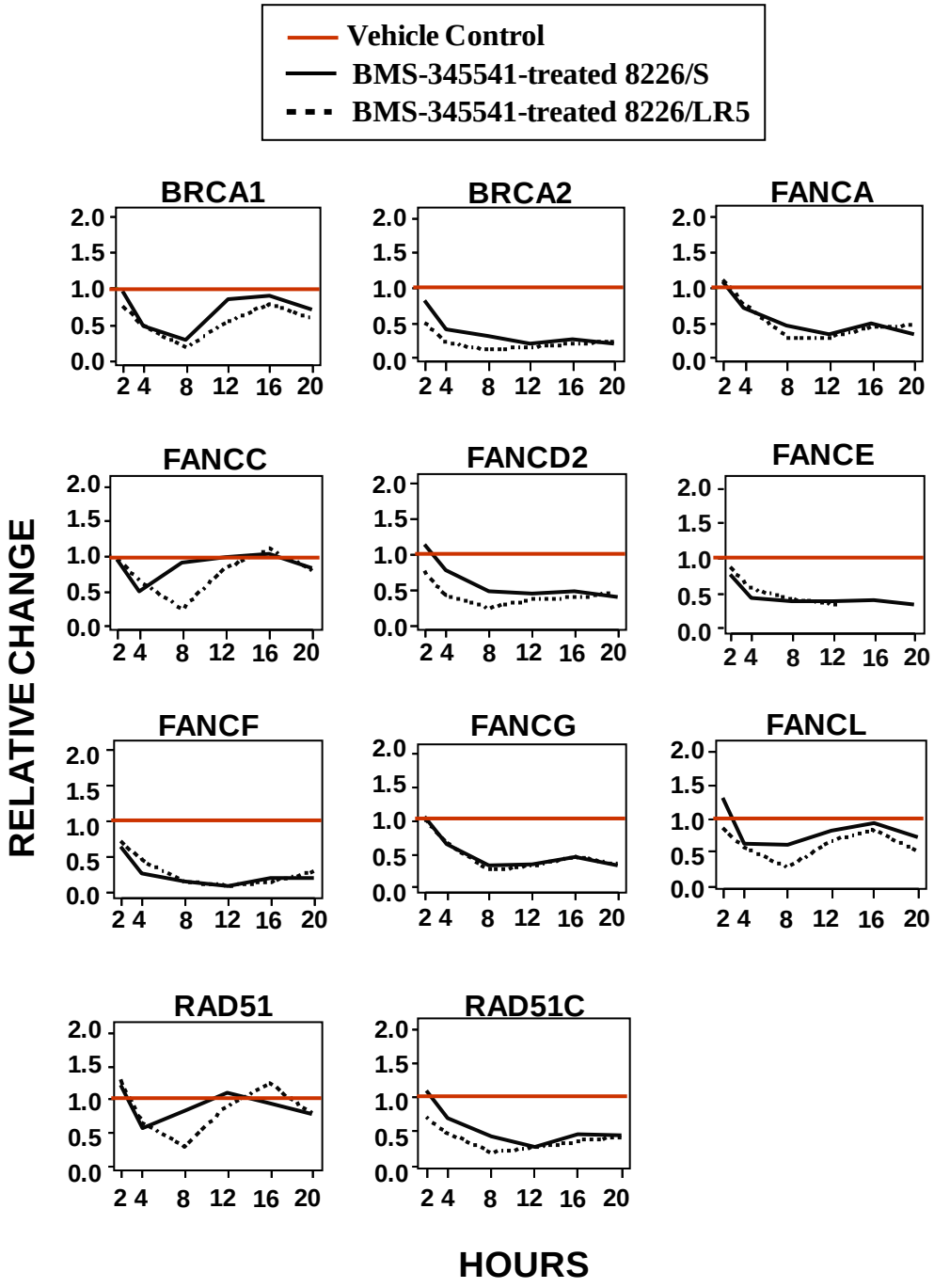

Figure 24. **BMS-345541 downregulates FA/BRCA pathway mRNA expression in melphalan sensitive and resistant myeloma cell lines**. 8226/S and 8226/LR5 cells were treated with 4 μM BMS-345541 and harvested at the indicated times. FA/BRCA gene expression was determined in quadruplicate samples by qPCR using a customized microfluidic card. Results depict fold change normalized to vehicle control (VC; i.e., DMSO) samples. Three independent experiments were performed. Statistical analysis results can be seen in Table 3.

A

Time (h)	2	4	8	12	16	20
BRCA1	0.9019	0.0908	0.0232	0.6050	0.7031	0.3794
BRCA2	0.3958	0.0038	0.0014	0.0002	0.0004	0.0004
FANCA	0.5649	0.0573	0.0015	0.0002	0.0010	0.0004
FANCC	0.8832	0.0348	0.7125	0.9441	0.8236	0.5191
FANCD2	0.5646	0.3120	0.0147	0.0043	0.0069	0.0064
FANCE	0.1443	0.0015	0.0009	0.0004	0.0004	0.0005
FANCF	0.0365	0.0004	0.0002	0.0001	0.0001	0.0003
FANCG	0.7314	0.0069	0.0001	0.0001	0.0002	0.0001
FANCL	0.5048	0.3810	0.3634	0.6050	0.8236	0.5191
RAD51	0.4089	0.0705	0.4959	0.6170	0.7551	0.3794
RAD51C	0.5646	0.0655	0.0017	0.0001	0.0009	0.0021

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B

Time (h)	2	4	8	12	16	20
BRCA1	0.2557	0.0313	0.0028	0.0509	0.3017	0.0783
BRCA2	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCA	0.3849	0.0509	0.0001	0.0001	0.0004	0.0006
FANCC	0.6332	0.0029	<0.0001	0.1172	0.2557	0.0617
FANCD2	0.1999	0.0064	0.0012	0.0037	0.0052	0.0084
FANCE	0.2557	0.0055	0.0007	0.0003	0.0007	0.0008
FANCF	0.0313	0.0008	<0.0001	<0.0001	<0.0001	0.0001
FANCG	0.8243	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCL	0.4414	0.0496	0.0029	0.1024	0.3715	0.0271
RAD51	0.2056	0.1207	0.0055	0.6132	0.2810	0.2965
RAD51C	0.0164	0.0002	<0.0001	<0.0001	<0.0001	0.0001

Table 3. **Statistical analysis of FA/BRCA gene expression after BMS-345541 treatment in 8226 cells.** To test the effect of BMS-345541 on FA/BRCA gene expression in 8226/S (A) and 8226/LR5 (B) cells, we examined whether fold change of gene expression was deviated away from 1. Since each gene showed a non-linear pattern of fold change over time (see Figure 19), a linear model with time as a categorical explanatory variable was used to test the null hypothesis of fold change=1 at each time point for each gene in each cell line. Since we tested 11 FA/BRCA related genes, we adjusted for p-value based on the false discovery rate to control for simultaneously testing (Benjamini, 1995).

also analyzed following exposure to 4 μM BMS-345541. As seen in Figure 25A, FANCD2 protein expression is reduced in both cell lines following exposure to BMS-345541.

We next analyzed FA/BRCA pathway mRNA expression in U266 cells using a newly designed microfluidic card to incorporate recently added FA members (this card allowed for the analysis of all 13 FA genes as well as *brca1* and *usp1*). Similar to results seen in the 8226 cells, exposing U266 cells to BMS-345541 resulted in a dramatic decrease in the expression of all 13 FA/BRCA pathway genes after 8 hours, followed by a significant recovery in the expression of *brca2, fancb, fancc, fancd2, fance, fancf, fancl, fancm,* and *fancn* in U266 and/or U266/LR6 cells at 12 hours post-BMS-345541 treatment (Figure 26; Table 4). The biological significance of the latter event remains unclear, and results suggest that compensatory mechanisms in response to a protracted loss of NF-κB activity are involved in the regulation of the expression of these genes.

To further examine the role of NF-κB in melphalan resistance, we treated 8226 cells with increasing amounts of BMS-345541 for 96 hours. Relative to sensitive cells, melphalan-resistant cells displayed a marked decrease in cell growth after BMS-345541 exposure (Figure 25B). Taken together, these results suggest that NF-κB regulates FA/BRCA expression, and that a sustained reduction in FA/BRCA gene expression is especially cytotoxic to melphalan-resistant myeloma cells.

