



Investigating the Mechanism and Species-Specificity of Thalidomide Derivatives

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Investigating the mechanism and species-specificity of thalidomide derivatives

A dissertation presented

by

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to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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Investigating the mechanism and species-specificity of thalidomide derivatives

Abstract

Thalidomide, once infamous for its teratogenic effects, and its derivatives lenalidomide and pomalidomide have found new clinical utility as highly effective treatments for multiple myeloma, other B cell neoplasms, and myelodysplastic syndrome (MDS) with del(5q). These drugs bind to cereblon (CRBN), the substrate adaptor of the CRL4^{CRBN} E3 ubiquitin ligase and induce the recruitment and ubiquitination of specific protein targets, ultimately resulting in their degradation by the proteasome. Prior work identified lenalidomide-induced ubiquitination and degradation of the lymphoid transcription factors Ikaros (IKZF1) and Aiolos (IKZF3). Degradation of IKZF1 and IKZF3 kills multiple myeloma cells and increases IL-2 release by T cells, explaining two aspects of lenalidomide's clinical efficacy, but does not account for lenalidomide's therapeutic window in MDS with del(5q).

Here, we demonstrated that lenalidomide induces the ubiquitination of casein kinase 1A1 (CK1 α) by CRL4^{CRBN}, resulting in CK1 α degradation. CK1 α is encoded by a gene, *CSNK1A1*, within the common deleted region for del(5q) MDS, and its haploinsufficiency sensitizes cells to lenalidomide therapy. Thus, lenalidomide-induced degradation of CK1 α provides a mechanistic basis for lenalidomide's therapeutic window in del(5q) MDS.

Despite their widespread clinical use, studies of thalidomide derivatives have been limited by their lack of effect in mouse models. While characterizing CK1 α as a lenalidomide-induced substrate, we found that mouse cells are resistant to thalidomide-derivatives because of species-specific differences in CRBN. To enable *in vivo* study of this class of drugs, we

developed a knock-in mouse model with a single amino acid change in Crbn, *Crbn*^{I391V}, which shows thalidomide-induced degradation of Ikzf1, Ikzf3, and Ckl1 α . Using this model, we demonstrated that haploinsufficiency for *Csnk1a1* confers lenalidomide sensitivity *in vivo* and that both lenalidomide-induced selection and *Trp53*-mediated resistance occur at the level of hematopoietic stem cells. We also demonstrated that *Crbn*^{I391V} is sufficient to confer thalidomide-induced fetal loss in mice. Further study of the *Crbn*^{I391V} model will provide valuable insights into the *in vivo* efficacy and toxicity of this class of drugs, as well as the opportunities to identify tissue-specific substrates which may contribute to efficacy in other conditions.

Table of Contents

List of Figures	vii
List of Tables.....	ix
Acknowledgements	x
Chapter 1 Introduction.....	1
Preface	2
A brief history of the thalidomide tragedy	2
Thalidomide embryopathy	5
The repurposing of thalidomide and development of the IMiDs	7
Mechanism of action	10
Therapeutic activity of IMiDs in multiple myeloma and other B cell neoplasms	12
Similar drugs	14
Pathogenesis of del(5q) MDS	15
Lenalidomide in del(5q) MDS	17
Mechanism of thalidomide's teratogenicity	19
IMiDs in rodent models.....	20
Aims of the thesis.....	22
Chapter 2 Lenalidomide induces the ubiquitination of CK1α by CRL4^{CRBN}	24
Contributions.....	25
Summary	26
Introduction	27
Results	27
Lenalidomide induces degradation of CK1 α	27
CK1 α is a substrate of CRL4 ^{CRBN}	31
Effect of <i>CSNK1A1</i> expression level.....	36
Species-specific effects of lenalidomide	42
Differential substrate specificity.....	51
Discussion	56
Methods.....	58
Chapter 3 Development of a thalidomide-sensitive mouse model via a single amino acid change in Cereblon	77
Contributions	78

Summary	79
Introduction	80
Design considerations.....	80
Results	82
Thalidomide derivatives induce degradation of substrates in <i>Crbn</i> ^{I391V} knock-in mice	82
Effect of lenalidomide treatment on hematopoiesis	95
<i>In vivo</i> modeling of efficacy of lenalidomide in cells haploinsufficient for <i>Csnk1a1</i>	101
Thalidomide-induced teratogenicity in <i>Crbn</i> ^{I391V} mice.....	109
Discussion	118
Methods.....	120
Chapter 4 Discussion	131
Drug-induced synthetic haploinsufficiency	133
Other therapeutic indications for CK1 α degradation	134
Contribution of other del(5q) genes to lenalidomide sensitivity in MDS with del(5q)	135
Potential mechanisms of lenalidomide in non-del(5q) MDS	137
Teratogenicity in the <i>Crbn</i> ^{I391V} model	139
Future applications of the <i>Crbn</i> ^{I391V} mouse model.....	141
Structural basis of the importance of V388 in IMiD-induced degradation of IKZF1, IKZF3, and CK1 α	144
Contribution of other Crbn residues to thalidomide derivatives' species-specific effects.....	145
Conclusion.....	149
References	150

List of Figures

Figure 1.1: The chemical structure of thalidomide.....	3
Figure 1.2: Critical period for thalidomide's teratogenic effects in humans.....	6
Figure 1.3: Chemical structures of IMiD compounds.....	9
Figure 1.4: Mechanism of lenalidomide in multiple myeloma.....	11
Figure 1.5: Commonly deleted regions in del(5q).....	17
Figure 1.6: Amino acid sequence alignment of human and mouse CRBN.....	21
Figure 1.7: Comparison of the IMiD-responsive regions of IKZF1 and IKZF3 in humans and mice.....	22
Figure 2.1: SILAC proteomics demonstrates lenalidomide-induced changes in ubiquitination and protein levels.....	28
Figure 2.2: Effect of lenalidomide on specific ubiquitination sites.....	29
Figure 2.3: Statistically significant SILAC results with 1 μ M lenalidomide.....	30
Figure 2.4: Lenalidomide treatment reduces protein levels of CK1 α in human KG-1 cells without altering <i>CSNK1A1</i> mRNA levels.....	31
Figure 2.5: Effect of lenalidomide in human cell lines.....	32
Figure 2.6: Lenalidomide induces the ubiquitination of CK1 α by CRL4 ^{CRBN}	34
Figure 2.7: Lenalidomide does not alter the subcellular localization of CK1 α	35
Figure 2.8: Narrowing the lenalidomide responsive region of CK1 α	36
Figure 2.9: <i>CSNK1A1</i> knockdown increases lenalidomide sensitivity in hematopoietic cells.....	38
Figure 2.10: Ectopic <i>CSNK1A1</i> overexpression reduces lenalidomide sensitivity in primary del(5q) MDS cells.....	40
Figure 2.11: Expression of <i>IKZF1</i> in patient samples.....	41
Figure 2.12: Lenalidomide does not induce degradation of Ck1 α in mouse cells.....	43
Figure 2.13: Amino acid changes in CRBN explain species-specific lenalidomide effects.....	45
Figure 2.14: Lenalidomide binds mCrbn and mCrbn ^{I391V}	46
Figure 2.15: Comparison of the IMiD-binding regions of human and mouse CRBN.....	48
Figure 2.16: Effects of lenalidomide treatment on <i>Csnk1a1</i> ^{+/-} mouse hematopoietic cells.....	50
Figure 2.17: Lenalidomide has a stronger effect on CK1 α than thalidomide or pomalidomide.....	52
Figure 2.18: Structures of thalidomide, lenalidomide, pomalidomide, and CC-122.....	53
Figure 2.19: Global proteomics with lenalidomide and CC-122.....	53
Figure 2.20: Western Blot verification of the effects of different IMiDs on CK1 α protein levels.....	55
Figure 2.21: Schematic presentation of the interaction of different thalidomide analogs with CRBN, substrates, and therapeutic indications.....	56
Figure 3.1: Design and generation of <i>Crbn</i> ^{I391V} knock-in mice.....	84
Figure 3.2: Genotype ratios and <i>Crbn</i> expression in <i>Crbn</i> ^{I391V} mice.....	85
Figure 3.3: Global proteome profiling identifies downregulated proteins in wild-type and knock-in cells treated with thalidomide derivatives <i>in vitro</i>	87

Figure 3.4: Proteins significantly altered by IMiD treatment in <i>Crbn</i> ^{I391V/I391V} cells.	88
Figure 3.5: IKZF3 and ZFP91 reporter assays in Hoxb8 cells.	90
Figure 3.6: Degradation of IKZF3 and CK1 α in T and c-Kit cells from <i>Crbn</i> ^{I391V} mice treated <i>in vitro</i>	91
Figure 3.7: Effects of <i>in vivo</i> IMiD treatment on cells from <i>Crbn</i> ^{I391V/I391V} mice.	92
Figure 3.8: Treatment with lenalidomide or pomalidomide increases IL-2 production by <i>Crbn</i> ^{I391V} T cells.	94
Figure 3.9: Effect of lenalidomide treatment on peripheral blood counts.	96
Figure 3.10: Effect of lenalidomide treatment on hematopoietic stem and progenitor cells.	97
Figure 3.11: Flow cytometry gating strategy for hematopoietic stem and progenitor compartments.	98
Figure 3.12: Effect of lenalidomide on hematopoietic progenitor compartments in <i>Crbn</i> ^{I391V} mice.	99
Figure 3.13: Effect of lenalidomide treatment on erythroid differentiation in <i>Crbn</i> ^{I391V} mice.	101
Figure 3.14: <i>Crbn</i> ^{I391V} cells heterozygous for <i>Csnk1a1</i> are more sensitive to lenalidomide <i>in vitro</i>	103
Figure 3.15: Lenalidomide induces <i>p21</i> and apoptotic cell death in <i>Crbn</i> ^{I391V} <i>Csnk1a1</i> ^{+/-} cells.	104
Figure 3.16: Design of <i>in vivo</i> transplant experiment.	105
Figure 3.17: <i>In vivo</i> lenalidomide treatment results in the depletion of <i>Csnk1a1</i> ^{+/-} cells but is rescued by deletion of a single allele of <i>Trp53</i>	106
Figure 3.18: Analysis of hematopoietic stem and progenitor cells in <i>in vivo</i> competition experiments.	107
Figure 3.19: Haploinsufficiency for <i>Rps14</i> contributes to lenalidomide sensitivity <i>in vitro</i> but not <i>in vivo</i>	109
Figure 3.20: Comparison of CRBN sequences in species with and without thalidomide teratogenicity.	111
Figure 3.21: Thalidomide-derivative induced fetal loss in <i>Crbn</i> ^{I391V} mice.	112
Figure 3.22: Effect of lenalidomide treatment on litter size at birth.	114
Figure 3.23: Determination of the critical period for IMiD-induced teratogenicity in <i>Crbn</i> ^{I391V/I391V} mice.	116
Figure 4.1: Structural visualization of additional human-mouse differences in CRBN.	148

List of Tables

Table 2.1: Characteristics of patient samples used for <i>CSNK1A1</i> and <i>IKZF1</i> expression experiments.	42
Table 3.1: Flow cytometry antibodies used in this study.....	128
Table 3.2: CRBN sequences used in alignment in Figure 3.18.	130

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Chapter 1 Introduction

Preface

The use of thalidomide as a treatment for morning sickness in the 1950s resulted in the birth of more than 10,000 infants with significant disabilities and changed how drugs are tested and approved. Since that time, thalidomide and its derivatives lenalidomide and pomalidomide have become clinically-effective and widely-used treatments for multiple myeloma, myelodysplastic syndrome (MDS) with del(5q), and complications of leprosy.

