Lenalidomide plays an important role in our chemotherapeutic armamentarium against multiple myeloma, in part by exerting direct anti-proliferative and pro-apoptotic effects. Unfortunately, long-term exposure leads to the development of drug resistance through unknown mechanisms, and we therefore sought to identify pathways that could be responsible for this phenotype. Chronic drug exposure produced myeloma cell lines that were tolerant of lenalidomide’s direct effects, with a degree of resistance of up to 2,500-fold. Gene expression profiling and pathway analysis identified dysregulation of the Wnt/β-catenin pathway as a consistent change across four independent cell isolates, and a pair of primary plasma cell samples. Acute drug treatment also increased β-catenin transcription by 3-fold or more, and both acute and chronic exposure resulted in enhanced accumulation of β-catenin protein by up to 20-fold or more. This produced Wnt/β-catenin pathway activation, as judged by increased activity of a lymphoid enhancer factor/T-cell factor promoter reporter, and enhanced accumulation of the downstream targets cyclin D1 and c-Myc. Components of the β-catenin destruction complex were also impacted by lenalidomide, which suppressed casein kinase 1α expression, while augmenting glycogen synthase kinase 3α/β phosphorylation. Stimulation of Wnt/β-catenin signaling with recombinant Wnt-3a, or by overexpression of β-catenin, reduced the anti-proliferative activity of lenalidomide. Conversely, suppression of β-catenin with small hairpin RNAs restored plasma cell sensitivity to lenalidomide. Together, these findings support the hypothesis that lenalidomide mediates activation of Wnt/β-catenin signaling in plasma cells as a mechanism of inducible chemoresistance through effects at the transcriptional and post-translational levels.

Introduction

Immunomodulatory drugs such as thalidomide and lenalidomide are being increasingly used in the treatment of multiple myeloma (1, 2). Thalidomide’s anti-angiogenic activity prompted the initial study in patients with advanced myeloma (3), and its encouraging activity there, and in further investigations, led to thalidomide-based regimens becoming a standard of care (1, 2). However, its unfavorable toxicity profile motivated efforts to develop derivatives with decreased toxicity (1, 2), resulting in the development of lenalidomide (CC-5013) (4, 5). Lenalidomide first showed clinical efficacy as a single agent (6, 7) and with dexamethasone (len/dex) it provided superior outcomes in the relapsed/refractory setting (8, 9). Later, len/dex showed activity against newly-diagnosed myeloma (10, 11), and early data suggest a benefit from maintenance therapy with lenalidomide after chemotherapy (12) and stem cell transplantation (13). Finally, len/dex may reduce the risk of progression from asymptomatic to symptomatic myeloma (14). Together, these developments argue that lenalidomide will play a crucial role in our chemotherapeutic armamentarium against myeloma for years to come.

The effects of immunomodulatory agents have been divided into three categories: modulation of the host immune system, modification of the tumor microenvironment, and direct inhibition of tumor proliferation and stimulation of apoptosis (2, 15). Initial studies to characterize the latter effects noted they inhibited...
DNA synthesis, promoted G1 arrest, activated apoptosis, and enhanced p21 expression (16). Induction of p21 correlated with cyclin-dependent kinase-2, -4, and -6 inhibition, and Retinoblastoma protein hypo-phosphorylation (17, 18). Lenalidomide may mediate this by epigenetically modifying p21 promoter chromatin structure through lysine-specific demethylase-1 (19). Other plasma cell effects included inhibition of protein kinase B/Akt (20), suppression of nuclear factor-kappaB (NF-κB), down-regulation of inhibitor of apoptosis protein 2 and FLICE, and priming of cells for CD95 receptor-mediated death (15, 21).

Thalidomide’s effects on in vivo models have been dissected to identify the mechanisms responsible for its teratogenicity (22, 23). Studies in chick embryo models proposed that thalidomide induced reactive oxygen species (ROS), which activated Bone morphogenetic protein (Bmp) through NF-κB inhibition. Dickkopf homolog (Dkk)-1 was induced through ROS and p53, and possibly from Bmp through c-Jun-N-terminal kinase (JNK)(24), and in aggregate this suppressed wingless-type MMTV integration site family member signaling. Inhibition of Wnt reduced β-catenin activity, in part through increased glycogen synthase kinase (GSK)-3β action, and downstream consequences included stimulation of apoptosis (25). This cell death may cause the limb truncation defects that were seen with thalidomide use in pregnant patients (22, 23).

Despite the pleiotropic anti-tumor effects of immunomodulatory agents, a minority of patients achieve complete remissions, most ultimately progress after an initial response, and few studies have evaluated potential mechanisms of resistance to thalidomide. In chick, human, and mouse embryo fibroblasts, thalidomide induced superoxide, and glutathione depletion enhanced thalidomide’s cytotoxicity (25). Conversely, free radical trapping agents, Dkk1 blocking antibodies, and inhibitors of Bmps, phosphatase and tensin homolog, or GSK-3β, attenuated apoptosis. The mechanisms of thalidomide resistance in myeloma cells have not been well delineated, however, and no studies of resistance to lenalidomide have been reported.

We therefore sought to examine the mechanisms responsible for lenalidomide resistance, and to do so we developed cell lines that were tolerant of its anti-proliferative and pro-apoptotic effects. Gene expression profiling and pathway analysis identified the Wnt/β-catenin pathway as being consistently dysregulated. Lenalidomide induced β-catenin transcription, activated the downstream lymphoid enhancer factor (LEF)/T-cell factor (TCF) promoter, and enhanced accumulation of β-catenin, cyclin D1, and c-Myc protein. Moreover, lenalidomide contributed to Wnt/β-catenin activation by suppressing casein kinase (CK)-1α expression and GSK-3α/β activity. Finally, targeted Wnt/β-catenin activation suppressed the activity of lenalidomide, while β-catenin inhibition restored its efficacy. Taken together, these data support the conclusion that, unlike thalidomide, which inhibits Wnt/β-catenin, lenalidomide induces Wnt/β-catenin in plasma cells, and this contributes to mechanisms of resistance to this agent.

**Experimental Procedures**

**Cell lines and primary sample** - Multiple myeloma cell lines were cultured in RPMI 1640 medium supplemented with L-glutamine, fetal bovine serum, penicillin, and streptomycin (all from Invitrogen; Carlsbad, CA). These cell lines were validated through The M. D. Anderson Cancer Center Characterized Cell Line Core Facility. Bone marrow aspirates were collected from patients under a protocol approved by the Institutional Review Board of The University of Texas M. D. Anderson Cancer Center, and informed consent was obtained in compliance with the Declaration of Helsinki. Primary plasma cells were purified by positive selection using magnetic-activated cell sorting with CD138+ MicroBeads (Miltenyi Biotec; Auburn, CA). These cells, as well as interleukin (IL)-6 dependent cell lines (ANBL-6 and KAS-6/1), were supplemented with recombinant human IL-6 (R&D Systems; Minneapolis, MN).