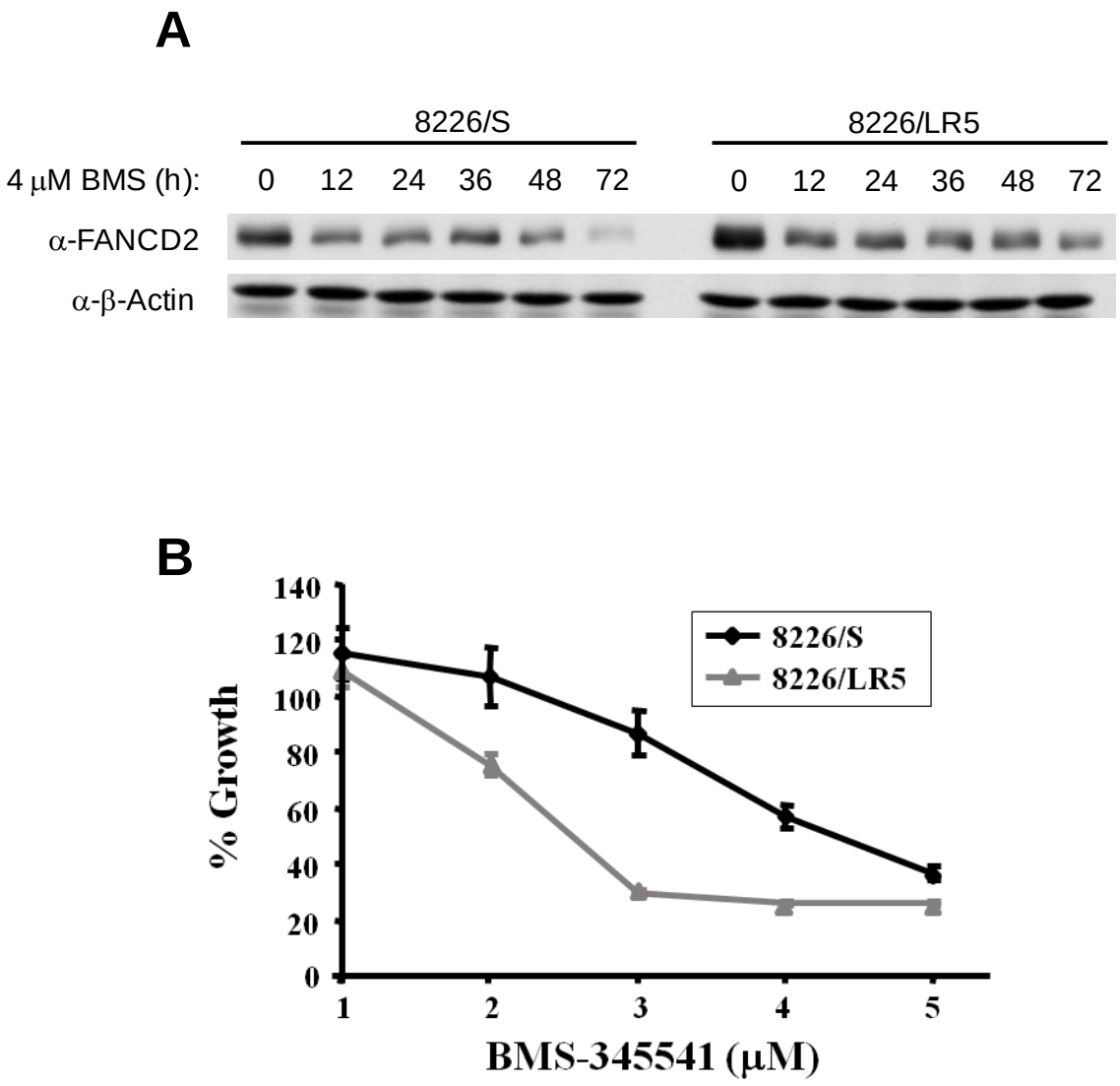

Figure 25. **BMS-345541 reduces FANCD2 protein expression and inhibits growth of 8226 cells.** (A) 8226/S and 8226/LR5 cells were treated with 4 μM BMS-345541 for 12, 24, 36, 48, and 72 h. Cellular extracts were harvested, resolved by SDS-PAGE, and immunoblotted with the indicated antibodies. β-Actin served as control for sample loading. (B) 8226/S and 8226/LR5 cells were treated with increasing amounts of BMS-345541 for 96 h and the percentage of cell growth was determined using a standard methyl-thiazol tetrazolium (MTT) colorimetric assay. Results (mean +/- SEM) were obtained from four independent trials.

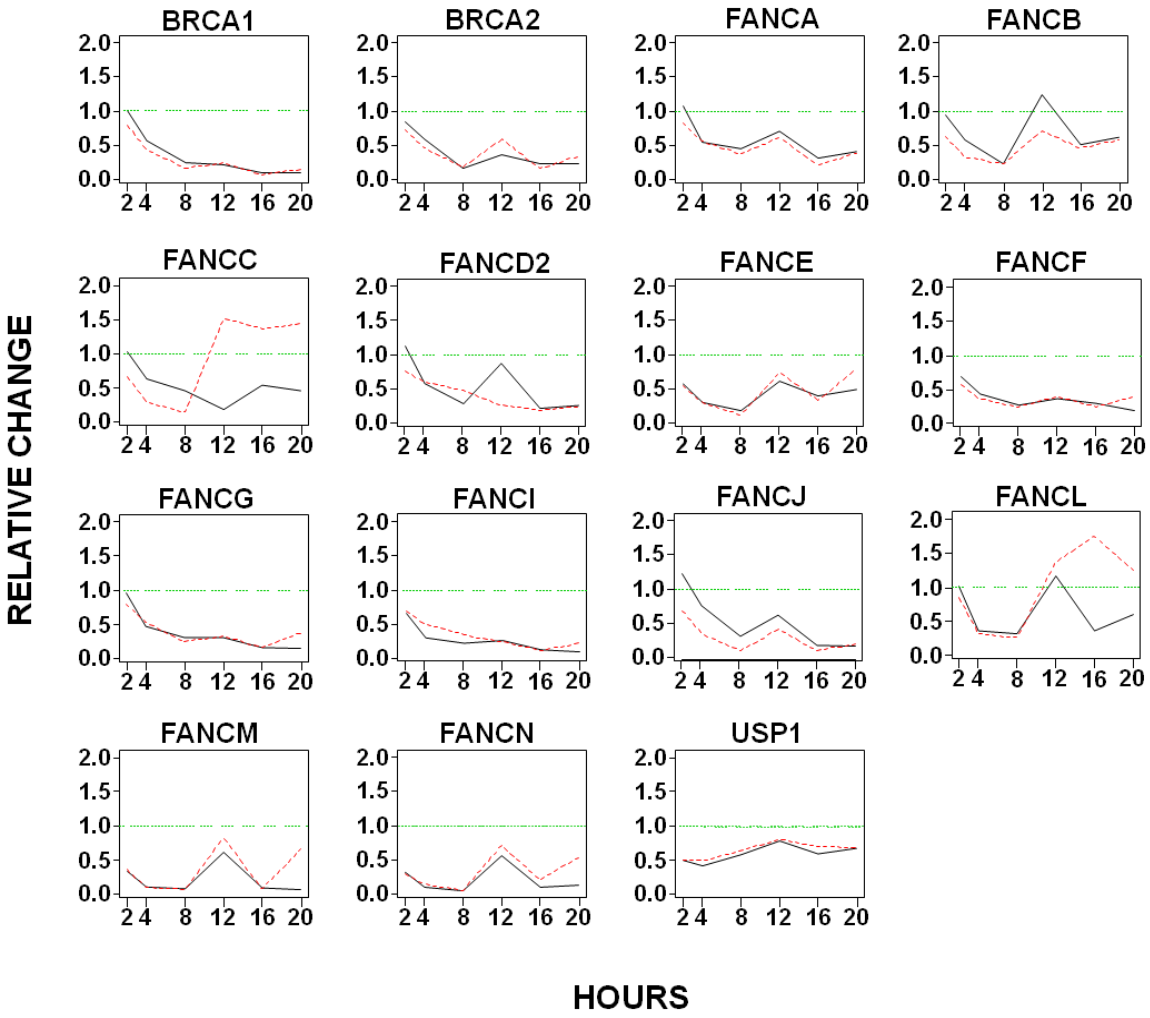

Figure 26. **BMS-345541 reduces FA/BRCA pathway mRNA expression in U266 and U266/LR6 cell lines.** U266 and U266/LR6 cells were treated with BMS-345541 as described in *Figure 24.* A newly designed microfluidic card was used to measure the expression of all 13 FA/BRCA genes, as well as brca1 and usp1. Results depict fold change normalized to vehicle control (VC; i.e., DMSO) in triplicate samples. Statistical analysis results can be seen in Table 4.

A

Time (h)	2	4	8	12	16	20
BRCA1	0.8900	0.0001	<0.0001	<0.0001	<0.0001	<0.0001
BRCA2	0.2013	0.0035	<0.0001	0.0001	<0.0001	<0.0001
FANCA	0.3032	0.0001	<0.0001	0.0026	<0.0001	<0.0001
FANCB	0.6186	0.0023	<0.0001	0.0398	0.0008	0.0039
FANCC	0.8028	0.0118	0.0008	<0.0001	0.0028	0.0009
FANCD2	0.1610	0.0005	<0.0001	0.2105	<0.0001	<0.0001
FANCE	0.0002	<0.0001	<0.0001	0.0004	<0.0001	<0.0001
FANCF	0.0022	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCG	0.8028	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCI	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCJ	0.0043	0.0057	<0.0001	0.0002	<0.0001	<0.0001
FANCL	0.8233	0.0001	<0.0001	0.1442	0.0001	0.0035
FANCM	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCN	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
USP1	<0.0001	<0.0001	<0.0001	0.0013	<0.0001	<0.0001

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B

Time (h)	2	4	8	12	16	20
BRCA1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
BRCA2	0.0009	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCA	0.0493	0.0001	<0.0001	0.0003	<0.0001	<0.0001
FANCB	0.0003	<0.0001	<0.0001	0.0017	<0.0001	0.0001
FANCC	0.0019	<0.0001	<0.0001	<0.0001	0.0007	0.0002
FANCD2	0.0022	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCE	0.0001	<0.0001	<0.0001	0.0070	<0.0001	0.0222
FANCF	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCG	0.0008	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCI	0.0010	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCJ	0.0012	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FANCL	0.3242	0.0005	0.0003	0.0202	0.0002	0.0898
FANCM	<0.0001	<0.0001	<0.0001	0.0358	<0.0001	0.0003
FANCN	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
USP1	<0.0001	<0.0001	0.0001	0.0069	0.0003	0.0002

Table 4. **Statistical analysis of FA/BRCA gene expression after BMS-345541 treatment in U266 cells.** To test the effect of BMS-345541 on FA/BRCA gene expression in U266 (A) and U266/LR6 (B) cells, we examined whether fold change of gene expression was deviated away from 1. Since each gene showed a non-linear pattern of fold change over time (see Figure 21), a linear model with time as a categorical explanatory variable was used to test the null hypothesis of fold change=1 at each time point for each gene in each cell line. Since we tested 15 FA/BRCA related genes, we adjusted for p value based on the false discovery rate to control for simultaneous testing (Benjamini, 1995).

Loss of RelB/p50 Reduces FANCD2 Expression and Re-Sensitizes 8226/LR5 Cells to Melphalan

To further examine the role of NF-κB in the regulation of FANCD2 expression and function following treatment with melphalan, we determined whether RelB/p50 double-knockdown was sufficient to sensitize 8226/LR5 cells to melphalan. Cells were transfected with control or RelB and p50 siRNAs, then exposed to varying doses of melphalan for 24 hours. Apoptosis, as indicated by the amount of DNA fragmentation, was determined using the Diffusion Apoptosis Slide Halo (DASH) assay. Treatment of 8226/LR5 cells with RelB and p50 siRNAs significantly sensitized these cells to melphalan-induced cell death (Figure 27A; Table 5). Furthermore, relative to wild type 8226/LR5 cells, RelB/p50-depleted 8226/LR5 cells displayed a dramatic increase in ICLs after exposure to melphalan, with levels of DNA damage significantly surpassing those observed in melphalan-sensitive cells (Figure 27B; Table 6). Statistical analysis also showed that the results presented in Figures 27A and 27B were positively correlated at each dose of melphalan tested, suggesting that loss of cell viability is causally linked to melphalan-induced ICL formation (Table 7). Importantly, consistent with the results obtained with RelB- and p50-depleted cells (Figure 22), RelB/p50 double-knockdown caused a striking decrease in FANCD2 protein expression (Figure 27C). Finally, re-expression of FANCD2 in the RelB/p50-depleted cells restored melphalan resistance (Figure 27D). These results indicate that RelB and p50 protect 8226/LR5 cells from melphalan-induced apoptosis, at least in part, by regulating expression of the DNA damage response protein FANCD2. Collectively, the results presented in this section demonstrate that NF-κB, specifically the RelB and p50 subunits, positively regulates

FA/BRCA pathway expression, and provide evidence for targeting this pathway as a means to overcome or even circumvent drug resistance.