The recent discovery of the novel mechanism of thalidomide derivatives has further increased interest in this class of drugs. Thalidomide derivatives induce targeted degradation of specific proteins, including several transcription factors, suggesting that this mechanism might be harnessed to target otherwise “undruggable” proteins. Despite advances in understanding the mechanism of thalidomide derivatives in multiple myeloma, the biochemical basis of their efficacy in del(5q) MDS and teratogenic effects remains unknown. Investigations into the mechanism and use of thalidomide derivatives have been further hampered by the lack of effect of these drugs in mouse models. This thesis sought to characterize the biochemical basis of lenalidomide response in del(5q) MDS, to characterize a thalidomide-responsive mouse model, and to use this model to investigate the *in vivo* effects of these drugs.

A brief history of the thalidomide tragedy

Thalidomide (**Figure 1.1**) was first developed by the German pharmaceutical company Chemie Grünenthal in the 1950s. Thalidomide was found to be an effective sedative that had no lethal dose in mice, making it an extremely attractive drug candidate in an era where demand for sedatives was high and barbiturates, with their significant safety problems, were the only other sedative class. In 1957, thalidomide was introduced to the market for numerous indications

including morning sickness, nausea, anxiety, influenza, and depression and it was advertised and marketed as completely safe. It was eventually sold in 46 countries around the world, under a variety of names, and became one of the best-selling drugs of the time [1].

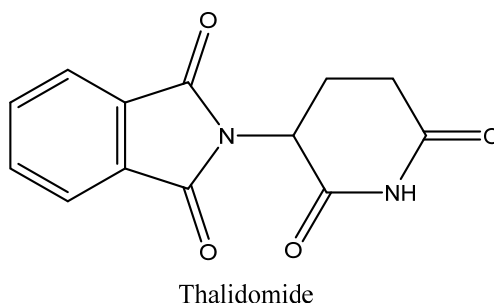


Figure 1.1: The chemical structure of thalidomide.

Concerns were first raised in 1959 about peripheral neuropathy [2], as patients taking thalidomide long-term experienced numbness, tingling, and shooting pains in their hands and feet. While an increase in the number of newborns with hypoplastic and aplastic abnormalities of the limbs was noted by local doctors [3], it was difficult to attribute these cases to any specific cause. This was made especially difficult as thalidomide was present in many different preparations and mothers often did not remember taking thalidomide during their pregnancy [1, 4]. It was only in 1961 that McBride [5] in Australia and Lenz [3] in Germany first independently suggested that thalidomide might be associated with birth defects. After the connection was made, many other groups quickly replicated the findings, and thalidomide was withdrawn from international markets in 1961-1962. However, significant damage had already been done, with an estimated 10,000 affected children born between 1957 and 1962 [1].

While thalidomide was tested in mice, whether thalidomide was tested in pregnant animals before use in humans remains a matter of debate. The successor of Chemie Grünenthal

states that it was tested in pregnant mice without effects, but documents related to the thalidomide tragedy were destroyed in advance of thalidomide-related legal proceedings and no contemporaneous reports of these experiments were published [6, 7].

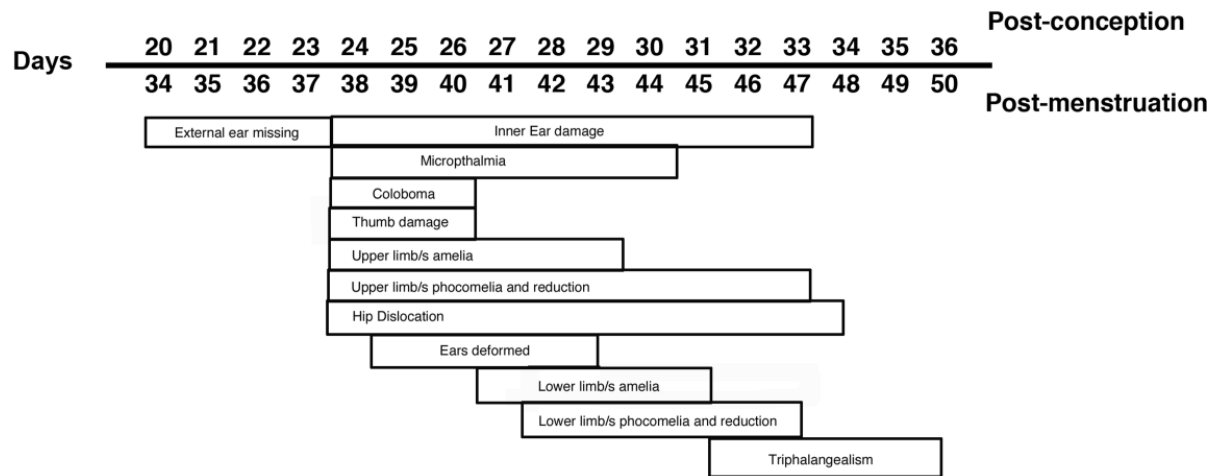
After the news of thalidomide's effects emerged, scientists rushed to test the effects in various animal models. Experiments in multiple primate models produced limb defects and other malformations seen in humans at clinically-relevant doses [8-17]. Thalidomide treatment of pregnant rabbits also produced limb and other defects [18-25]. In contrast, treatment of pregnant mice and rats generally did not produce malformations [20, 21, 24, 26, 27], even when pregnant rodents were treated with doses of thalidomide as high as 4,000 mg per kg [28]. Thus, thalidomide provided the first suggestion that drugs could have distinct effects in different species.

The incidence of thalidomide malformations was markedly lower in the United States, where Food and Drug Administration (FDA) reviewer Frances Kelsey had delayed the drug's application because of concerns over peripheral neuropathy and the lack of sufficient testing in pregnancy [29, 30]. Despite not being approved by the FDA, some cases of thalidomide embryopathy did occur in the United States, as samples of the drug were distributed to physicians who were not formally involved in clinical trials. The thalidomide tragedy provided political impetus to tighten regulation of new drug applications, culminating in 1962 passage of the Kefauver Harris Amendment to the Federal Food, Drug, and Cosmetic Act. These new rules required drug companies to demonstrate efficacy as well as safety, test drugs on animals, keep accurate records, and report the results of all pre-clinical trials to the FDA. Based on experiences with the species-specificity of thalidomide, companies were required to test for teratogenicity in multiple animal species if the drug was to be used in women of childbearing age [29].

Thalidomide embryopathy

Thalidomide embryopathy refers to the wide range of developmental defects that can arise following *in utero* thalidomide exposure. The critical period for thalidomide-induced malformations in humans is between 20 and 36 days after fertilization (**Figure 1.2**) and maternal ingestion of a single 50 mg thalidomide tablet during this period may be sufficient to alter development [1]. Thalidomide embryopathy affects a wide range of tissues and organ systems, resulting in infant mortality as high as 40% and an increased rate of stillbirths and *in utero* fetal death. Although early pregnancy detection was limited in the 1950s, an increased rate of miscarriages was noted in the time [1, 31] and some studies have found that maternal exposure to thalidomide before the 20-36 day critical period or at high doses can cause spontaneous abortion [32]. Experiments in many non-human primates [8-17], in which early pregnancy can be closely monitored, find an increased rate of spontaneous abortions in addition to malformations, especially when thalidomide is administered in high doses or early in development. Thus, the total number of fetuses affected by both the abortifacient and malformation-inducing effects of thalidomide, while significant, will never be exactly known.

Appearance of outward damage caused by thalidomide



From: Lenz and Knapp, 1962, Nowack, 1965, Ruffing, 1973, Smithells and Newman, 1992, Miller and Stromland, 1999

Figure 1.2: Critical period for thalidomide's teratogenic effects in humans.

Excerpted from Vargesson [1].

Limb defects, including phocomelia and amelia, are the most common and characteristic thalidomide-induced malformations. These malformations are often bilateral and most commonly affect the upper limbs, although all four limbs can be affected. Phocomelia is a shortening of the proximal elements of a limb (the long bones), with preservation of the distal elements (the hands or feet), while amelia refers to a complete absence of limb elements. Thalidomide classically affects the thumb first, then the radius, humerus, and ulna, often resulting in combined loss of the radius and thumb known as radial dysplasia. In addition to the limbs, thalidomide causes other skeletal issues including characteristic hip and shoulder malformations, hypoplastic or absent hips, and abnormal development of the vertebral column [1].

Thalidomide-induced malformations are not limited to the skeletal system. Ear and eye damage is a hallmark of thalidomide embryopathy, and microphthalmia, anophthalmia, anotia, and microtia are often observed. Malformations of the heart, kidneys, gastrointestinal tract, and genitals have been noted, and this severe multi-organ damage likely contributed to the high rate of infant mortality among affected children. Infants exposed to thalidomide *in utero* also characteristically have a “storkmark” or enlarged facial nevus or hemangioma at birth [1].