**Lenalidomide-resistant cell lines** - Drug-naïve ANBL-6, KAS-6/1, U266, and MM1.S myeloma cells were initially exposed to lenalidomide (Celgene Corporation; Summit, NJ) concentrations that were 10% of the drug’s maximal inhibitory concentration (IC10). Over several months, drug concentrations were serially increased following the verification of outgrowth
of cells that retained membrane integrity, as judged by Trypan blue exclusion, and proliferative ability. The maximal lenalidomide concentration sustained in standard culture conditions was 10 µM, establishing the lenalidomide-tolerant cell lines ANBL-6/R10R, KAS-6/R10R, U266/R10R, and MM1/R10R. In some experiments, lenalidomide-containing culture media was removed for seven days to provide a drug-free interval (ANBL-6/RR, KAS-6/RR, U266/RR, and MM1/RR).

**Proliferation and viability assay** - Cell lines were plated in triplicate in 96-well round-bottom plates with the indicated lenalidomide concentrations. These were incubated for 72 hours, at which time the media was replaced with fresh lenalidomide-supplemented media, and allowed to incubate for another 96 hours. In experiments utilizing human recombinant Wnt-3a (rWnt-3a)(R&D Systems), this was added at 25 ng/mL for two hours prior to lenalidomide. The WST-1 tetrazolium salt (Roche Applied Science; Indianapolis, IN) was then added and used according to the manufacturer’s specifications. Cleavage of WST-1 occurs through the action of mitochondrial succinate-tetrazolium reductase in viable cells, and the quantity of formazan dye generated is directly related to the number of metabolically active cells. Colorimetric measurements were taken at 450 nm on a Victor 3V (PerkinElmer; Waltham, MA) plate reader, and analyzed using GraphPad Prism 5 software (GraphPad Software; La Jolla, CA). All data points were represented as a mean with the standard deviation (SD) from the number of determinations indicated in the figure legends.

**Gene expression analysis** - Cultures of drug-naïve parental cell lines and their lenalidomide-tolerant counterparts were harvested in triplicate. In addition, paired primary samples were obtained from a patient prior to initiating lenalidomide therapy, and after the patient had developed clinical lenalidomide resistance. Total RNA was extracted and purified using the RNeasy purification kit (Qiagen; Valencia, CA), and then utilized for labeling and microarray hybridization to U133 plus 2.0 human whole genome microarrays (Affymetrix; Santa Clara, CA) by Genome Explorations, Inc. (Memphis, TN). Quantification of expression levels was determined with Affymetrix Microarray Suite (MAS) 5.0. Alterations in mRNA transcript levels were compared as the fold difference for drug-naive vs. lenalidomide-tolerant samples. These fold changes were then mapped and analyzed using Ingenuity Pathway Analysis Software (Ingenuity Systems; Redwood City, CA).

**Immunoblotting** - Cells were harvested and lysed in 1x Cell Lysis Buffer (Cell Signaling Technology; Danvers, MA) supplemented with 1x Protease Inhibitor Cocktail, 1x Phosphatase Inhibitor Cocktail, and PMSF (Sigma-Aldrich; St. Louis, MO). Cell lysates were clarified, protein concentrations were determined using the DC Protein Assay (BioRad; Hercules, CA), samples were subjected to electrophoresis on 4-12% (Invitrogen) or 8-10% gradient (BioRad) acrylamide gels, and then transferred to nitrocellulose membranes (BioRad). After blocking, they were probed with the respective primary antibodies overnight, then washed and incubated with peroxidase-conjugated secondary antibodies, and protein bands were visualized using the ECL Chemiluminescence Kit (all from GE Healthcare Life Sciences; Piscataway, NJ). Primary antibodies included anti-β-catenin, anti-CK1α, and anti-phospho-GSK3α/β (Cell Signaling Technology), and anti-β-actin (Sigma-Aldrich). Protein bands were measured using ImageJ software (National Institutes of Health; http://rsbweb.nih.gov/ij/), normalized to β-actin loading controls, and controls in cells that were either vehicle-treated or wild-type, as indicated.

**Semi-quantitative RT-PCR** - Total RNA was isolated using RNeasy (Qiagen), and cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen) and Choice-Taq Blue Mastermix (Denville Scientific Inc.; Metuchen, NJ). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the following primers: β-catenin, forward 5’-GTCTGAGGACAACAGCCAACAGAACAAGATA-3’ and reverse 5’-CCATCAACTGGATAGTCAAGCACCAG -3’; β-actin, forward 5’-GGCATCCTCACCCTGAAGTA-3’ and reverse 5’-GGGGTGTTGAAGGTCTCAAA-3’. Reaction products were separated by agarose gel electrophoresis, DNA bands were measured using ImageJ software, and normalized to β-actin.

**Promoter assays** - Cells were infected with Lentiviral particles containing an LEF/TCF
promoter combined with a green fluorescent protein (GFP) reporter construct (SABiosciences™; Frederick, MD) in polybrene (Sigma-Aldrich). Control cells were infected with Lentiviral particles containing GFP under the control of the cytomegalovirus (CMV) promoter (Sigma-Aldrich). After infection, cells were washed, selected using puromycin (InvivoGen; San Diego, CA), treated with lenalidomide or vehicle for the indicated times, and GFP expression was analyzed using a FACS Calibur flow cytometer (BD Biosciences; San Jose, CA) and FlowJo software (Tree Star, Inc.; Ashland, OR). Measurements were made in triplicate, and are presented as the mean ± SD, while statistical comparisons were made using the paired student t-test, with p<0.05 considered as significant, and p<0.01 as highly significant.

**Flow cytometry** - Cells were collected and fixed in 3% formaldehyde, and either subjected to flow analysis immediately, or stored until later use. Prior to analysis, all samples were permeabilized in ice-cold 100% methanol, and then resuspended in buffer with either anti-β-catenin or anti-cyclin D1 antibody (Cell Signaling Technology), or anti-c-Myc antibody (Santa Cruz Biotechnology; Santa Cruz, CA). After washing, cells were incubated with Alexa Fluor-488- or Alexa Fluor-647-conjugated secondary antibodies (Invitrogen), and analyzed by flow cytometry with collection of 20,000 events.

**Adenoviral and Lentiviral Transduction** - Adenoviruses (Ad) directing the expression of GFP as a negative control, or β-catenin (Vector Biolabs; Philadelphia, PA), were infected into myeloma cell lines in triplicate experiments prior to exposure to lenalidomide. Lentiviral particles (Sigma-Aldrich) containing either a control, scrambled sequence shRNA (scr-shRNA), or β-catenin-targeting shRNAs (β-cat-shRNA1-2), were transduced overnight into lenalidomide-tolerant cells. These transiently transduced wild-type and drug-resistant cells were then exposed to lenalidomide and analyzed as described in the text.