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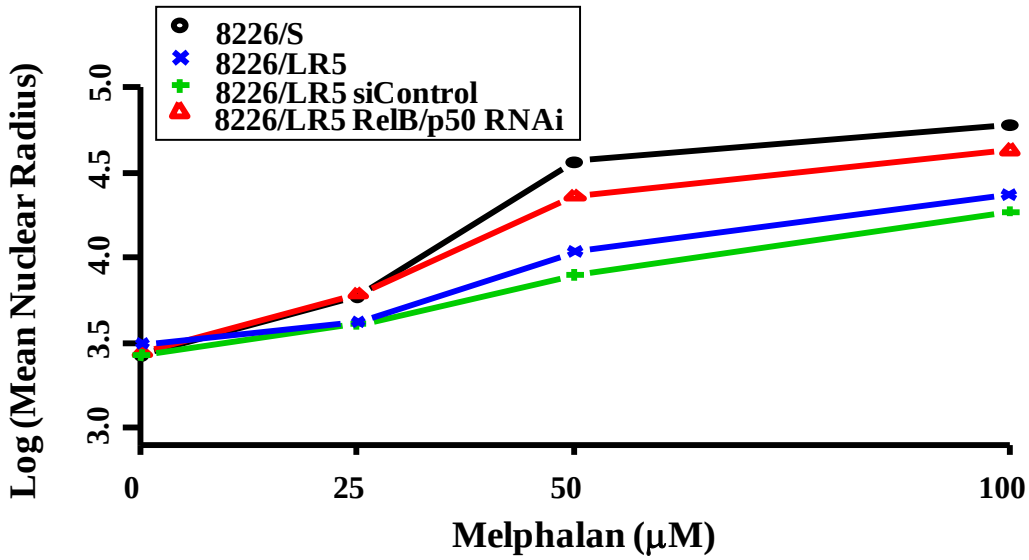

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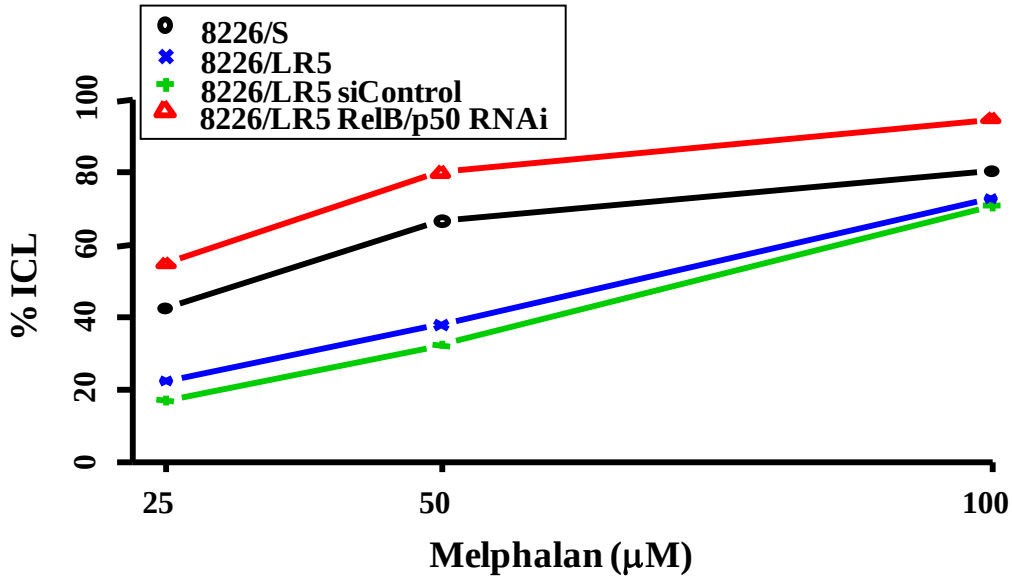

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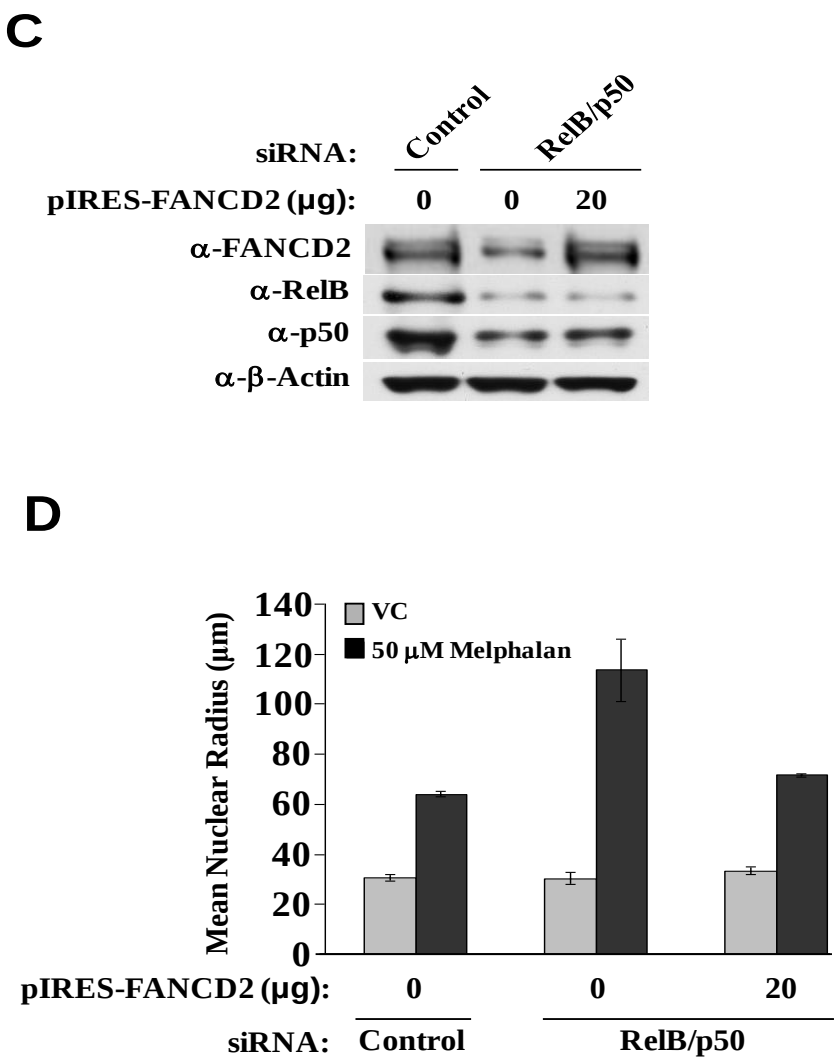

Figure 27. **Loss of RelB and p50 re-sensitizes 8226/LR5 cells to melphalan treatment and reduces FANCD2 expression.** (A) 8226/LR5 cells were treated with the indicated siRNAs for 48 h, stimulated with 25-100 μM melphalan or vehicle control for 24 h, and the relative amount of DNA fragmentation was determined by DASH Assay. Wild-type 8226/S and 8226/LR5 cells were used as reference samples. (B) At 48 h post-transfection, the cells were exposed to 25-100 μM melphalan for 2 h, damaged with 9 Gy ionizing radiation, and interstrand cross-links were evaluated using the Alkaline Comet Assay. Wild-type 8226/S and 8226/LR5 cells were used as reference samples. (C) Representative immunoblots from 8226/LR5 cells transfected with siControl (control) or RelB and p50 siRNAs, as well as from cells transfected with RelB/p50 siRNAs plus pIRES-FANCD2. (D) 8226/LR5 cells were treated with the indicated siRNAs, and after 24 h, the cells were transfected with empty vector or untagged, full-length FANCD2 (pIRES-FANCD2). At 48 h post-siRNA transfection, the cells were stimulated for 24 h with either vehicle control (VC) or 50 μM melphalan, and the relative amount of DNA fragmentation was determined as described in *panel A*. Results (mean ± S.E) were obtained from three independent trials.

Adjusted P-value (Tukey's Method)	Melphalan (μM)			
	0	25	50	100
LR5 siControl - LR5	0.5300	0.9856	0.3302	0.3053
LR5 RelB/p50 - LR5	0.6548	0.0064	0.0098	0.0038
8226/S - LR5	0.4897	0.0078	0.0004	0.0002
LR5 RelB/p50 - LR5 siControl	0.9956	0.0042	0.0011	0.0005
8226/S - LR5 siControl	0.9998	0.0051	0.0001	<0.0001
8226/S - LR5 RelB/p50	0.9898	0.9983	0.0966	0.0715

Table 5. **Statistical analysis of melphalan-induced DNA fragmentation in RelB/p50 siRNA-treated cells.** Analysis of variance (ANOVA) was used to compare the radius difference (log scale) of cell death between four groups: (8226/S, LR5 RelB/p50, LR5 siControl, and LR5), at each dose level (0, 25, 50, and 100 μM) in Figure 22A. Multiple comparisons of nuclear DNA diffusion were adjusted with the Tukey's Honest Significance Difference method (Miller, 1981). A P-value <0.05 was considered significant. These results show that siRNA-mediated depletion of RelB and p50 in 8226/LR5 cells significantly increases the magnitude of cell death induced by melphalan stimulation.

	Estimate	P-value
Intercept Difference (Melphalan = 25 μM)		
LR5 siControl – LR5	-0.060	0.0747
LR5 RelB/p50 – LR5	0.377	<0.0001
8226/S – LR5	0.254	<0.0001
Linear Slope Difference		
LR5 siControl – LR5	0.001	0.5015
LR5 RelB/p50 – LR5	-0.002	0.0202
8226/S – LR5	-0.002	0.0082

Table 6 **Statistical analysis of melphalan-induced ICL in RelB/p50 depleted cells.** A linear regression model was used to analyze the percentage of ICLs obtained from Figure 22B cell populations. Intercept and slope differences were determined using 8226/LR5 cells as a reference cell line. A P-value <0.05 was considered significant. These results show that RelB/p50-depleted cells display a significant increase in ICL formation after exposure to melphalan relative to wild-type or siControl-transfected 8226/LR5 cells.

		Dash Data			
	Melphalan (μM)	0	25	50	100
Comet Data	25	-0.22	0.85	0.8	0.83
	50	-0.21	0.89	0.83	0.85
	100	-0.16	0.78	0.65	0.67

Table 7. **Correlation between melphalan-induced ICL and DNA fragmentation.** Relationship of DNA fragmentation and ICL was assessed by Pearson correlation. Note that a zero correlation indicates no association between the two variables while a correlation close to 1 suggests a strong positive correlation. These results show that ICL formation and DNA fragmentation can be positively correlated in 8226 cells after melphalan-induced stress.