The repurposing of thalidomide and development of the IMiDs

After the thalidomide tragedy, thalidomide was tested in several cancers based on the idea that its teratogenicity might result from a propensity to kill rapidly dividing cells [33]. While these tests were largely negative, thalidomide was incidentally found to be effective in the treatment of erythema nodosum leprosum (ENL) in a series of patients given the drug as a sedative during their illness [34], and these results were later confirmed in several randomized trials [35, 36]. ENL is an acute inflammatory complication of lepromatous leprosy characterized by severe systemic inflammation, fever, cutaneous lesions, arthritis, glomerulonephritis, and circulating immune complexes [37]. Thalidomide’s success in ENL led to its experimental use throughout the 1970s in a variety of other dermatologic and inflammatory conditions including sarcoidosis, Bechet’s disease, inflammatory bowel disease, ankylosing spondylitis, and rheumatoid arthritis [38]. Its anti-emetic and sedative properties were also employed to treat cancer cachexia and HIV wasting syndrome [38].

In 1990, circulating levels of tumor necrosis factor α (TNF α) and interleukin-1 were found to be increased the serum of patients with ENL, suggesting that cytokine alterations were a key feature of leprosy reactions [39]. This finding also suggested that thalidomide’s efficacy in

ENL might arise from immunomodulatory effects and soon thalidomide was shown to inhibit lipopolysaccharide-stimulated production of TNF α from human blood monocytes treated *in vitro* [40]. In 1998, the new manufacturer of thalidomide, Celgene, secured FDA approval for use of the drug in ENL, redeeming the infamous drug for legitimate clinical use [41].

In the late 1990s, Celgene created and tested thalidomide derivatives in an attempt to identify compounds with enhanced effects on TNF α and improved stability [42-44]. The two most successful of these derivatives were later independently marketed as lenalidomide and pomalidomide (**Figure 1.3**). In addition to inhibition of TNF α release from monocytes, these drugs were found to alter NK cell function and increase T cell proliferation and production of IL-2 and interferon gamma [45, 46]. Because of these shared effects on the immune system, lenalidomide, thalidomide, and pomalidomide were deemed to form a new class of immunomodulatory drugs (IMiDs).

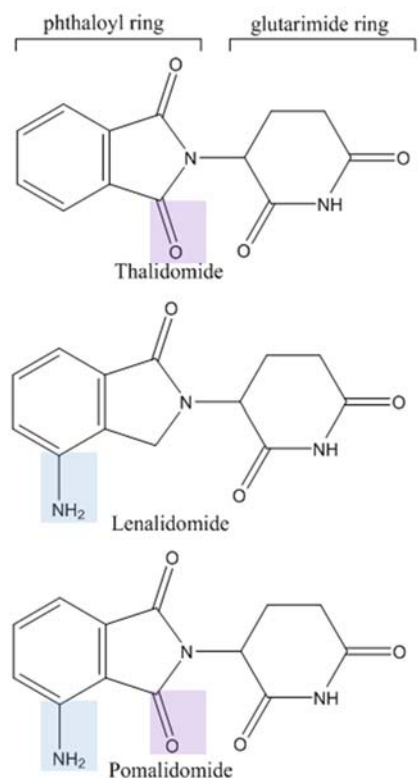


Figure 1.3: Chemical structures of IMiD compounds. Chemical structures of thalidomide, lenalidomide, and pomalidomide, with phthaloyl and glutarimide rings indicated and differences between the structures highlighted.

From the 1950s onward, many scientists studied the cellular effects of thalidomide in an attempt to understand the basis of its teratogenicity. In the wake of Judah Folkman's work on the role of angiogenesis in cancer progression [47], thalidomide was reported to have antiangiogenic effects [48, 49], triggering a flurry of research. Thalidomide was tested in multiple myeloma, a malignancy of plasma cells, because of the prominent bone marrow vascularity and elevated levels of angiogenic cytokines seen in this condition, as well as the correlation between elevated bone marrow vascularity and worse prognosis [50]. Thalidomide had a remarkable effect in multiple myeloma, launching the widespread clinical use of this drug and its derivatives.

However, thalidomide's rapid effect in multiple myeloma and lack of a clear reduction in bone marrow vascularity in responders [50, 51] may have foreshadowed that it operates through a distinct mechanism.

Thalidomide's reported anti-cytokine and anti-angiogenic effects also motivated testing in myelodysplastic syndrome and thalidomide was found to improve blood counts in MDS patients [52]. When these findings were replicated with lenalidomide, subgroup analyses found that patients with del(5q) MDS had particularly profound responses, often with complete disappearance of the del(5q) clone [53]. These findings were replicated in larger trials [54] and lenalidomide was approved for use in del(5q) MDS.

Today, treatment with IMiDs is the standard of care in multiple myeloma and myelodysplastic syndrome with del(5q) [55, 56]. IMiDs are also used in the treatment of ENL [37] as well as B cell neoplasms like mantle cell lymphoma [57], chronic lymphocytic leukemia [58, 59], and diffuse large B cell lymphoma [60]. Currently, clinical trials are investigating the role of the IMiDs in the treatment of acute myeloid leukemia, systemic light chain amyloidosis, systemic lupus erythematosus, and inflammatory bowel disease [46, 61, 62].

Mechanism of action

In 2010, Ito et al. [63] identified cereblon (CRBN) as the primary molecular target of thalidomide (**Figure 1.4**). CRBN is the substrate adaptor of the CRL4^{CRBN} E3 ubiquitin ligase, a cullin-ring ligase composed of damaged DNA binding protein 1 (DDB1), cullin 4a (CUL4A), and regulator of cullins 1 (ROC1). While thalidomide was initially thought to inhibit the activity of CRL4^{CRBN}, subsequent work revealed that thalidomide derivatives work by a novel drug mechanism—modulation of the substrate specificity of an E3 ubiquitin ligase.

Proteome-wide surveys revealed that the lymphoid transcription factors IKZF1 and IKZF3 are selectively ubiquitinated and degraded in the presence of lenalidomide in multiple myeloma cells [64-66]. Biochemical investigation demonstrated that lenalidomide induces the recruitment of IKZF1 and IKZF3 to CRL4^{CRBN} and promotes their ubiquitination by this complex, ultimately resulting in targeted ubiquitination of these transcription factors by the proteasome.

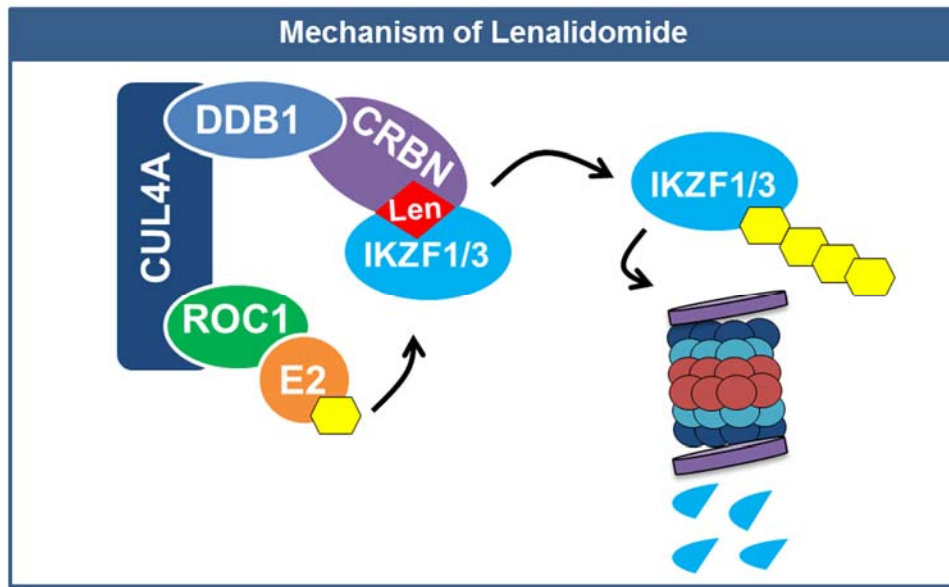


Figure 1.4: Mechanism of lenalidomide in multiple myeloma. Lenalidomide binds to CRBN, the substrate adaptor of the CRL4^{CRBN} E3 ubiquitin ligase. Lenalidomide induces the recruitment of specific substrates to CRL4^{CRBN} and their ubiquitination by this ligase. In multiple myeloma, the ubiquitination and subsequent proteasomal degradation of two B cell transcription factors, IKZF1 and IKZF3, kills multiple myeloma cells. Yellow hexagons represent ubiquitin molecules. Modified from Fink and Ebert [67].

Lenalidomide-induced ubiquitination of these substrates can be recapitulated *in vitro*, confirming direct interaction between these targets and CRBN. Lenalidomide-induced substrate recognition is exquisitely specific, as other highly homologous members of the Ikaros family are not affected. A 60 amino-acid region within the IKZF3 protein constitutes a lenalidomide-inducible degron. Fusion of this sequence to a heterologous protein is sufficient for lenalidomide-induced degradation, but single point mutations in this region can abrogate the lenalidomide response [65].

The crystal structure of CRBN-DDB1 bound to lenalidomide [68, 69] provides further mechanistic insight into how IMiDs act on CRL4^{CRBN}. The IMiD compounds bind CRBN through their shared glutarimide ring, leaving portions of their variable phthaloyl ring solvent-exposed (**Figure 1.3**). Substrates are recruited to the CRBN-IMiD interface only in the presence of drug and they make contacts with both the surface of CRBN and the IMiD. Thus, IMiDs appear to act as a “molecular glue,” bridging the interface between CRBN and recruited substrates [70].

Therapeutic activity of IMiDs in multiple myeloma and other B cell neoplasms

IMiDs are remarkably effective in multiple myeloma, with response rates for combined lenalidomide and dexamethasone exceeding 70% as a first-line therapy [71-75]. CRBN-mediated degradation of IKZF1 and IKZF3 provides a mechanism for the effects of thalidomide derivatives in multiple myeloma. IKZF1 and IKZF3 are transcription factors that are essential for B cell differentiation, and multiple myeloma cells require ongoing expression of IKZF1 and IKZF3 for survival. In cell lines, lenalidomide response is associated with sensitivity to IKZF1/3 knockdown. Addition to IKZF1 and IKZF3 expression may also explain the efficacy of

lenalidomide in other B cell malignancies including mantle cell lymphoma and chronic lymphocytic leukemia (CLL).