**RESULTS**

**Development of lenalidomide-resistant cell lines.** Lenalidomide-induced anti-proliferative effects were evaluated initially in interleukin (IL)-6-dependent (ANBL-6) and IL-6-independent (U266) multiple myeloma cell lines (Fig. 1A, B). Cellular viability measurements, determined using the WST-1 reagent as a function of mitochondrial activity and thus cellular proliferation, indicated a strong time-dependence for the activity of lenalidomide when plasma cells were exposed continuously to this agent at 1 µM. Modest anti-proliferative activity was seen after 24, 48, and 72 hours of exposure (Fig. 1A, B), and substantial effects were not evident until plasma cells had been treated for 96 to 120 hours. When a lenalidomide concentration of 10 µM was used instead, the time-dependence was comparable, although the magnitude of the anti-proliferative activity, as expected, was greater (Supplementary Fig. 1). These effects are likely to be due to lenalidomide itself as opposed to any of its metabolites, since incubation of the drug in the presence of either human liver microsomes, recombinant cytochrome P450 isoenzymes, or human hepatocytes did not result in significant drug metabolism, confirming data from human studies (26). Also, the activity of lenalidomide was associated with an increased staining of myeloma cells with an antibody to Annexin V (Supplementary Fig. 2), suggesting that its effect was not just anti-proliferative, but also pro-apoptotic. Notably, patient-derived plasma cells typically undergo spontaneous programmed cell death within 48 to 72 hours of being removed from the permissive bone marrow microenvironment even in the absence of drug treatment. These observations suggested that primary plasma cells could not be effectively used to model resistance to lenalidomide given its slow onset of action. We therefore chose to utilize thoroughly characterized and established multiple myeloma cell lines to further investigate mechanisms of resistance to lenalidomide.

IL-6-dependent ANBL-6 and KAS-6/1 cells, as well as IL-6-independent MM1.S and U266 cells, were exposed to lenalidomide concentrations at the IC_{10} for this agent. Concentrations were then serially increased until each cell line maintained membrane integrity during continuous exposure to 10 µM, as judged by Trypan Blue exclusion (data not shown). All cell lines retained their viability (Fig. 1C-F), and relative fold resistance was calculated based upon the IC_{50} values. For drug-naïve ANBL-6 (ANBL-6.wt) cells, for example, the IC_{50} was 0.17 µM,
while their drug-resistant counterparts (ANBL-6/R10R) showed an IC_{50} >100 \mu M, indicating an at least 500-fold increased tolerance. Calculations for KAS-6/1, U266, and MM1.S cells indicated an at least 350-, 83-, and 2,500-fold increased resistance, respectively. Interestingly, in ANBL-6/R10R and U266/R10R cells, lenalidomide even seemed to stimulate cell growth at some of the drug concentrations tested, as indicated by an increase in cell viability above 100%. To evaluate the stability of the drug-resistant phenotype, cells were grown in lenalidomide-free media, first for seven days, and later for ninety days. When lenalidomide was added back, comparable levels of tolerance were seen compared to cells propagated continuously with lenalidomide using wild-type, drug-naïve cells as controls (data not shown). These findings suggested that lenalidomide resistance was a stable phenotype, though the possibility remained that a rapidly inducible, and then reversible event was responsible. Interestingly, lenalidomide-resistant cells displayed a subjective morphological change, and had a greater tendency to form multi-cellular aggregates than their drug-naïve counterparts (data not shown). Also of note, the drug-resistant phenotype appeared to be specific to lenalidomide, since these cells did not show significant cross-resistance to other commonly used anti-myeloma drugs, such as dexamethasone and bortezomib (data not shown).

*Lenalidomide induces Wnt/\(\beta\)-catenin pathway dysregulation.* To identify mechanisms of lenalidomide resistance, gene expression profiling was performed on the lenalidomide-tolerant myeloma cells and their vehicle-treated, drug-naïve counterparts. These data were then analyzed and mapped using Ingenuity Pathway Analysis Software, and this approach identified the Wnt/\(\beta\)-catenin cascade as the most commonly dysregulated pathway. Indeed, a number of Wnt/\(\beta\)-catenin intermediates were consistently induced at the messenger level in all four independently derived cell lines (Table 1). These included secreted components such as Wnt-3, receptor components such as frizzled Drosophila homolog (Fzd)-4, and downstream intermediates, such as \(\beta\)-catenin itself. Consistent with a role for lenalidomide in dysregulating Wnt/\(\beta\)-catenin, qualitatively similar changes were seen in primary samples from a patient who had lenalidomide-refractory disease (Table 1).

Following the identification of the dysregulation of the Wnt signaling pathway by microarray analysis, we first sought to validate this induction by performing RT-PCR with \(\beta\)-catenin-specific probes, and an increased content of \(\beta\)-catenin mRNA was seen in the lenalidomide-resistant cells (Fig. 2A). To determine if this occurred during acute exposure as well, RT-PCR was performed on previously drug-naïve myeloma cells. ANBL-6 cells treated in this fashion for three days revealed a lenalidomide dose-dependent increase in \(\beta\)-catenin mRNA (Fig. 2B) of up to 3-fold or more. As was the case for both chronically exposed myeloma cells and acutely treated ANBL-6 cells, KAS-6/1 (Fig. 2C) and MM1.S (Fig. 2D) cells treated with lenalidomide for seven days showed an up to 2-fold \(\beta\)-catenin induction.

*Wnt pathway dysregulation induces accumulation of \(\beta\)-catenin.* Dysregulation of Wnt often leads to \(\beta\)-catenin stabilization, and we therefore sought to determine if lenalidomide increased accumulation of this key protein. For all three cell lines examined, total \(\beta\)-catenin levels were increased in the drug-resistant cells propagated continuously in 10 \(\mu\)M lenalidomide (R10R) compared to controls (Fig. 3A). Increased \(\beta\)-catenin accumulation was also seen in drug-resistant cells grown in the absence of lenalidomide for seven days (RR), though these levels were lower than in the R10R cells (Fig. 3A). Using the U266 model as an example, compared to wild-type vehicle-treated cells, U266/R10R cells showed a >6-fold increase in \(\beta\)-catenin content, while U266.RR cells showed a >2-fold increase. The acute effects of drug exposure on \(\beta\)-catenin content were also studied in ANBL-6 (Fig. 3B), KAS-6/1 (Fig. 3C), and MM1.S (Fig. 3D) cells. When lenalidomide was added, \(\beta\)-catenin content increased in all three lines in a dose-dependent fashion. In KAS-6/1 cells, for example, \(\beta\)-catenin accumulation was seen starting at a lenalidomide concentration of 0.1 \(\mu\)M, and increased further through the evaluated range, with a maximal, ~20-fold induction over controls.