Part IV:

Post-Transcriptional Regulation of FANCD2 by Bortezomib

The results presented in Parts II and III of this dissertation show that NF-κB transcriptionally regulates members of the FA/BRCA DNA damage repair pathway, and that bortezomib can inhibit FA/BRCA pathway mRNA expression, as well as FANCD2 protein expression and foci formation. We also show that bortezomib enhances melphalan-induced DNA damage, likely via inhibition of FA/BRCA pathway-mediated DNA damage repair. Since we found that FA/BRCA pathway mRNA expression was attenuated by bortezomib, but not to the same extent as the inhibition of FANCD2 protein expression, we analyzed regulation of the FA/BRCA pathway downstream of transcriptional regulation. We hypothesized that bortezomib not only reduces FANCD2 protein expression via reduction of FANCD2 mRNA levels, but also by directly targeting FANCD2 protein. Therefore, we analyzed FANCD2 protein stability via stable isotopic labeling of amino acids in cell culture (SILAC) and mass spectrometry. We also analyzed the effects of various miRNAs on the expression of FANCD2, since miRNAs have been shown to repress translation without cleavage of the mRNA (193-194). Finally, we studied ATR activation and cell cycle progression in cells treated with bortezomib. In this section, we show that bortezomib inhibits FANCD2 synthesis and ATR activation, and also overcomes melphalan-induced S-phase arrest.

Bortezomib Inhibits FANCD2 Synthesis

FANCD2 protein stability was analyzed in bortezomib-treated 8226/LR5 cells via stable isotopic labeling of amino acids in cell culture (SILAC) and mass spectrometry.

Cells were labeled with “heavy” media containing stable isotopes of lysine and arginine for six doubling times (approximately seven days). The media was then replaced with “light” (regular) media, containing the naturally-occurring forms of lysine and arginine. At the time of addition of the light media, cells were also treated with vehicle control or 3 nM bortezomib, and samples were collected at 0, 4, 8, 12, 24 and 36 hours post-bortezomib treatment. Incorporation of “heavy” lysine and arginine during protein synthesis causes a shift in the molecular weight of the protein that can be detected by mass spectrometry. Therefore, we were able to analyze FANCD2 degradation (as indicated by reduction in “heavy” label over time) and synthesis (as indicated by increased “light” expression over time) following bortezomib treatment, based on seven FANCD2-specific peptides. Control- and bortezomib- treated lysates were separated on a gel (Figure 28A; shows equal loading of lysates), and the FANCD2 bands were excised and analyzed via mass spectrometry.

Total FANCD2 protein expression, as indicated by the sum of the heavy and light labeled FANCD2, is reduced at 24 hours in bortezomib-treated cells when compared to control-treated cells (Figure 28B). These findings are similar to the results seen via Western blot analysis of FANCD2 protein expression following bortezomib treatment (Figure 10). Degradation of FANCD2 protein, as indicated by reduction in the “heavy” protein, is similar in control- and bortezomib-treated cells up to 12 hours (Figure 28C). At 24 and 36 hours, however, a slight increase in FANCD2 degradation is seen in the bortezomib-treated cells. FANCD2 protein synthesis, on the other hand, is drastically inhibited by bortezomib at 24 and 36 hours post-treatment (Figure 28D). In total, these

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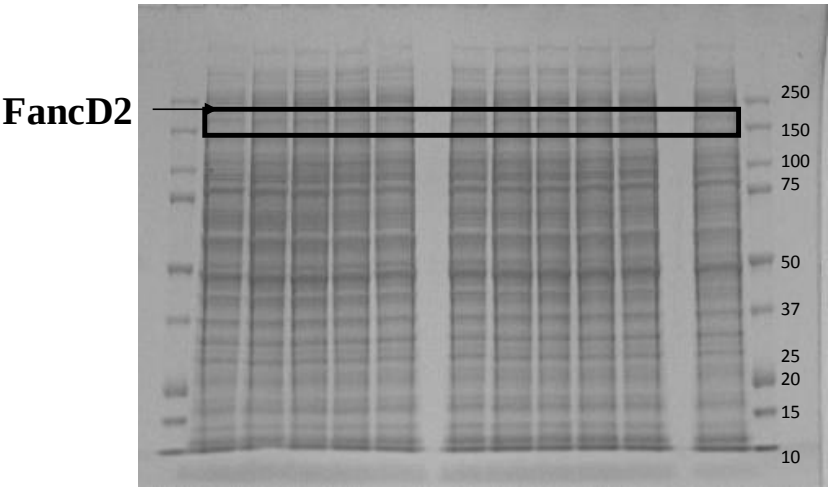

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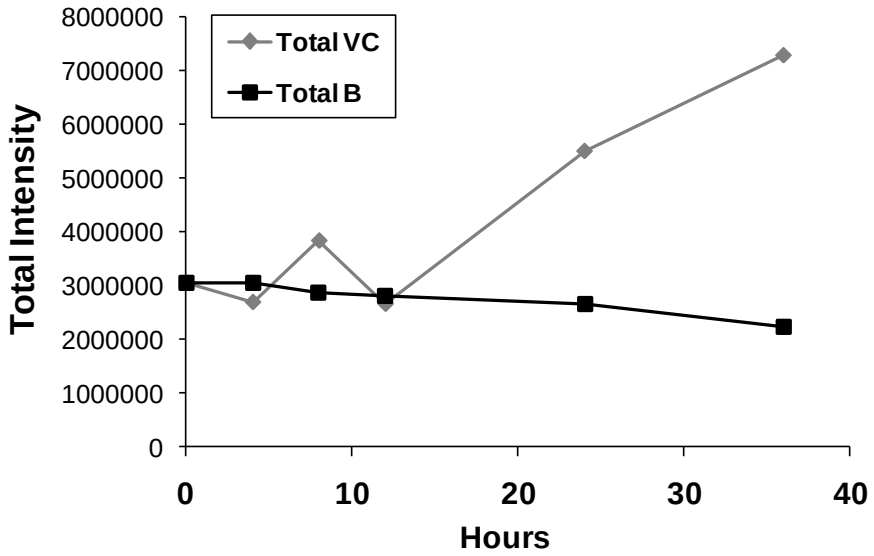

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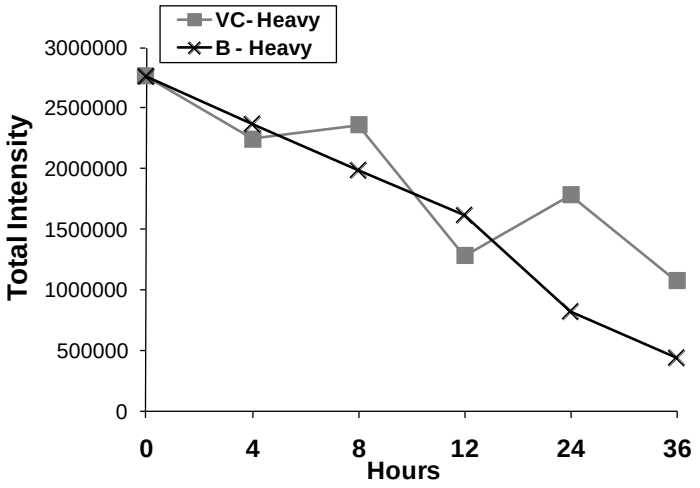

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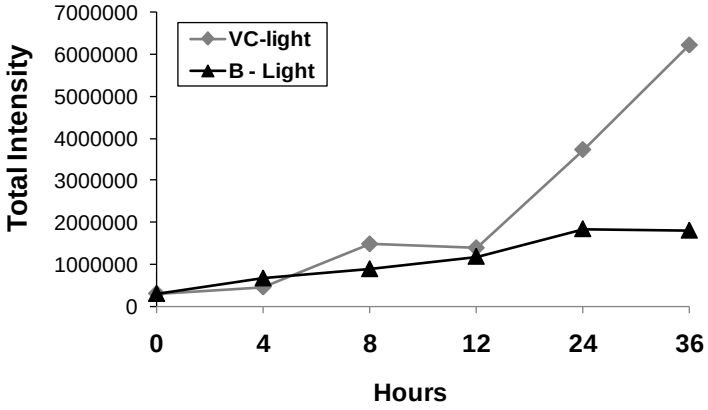


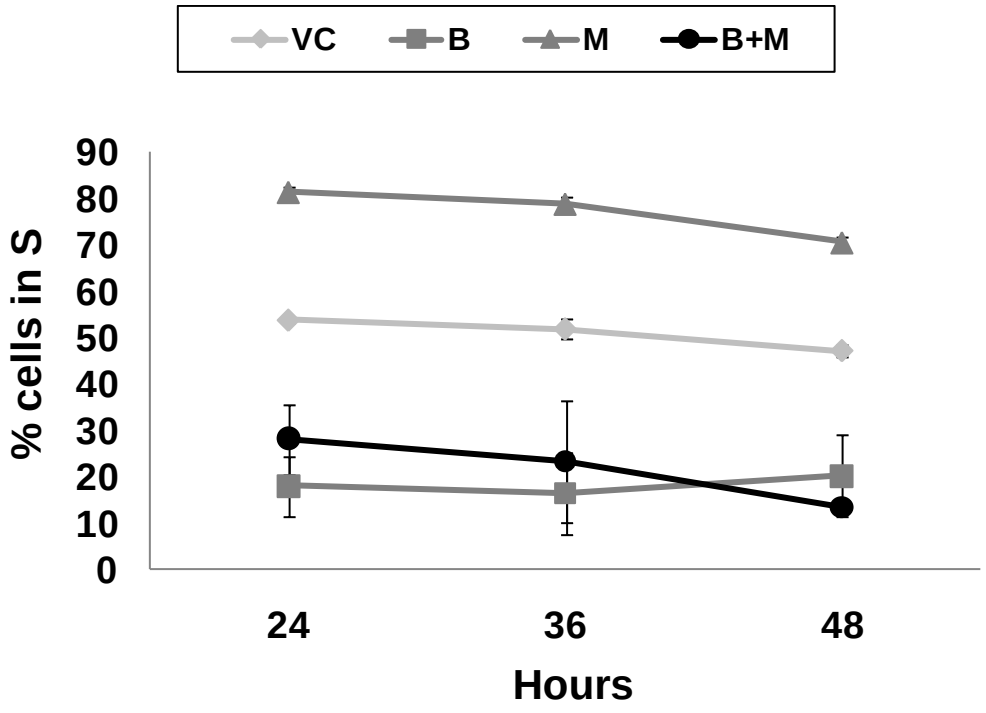
Figure 28. **Bortezomib inhibits FANCD2 protein synthesis.** Stable isotopic labeling of amino acids in cell culture (SILAC) was performed on 8226/LR5 cells to analyze FANCD2 synthesis and degradation following treatment with bortezomib. Cells were labeled with "heavy" media containing stable isotopes of lysine and arginine for six doubling times (approximately seven days). The media was then replaced with regular media and cells were immediately treated with control or 3 nM bortezomib for 4,8, 12, 24 and 36 hours. Mass spectrometry was performed using FANCD2-specific peptides. Two independent experiments were performed, both showed the same trend, and a representative is shown. (A) Coomassie Brilliant Blue stained gel to show equal loading. Bands from this gel were excised for analysis. (B) Total FANCD2 protein expression, as detected by mass spectrometry, following bortezomib treatment for indicated times. (C) FANCD2 degradation (as indicated by reduction in "heavy" label over time) and synthesis (as indicated by increased "light" expression over time) following bortezomib treatment and based on seven FANCD2-specific peptides.