IKZF1 and IKZF3 regulate a transcriptional network that is essential for multiple myeloma cells. In particular, IKZF3 regulates expression of interferon regulatory factor 4 (IRF4), a transcription factor that has previously been demonstrated to be an essential gene in multiple myeloma [76] and is linked with lenalidomide's activity in disease [77]. IRF4 is a master regulator of an aberrant myeloma-specific gene program, which encompasses a positive feedback loop with the oncogene *MYC* as well as many other genes essential to myeloma survival. Regardless of their underlying genetic alterations, myeloma cells are addicted to this IRF4-controlled expression profile [76]. Through targeted degradation of IKZF3 and the subsequent decrease in IRF4 expression, lenalidomide exploits the IRF4 addiction of multiple myeloma cells to contribute to its overall therapeutic window.

IKZF3 is also a transcriptional repressor of the interleukin 2 gene (*IL2*). Lenalidomide-induced degradation of IKZF3 is associated with increased *IL2* transcription and production, providing an explanation for at least some of lenalidomide's immunomodulatory effects. Increased IL-2 production has been shown to increase the proliferation of NK, NKT, and CD4⁺ T cells in CLL [78]. This lenalidomide-induced activation of an anti-leukemic immune response may contribute to lenalidomide's therapeutic efficacy in this condition.

Given lenalidomide's mechanism of action, it is intriguing that IMiDs and proteasome inhibitors are synergistic in the treatment of multiple myeloma [79]. Recent evidence suggests that this synergy may result from the pharmacokinetic properties and dosing schedules of these drugs. While treatment with proteasome inhibitors can block lenalidomide-induced degradation

of IKZF1 and IKZF3 *in vitro* [65], this effect depends on both the order of administration and dose. When the drugs are administered at the same time, lenalidomide-induced degradation occurs before the onset of proteasomal blockade [80]. Similarly, low doses of proteasome inhibitor cause the death of myeloma cells while still allowing proteasomal degradation of lenalidomide's substrates [80]. Such dose effects may be particularly relevant in the clinic since lenalidomide is given daily, while proteasome inhibitors are administered as pulse infusions. These findings remain to be confirmed clinically but provide a biologically plausible explanation for these synergistic effects.

Similar drugs

Thalidomide derivatives are the first drug class that works by altering the substrate specificity of an E3 ubiquitin ligase. If this approach can be applied more broadly, it may provide a powerful way to alter previously “undruggable targets,” such as transcription factors essential in cancer. As we describe in Chapter 2, our work provided a proof of concept that changes in the chemical structure of thalidomide derivatives could alter their substrate specificity. Recent work has focused on the synthesis and characterization of novel thalidomide derivatives, such as CC-122, CC-220, and CC-885 [81-83]. These molecules all bind CRBN but differ in their substrate specificity. These derivatives are currently in clinical trials for a variety of conditions, including hepatocellular carcinoma, systemic lupus erythematosus, and B cell neoplasms [83-86]. Several groups have also generated bifunctional thalidomide-derivatives which consist of the CRBN-binding portion of thalidomide chemically linked to a small molecule that binds to a protein of interest [87-89]. Through this approach, a variety of novel protein targets can be recruited to CRL4^{CRBN} for ubiquitination and are ultimately degraded by the proteasome.

Recently, a second class of drugs was reported to work by inducing the ubiquitination of specific protein targets. Indisulam and related drugs bind to DCAF15, the substrate adaptor of the CRL4^{DCAF15} ubiquitin ligase [90, 91]. This binding induces the recruitment and ubiquitination of the splicing factor RBM39. Subsequent degradation of RBM39 explains the activity of indisulam and its derivatives in cancer cells. The mechanism of indisulam derivatives is strikingly similar to that of thalidomide derivatives and suggests that modulation of E3 ubiquitin ligase activity is a powerful pharmacologic mechanism that may be broadly applicable.

Pathogenesis of del(5q) MDS

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, peripheral cytopenias, and a risk of progression to acute myeloid leukemia (AML) [92-94]. MDS commonly affects the elderly, causing anemia, infections, and bleeding that necessitate transfusions and reduce life expectancy. Sequencing studies have identified recurrent mutations in essential cellular processes, such as RNA splicing, epigenetic regulation, the DNA-damage response, and tyrosine kinase signaling, suggesting that these pathways play a role in the pathogenesis of MDS [95]. Fifty percent of patients also have identifiable cytogenetic abnormalities, although in many cases the pathological significance of these rearrangements remains unknown [94]. The most common and best understood cytogenetic abnormality in MDS is deletion of the large arm of chromosome 5 (del(5q)). MDS with an isolated 5q deletion was first described in 1974 in patients with macrocytic anemia, erythroid hypoplasia, normal to elevated platelet count, hypolobulated megakaryocytes, and low blast counts [96]. This constellation of clinical findings was dubbed the “5q- syndrome,” although further work revealed that up to 25% of patients with MDS have

abnormalities in 5q without the classical syndrome. Together, these entities are referred to as del(5q) MDS [93].

The phenotype of del(5q) MDS is thought to result from haploinsufficiency of critical genes on 5q. Mapping studies have defined two common deleted regions (CDR) on 5q: 5q32-33, which is associated with 5q- syndrome [97], and 5q31, which is more often found in AML with del(5q) [98] (**Figure 1.5**). Del(5q) cells express genes within these CDRs at approximately 50% of normal levels and homozygous deletions or mutations are uncommon, consistent with a haploinsufficient mechanism [99]. Indeed, heterozygous deletion of *RPS14*, a component of the 40S ribosome encoded within the CDR, has been shown to cause the hypoplastic anemia seen in the 5q- syndrome [100, 101]. Additionally, haploinsufficiency of two microRNAs, miR-145 and miR-146a, may explain the accompanying dysplastic megakaryopoiesis and thrombocytosis [102, 103]. Thus, del(5q) MDS is a disease of haploinsufficiency.

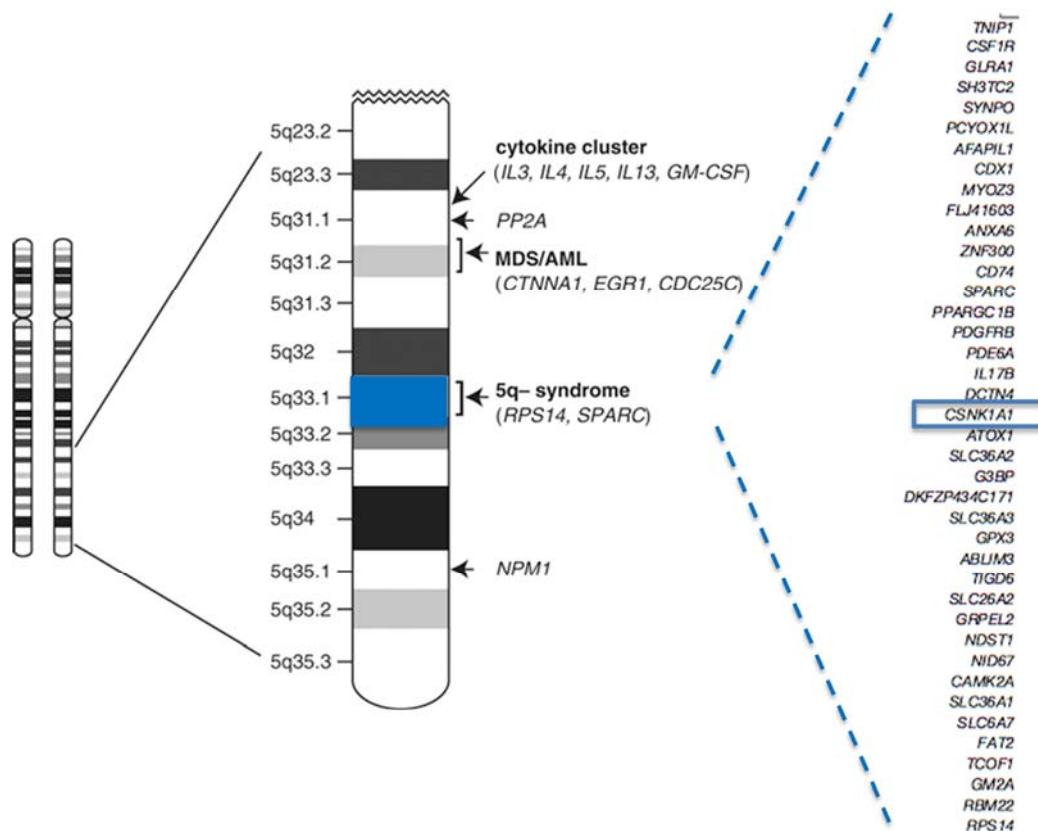


Figure 1.5: Commonly deleted regions in del(5q). Genes in the 5q32-33 region, which is most associated with 5q- syndrome, are shown to the right. *CSNK1A1*, the putative target of lenalidomide in del(5q) MDS discussed in Chapter 2, is boxed. Modified from Ebert [104].

Lenalidomide in del(5q) MDS

Lenalidomide is a remarkably effective treatment for del(5q) MDS and is unique in that it specifically targets the del(5q) clone [105]. In clinical trials, lenalidomide reduced the transfusion requirement and increased the hemoglobin level of 76% of del(5q) patients, with 67% becoming transfusion-independent [53, 54, 106, 107]. Erythroid responses occurred rapidly (median, 4.6 weeks), and were quite durable, with a median response time of over two years [53, 54]. Remarkably, cytogenetic responses were seen in up to 73% of patients and 45% of patients

had a complete disappearance of the del(5q) clone [53, 54, 106, 107]. Unfortunately, resistance to lenalidomide ultimately develops and it is associated with selection of a pre-existing p53 mutant clone [108, 109].

Several targets on chromosome 5q have been proposed to explain lenalidomide's effect in del(5q) MDS [99, 110]. Pellagati et al. [111] reported that lenalidomide treatment of MDS cells results in increased expression of the tumor suppressor *SPARC*, a gene located within the del(5q) CDR. However, there is no direct biochemical link between lenalidomide and *SPARC* expression, suggesting that *SPARC* upregulation is a downstream effect. Wei et al. [110] reported that lenalidomide inhibits the activity of two dual specificity phosphatases, CDC25C and PP2A, which are encoded on 5q and expressed at heterozygous levels in del(5q) MDS cells. However, these genes are not encoded within the del(5q) CDR and there are reports of lenalidomide response in patients with small del(5q) deletions that do not include these genes [112]. Additionally, while Wei et al. [110] proposed that lenalidomide directly inhibits CDC25C, these results are difficult to reconcile with Ito et al.'s [63] finding that CRBN is the direct molecular target of thalidomide derivatives.