*Accumulation of \(\beta\)-catenin induces LEF/TCF activity.* Canonical Wnt signaling induces cytoplasmic \(\beta\)-catenin, which translocates to the nucleus and acts as a co-activator for the
LEF/TCF family of DNA binding proteins. Myeloma cells were therefore challenged with lenalidomide after infection with a Lentiviral LEF/TCF promoter-GFP reporter construct. When control ANBL-6 (Fig. 4A) or U266 (Fig. 4B) cells infected with a CMV promoter-GFP reporter construct were treated with lenalidomide, no significant promoter activation was seen. In contrast, both lines showed a concentration- and time-dependent LEF/TCF promoter induction with lenalidomide. Using ANBL-6 cells as an example, lenalidomide concentrations as low as 0.1 μM for three or seven days induced LEF/TCF activity by almost 2-fold, while concentrations up to 100 μM increased activity by >5-fold (Fig. 3A).

Activation of LEF/TCF can lead to induction of downstream targets such as cyclin D1 and c-Myc, and myeloma cells were therefore examined using a FACS-based assay. Consistent with earlier findings (Fig. 4), U266/R10R and MM1/R10R lenalidomide-resistant cells showed a 3.6- and 5.8-fold increased β-catenin content, respectively, compared to drug-naïve cells (Fig. 5A). When propagated in 10 μM lenalidomide, drug-resistant MM1.S cells showed a 2.3-fold increase in cyclin D1 protein (Fig. 5B). A smaller, 1.2-fold increase was seen in lenalidomide-treated U266 cells, possibly since they harbor the t(11;14) translocation, which induces high cyclin D1 levels at baseline, and may disrupt the input of the Wnt/β-catenin pathway. Finally, MM1/R10R and U266/R10R cells had an increased content of c-Myc by 3.1- and 3.9-fold, respectively, compared to controls (Fig. 5C). These data support the hypothesis that lenalidomide induces the Wnt/β-catenin pathway, and activates LEF/TCF with consequences on downstream targets such as cyclin D1 and c-Myc.

Lenalidomide suppresses CK1α and GSK3α/β. Increased β-catenin transcription would contribute to its intracellular accumulation, but the induction at the protein level seemed out of proportion to the increase in mRNA, suggesting contributions from other mechanisms. Constitutive cytoplasmic β-catenin levels are controlled by a ‘destruction complex’ that includes CK1α and GSK3α/β. In the absence of Wnt, β-catenin is phosphorylated by CK1α at Ser45, which primes it for phosphorylation by GSK3α/β at Thr41/Ser37/Ser33, triggering β-catenin ubiquitination and proteasome-mediated degradation (27). We therefore examined the possibility that lenalidomide was modulating β-catenin levels by altering components of the destruction complex. Compared to drug-naïve cells, drug-resistant ANBL-6/R10R, U266/R10R, and MM1/R10R cells propagated in 10 μM lenalidomide showed decreased expression levels of CK1α (Fig. 6A). Notably, CK1α levels did not seem to recover substantially even after drug removal in drug-resistant ANBL-6/RR, U266/RR, and MM1/RR cells, suggesting this was a stable phenotype. To determine if CK1α suppression was also an acute effect of lenalidomide, ANBL-6.wt, KAS-6.wt, and U266.wt drug-naïve cells were examined, and acute lenalidomide exposure also suppressed CK1α (Fig. 6B). This effect was most pronounced in the ANBL-6 models, where acute and chronic drug exposure induced an almost quantitative disappearance of CK1α.

We next examined GSK3α/β phosphorylation status at residues Ser21/Ser9, since phosphorylation at these sites inhibits GSK3α/β kinase activity toward β-catenin. Compared to drug-naïve cells, ANBL-6, U266, and MM1.S cells propagated with lenalidomide showed an increase in phosphorylated, inactivated GSK3α/β (Fig. 7A). Previously drug-naïve ANBL-6, KAS-6/1, and U266 cells acutely exposed to lenalidomide also showed increased levels of phospho-GSK3α/β (Fig. 7B). These studies support the hypothesis that lenalidomide induced Wnt/β-catenin in part by suppressing the stability of CK1α and activity of GSK3α/β, resulting in decreased β-catenin turnover.

Wnt/β-catenin modulates lenalidomide sensitivity. To more directly examine the role of the Wnt/β-catenin pathway in lenalidomide resistance, we sought to use molecular approaches to induce its activity. Extracellular Wnt acts by binding to the Fzd receptor and LRP co-receptor, which then propagate a signal to disrupt the β-catenin ‘destruction complex’ (28). We therefore pre-incubated drug naïve KAS-6/1 and ANBL-6 (Fig. 8A, right and left panels, respectively) for two hours with rWnt-3a, and then exposed them to lenalidomide. Such Wnt/β-catenin pathway stimulation significantly reduced the ability of lenalidomide to exert anti-proliferative effects, as judged by an increase in the IC₅₀ values of at least
10-fold for these cell lines. Since Wnt signaling can occur through non-canonical, β-catenin-independent pathways (28), it was also of interest to verify that β-catenin accumulation was by itself sufficient to mediate lenalidomide resistance. Adenoviruses inducing expression of GFP or β-catenin were therefore transduced into drug naïve KAS-6/1 and U266 cells (Fig. 8B, left and right panels, respectively), which were then treated with lenalidomide. In both lines, β-catenin overexpression preserved cellular viability, with an increase in the IC50 values of at least 10-fold.

We next wondered if we could reverse the lenalidomide-resistant phenotype by reducing β-catenin expression. Lentiviral particles containing constructs for a non-specific scrambled sequence shRNA (scr.shRNA), or two different shRNA constructs targeting β-catenin (β-cat-shRNA.1 and β-cat-shRNA.2) were therefore used. For ANBL-6/R10R cells (Fig. 8C, left panel), both β-cat-shRNA constructs restored the anti-proliferative activity of lenalidomide, as judged by a reduction in viability of approximately 30% and 50% following 3 and 7 day drug exposures, respectively, compared to scr.shRNA controls. Similarly, KAS-6/R10R cells showed a reduction in cellular viability of 20% and 30% to lenalidomide, respectively, compared to controls. These findings together support the possibility that β-catenin activation mediates resistance to lenalidomide, and that approaches which suppress Wnt/β-catenin may be of benefit in restoring sensitivity to this agent.