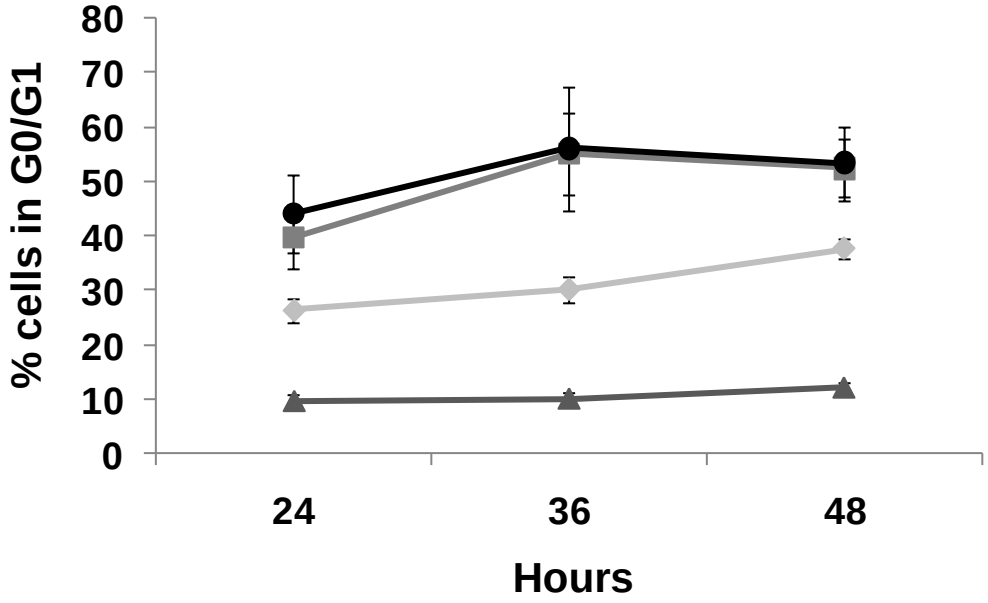
results show that bortezomib reduces FANCD2 protein expression by inhibiting FANCD2 synthesis.

Bortezomib Overcomes Melphalan-Induced S-Phase Arrest

We next wanted to determine if the reduction of FANCD2 protein expression and foci formation by bortezomib could be due to induction of a cell cycle arrest. Previous reports have shown that FANCD2 monoubiquitination and foci formation is S-phase specific (156), and bortezomib is known to reduce the percentage of cells in S-phase (188-189). As shown in Figure 12, melphalan drastically increases the percentage of cells in S-phase at 24 hours, but pre-treatment with bortezomib reduces this number. We next wanted to determine if bortezomib was arresting these cells at a specific phase of the cell cycle, and so analyzed cell cycle progression at later time points. As shown in Figure 29, melphalan arrests cells in the S-phase of the cell cycle. Bortezomib, on the other hand, induces a G0/G1 arrest. Importantly, the melphalan-induced S-phase arrest is overcome by bortezomib, and cells treated with the combination of these two drugs also arrest at G0/G1 (Figure 29).

Following DNA damage, the ATR kinase can be activated and induce an S-phase cell cycle checkpoint (195). ATR has also been shown to phosphorylate FANCD2, and this event promotes the monoubiquitination/activation of FANCD2 (64). Based on these studies, we hypothesized that bortezomib inhibits ATR activation as a mechanism to overcome the S-phase arrest induced by melphalan. We therefore treated cells with 3 nM bortezomib, and analyzed ATR activation (as indicated by phosphorylated ATR). As shown in Figure 30, ATR phosphorylation is reduced in cells treated with bortezomib for





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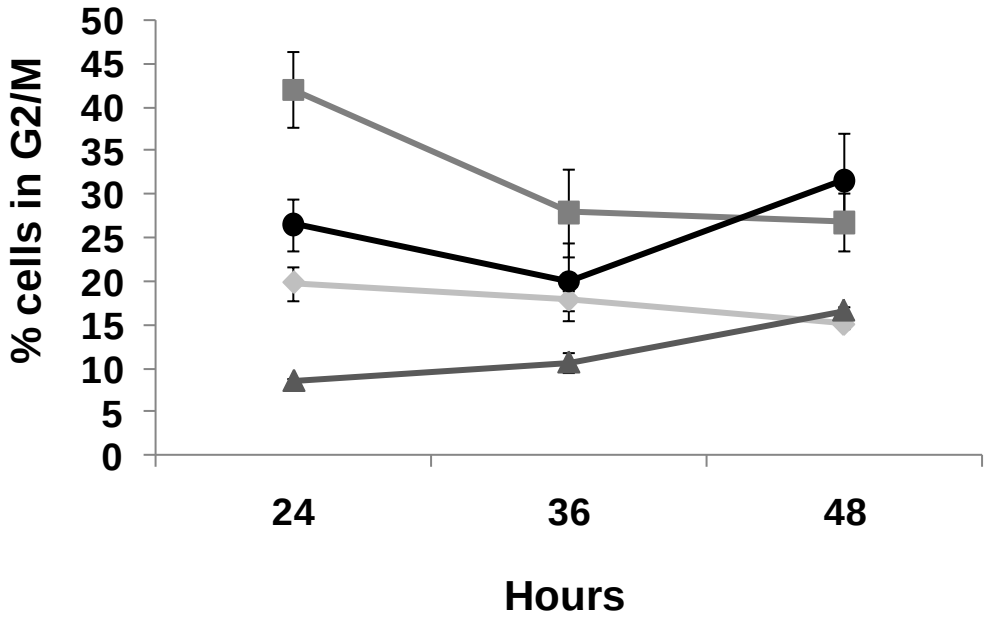

Figure 29. **Bortezomib overcomes melphalan-induced S-phase arrest and arrests cells in G0/G1.** 8226/LR5 cells were treated with control or 3 nM bortezomib for 8 hours, followed by treatment with 25 mM melphalan, and collected from 24-48 hours post-treatment. Cell cycle analysis was performed by labeling with BrdU and PI. The average and SEM of four independent experiments are shown.

eight hours. Futhermore, ATR is known to phosphorylate p53 at serine 15, and this phosphorylation event is also slightly inhibited following eight hours treatment with bortezomib, when compared to control-treated cells (Figure 30). Importantly, in the combination studies of bortezomib plus melphalan described above, cells are pre-treated with bortezomib for eight hours before the addition of melphalan. The results presented in this section show that low-dose bortezomib arrests cells in G0/G1 and also overcomes the S-phase arrest induced by melphalan, likely via inhibition of ATR activity.

FANCD2 Expression is Not Regulated by Hsa-miR-23a or Hsa-miR-27

MicroRNAs (miRNAs) are short RNAs, approximately 22 nucleotides in length, that do not code for protein, but are known to regulate gene expression post-transcriptionally (196-197). MiRNAs bind to the untranslated regions of mRNAs and inhibit translation (194). We hypothesized that bortezomib enhances expression of miRNAs that inhibit the translation of FANCD2. To this end, we treated 8226/LR5 cells with vehicle control or 3 nM bortezomib for eight hours, and a microarray of miRNA expression was analyzed. The results revealed that 33 miRNAs were differentially expressed in bortezomib-treated versus control-treated samples (Figure 31).

We next wanted to determine if any of the miRNAs altered by bortezomib treatment targeted FANCD2. We analyzed these 33 miRNAs using TARGETSCAN software and determined, based on sequence homology, that hsa-miR-23a could potentially target FANCD2. We next transfected 8226/LR5 cells with pre-miRNAs specific for hsa-miR-23a. Pre-miRNAs are cleaved inside the cell to generate the short, functional miRNA. As indicated by q-PCR analysis of hsa-miR-23a, transfection of

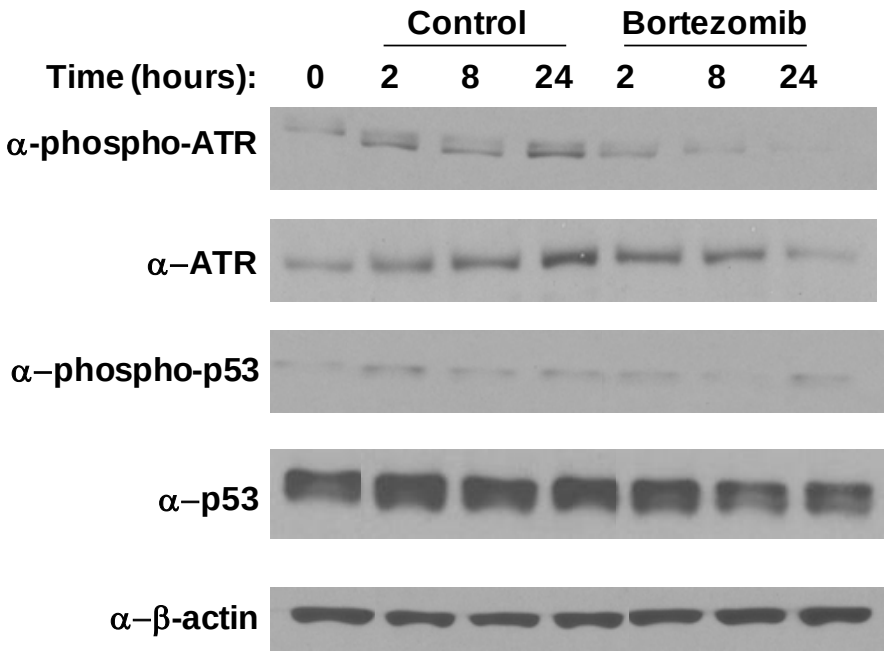

Figure 30. **Bortezomib inhibits ATR activation.** 8226/LR5 myeloma cells were treated with 3 nM bortezomib and samples were collected at 2, 8 and 24 hours post-treatment. ATR activation, as indicated by phospho-ATR and phospho-p53, was analyzed. Three independent experiments were performed and a representative blot is shown.

8226/LR5 cells with pre-miR-23a increased expression of miR-23a by approximately 100-fold or greater when compared to control-transfected cells. This miRNA did not, however, affect expression of FANCD2 protein (Figure 32A). As expected, transfection of cells with pre-miR-23a did, however, reduce expression of GLS, a known target of miR-23a.

We also analyzed the effect of hsa-miR-27 on FANCD2 expression. This miRNA was one of the most highly expressed following bortezomib treatment. In these experiments, 8226/LR5 cells were transfected with an inhibitor of miR-27. Transfection with the anti-miR-27 caused a 2- to 4-fold reduction in hsa-miR-27 expression. Western blot analysis revealed overexpression FADD (the positive control) in anti-miR-27 transfected cells, but no alteration of FANCD2 expression was observed (Figure 32B). In total, these results show that treatment with bortezomib modulates miRNA expression, but FANCD2 is not a target of hsa-miR-23a or hsa-miR-27.