CRBN-mediated degradation of IKZF1 and IKZF3 is unlikely to explain the efficacy of lenalidomide in del(5q) MDS since IKZF1 and IKZF3 are primarily lymphoid transcription factors and are not highly expressed in the myeloid cells that cause MDS [113]. Disruption of IKZF1 and IKZF3 has been shown to lead to lymphoid, not myeloid, malignancies [113]. Additionally, neither IKZF1 nor IKZF3 is encoded in the del(5q) CDR and there is no evidence that del(5q) cells would be specifically sensitive to their degradation. However, the characterization of lenalidomide-induced degradation of IKZF1 and IKZF3 led to the hypothesis

that lenalidomide-induced ubiquitination and degradation of a unique protein could explain lenalidomide's efficacy in del(5q) MDS. We investigate this hypothesis in Chapter 2.

Mechanism of thalidomide's teratogenicity

Despite 60 years of intensive study, no single mechanism of thalidomide's teratogenicity has been fully accepted by the field. There are currently three leading hypotheses for the mechanism of thalidomide's limb deformations: anti-angiogenesis [48], oxidative stress [114], and inhibition of Fgf8 [63]. Since thalidomide and lenalidomide are not teratogenic in rodents [20, 21, 24, 26-28, 115], most of these experiments have been conducted in rabbits, chicks, or zebrafish [116].

The anti-angiogenesis hypothesis rests on the same experimental basis that led to thalidomide's initial testing in multiple myeloma. The underlying idea is that blood vessels are essential to development and that thalidomide or its metabolites specifically affect blood vessels which have not yet acquired their smooth muscle coats. In this model, limb development is particularly sensitive to the anti-angiogenic effects of thalidomide because the limb vasculature does not acquire smooth muscle coverage until late in development [1].

Proponents of the oxidative stress hypothesis suggest that thalidomide induces the formation of reactive oxygen species which cause the death of limb bud cells through oxidative stress. This model can be merged to some extent with the third hypothesis, as thalidomide treatment of rabbit limbs reduces expression of the key patterning growth factors Fgf8 and Fgf10, but blocking reactive oxygen species restores their expression [1]. These growth factors are also central to Ito et al.'s [63] model that inhibition of CRBN by thalidomide triggers downregulation of Fgf8 and Fgf10 through unknown mechanisms.

Thus, while more than 30 models have been proposed to explain the mechanism of thalidomide's teratogenicity [1], none provides a satisfying biochemical explanation that fits our current understanding of the molecular targets of thalidomide derivatives.

IMiDs in rodent models

Thalidomide's lack of effect in rodent models was the first large-scale demonstration of a species-specific effect of a drug. With the development of thalidomide derivatives, it was subsequently shown that lenalidomide is ineffective in mouse multiple myeloma models [117]. Historically, the lack of thalidomide-induced teratogenicity in rodent models was most commonly attributed to differences in the production of key active thalidomide metabolites in mice and humans [118-123]. Now with the identification of CRBN, IKZF1, and IKZF3 as direct molecular partners of IMiDs we can ask whether differences in these proteins underlies IMiD's species-specific effects.

Several sequence differences exist between mouse and human CRBN (**Figure 1.6**). Despite these differences, both proteins bind thalidomide [63] and lenalidomide (**Figure 2.14**), so a lack of CRBN-drug binding does not explain lack of response. While Ito et al.'s [63] model posits that inhibition of CRBN leads to thalidomide-induced teratogenicity, it does not explain the molecular basis of the species-specific response. While differences in CRBN could alter sensitivity to inhibition by IMiDs, Ito et al.'s [63] model predicts that knockout of *Crbn* should cause teratogenic effects. In fact, *Crbn* null mice have normal limb development [124, 125].

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Human/1-442  1 MAGEGDQQDAAHNMGNHLLPAAESEEED - - - EMEVEDQDSKEAKKPNIIIN  48
Mouse/1-445  1 MAGEGDQQDAAHNMGNHLLPADSEDEDDEIEMEVEDQDSKEARKPNIIIN  51

Human/1-442  49 FDTSLPTSHTYLGADMEEFHGRTLHDDDDSCQVIPVLPQVMMILIPGQTLPL  99
Mouse/1-445  52 FDTSLPTSHTYLGADMEEFHGRTLHDDDDSCQVIPVLPQVLMILIPGQTLPL 102

Human/1-442 100 QLFHPQEVSMVRNLIQKDRTFAYLAYSNVQEREAQFGTTAEIYAYREEQDF 150
Mouse/1-445 103 QLSHPQEVSMVRNLIQKDRTFAYLAYSNVQEREAQFGTTAEIYAYREEQEF 153

Human/1-442 151 GIEIVKVKAIQRQRFKVLELRTQSDGIQQAQVQILPECVLPSTMSAVQLES 201
Mouse/1-445 154 GIEIVKVKAIQRQRFKVLELRTQSDGIQQAQVQILPECVLPSTMSAVQLES 204

Human/1-442 202 LNKCCIIFPSKPVSRDQCSEYKWWQKYQKRKFHCANLTSWPRWLYSLYDAET 252
Mouse/1-445 205 LNKCCVVFPSKPISWEDQYSCYKWWQKYQKRKFHCANLTSWPRWLYSLYDAET 255

Human/1-442 253 LMDRIKKQLREWDENLKDDSLPSNPIDFSYRVAACLPIDDVLR IQLLKIGS 303
Mouse/1-445 256 LMDRIKKQLREWDENLKDDSLPENPIDFSYRVAACLPIDDVLR IQLLKIGS 306

Human/1-442 304 AIQRLRCELD IMNKCTSLCKQCQETEITTKNEIFSLSLCGPMAAYVNPHG 354
Mouse/1-445 307 AIQRLRCELD IMNKCTSLCKQCQETEITTKNEIFSLSLCGPMAAYVNPHG 357

Human/1-442 355 YVHETLTVYKACNLNLIGRPSTEHWSWFGYAWTV AQCKICASHIGWKFTAT 405
Mouse/1-445 358 YVHETLTVYKASNLNLIGRPSTVHSWFGYAWTV AQCKICASHIGWKFTAT 408

Human/1-442 406 KKDMSPQKFWGLTRSALLPTIPDTEDEISPDKVILCL 442
Mouse/1-445 409 KKDMSPQKFWGLTRSALLPTIPEDEDEISPDKVILCL 445

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Figure 1.6: Amino acid sequence alignment of human and mouse CRBN. Residues that differ between human CRBN and mouse Crbn are shaded in red, with the degree of shading determined by the similarity of the amino acids. Residues which contact IMiDs according to a crystal structure [68] are highlighted in teal. Sequences shown are human CRBN isoform 1 (UniProt Q96SW2) and mouse CRBN isoform 2 (UniProt Q8C7D2).

The lenalidomide-responsive regions of IKZF1 and IKZF3 are identical in mice and humans (**Figure 1.7**), ruling out differences in target protein sequences as an explanation for lenalidomide’s failure in mouse myeloma models [65]. This finding focused our attention on how species-specific differences in CRBN might affect the IMiD-induced recruitment of substrates to CRL4^{CRBN} without disrupting CRBN-IMiD binding. Understanding how differences in CRBN affect species-specific responses could allow the development of

humanized mouse models that promote understanding of the biochemical basis of thalidomide derivatives' clinical efficacy and teratogenic effects. Chapter 2 investigates this hypothesis and Chapter 3 reports the characterization and use of such an *in vivo* model.

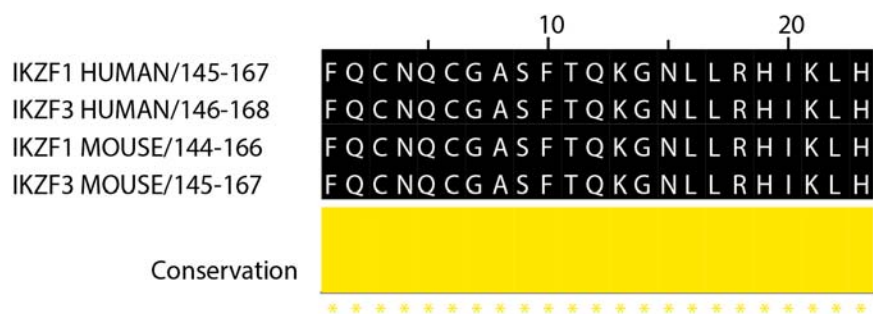


Figure 1.7: Comparison of the IMiD-responsive regions of IKZF1 and IKZF3 in humans and mice. An alignment of the second zinc finger of IKZF1 and IKZF3 of human and mouse origin is shown. This region of IKZF1/IKZF3 has recently been shown to be sufficient for IMiD-induced degradation [126].

Aims of the thesis

This thesis sought to build upon recent advances in our understanding of the molecular mechanism of thalidomide derivatives. Because degradation of IKZF1 and IKZF3 is unlikely to explain lenalidomide's therapeutic response in del(5q) MDS, we sought to identify additional lenalidomide-induced targets of CRL4^{CRBN} that explain lenalidomide's therapeutic window in this condition (Chapter 2). After identifying CK1 α as a lenalidomide-induced CRL4^{CRBN} target, we noted that degradation of Ikzf1, Ikzf3, and Ck1 α was not seen in mouse cells. We asked whether species-specific differences in CRBN could explain this response and identified a single amino acid change in mouse Crbn, Crbn^{I391V}, that sensitized mouse cells to IMiD treatment.

In Chapter 3, we generated a knock-in mouse model with this single amino acid change in Crbn to determine if it confers *in vivo* lenalidomide response. Characterization of this model revealed that *Crbn*^{I391V} is sufficient for IMiD-induced degradation of Ikaros, IKZF3, and Cks1α in mouse cells, along with the recently identified target Zfp91. Using the *Crbn*^{I391V} model, we demonstrate that haploinsufficiency for *Csnk1a1* explains lenalidomide's therapeutic window *in vivo*. Additionally, we show that this single amino acid change confers thalidomide-induced fetal loss in mice.

Overall, the work presented here has enhanced our understanding of the mechanisms underlying the efficacy and toxicity of these clinically important drugs and has generated new tools to support their future study.

Chapter 2 Lenalidomide induces the ubiquitination of CK1 α by CRL4^{CRBN}

Contributions

The work presented in this chapter was a collaboration with a post-doctoral fellow in the lab, Jan Krönke, and has been published a co-first author publication [82], although not all data from that publication is included here. Throughout, I have noted in the figure legends which experiments Jan performed working with his technician, Slater Hurst.