**DISCUSSION**

Clinical drug development in multiple myeloma usually begins by the evaluation of promising agents in patients with relapsed/refractory disease, and those that show substantial activity, such as bortezomib (29) and lenalidomide (1), are often later incorporated into front line therapy. One potential disadvantage to the earlier use of our best agents is the risk that drug-resistant disease clones will develop, thereby reducing options for treatment of patients with relapsed disease. We therefore sought to develop a model of plasma cells that were resistant to the direct anti-proliferative and pro-apoptotic effects of lenalidomide. This was with the rationale that these could ultimately help in understanding the mechanisms by which lenalidomide exerts its effects, identifying biomarkers of sensitivity, characterizing the mechanisms of lenalidomide resistance, and validating novel drugs or approaches to overcome or prevent the emergence of resistance.

In the current work, which to our knowledge is the first to present such a model, we report our initial characterization of these cells, which were tolerant of clinically relevant lenalidomide drug concentrations (6, 13)(Fig. 1). Microarray alignment of lenalidomide-resistant cells compared with their drug-naïve counterparts identified the Wnt/β-catenin signaling pathway as having multiple conserved changes (Table 1). Canonical Wnt/β-catenin signaling starts with the extracellular binding of Wnts to their receptors and co-receptors, thereby destabilizing a destruction complex that normally targets β-catenin for proteasome-mediated degradation (28). This promotes β-catenin accumulation and its nuclear translocation, and participation as a co-activator in transcription of target genes responsible for a variety of cellular processes. A growing body of evidence has implicated Wnt/β-catenin activation or hyperactivation through genetic (30) or epigenetic (31) mechanisms as contributing to, or even initiating oncogenesis. β-catenin gain of function mutations are commonly found in colorectal and prostate carcinomas, among others (28). Overexpression of positive regulators such as secreted Wnts, the receptor protein Fzd, or signal mediators Disheveled and Frat1 occur in a variety of tumors as well, such as ovarian and breast cancers (28). Conversely, loss of function mutations in the negative regulators APC and Axin occur in several cancers, including colorectal and liver tumors (28). Moreover, emerging data also support an important role for Wnt/β-catenin activation as a mediator of chemoresistance. For example, resistance to interferon-α/5-fluorouracil-mediated cell death in hepatocellular carcinoma required Wnt/β-catenin activation (32). Also, studies of cisplatin resistance in laryngeal carcinoma identified a link between cell adhesion mediated drug resistance (CAM-DR)(33) and β-catenin and plakoglobin levels. Activation of Wnt/β-catenin was also responsible for CAM-DR to anthracyclines in neuroblastomas (34), acute myeloid leukemia (35),...
and myeloma (36). Given this background, and the important role of CAM-DR in the natural history of myeloma (37, 38), it is tempting to speculate that Wnt/β-catenin signaling mediates lenalidomide resistance by impacting on plasma cell adhesive properties. This possibility is supported by the observation that lenalidomide-resistant cells appear to form aggregates to a greater extent than their drug-naïve counterparts (data not shown), and we are currently performing further studies to identify the mechanisms behind this morphologic change. Additional studies will also be needed to examine the impact of Wnt/β-catenin activation on the other properties of lenalidomide that contribute to its anti-myeloma activity, including the ability of this drug to act as an immune modulator, and to modify the tumor microenvironment, ideally using both murine in vivo models, as well as patient-derived clinically annotated primary samples.

Interestingly, activation of Wnt/β-catenin was seen both in drug-naïve cells acutely exposed to lenalidomide (Fig. 3B-D), and in drug-resistant cells chronically treated with this agent (Fig. 3A). Since the former were undergoing lenalidomide-induced apoptosis, while the latter proliferated in the presence of drug, and under some conditions to an even greater extent than with vehicle alone (Fig. 1 C, E), this may at first seem to undermine the role of Wnt/β-catenin in drug resistance. However, a direct link between pathway activation and lenalidomide resistance was convincingly supported by studies in which Wnt/β-catenin was manipulated using targeted, molecular approaches. These showed that pathway activation with recombinant Wnt (Fig. 8A), or over-expression of β-catenin (Fig. 8B), reduced lenalidomide sensitivity in drug-naïve cells, while shRNA-mediated suppression in lenalidomide-resistant cells enhanced drug activity (Fig. 8C). It is also notable in this regard that time-course experiments with ANBL-6 (Fig. 1A) and U266 cells (Fig. 1B) showed that the majority of the anti-proliferative and pro-apoptotic effects of lenalidomide were fully expressed by day 5 of exposure. Thus, by day 7, which was the timepoint used in our studies, the vast majority of the drug-sensitive cells have already been eliminated, and the remaining cells are already functionally drug-resistant, but may have not yet acquired this property in a stable phenotype, which takes a longer period of exposure. This rapid emergence of drug-resistant cells also suggests the possibility that these cell lines, and possibly primary tumors as well, consist of a mixture of inherently lenalidomide-sensitive and –resistant populations, and that it is the ability of the latter to better activate Wnt/β-catenin and survive drug treatment that leads to disease persistence and subsequent relapse. Studies are underway to determine by immunophenotyping if two such populations can indeed be identified. If this could be done, it would be interesting to compare these with genomic and proteomic techniques to provide further insights into the mechanisms of drug resistance, as well as to possibly provide markers of drug sensitivity that could be useful clinically to predict which patients would most benefit from therapy.

Our current findings suggest a role for two mechanisms in the induction of the Wnt/β-catenin pathway by lenalidomide. One of these appears to be enhanced β-catenin transcription, since we observed increased transcript levels in chronically treated cells (Table 1), and in acutely exposed cells (Fig. 2). These findings are interesting considering that β-catenin has been felt to be primarily regulated at the protein level, while transcriptional control was under a constitutive promoter. However, Bandapali et.al. (39) recently showed that β-catenin, in conjunction with TCF4, bound to and regulated its own promoter. Moreover, previous β-catenin promoter mapping studies showed an LEF/TCF binding site, and high affinity sites for E2F1 and NF-κB (40). Since the former is regulated by cyclin D1, a downstream Wnt/β-catenin target, it is possible that induction of β-catenin transcription is a secondary event stimulated by the accumulation of β-catenin protein itself (Fig. 3), and activation of the pathway as a whole (Fig. 4), in a positive feedback loop. However, our experiments have not probed for the possibility that lenalidomide may have a direct epigenetic effect on the β-catenin promoter, as it does on p21 (19), or that it may directly or indirectly influence other transcriptional activators and/or repressors that control β-catenin transcription. Further studies will be needed to determine the contribution of these various possible mechanisms to the actions of lenalidomide.
The second mechanism of β-catenin activation suggested by our findings is through suppression of expression of CK1α (Fig. 6) and activity of GSK3α/β (Fig. 7). CK1α, a serine/threonine kinase, is responsible for the initial phosphorylation priming step at Ser45 of β-catenin. Casein kinases are ubiquitous (41), but less is understood about their regulation or turnover. Unlike other casein kinases that are regulated by phosphorylation events, CK1α lacks the consensus sequences needed to submit to this type of regulation. Potential mechanisms for a reduction of CK1α could therefore be similar to those discussed above for β-catenin, including a suppression of transcription, or enhanced protein turnover. Interestingly, our microarray data indicated that there was a consistent increase in CK1α mRNA levels (not shown), suggesting that lenalidomide both activated CK1α transcription, and either suppressed its translation, or enhanced its turnover.