Based on the work presented in this section of the dissertation, we conclude that reduced FANCD2 protein expression following bortezomib treatment is not due solely to inhibition of transcription by NF-κB and reduction of FANCD2 mRNA expression. Bortezomib also inhibits FANCD2 protein synthesis and inhibits ATR activation, overcoming melphalan-induced S-phase arrest.

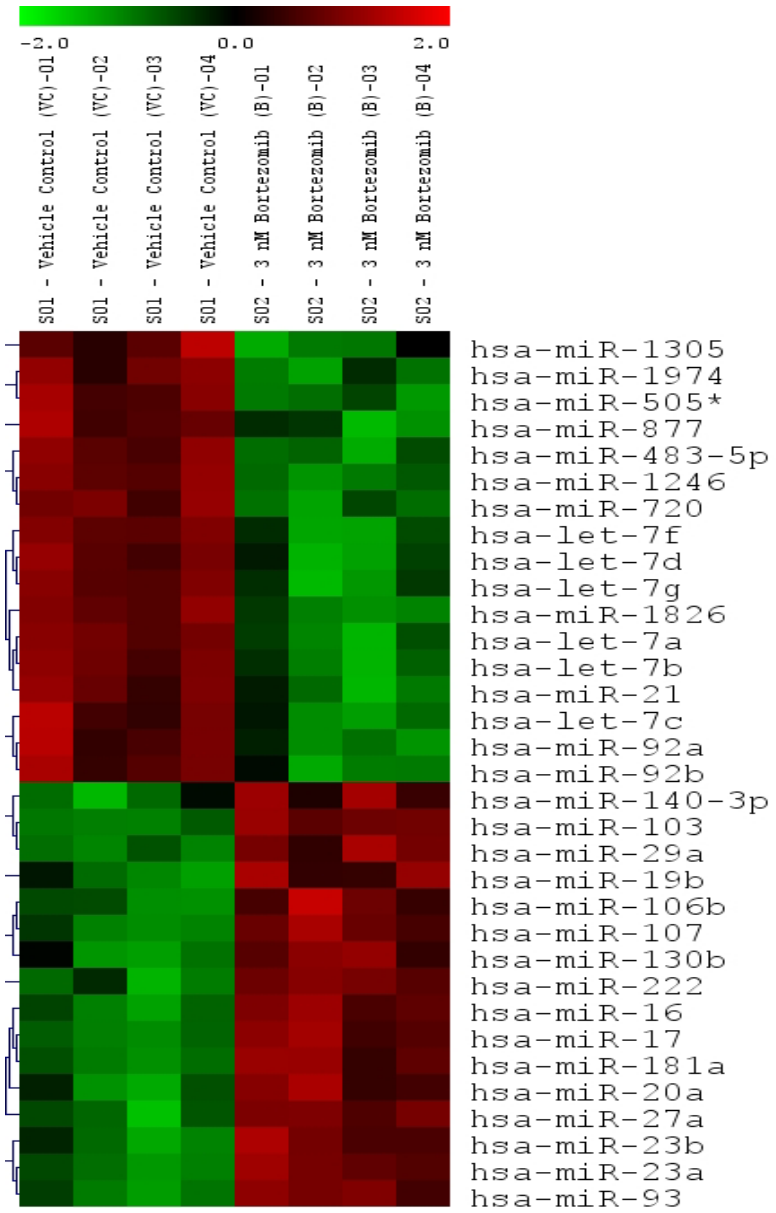

Figure 31. **miRNA analysis of bortezomib-treated 8226/LR5 myeloma cells.** miRNA expression was analyzed in 8226/LR5 cells treated with vehicle control or 3 nM bortezomib for eight hours. Microarray analysis of miRNAs revealed 33 differentially expressed miRNAs in bortezomib-treated cells when compared to control-treated cells.

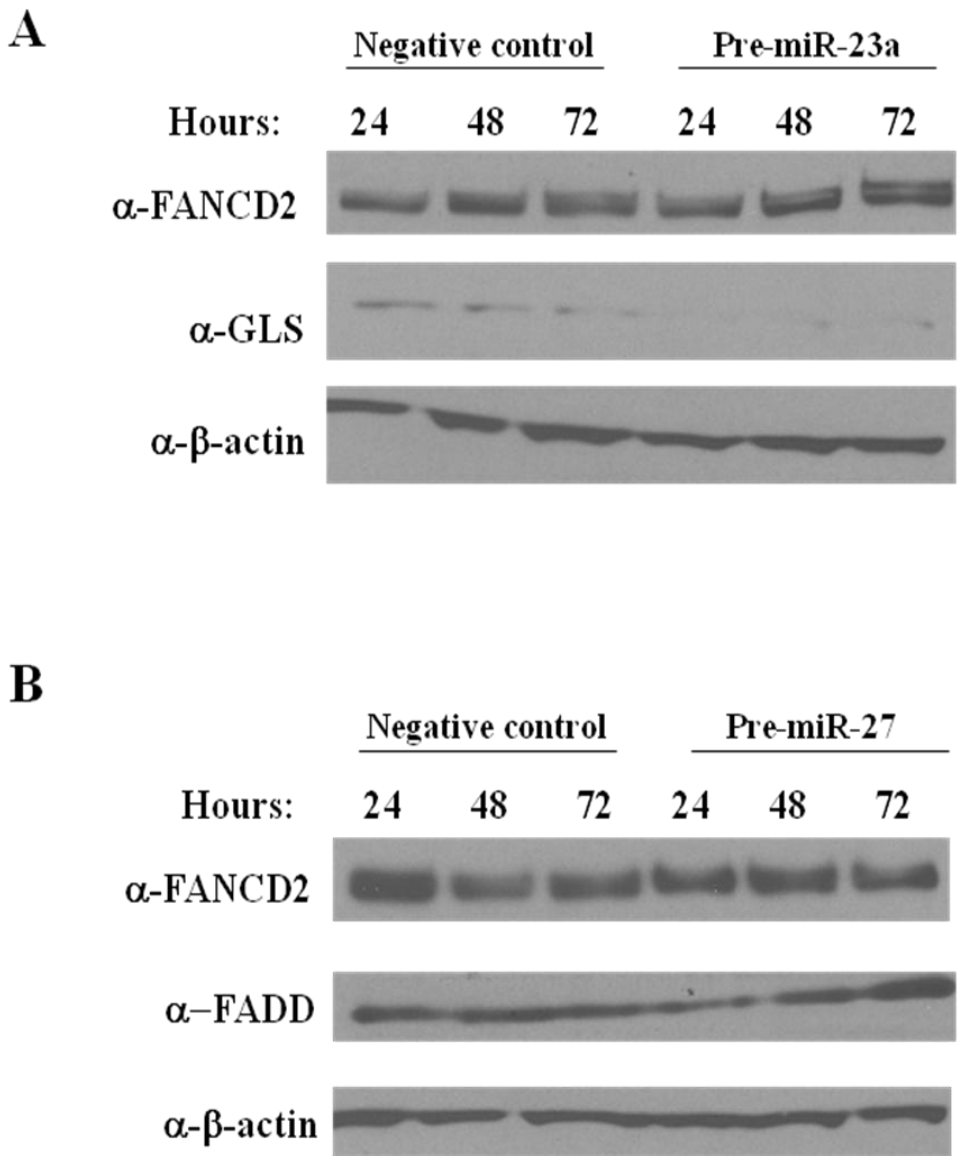

Figure 32. **FANCD2 is not targeted by hsa-miR-23a or hsa-miR-27.** (A) 8226/LR5 cells were transfected with 50 nM pre-miR-23a oligonucleotide, as a means to overexpress hsa-miR-23a in these cells. Western blot analysis of FANCD2 following transfection shows no change in FANCD2 expression in miR-23a overexpressing 8226/LR5 cells when compared to control-transfected cells. Lysates were also probed with α-glutaminase (GLS) antibody as a positive control, and reductions in GLS expression were seen 24-72 hours post-transfection. (B) Western blot analysis of FANCD2 following transfection shows no change in FANCD2 expression in miR-27 overexpressing 8226/LR5 cells when compared to control-transfected cells. Lysates were also probed with α-FADD antibody as a positive control, and and increase in FADD expression were seen 72 hours post-transfection.

DISCUSSION

Members of the FA/BRCA Pathway are Overexpressed in Drug Resistant Cancer Cells and Can be Inhibited by Bortezomib

We recently reported that the FA/BRCA DNA damage repair pathway is significantly involved in melphalan resistance in MM cells (3-4). Specifically, we showed overexpression of FA/BRCA pathway genes in the melphalan resistant 8226/LR5 and U266/LR6 cell lines when compared to their respective drug sensitive parental cell lines. Futhermore, this overexpression was determined to be causative for resistance, as inhibition of FANCF in the drug resistant8226/LR5 cells reversed resistance, and overexpression of FANCF in the drug sensitive 8226/S cells conferred resistance to melphalan (3). Similarly, Dr. Alan D'Andrea's group reported that the FA/BRCA pathway is responsible for resistance of glioma cells to the alkylating agents TMZ and BCNU, and activation of FANCF has been implicated in cisplatin resistance as well (170, 173). In the first section of this dissertation, we extend the work performed by our lab and others by analyzing FA/BRCA pathway mRNA expression in drug sensitive and drug resistant tumor cell lines. Consistent with previous reports, prostate and ovarian cancer cell lines selected for resistance to the alkylating agents melphalan and cisplatin were found to overexpress many FA/BRCA pathway genes. These results suggest that the FA/BRCA pathway mediates resistance to alkylating agents in a variety of tumor types,

and provide impetus for discovering new agents that target this pathway as a means of overcoming or circumventing drug resistance.

FA/BRCA pathway mRNA expression was also analyzed in 8226 cells selected for resistance to doxorubicin and mitoxantrone. These drugs inhibit the function of topoisomerase II, resulting in the accumulation of double strand breaks (DSBs). Interestingly, acquired resistance to doxorubicin and mitoxantrone did not result in global overexpression of FA/BRCA pathway genes, but rather a specific enhancement of FANCF expression. The consequence of overexpression of a single FA protein is unknown. All FA proteins are required for core complex function and subsequent activation of the ID complex, but the stoichiometry of FA core complex subunits is still undetermined (198). FANCF is a flexible adaptor protein, responsible for stabilizing the interaction between FANCA and FANCG and between FANCC and FANCE (145). It is possible that enhanced FANCF expression results in more efficient and/or increased formation of the core complex by increasing the binding of uncomplexed FANC proteins within the nucleus, leading ultimately to enhanced DNA damage repair and drug resistance. Alternatively, FANCF may function outside of the FA/BRCA pathway, interacting with other proteins to mediate drug resistance in these cells. Finally, it is also plausible that overexpression of a single FA/BRCA pathway subunit may not be causative for resistance to topoisomerase II inhibitors, and FANCF overexpression is not responsible for the drug resistant phenotype observed in these cells.