Many other people also contributed to this work. Proteomics experiments were performed by the Carr lab at the Broad Institute, specifically Namrata Udeshi, Tanya Svinkina, and D.R. Mani.

Philip Chamberlain (Celgene) provided structural insights and renderings based on published structures [68]. Experiments with CC-122 were performed by Kyle MacBeth and his team at Celgene, as we did not have access to this compound.

Rebecca Schneider provided the initial *Csnk1a1*^{+/-} mice and valuable advice on bone marrow infections and flow cytometry. She also performed *in vivo* treatments of wild-type mice. Marie McConkey assisted with breeding, colony management, and technical support. Eamon Comer provided lenalidomide-conjugated affigel.

Summary

Lenalidomide is a highly effective treatment for myelodysplastic syndrome (MDS) with deletion of chromosome 5q (del(5q)). Here, we demonstrate that lenalidomide induces the ubiquitination of casein kinase 1A1 (CK1 α) by the CRL4^{CRBN} E3 ubiquitin ligase, resulting in CK1 α degradation. CK1 α is encoded by a gene within the common deleted region for del(5q) MDS and haploinsufficient expression sensitizes cells to lenalidomide therapy, providing a mechanistic basis for lenalidomide's therapeutic window in del(5q) MDS. We found that mouse cells are resistant to lenalidomide but that changing a single amino acid in mouse Crbn to the corresponding human residue enables lenalidomide-dependent degradation of CK1 α . We further demonstrate that minor side chain modifications in thalidomide and a novel analog, CC-122, can modulate the spectrum of substrates targeted by CRL4^{CRBN}. These findings have implications for the clinical activity of lenalidomide and related compounds and demonstrate the therapeutic potential of novel modulators of E3 ubiquitin ligases.

Introduction

The immunomodulatory (IMiD) agents lenalidomide, thalidomide, and pomalidomide are the first drugs identified that promote the ubiquitination and degradation of specific substrates by an E3 ubiquitin ligase. These compounds bind CRBN [63], the substrate adaptor for the CRL4^{CRBN} E3 ubiquitin ligase, and modulate the substrate specificity of the enzyme. Each of these drugs induces degradation of two lymphoid transcription factors—IKZF1 and IKZF3—leading to dramatic clinical efficacy in multiple myeloma and increased interleukin-2 release from T-cells [64-66]. However, it has not yet been determined whether degradation of distinct substrates may mediate additional activities and whether all IMiD compounds have the same substrate specificity.

Lenalidomide is also a highly effective treatment for myelodysplastic syndrome (MDS) with deletion of chromosome 5q (del(5q)), inducing cytogenetic remission in more than 50% of patients [53, 54, 107]. *In vitro*, lenalidomide selectively induces apoptosis of del(5q) MDS cells [110, 111]. No biallelic deletions or loss of function mutations on the remaining allele have been detected in any of the genes in the del(5q) common deleted region, implying that MDS with del(5q) is a disease of haploinsufficiency [97, 127]. We hypothesized that ubiquitination of a distinct CRBN substrate explains the efficacy of lenalidomide in del(5q) MDS.

Results

Lenalidomide induces degradation of CK1α

In order to identify lenalidomide-regulated CRL4^{CRBN} substrates in myeloid cells, we applied stable isotope labeling of amino acids in cell culture (SILAC)-based quantitative mass spectrometry [128] to assess global changes in ubiquitination [129] and protein levels in the

del(5q) myeloid cell line KG-1 (**Figure 2.1, Figure 2.2, Figure 2.3**) [129]. Treatment with 1 μ M lenalidomide significantly altered only 7 K- ϵ -GG sites from 5 proteins out of 13,061 reproducible sites, demonstrating the highly specific effects of this drug. Moreover, lenalidomide significantly altered the protein abundance of 3 out of 5 differentially ubiquitinated proteins. Consistent with previous studies, lenalidomide treatment decreased ubiquitination of CRBN ($P=0.026$) and increased ubiquitination of IKZF1 ($P=7.23 \times 10^{-6}$ and $P=4.97 \times 10^{-4}$ for two distinct sites), with a reciprocal decrease in IKZF1 protein abundance ($P=0.006$) [64, 65].

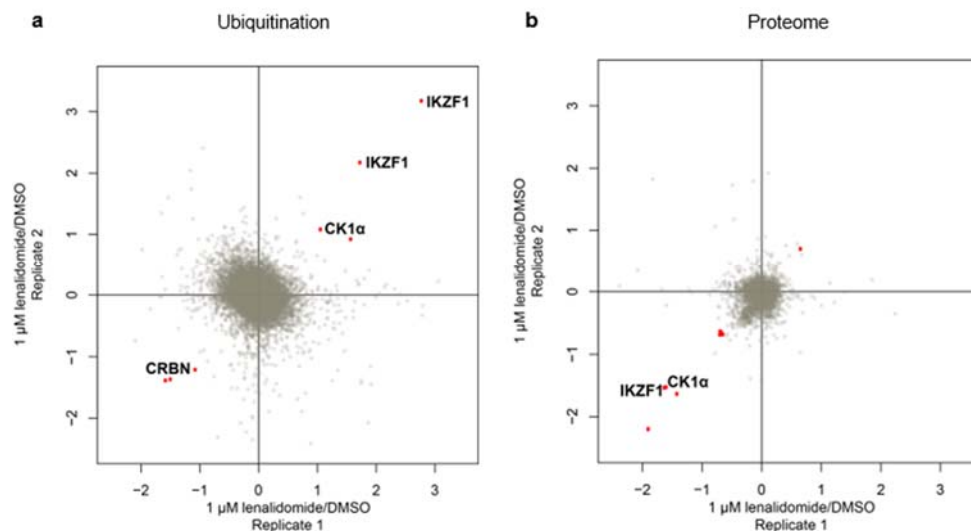


Figure 2.1: SILAC proteomics demonstrates lenalidomide-induced changes in ubiquitination and protein levels. a, Log₂ ratios for individual K- ϵ -GG sites of lenalidomide- (1 μ M) versus DMSO-treated KG-1 cells for biological replicates 1 and 2. Each point represents a unique K- ϵ -GG site. Significantly regulated sites ($P < 0.05$) are red. **b,** Log₂ ratios of protein abundance for lenalidomide- (1 μ M) versus DMSO-treated KG-1 cells for biological replicates 1 and 2. Each point represents a unique protein group. Significantly regulated proteins ($P < 0.05$) are red. Data are from Namrata Udeshi, Tanya Svinkina, and D.R. Mani.

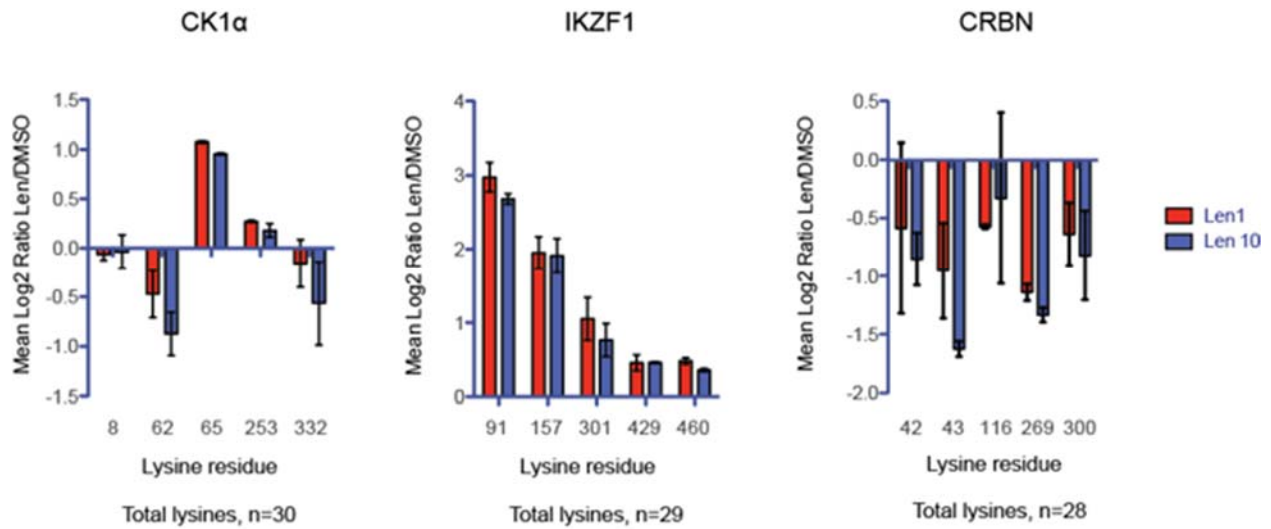


Figure 2.2: Effect of lenalidomide on specific ubiquitination sites. Median log₂ ratios for different lysine residues in CK1α isoform 2, IKZF1 isoform 1, and CRBN isoform 2 for 1 or 10 μM lenalidomide treated KG-1 cells versus DMSO treated cells. SILAC experiments were performed in two biological replicates with flipped SILAC labeling. Only lysine residues detected in both replicates are shown. Error bars are range. Data are from Namrata Udeshi, Tanya Svinkina, and D.R. Mani.

a

K-ε-GG site	Replicate 1	Replicate 2	Average Log ₂ fold change	adj.P.Val	Direction of change
IKZF1	2.78	3.18	2.98	7.23E-06	up
IKZF1	1.73	2.17	1.95	0.000497	up
MARCH8	1.58	0.92	1.25	0.036986	up
CK1α	1.06	1.08	1.07	0.035843	up
CRBN	-1.07	-1.21	-1.14	0.026006	down
RNF166	-1.49	-1.37	-1.43	0.003102	down
RNF166	-1.58	-1.39	-1.48	0.003102	down

b

Protein	Replicate 1	Replicate 2	Average Log ₂ fold change	adj.P.Val	Direction of change
ZNF692	-1.89	-2.20	-2.05	0.013806	down
IKZF1	-1.62	-1.54	-1.58	0.005638	down
CK1α	-1.59	-1.53	-1.56	0.005638	down
RNF166	-1.41	-1.64	-1.52	0.015257	down
ZFP91	-0.69	-0.69	-0.69	0.047677	down
LEMD3	-0.66	-0.68	-0.67	0.047677	down
NRM	-0.64	-0.68	-0.66	0.047677	down
LBR	-0.67	-0.65	-0.66	0.047677	down
UNC84A.SUN1	-0.68	-0.64	-0.66	0.047677	down
C12orf57	0.66	0.70	0.68	0.047677	up

Figure 2.3: Statistically significant SILAC results with 1 μM lenalidomide. **a**, List of significantly regulated K-ε-GG sites with 1 μM lenalidomide vs. DMSO. *P* value is adjusted as described in the methods section. **b**, List of significantly regulated proteins with 1 μM lenalidomide vs. DMSO. *P* value is adjusted as described in the methods section. Average log₂ fold change of two biological replicates. Data are from Namrata Udeshi, Tanya Svinkina, and D.R. Mani.