GSK3α/β is also a common threonine/serine kinase made up of a heterodimer of the α and β subunits. It is unphosphorylated when active, and is responsible for the phosphorylation of β-catenin at Thr41/Ser37/Ser33. This generates the recognition signal that allows the E3 ubiquitin ligase β-TRCP to bind and poly-ubiquitinate β-catenin, leading to its proteasome-mediated degradation. Following an array of stimulatory responses, GSK3α/β is phosphorylated at Ser21 of the α subunit, and Ser9 of the β subunit, by either protein kinase A (PKA)(42) or protein kinase B (PKB)/Akt (43). This phosphorylation leads to inactivation of GSK3α/β, and is associated with a pro-survival cellular response. Our data indicate that phosphorylated GSK3α/β levels are increased upon acute and chronic lenalidomide exposure. Consistent with an inhibition of GSK3α/β activity, this resulted in decreased abundance of β-catenin phosphorylated at Thr41/Ser37/Ser33 (not shown). This could be occurring through a Wnt-dependent autocrine or paracrine process resulting in dissolution of the destruction complex, given that our microarray data showed increased transcription of several Wnt isoforms. However, we were unable to confirm the presence of increased Wnt production and secretion into the media (not shown), providing an interesting parallel with the data for CK1α, above. It is also possible that lenalidomide directly stimulates PKA or PKB/Akt, resulting in enhanced GSK3α/β phosphorylation, though this is not supported by previous studies suggesting that lenalidomide suppressed Akt (20). Once again, additional studies will be needed to define all of the mechanisms by which this compound exerts such pleiotropic effects.

Taken together, our experimental efforts have identified activation of the Wnt/β-catenin pathway as a conserved effect of acute and chronic exposure of plasma cells to lenalidomide. Activation is mediated by decreased β-catenin destruction and increased transcription, and results in downstream activation of targets such as LEF/TCF, cyclin D1, and c-Myc. Additionally, we have shown that selective modulation of this pathway can alter the anti-proliferative potency of this drug against myeloma cells. These drug-resistant models may also allow us in the future to examine whether Wnt/β-catenin activation can be a biomarker for lenalidomide sensitivity, and whether it can be targeted through pharmacological means to induce chemosensitization and reverse resistance. If validated, these approaches will also need to be considered in the context of the role of the Wnt/β-catenin pathway in myeloma bone disease. Overproduction of Dkk, a secreted LRP inhibitor of produced by myeloma cells, induces bone resorption through its effects on marrow osteoblasts (44). Binding of Dkk to LRP extinguishes Wnt signaling, and Dkk antibodies, which would enhance Wnt/β-catenin activity, are being evaluated as bone anabolic agents (45). Thus, there is the risk that approaches that would inhibit Wnt/β-catenin could enhance the efficacy of lenalidomide, but also enhance bone resorption, unless they could somehow be targeted only to myeloma cells.

Acknowledgements
R.Z.O., a Leukemia & Lymphoma Society Scholar in Clinical Research, would like to acknowledge support from the Leukemia & Lymphoma Society (6096-07), and the National Cancer Institute (R01 CA102278). All flow cytometry experiments were performed at The University of Texas M. D. Anderson Cancer Center Flow Cytometry and Cellular Imaging Center, which is funded by NCI # CA16672.

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Abbreviations
Ad, Adenoviral; APC, adenomatous polyposis C; Akt, AKT8 virus oncogene cellular homolog; β-cat, β-catenin; Bmp, Bone morphogenetic protein; CAM-DR, cell adhesion mediated drug resistance; CK, casein kinase; CMV, cytomegalovirus; Dkk, Dickkopf homolog; Dvl, Disheveled; FACS, fluorescence-activated cell sorting; Fzd, frizzled Drosophila homolog; GFP, green fluorescent protein; GSK, glycogen synthase kinase; IC, inhibitory concentration; IL, interleukin; IMiD, immunomodulatory agent; JNK, c-Jun-N-terminal kinase; LEF, lymphoid enhancer factor; Len, lenalidomide; len/dex, lenalidomide with dexamethasone; LR, lenalidomide resistant; LRP, lipoprotein related protein; MM, multiple myeloma; MOI, multiplicity of infection; NF-κB, nuclear factor-kappaB; PKA, protein kinase A; PKB, protein kinase B; ROS, reactive oxygen species; RR, lenalidomide resistant cells propagated in 10µM lenalidomide that have had drug removed for the period indicated in the text; R10R, lenalidomide resistant cells propagated continuously in 10µM lenalidomide; RT-PCR, reverse transcriptase-polymerase chain reaction; scr, scrambled sequence; SD, standard deviation; shRNA, short hairpin RNA; TCF, T-cell factor; Wnt, wingless MMTV integration type

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**Figure Legends**

**Figure 1**: Cellular viability of multiple myeloma (MM) cell lines following lenalidomide exposure. The drug naïve IL-6-dependent (ANBL-6)(A), and IL-6-independent (U266)(B) cell lines were evaluated for time-dependent viability following continuous exposure to 1 μM lenalidomide for up to seven days. Lenalidomide-resistant cell lines were generated through a serial increase in lenalidomide concentrations added to the culture medium over several months, until the cell lines were capable of maintaining proliferation and cell membrane integrity at 10 μM lenalidomide. These new cell lines were named (C) ANBL-6/R10R, (D) KAS-6/R10R, (E) U266/R10R, and (F) MM1/R10R. Comparison of the sensitivity of lenalidomide-resistant cell lines vs. their wild-type counterparts to lenalidomide is shown here in relative IC50 curves that are displayed as a percentage of cellular viability via mitochondrial reduction of the tetrazolium salt, WST-1. All data points were normalized to the vehicle control (0.01% DMSO), which was arbitrarily set at 100% viability. Samples were run in triplicate, and are represented as the mean ± the S.D.