The emergence of drug resistance remains the largest hurdle in the effective treatment of myeloma. Thus, preventing or reversing drug resistance via combination therapy is likely vital for curing this disease. To this end, it has been reported that

bortezomib enhances sensitivity to chemotherapeutic agents, including melphalan, both *in vitro* and in patient specimens (28, 59, 78). Based on these observations and our knowledge that FA/BRCA pathway overexpression is responsible for melphalan resistance, we hypothesized that bortezomib enhances melphalan cytotoxicity by inhibiting DNA repair associated with the FA/BRCA pathway. In support of this hypothesis, the experiments presented in Part II of this dissertation show that bortezomib inhibits FA/BRCA pathway expression and function. First, we show that bortezomib attenuates mRNA expression of FA/BRCA pathway family members in the drug sensitive and drug resistant 8226/S and 8226/LR5 cell lines. In addition to reducing gene expression, bortezomib inhibited FANCD2 protein expression and foci formation, even in the presence of melphalan, in two melphalan resistant cell lines. Bortezomib also enhanced melphalan-induced DNA damage, likely through inhibition of FANCD2 and thus abrogation of DNA repair. Similar to these results, Jacquemont *et. al*. reported that proteasome function is required for the monoubiquitination of FANCD2 and FANCD2 foci formation (176). These results provide insight into the mechanism by which bortezomib potentiates the cytotoxicity of DNA damaging agents.

Based on our results, we believe that the dose scheduling of bortezomib plus melphalan needs to be considered when attempting to improve therapeutic results. We speculate that pre-treatment with low dose bortezomib followed by melphalan may be necessary to capitalize on the ability of bortezomib to inhibit the FA/BRCA DNA damage response pathway. Conceptually, the cytotoxic effects of melphalan due to interstrand crosslink formation may be enhanced if DNA damage repair function via the

FA/BRCA pathway is first inhibited. We believe that this dosing schedule will result in more durable remissions and thus should be explored further.

Exploiting the FA/BRCA pathway-inhibitory effects of bortezomib by combining this drug with chemotherapeutic agents other than melphalan may also prove effective in treating myeloma. For example, *BRCA1* and *BRCA2*-deficient tumor cells are sensitive to inhibiton of poly(ADP-ribose) polymerase (PARP) (199-200). PARP is a key enzyme involved in base excision repair, and its inhibition leads to the accumulation of double strand breaks (DSBs), which cannot be repaired in cells that are deficient in homologous recombination (HR) repair (201). Therefore, combining PARP inhibitors with bortezomib, a drug that we show inhibits BRCA1, BRCA2, and other FA proteins involved in HR repair, may prove to be an effective strategy to treat patients with myeloma. As another example, combining bortezomib with Imexon may also prove effective. Imexon is a small molecule that induces accumulation of reactive oxygen species (ROS) (202). ROS accumulation has also been observed in bortezomib-treated cells (203), and FA patient cells are hypersensitive to ROS (204). The cytotoxic effects of bortezomib plus Imexon are synergistic in myeloma tumor cell lines (202), and we postulate that this enhanced cytotoxicity may be due to the detrimental effects of ROS accumulation in FA/BRCA pathway-inhibited cells.

In summary, the findings presented in Parts I and II of this dissertation show that the FA/BRCA DNA damage repair pathway is upregulated in a variety of tumor types selected for resistance to alkylating agents, and that bortezomib potentiates melphalan cytotoxicity, at least in part, via suppression of this pathway. Based on these studies, we propose that the therapeutic index of melphalan, and potentially other agents, in myeloma

patients can be greatly enhanced by targeting the FA/BRCA DNA damage repair pathway.

Mechanisms by Which Bortezomib Inhibits FA/BRCA Pathway Expression

The studies presented in Part II of this dissertation, and as described in the discussion above, show that bortezomib treatment decreases expression of many FA/BRCA pathway genes in both melphalan sensitive and melphalan resistant cell lines, as well as in MM patient specimens, suggesting that these genes may be co-regulated. Furthermore, we and others have shown that bortezomib inhibits NF-κB activity (59, 78), and combination index analysis studies performed by our lab revealed antagonism when myeloma cells were treated simultaneously with bortezomib and BMS-345541, a specific inhibitor of the NF-κB pathway. The results suggest that both drugs exact their cytotoxic effects via inhibition of this pathway, and we therefore hypothesized that NF-κB is an upstream mediator of the FA/BRCA pathway.

The transcriptional regulation of FA/BRCA pathway genes is largely unknown. Analysis of the promoter regions of FA/BRCA pathway genes revealed putative NF-κB binding sites on 12 of these genes (*brca1, brca2, Fanca, fancc, fancd2, fance, fancf, fancg, fanci, fancj, fancl, fancm,* and *fancn*). In Part III of this dissertation, we report for the first time that NF-κB functions as a transcriptional regulator of the FA/BRCA pathway.

Our lab previously reported that FA/BRCA pathway mRNA levels are overexpressed in melphalan resistant myeloma cells. Interestingly, the melphalan resistant 8226/LR5 cells also exhibit an increase in basal NF-κB DNA-binding activity

when compared to the 8226/S cells, which we hypothesized could explain the enhanced expression of FA/BRCA pathway genes. Gel shift assays and siRNA inhibition of NF-κB subunits identified RelB and p50 as the subunits responsible for this enhanced binding activity. Additionally, chromatin immunoprecipitation (ChIP) analysis revealed that the level of NF-κB activity observed in the melphalan resistant 8226/LR5 cells is attributable to increased binding of RelB and p50 to the FANCD2 promoter. As expected, FANCD2 protein expression was drastically decreased in RelB/p50-depleted 8226/LR5. Importantly, these RelB/p50-depeleted cells were no longer melphalan resistant, as indicated by an increase in ICL and DNA fragmentation and the onset of apoptosis following acute exposure to melphalan. Conversely, re-expressing FANCD2 in RelB/p50-depleted cells conferred resistance to melphalan. Collectively, these findings indicate that attenuated FANCD2 expression and function in RelB/p50-depleted cells is responsible for the enhanced melphalan sensitivity seen in these cells.

Interestingly, we demonstrate that chronic exposure of 8226 cells to melphalan engages the specific phosphorylation of IKKα, which in turn leads to augmented basal NF-κB DNA-binding activity in these cells. Based on the finding that IKKα is constitutively phosphorylated in melphalan resistant cells, our results suggest that the alternative pathway of NF-κB activation protects these cells against apoptotic stress imposed by the alkylating agent melphalan.

FANCD2-specific NF-κB activity was greatly enhanced in 8226/LR5 cells relative to the drug sensitive 8226/S cell line, and our results implicate the RelB and p50 subunits in this enhanced binding activity. Depletion of RelB or p50 expression abolished the basal DNA-binding activity of NF-κB in 8226 cells. We hypothesize that

other NF-κB subunits may compensate for loss of RelB or p50 in the regulation of FANCD2 expression in myeloma cells. This model is consistent with our finding that p65, c-Rel, and p52 can also bind to the FANCD2 promoter in 8226 cells. Moreover, relative to control cells, 8226/LR5 cells also exhibited a higher degree of coimmunoprecipitation of the FANCD2 promoter with p52. Since RelB stability requires multi-domain interactions with p100/p52 (205), it is also plausible that this NF-κB precursor/product pair promotes the stable formation of a transcriptionally active RelB/p50 heterodimer in melphalan resistant cells. The mechanism of p50 activation in these cells is unknown, although a recent report indicates that p100 mediates p65/p50 activation in response to noncanonical NF-κB signaling pathways (206).

We speculate that prolonged or high-intensity replicative stress in myeloma cells triggers the transcriptional activation of FA/BRCA genes as a means to ameliorate fork progression and repair. In Part III of this dissertation, we demonstrate that NF-κB can transcriptionally regulate FA/BRCA pathway genes. Stimulation of melphalan sensitive and resistant 8226 and U266 cells with BMS-345541, a known inhibitor of NF-κB activation, elicited a rapid decline in FA/BRCA mRNA levels. However, at later time points and in some of the FA/BRCA genes analyzed, gene expression was seen to either transiently or stably recover to close to baseline levels. It is likely that acquired melphalan resistance involves the coordinated effects of a number of DNA damage-induced transcription factors, resulting in transient oscillations of FA/BRCA gene expression. For example, Hoskins *et. al.* reported that FA gene expression is regulated by members of the Rb/E2F family (207). It is plausible that this family of transcription factors can compensate for the loss of NF-κB following BMS-345541 treatment and

induce transcription of FA/BRCA pathway genes. Alternatively, it is possible that FA/BRCA gene expression is regulated by epigenetic modifications of promoter regions. In this regard, chronic exposure of myeloma cells to melphalan may promote demethylation of FA/BRCA promoter regions, granting DNA damage-induced transcriptional regulators, such as NF-κB, free access to transcriptional start sites, eventually resulting in enhanced FA/BRCA pathway expression.

NF-κB is known to play a pivotal role in MM tumorigenesis and drug resistance. NF-κB is constitutively activated in multiple myeloma, is overexpressed in drug resistant myeloma cell lines, and is elevated in patient specimens following chemotherapeutic treatment and at time of relapse (37, 55-57, 59-60, 62). We postulate that genetic activation of the alternative NF-κB pathway, specifically the RelB and p50 subunits, allows malignant plasma cells to gain independence from the rich milieu of growth factors and cytokines produced in the bone marrow microenvironment. Interestingly, Landowski *et. al.* reported that these same subunits are upregulated following MM cell adhesion to fibronectin (FN), a component of the bone marrow microenvironment (58). Adhesion to this extracellular matrix protein induces a transient form of drug resistance in the adhered tumor cells (208). Based on the specific enhancement of RelB and p50 in adhered cells, it is plausible that the FA/BRCA DNA damage repair pathway is important not only for acquired resistance, but also contributes to the *de novo* drug resistance phenotype. Furthermore, a recent report demonstrated that HES1, the downstream effector protein of Notch1, interacts with the FA core complex and is required for FANCD2 monoubiquitination (127). Notch1 signaling is upregulated following cellular adhesion to bone marrow stromal cells and confers resistance in these tumor cells (41).

These reports provide another potential link between the FA/BRCA pathway and *de novo* drug resistance; upregulation of Notch1 in adhered cells enhances HES1 signaling, and may result in enhanced expression of the FA/BRCA pathway leading to drug resistance.

In summary, the results presented in Part III of this dissertation show for the first time that NF-κB plays an important role in the regulation of FA/BRCA gene expression in human multiple myeloma cells. This conclusion is further substantiated by a recent report that curcumin analogs inhibit the FA pathway via inhibition of NF-kB activity (209). The importance of this work is best illustrated by the observation that a sustained reduction in FA/BRCA gene expression is especially cytotoxic to melphalan-resistant cells. It is likely that these cells have become addicted to FA/BRCA-mediated genome surveillance and DNA repair. We therefore postulate that targeting the FA/BRCA pathway directly, or indirectly via inhibition of NF-κB, will enhance the cytotoxic effects of commonly used chemotherapeutic agents.