In addition to IKZF1, we detected increased ubiquitination ($P=0.04$) and decreased protein abundance ($P=0.006$) of casein kinase 1A1 (CK1α) following treatment with 1 μM lenalidomide (**Figure 2.1**, **Figure 2.2**, **Figure 2.3**). CK1α is encoded by the *CSNK1A1* gene, which is located in the del(5q) common deleted region and is expressed at haploinsufficient

levels in del(5q) MDS [97, 99]. CK1 α has been implicated in the biology of del(5q) MDS [130] and has been shown to be a therapeutic target in myeloid malignancies [131], and is therefore an attractive candidate for mediating the effects of lenalidomide in del(5q) MDS.

CK1 α is a substrate of CRL4^{CRBN}

We sought to determine whether CK1 α is a lenalidomide-dependent substrate of the CRL4^{CRBN} E3 ubiquitin ligase. We confirmed that lenalidomide treatment decreases CK1 α protein levels in multiple human cell lines (**Figure 2.4, Figure 2.5**).

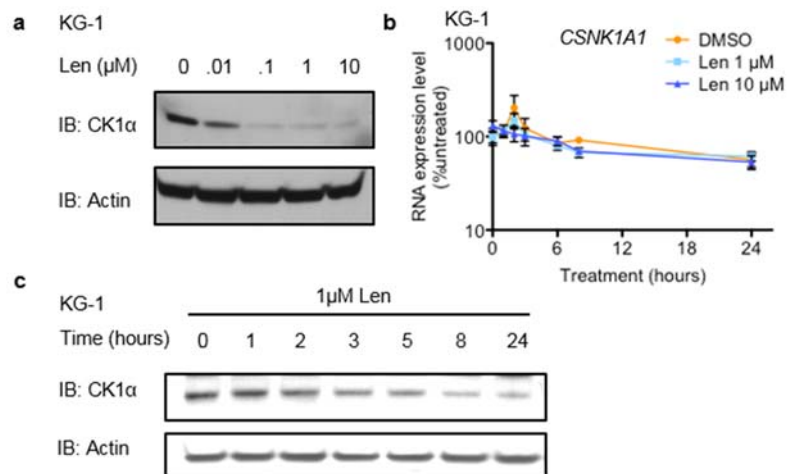


Figure 2.4: Lenalidomide treatment reduces protein levels of CK1 α in human KG-1 cells without altering *CSNK1A1* mRNA levels. **a**, Effects of lenalidomide (Len) on endogenous CK1 α protein levels in KG-1 cells after 24-h treatment. IB, immunoblot. **b**, *CSNK1A1* mRNA levels in KG-1 cells following lenalidomide treatment. Data are mean $\pm$ s.d. **c**, Time course of effect of lenalidomide treatment on CK1 α protein levels in KG-1 cells. Data are from Jan Krönke and Slater Hurst.

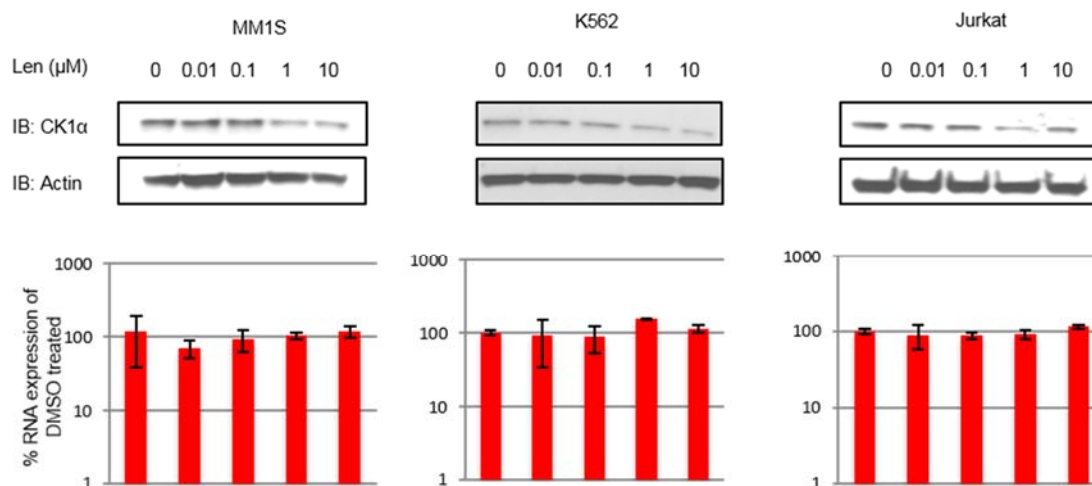


Figure 2.5: Effect of lenalidomide in human cell lines. MM1S, K562, and Jurkat cells were treated with different concentrations of lenalidomide for 24 hours. CK1α protein levels were detected by western blot and *CSNK1A1* mRNA expression levels were measured by RQ-PCR. Data are mean ± s.d. Data are from Jan Krönke and Slater Hurst.

Lenalidomide decreased CK1α protein levels without altering *CSNK1A1* mRNA expression (**Figure 2.4, Figure 2.5**), consistent with a post-translational mechanism of regulation. The lenalidomide-dependent decrease in CK1α protein level was abrogated by treatment with the proteasome inhibitor MG132 and the NEDD8-activating enzyme inhibitor MLN4924, which interferes with the activity of cullin-RING E3 ubiquitin ligases, implicating proteasome- and cullin-dependent degradation of CK1α (**Figure 2.6a**). Homozygous genetic inactivation of CRBN by CRISPR-Cas9 genome editing eliminated lenalidomide-dependent degradation of CK1α, demonstrating CRBN-dependent degradation of CK1α (**Figure 2.6b**).

We next examined whether CK1α binds CRBN and is ubiquitinated by the CRL4^{CRBN} E3 ubiquitin ligase. We observed co-immunoprecipitation of CK1α with endogenous and FLAG-tagged CRBN only in the presence of lenalidomide (**Figure 2.6d**). Lenalidomide treatment increased the ubiquitination of endogenous CK1α in KG-1 cells (**Figure 2.6c**) and in the

presence of CRBN *in vitro* (**Figure 2.6e**), confirming that CK1 α is a direct target of CRL4^{CRBN}. Lenalidomide treatment did not alter the subcellular localization of CK1 α (**Figure 2.7**). Using a chimeric protein of CK1 α and CK1 ϵ , which shares significant homology with CK1 α but is not responsive to lenalidomide, we found that the N-terminal half (amino acids 1-177) of CK1 α is essential for lenalidomide-induced degradation (**Figure 2.8**). Sequence alignment with the previously delineated lenalidomide-responsive degron in IKZF1/3 did not reveal any evident homology, suggesting that CK1 α and IKZF1/3 may interact with the CRBN-lenalidomide complex in distinct manners.

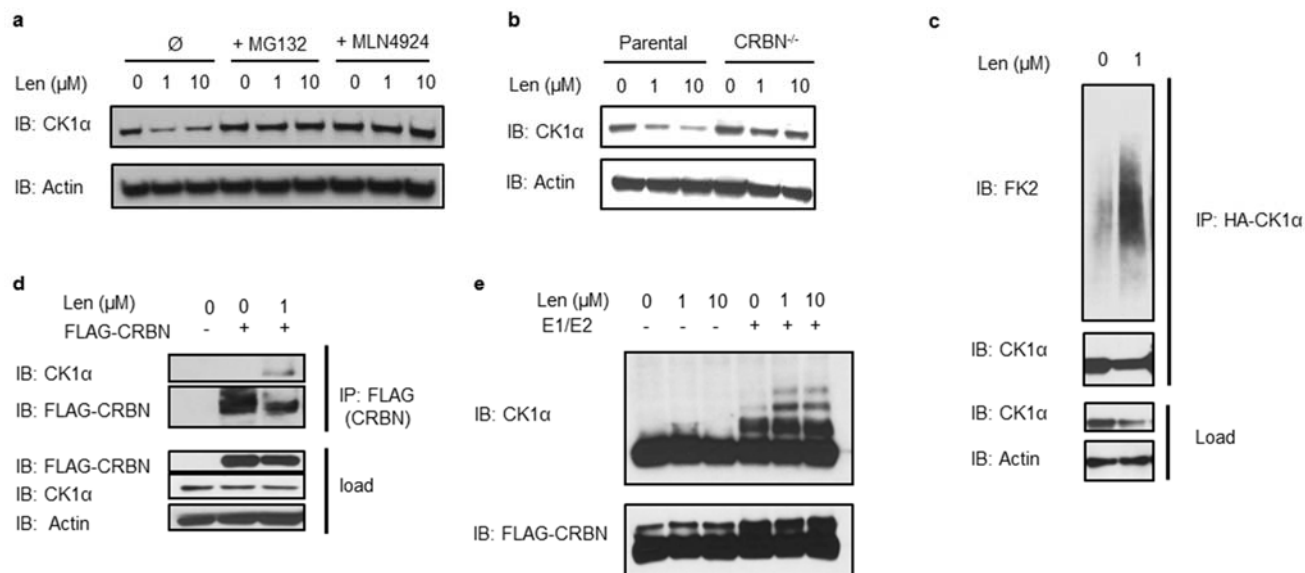


Figure 2.6: Lenalidomide induces the ubiquitination of CK1α by CRL4^{CRBN}. **a**, CK1α protein levels in KG-1 cells treated with DMSO, lenalidomide alone, or lenalidomide with MG132 or MLN4924 for 6 hours. **b**, CK1α protein levels in CRBN knockout 293T cells treated with lenalidomide. **c**, *In vivo* ubiquitination analysis of HA-tagged CK1α transiently expressed in 293T cells. **d**, Immunoprecipitation of FLAG-CRBN in 293T cells treated with DMSO or 1 μM lenalidomide in the presence of MG132. **e**, Ubiquitination of CK1α by CRBN *in vitro*. Lysine-free (K0) ubiquitin was used in the reaction. Panels **a** and **b** are from Jan Krönke and Slater Hurst.