**Figure 2**: Acute exposure to lenalidomide induces β-catenin transcription.
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RT-PCR was performed to detect β-catenin or β-actin transcripts from cells propagated continuously in lenalidomide (R10R), or R10R cells that were removed from lenalidomide for one week (RR), to detect β-catenin, and comparisons were made to vehicle-treated, drug-naïve cells (wt(A)). RT-PCR products were separated by acrylamide gel electrophoresis, and detected by ethidium bromide staining. The left panel shows representative agarose gels, and densitometry was performed to calculate the increase in β-catenin after correcting for β-actin, which was normalized to the wt cells, which were set at 1.0. Drug-naïve ANBL-6 (B), KAS-6/1 (C), and MM1.S (D) cells were exposed to the indicated lenalidomide concentrations for 72 (ANBL-6) or 168 hours (KAS-6/1, MM1.S). Each top panel provides a representative photomicrograph, and densitometry was performed to calculate the β-catenin/β-actin ratio. Values for each cell line are provided in the lower panels after normalization to the vehicle-treated control, which was arbitrarily set at 1.0.

Figure 3. Lenalidomide induces β-catenin protein accumulation.
Protein extracts from myeloma cells propagated continuously in lenalidomide (R10R), or R10R cells that were removed from lenalidomide for one week (RR), were probed to detect β-catenin, and compared to vehicle-treated drug-naïve cells (wt(A)). The left panel shows a representative Western blot, and densitometry was performed to calculate the increase in β-catenin in each cell line after correcting for β-actin, which was normalized to the wt cells, which were set at 1.0. Acute exposure was studied in drug-naïve ANBL-6 (B), KAS-6/1 (C), and MM1.S (D) cells, which were treated with the indicated lenalidomide concentrations for 72 (ANBL-6) or 168 hours (KAS-6/1, MM1.S).

Figure 4. Lenalidomide stimulates LEF/TCF-dependent transcriptional activity.
LEF/TCF reporter activity was measured in ANBL-6 (A) and U266 (B) cells following exposure to the indicated lenalidomide concentrations for 3 or 7 days. Triplicate measurements of GFP fluorescence were normalized to the vehicle-treated controls, and expressed as the mean signal (A.U.±S.D). The student’s paired t-test was used to determine statistical significance, and “**” denotes p<0.005, while “***” denotes p<0.001. ANBL-6 (A) and U266 (B) cells were also infected with Lentiviral particles directing expression of a CMV-GFP control reporter, and the impact of lenalidomide is shown.

Figure 5. Activation of β-catenin induces cyclin D1 and c-Myc levels.
Wild-type drug-naïve U266 (upper profiles) and MM1.S (middle profiles) cell populations (dashed lines), and their comparable R10R variants (solid lines), were evaluated by FACS for expression of β-catenin (A), cyclin D1 (B), and c-Myc (C). The lower panels display semiquantitative analyses of the mean fluorescent intensity for each R10R cell line compared to the wt controls, which were arbitrarily set at 1.0. Each dotted line represents 1.0, and values above that level represent increased expression of the proteins of interest. The student’s paired t-test was used to determine statistical significance, and “*” denotes p<0.05, while “**” denotes p<0.01.

Figure 6. Lenalidomide suppresses expression of CK1α.
Protein extracts from R10R or RR myeloma cell lines were probed to detect CK1α, and compared to vehicle-treated drug-naïve cells (wt(A)). The left panel shows a representative Western blot, and densitometry was performed to calculate the decrease in CK1α in each cell line after correcting for β-actin, which was normalized to the wt cells, which were set at 1.0. Acute exposure was studied in drug-naïve cells (B), which were treated with the indicated lenalidomide concentrations for 72 (ANBL-6) or 168 hours (KAS-6/1, U266). The left panels show representative Western blots, while the right panels show densitometry performed to evaluate CK1α levels corrected for β-actin loading, and normalized to the wt controls.

Figure 7. Lenalidomide enhances phosphorylation of GSK3α/β.
Protein extracts from R10R or RR myeloma cell lines were probed to detect GSK3α/β, and compared to
vehicle-treated drug-naïve cells (wt). Densitometry was performed to calculate the increase in phospho-GSK3α/β after correcting for β-actin, which was normalized to the wt cells, which were set at 1.0. Acute exposure was studied in drug-naïve ANBL-6, KAS-6/1, and U266 cells, which were treated with the indicated lenalidomide concentrations for 72 (ANBL-6) or 168 hours (KAS-6/1, U266). The left panels show representative Western blots, while the right panels show densitometry performed to evaluate phospho-GSK3α/β levels corrected for β-actin normalized to the wt controls.

**Figure 8. Modulation of Wnt/β-catenin pathway alters lenalidomide sensitivity.**

Drug naïve KAS-6.wt and ANBL-6.wt cells were exposed to vehicle or 25 ng/mL human recombinant Wnt-3a (rWnt-3a) prior to exposure to lenalidomide for 168 hours. Cellular viability measurements were performed using the WST-1 assay, and all data points were normalized to the vehicle control (0.01% dimethyl sulfoxide), which was set at 100%. Mean viability values are provided from three independent experiments, along with the S.D. KAS-6.wt and U266.wt were then infected with Adenoviral particles containing CMV promoter-driven expression vectors for GFP (Ad-GFP) or β-catenin (Ad-β-cat) for 24 hours. They were then exposed to lenalidomide, analyzed, and are presented as described in panel A. Finally, ANBL-6/R10R and KAS-6/R10R cells were infected with Lentiviral particles containing shRNA constructs targeted to a scrambled sequence (scr.shRNA), or an shRNA targeting β-catenin (β-cat-shRNA-1 and -2). Twenty-four hours later they were exposed to 10 µM lenalidomide for 72 or 168 hours, and analyzed for viability. All values are normalized to the vehicle control, and presented as the average of triplicate measurements ± S.D. The student’s paired t-test was used to determine statistical significance, and “**” denotes p<0.005, while “***” denotes p<0.001 for part (C).
**Table 1. Impact of Chronic Lenalidomide Exposure on Selected Components of the Wnt/β-catenin Pathway**