Based on the studies presented in this dissertation, we propose that targeting the proteasome accentuates melphalan response by reducing FA/BRCA gene expression via inhibition of NF-κB, and blocking activation of FANCD2, thereby inhibiting DNA damage repair following drug treatment. The observation that FA/BRCA pathway mRNA expression was attenuated by bortezomib, but not to the same degree as the inhibition of FANCD2 protein expression, suggests that bortezomib not only reduces FANCD2 protein expression via inhibition of NF-κB activity, but also by regulating FANCD2 post-transcriptionally. We therefore analyzed bortezomib-mediated regulation of the FA/BRCA pathway downstream of transcription.

We first analyzed FANCD2 protein stability in bortezomib-treated cells. Analysis of FANCD2 synthesis and degradation using stable isotopic labeling of amino acids in cell culture (SILAC) and mass spectrometry revealed that the reduction of FANCD2 protein levels exhibited in bortezomib-treated cells is due to a drastic suppression of FANCD2 protein synthesis. Of note, however, is that enhanced degradation of FANCD2 protein was observed in bortezomib-treated cells as well, although the contribution of degradation to the decrease in overall FANCD2 protein levels after bortezomib treatment was minor compared to the contribution of inhibited synthesis. We postulate that FANCD2 degradation in bortezomib-treated cells is due to incomplete inhibition of proteasomal activity by the low dose of drug utilized in these experiments or due to a non-proteasomal mechanism.

We next analyzed the contribution of cell cycle regulation to the inhibition of FANCD2. FANCD2 monoubiquitination and foci formation is reported to be S-phase specific (156), and bortezomib is known to reduce the percentage of cells in S-phase (188-189). Melphalan was found to arrest cells in the S-phase of the cell cycle. Bortezomib, on the other hand, induces a G0/G1 arrest, and can overcome the melphalan-induced S-phase arrest. Furthermore, ATR activation was inhibited after eight hours treatment with bortezomib, which, incidentally, is the same amount of time that cells are pre-treated with bortezomib before the addition of melphalan in the studies described above. ATR is typically activated following DNA damage and induces an S-phase cell cycle checkpoint, leading to cell cycle arrest (195). This kinase also phosphorylates FANCA, FANCD2, FANCI, and FANCM, and these phosphorylation events are critical for the activation of the FA/BRCA pathway (64, 151, 155, 210). Based on these results,

we conclude that bortezomib overcomes melphalan-induced S-phase arrest via inhibition of ATR activation. Curiously, bortezomib-treated cells do arrest, and we speculate that ATM kinase activity is not inhibited by bortezomib and can therefore compensate for the lack of ATR function and induce the observed G0/G1 cell cycle arrest. In support of this theory, two separate labs have reported that ATM activity is not affected by proteasome inhibition (176, 211).

Finally, in a separate set of studies, we analyzed miRNA expression following bortezomib treatment. MiRNAs bind to mRNA and repress translation without actually cleaving the mRNA itself (193-194). We therefore hypothesized that the drastic decrease in FANCD2 expression could be due to upregulation of a miRNA that targets and thus represses FANCD2. Based on microarray analysis of miRNA expression in bortezomib- versus control-treated cells, we identified two miRNAs that could potentially target FANCD2 mRNA, specifically, hsa-miR-23a and hsa-miR-27. Further analysis of these miRNAs revealed that they do not function by targeting FANCD2. A total of 33 miRNAs were differentially expressed in bortezomib-treated cells, however, and it is plausible that these miRNAs may indirectly inhibit FANCD2 protein expression by targeting upstream mediators of the FA/BRCA pathway.

In summary, we have shown that bortezomib inhibits FA/BRCA pathway expression. FANCD2 protein synthesis is inhibited in bortezomib-treated cells, and this inhibition is likely due to the combined effects of inhibition of NF-κB, which we determined is a transcriptional activator of the FA/BRCA pathway, and bortezomib-induced cell cycle arrest. Overall, the results presented in this dissertation show that drug response and resistance in MM, and possibly other cancers, is mediated by the FA/BRCA

DNA damage repair pathway. Therefore, we believe that targeting the FA/BRCA pathway to inhibit DNA damage repair, either directly or indirectly via inhibition of NF-κB or ATR activation, is vital for reversing, or possibly circumventing, drug resistance in multiple myeloma and possibly other cancers.

Future Directions

We recently demonstrated that FA/BRCA pathway members are overexpressed in melphalan resistant cells, and causative for this resistance (3-4). Additionally, other labs have reported that members of the FA/BRCA pathway mediate sensitivity to cisplatin, BCNU, and TMZ in different tumor types as well (170, 172-175). In the Part I of this dissertation, we show that FA/BRCA pathway genes are also overexpressed in prostate and ovarian cancer cell lines selected for resistance to melphalan and cisplatin, respectively. Conversely, we found that cells selected for resistance to topoisomerase II inhibitors do not show this same pattern of global overexpression of FA/BRCA pathway genes. Instead, these resistant cells selectively overexpress FANCF. It is known that all members of the FA core complex must be functional in order for ID complex activation, but the relative contribution of upregulation of one FA protein is unclear. Therefore, we propose experiments that will examine the contribution of overexpression of FANCF to acquired resistance to topoisomerase II inhibitors. First, we propose to transfect 8226/Dox40 and 8226/MR20 cells with siRNAs specific to FANCF, and subsequently analyze sensitivity to doxorubicin and mitoxantrone, respectively, in siRNA-transfected cells compared to control-transfected cells and the drug sensitive 8226/S cells. If FANCF

does contribute to acquired drug resistance in the 8226/Dox 40 and 8226/MR20 cells, depletion of FANCF should enhance drug sensitivity.

It is possible that FANCF is functioning both inside and outside of the FA/BRCA pathway. Therefore, we also propose to examine FANCF protein interactions in drug sensitive and resistant cells by immuno-precipitation using a FANCF-specific antibody. Since FANCF is a flexible adaptor protein important for stabilizing interactions between other core complex proteins, the results may reveal enhanced interactions between FANCF and other FANC proteins in the drug resistant cells. Alternatively, comparison of FANCF binding partners in the drug sensitive and resistant cells may reveal novel interactions between FANCF and non-FA pathway proteins that contribute to drug resistance. Additionally, since demethylation of FANCF is known to contribute to drug resistance (173), we propose to examine the methylation status of FANCF in the drug sensitive and resistant cell lines.

Studies are also proposed to analyze the role of the FA/BRCA pathway in cell adhesion-mediated drug resistance (CAM-DR). In this dissertation, we demonstrate that the NF-κB subunits RelB and p50 regulate FA/BRCA pathway members. Furthermore, RelB and p50 DNA binding activity is enhanced in cells adhered to fibronectin (FN) (58). We therefore hypothesize that FA/BRCA pathway expression is augmented when cells are adhered to FN, and upregulation of this pathway contributes to the CAM-DR phenotype. First, we propose analyzing FA/BRCA pathway protein expression in cells adhered to FN to determine if basal expression of FA proteins is enhanced upon adhesion. Also, since melphalan activates the FA/BRCA pathway, it will be important to analyze FA protein levels in FN-adhered myeloma cells treated with melphalan. If FA

proteins are indeed upregulated by FN adhesion, using siRNAs to inhibit expression of these proteins is proposed to determine the contribution of this overexpression to drug resistance.

We also propose to analyze the factors that regulate FA/BRCA pathway expression in the absence of NF-κB. As seen in Part III of this dissertation, inhibition of NF-κB with the BMS-345541 compound transiently reduced FA/BRCA pathway mRNA expression, but levels of expression oscillated throughout time. Theoretically, once NF-κB is inhibited, other transcription factors will take over and transcriptionally activate this pathway. Therefore, we propose stably expressing IκB in 8226/S and 8226/LR5 cells to inhibit NF-κB activation, and analyzing the transcriptional regulation of FA/BRCA pathway members in NF-κB-inhibited cells. It will also be important to determine if these transcriptional regulators can fully compensate for NF-κB loss, and we propose analyzing sensitivity to melphalan in the NF-κB inhibited cells. We first propose analyzing Rb/E2F function in these cells, as it has been reported that this family of transcription factors regulates transcription of members of the FA/BRCA pathway (207). Furthermore, inhibiting E2F in the NF-κB-inactive cells would be interesting to determine if FA/BRCA pathway expression can be completely abolished and if inhibition of both NF-κB and E2F/Rb greatly enhances drug sensitivity.

In Part IV of this dissertation, we show that melphalan induces an S-phase arrest, whereas bortezomib causes an arrest in G0/G1. Furthermore, pre-treatment with bortezomib is able to overcome the melphalan-induced S-phase arrest, and combination-treated cells also arrest in G0/G1. We also show that ATR activation is inhibited in bortezomib-treated cells. We hypothesize that ATM is activated by bortezomib, and that

this kinase is responsible for activation of the G1 cell cycle checkpoint leading to the G0/G1 arrest seen in bortezomib-treated cells. It will be important to look at ATM activity in bortezomib-treated cells. Likewise, ATM and ATR activation should be analyzed in melphalan-stimulated cells as well as in cells treated with the combination of bortezomib plus melphalan. Similarly, the activity of Chk1 and Chk2 kinases, two downstream mediators of ATM and ATR, should also be analyzed in cells treated with bortezomib and melphalan.

An interesting report by Alan D'Andrea's group showed that inhibition of the FA/BRCA pathway led to "hyper-activation" of Chk1, and this activation compensates for lack of FA-mediated DNA repair. Therefore, we hypothesize that combining bortezomib with Chk1 inhibitors will greatly enhance cytotoxicity by inhibiting both FA- and Chk1-mediated repair. We propose analyzing the combined effects of bortezomib with the Chk1-inhibitor Go6976 via combination index analysis in 8226/S and 8226/LR5 drug-treated cells. Along similar lines, we also propose to analyze the combinatorial effects of bortezomib and PARP inhibitors. As mentioned in the discussion, *BRCA1*- and *BRCA2*-deficient cells are extremely sensitive to PARP inhibition (199-200), and we believe that exploiting the FA/BRCA pathway-inhibitory effects of bortezomib by treatment with PARP inhibitors will greatly enhance the cyctotoxic effects of bortezomib.

Analysis of FA/BRCA pathway expression in myeloma cells treated with the second-generation proteasome inhibitors carfilzomib and NPI-0052 may also be important. These agents irreversibly inhibit the proteasome and can overcome boretezomib resistance (29, 81). It is plausible that these drugs can inhibit the FA/BRCA pathway more effectively and for a longer period of time than can bortezomib.

Finally, it may also prove worthwhile to further analyze miRNA expression following bortezomib treatment. Although bortezomib treatment may not alter expression of miRNAs that directly target FA mRNAs, it is conceivable that bortezomib treatment changes expression of miRNAs that target proteins upstream of this pathway. Furthermore, miRNA analysis of drug sensitive versus drug resistant cells may be important for determining if differential expression of specific miRNAs contributes to acquired drug resistance.

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