|          | ANBL-6 | KAS-6 | U266 | MM1.S | Primary ‡ |
|----------|--------|-------|------|-------|-----------|
| WNT3     | 0.500  | 5.300 | 2.800| 3.400 | 5.500     |
| WNT7B    | 0.200  | 3.500 | 1.300| 0.400 | 1.300     |
| WNT8B    | -0.200 | 1.100 | 0.000| 0.300 | 2.500     |
| WNT4     | -1.500 | 2.500 | 0.400| 2.200 | 2.200     |
| WNT9A    | -1.300 | 1.200 | 0.300| 2.000 | 2.200     |
| WNT10A   | 1.200  | 2.000 | -2.600| 1.200 | 1.800     |
| WNT10B   | 2.700  | 1.600 | -2.100| 2.000 | 0.800     |
| WNT2B    | 1.900  | 2.500 | -2.000| 0.600 | 1.800     |
| WNT1     | -0.600 | 0.300 | 0.100| 0.000 | 5.200     |
| WNT11    | -0.100 | 2.100 | 0.700| 0.300 | 1.900     |
| LRP1     | -0.400 | 3.100 | 2.700| 3.200 | 5.100     |
| LRP5     | -0.200 | 0.600 | 3.000| 3.800 | 3.300     |
| LRP6     | -0.900 | 2.000 | -2.600| 0.800 | 1.900     |
| FZD4     | 1.400  | 2.000 | 1.000| 0.600 | 1.500     |
| FZD2     | -0.600 | 1.000 | 3.100| 1.900 | 2.900     |
| FZD5     | 0.800  | 1.800 | 1.000| -0.500| 2.400     |
| FZD7     | -0.900 | 2.100 | 3.100| 0.200 | 1.900     |
| FZD10    | 1.800  | 3.900 | -0.600| 0.400 | 2.600     |
| DVL1     | 0.000  | 1.500 | 0.200| 1.400 | 2.600     |
| DVL3     | 0.500  | 1.000 | 0.400| 0.400 | 2.400     |
| CTNNB1   | 0.800  | 2.500 | 0.700| 2.900 | 1.700     |
| LEF1     | 0.300  | 2.500 | -0.400| -1.500| 4.200     |
| TCF7L2   | -1.100 | 2.300 | 3.600| 3.500 | 4.300     |
| TCF7L1   | -0.600 | 1.600 | -0.200| 2.600 | 1.000     |
| TCF3     | 1.700  | 1.900 |-1.800| -1.500| 2.900     |
| TCF4     | 0.300  | -4.000| 0.600| 1.000 | -1.300    |

*All data are expressed as the log change in expression of the chronically lenalidomide-exposed cells compared to their vehicle-treated, drug-naïve controls

†One primary sample was obtained from a patient who was clinically refractory to lenalidomide therapy as defined in the “Materials and Methods,” and compared to the profile of plasma cells from this same patient obtained at a point prior to therapy with lenalidomide

Abbreviations: CTNNB1, catenin (cadherin-associated protein), beta 1, 88kDa; DVL, dishevelled, dsh homolog (Drosophila); FZD, frizzled homolog (Drosophila); LEF1, lymphoid enhancer-binding factor 1; LRP, low density lipoprotein-related protein; TCF3, transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47); TCF7L2, transcription factor 7-like 2 (T-cell specific, HMG-box) transcription factor 7-like 2 (T-cell specific, HMG-box); WNT, wingless-type MMTV integration site family, member
Figure 1

A. ANBL-6.wt

B. U266.wt

C.

D.

E.

F.

\( \beta \)-catenin Signaling Mediates Lenalidomide Resistance

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Figure 2

A. 

|       | ANBL-6 | U266 | MM1.S |
|-------|--------|------|-------|
| β-catenin | wt | R | RR | wt | R | RR | wt | R | RR |
| β-actin |      |     |     |     |     |     |     |     |     |

B. 

|     | ANBL-6,wt | Lenalidomide |
|-----|------------|--------------|
| β-catenin |       |              |
| β-actin |      |               |

C. 

|     | KAS-6,wt | Lenalidomide |
|-----|----------|--------------|
| β-catenin |       |              |
| β-actin |      |               |

D. 

|     | MM1.S,wt | Lenalidomide |
|-----|----------|--------------|
| β-catenin |       |              |
| β-actin |      |               |
**Figure 3**

A.  

|          | ANBL-6 | U266 | MM1.S |
|----------|--------|------|-------|
| β-catenin| wt     | R10R | RR    |
| β-actin  |        |      |       |

B.  

|          | ANBL-6.wt | Lenalidomide |
|----------|------------|--------------|
| β-catenin|            |              |
| β-actin  |            |              |

C.  

|          | KAS-6.wt | Lenalidomide |
|----------|----------|--------------|
| β-catenin|          |              |
| β-actin  |          |              |

D.  

|          | MM1.S.wt | Lenalidomide |
|----------|----------|--------------|
| β-catenin|          |              |
| β-actin  |          |              |
**Figure 4**

A. ANBL-6.wt  

| Lenalidomide (µM) | LEF/TCF Reporter Signal (A.U.) |
|-------------------|-------------------------------|
| Vehicle           | 0                             |
| 0.1               | **                         |
| 1                 | ***                        |
| 10                | **                       |
| 100               | ***                      |

B. U266.wt  

| Lenalidomide (µM) | LEF/TCF Reporter Signal (A.U.) |
|-------------------|-------------------------------|
| Vehicle           | 0                             |
| 0.1               | *                         |
| 1                 | ***                        |
| 10                | **                       |
| 100               | ***                      |
Figure 5

A. β-catenin
B. cyclin D1
C. c-Myc

U266

MML S

Normalized Fold Increase (U266 vs. MM1)

* p < 0.05
** p < 0.01
### Figure 6

#### A.

|       | ANBL-6 | U266 | MM1S |
|-------|--------|------|------|
| wt    |        |      |      |
| R10R  |        |      |      |
| RR    |        |      |      |

**CK1α**

![CK1α blots](image1)

**β-actin**

![β-actin blots](image2)

#### B.

![Vehicle vs Lenalidomide blots](image3)

**ANBL-6.wt**

**KAS-6.wt**

**U266.wt**

**Lenalidomide (μM)**

| Lenalidomide (μM) | Vehicle | 0.01 | 0.1  | 1    | 10   | 100  |
|-------------------|---------|------|------|------|------|------|
| ANBL-6            |         |      |      |      |      |      |
| U266              |         |      |      |      |      |      |
| MM1S              |         |      |      |      |      |      |

**Normalized Decrease (CK1α/β-actin)**

![Normalized Decrease Graph](image4)
Figure 7

A.

|       | ANBL-6 | U266 | MM1.S |
|-------|--------|------|--------|
| p-GSK3α/β |        |      |        |
| β-actin   |        |      |        |
| wt       | R10R   | RR   | wt     | R10R   | RR   |
|          |        |      |        |        |      |

B.

[Graph showing normalized increase in R10R/WT for ANBL-6, U266, and MM1.S]

[Graph showing normalized increase in p-GSK3α/β/β-actin for ANBL-6, KAS-6, and U266 across different concentrations of Lenalidomide (μM)]
Figure 8

A. ANBL-6.wt and KAS-6.wt cell viability in response to different concentrations of lenalidomide with and without Wnt3a signaling.

B. KAS-6.wt and U266.wt cell viability showing the effect of adenovirus expression of GFP and β-catenin on lenalidomide treatment.

C. ANBL-6/R10R and KAS-6/R10R cell viability with different shRNA treatments and lenalidomide treatment duration.
Evidence of a role for activation of Wnt/β-catenin signaling in the resistance of plasma cells to lenalidomide

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J. Biol. Chem. published online December 28, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.180208

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