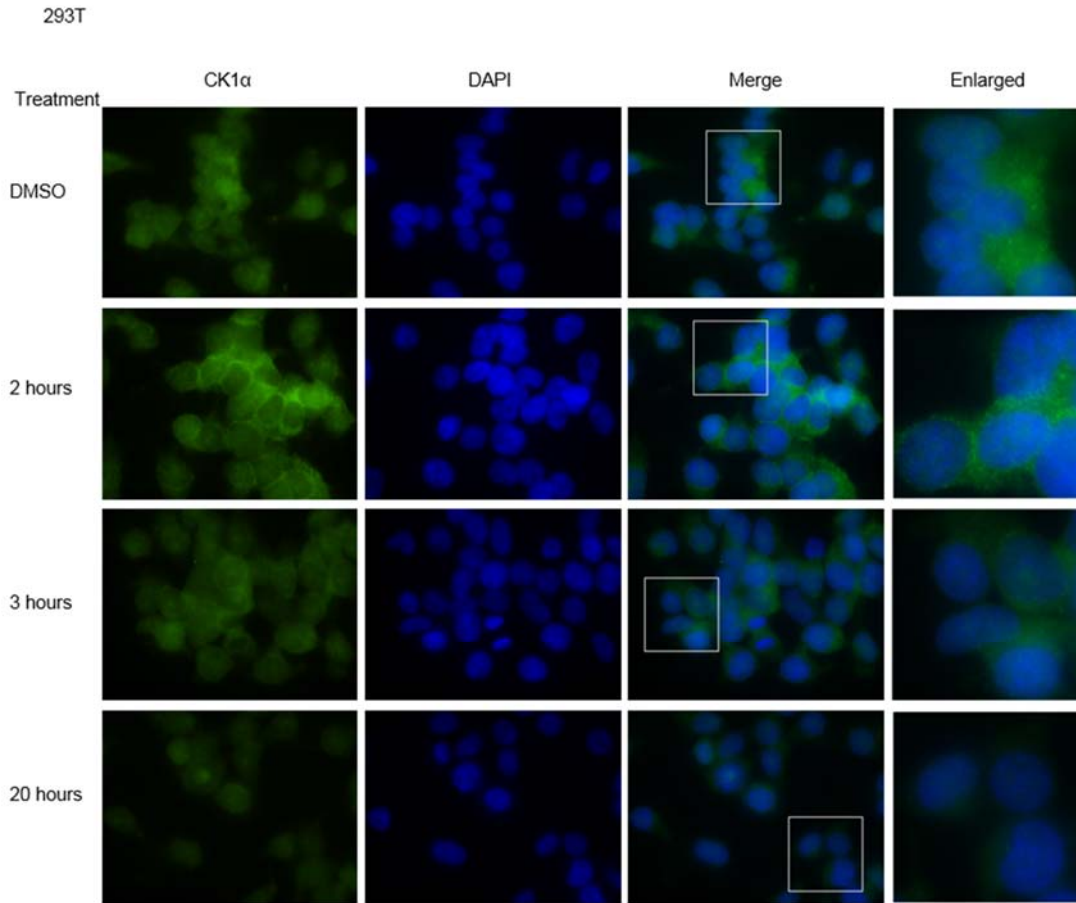


Figure 2.7: Lenalidomide does not alter the subcellular localization of CK1 α .

Immunofluorescence for CK1 α after treatment with DMSO or 10 μ M lenalidomide. Enlarged area is indicated by a box in Merge. FITC channel represents staining for CK1 α . No changes in CK1 α localization are seen upon lenalidomide treatment.

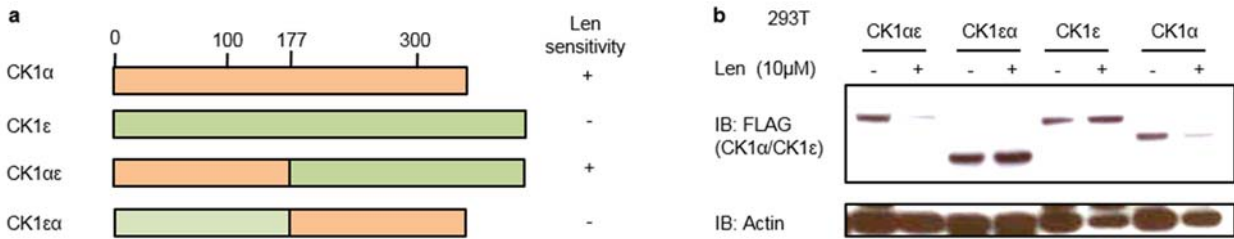


Figure 2.8: Narrowing the lenalidomide responsive region of CK1α. **a**, Chimeric proteins of casein kinase 1A1 (CK1α) and casein kinase 1E (CK1ε), which shares significant homology with CK1α but is not responsive to lenalidomide, that were used to determine the lenalidomide-responsive region in CK1α. **b**, FLAG-tagged (chimeric) proteins from **a** were transfected in 293T cells together with a CRBN-expressing plasmid. Cells were treated with 1 μM lenalidomide for 24 hours and protein was detected with a FLAG-specific antibody. Data are from Jan Krönke and Slater Hurst.

Effect of CSNK1A1 expression level

We next explored the biological effects of CK1α depletion. CK1α is a widely expressed serine/threonine kinase involved in many essential cellular pathways, including cell cycle regulation, DNA damage repair, and the β-catenin and NFκB pathways [132, 133]. Most notably, CK1α inhibits p53 through MDM2 and MDMX and negatively regulates Wnt signaling as a component of the β-catenin destruction complex [132, 134-137]. It has recently been implicated as a tumor suppressor and loss of casein kinase expression drives invasion and metastasis in colon cancer [132] and melanoma [138]. In a hematopoietic-specific conditional knockout mouse model, homozygous inactivation of *Csnk1a1* induces apoptosis and bone marrow failure via p53 activation, while heterozygous loss of *Csnk1a1* causes β-catenin accumulation and stem cell expansion [139]. Similarly, cells haploinsufficient for *Csnk1a1* preferentially undergo apoptosis in response to the casein kinase 1 inhibitor D4476 [131]. Since del(5q) cells express about 50%

of normal levels of *CSNK1A1* [99], these results led us to hypothesize that del(5q) cells would be more sensitive to the effects of lenalidomide-induced degradation of CK1 α as compared to normal cells with two copies of the gene.

To evaluate whether decreased *CSNK1A1* expression sensitizes cells to lenalidomide, we transduced primary human CD34⁺ hematopoietic stem and progenitor cells with GFP-tagged lentiviral vectors expressing *CSNK1A1* or control shRNAs. Cells expressing *CSNK1A1* shRNAs were depleted in the absence of treatment, demonstrating that knockdown of *CSNK1A1* inhibits the growth or survival of hematopoietic cells (**Figure 2.9**). Treatment with lenalidomide enhanced the depletion of cells expressing *CSNK1A1* shRNAs but had no effect on cells expressing control shRNAs, demonstrating that reduced *CSNK1A1* levels sensitize hematopoietic cells to lenalidomide.

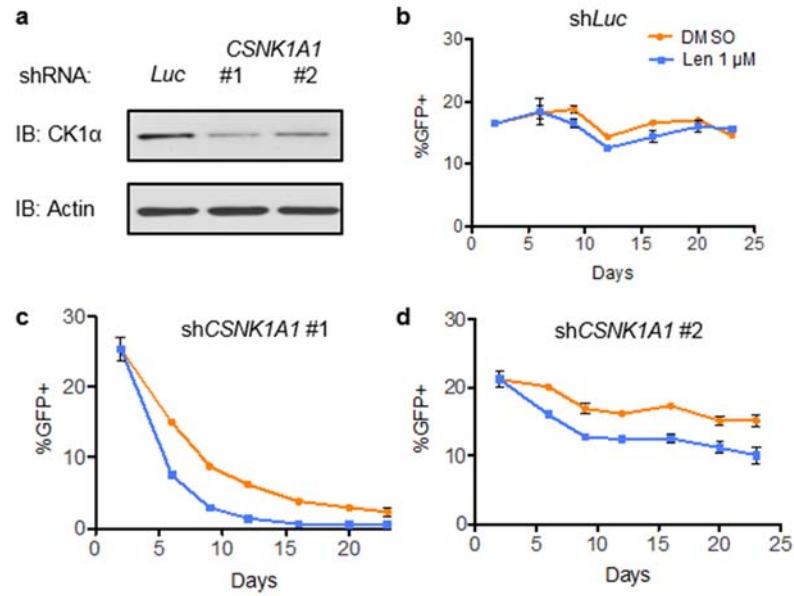


Figure 2.9: *CSNK1A1* knockdown increases lenalidomide sensitivity in hematopoietic cells.

a, Knockdown validation by western blot. **b-d**, CD34⁺ cells were transduced with GFP-labeled lentivirus expressing either control shRNA targeting *luciferase* (**b**) or shRNA targeting *CSNK1A1* (**c, d**) and treated with DMSO or 1 μM lenalidomide. The percentage of GFP⁺ cells was assessed by flow cytometry over time. Data are from Jan Krönke and Slater Hurst.

We next evaluated whether overexpression of *CSNK1A1* could reduce the lenalidomide sensitivity of del(5q) MDS cells. We obtained bone marrow samples from MDS patients with heterozygous deletions of chromosome 5q, including heterozygous deletion of *CSNK1A1*, prior to treatment with lenalidomide. We isolated CD34⁺ cells from these samples and transduced them with a GFP-tagged lentivirus expressing *CSNK1A1* cDNA or empty vector (**Figure 2.10**). Overexpression of *CSNK1A1* reduced the lenalidomide sensitivity of CD34⁺ cells from three out of five del(5q) MDS patients, which compares well with the clinically-observed cytogenetic response rate of about 50% [53, 54, 107]. In contrast, overexpression of *CSNK1A1* had no effect

on CD34⁺ cells from patients with normal karyotype MDS or normal donors. Although lenalidomide also induced the degradation of IKZF1 in myeloid cells (**Figure 2.1a,b**), overexpression of *IKZF1* had a similar effect on del(5q) MDS, normal karyotype MDS, and normal donor CD34⁺ cells, suggesting that degradation of IKZF1 does not explain lenalidomide's therapeutic window in del(5q) MDS (**Figure 2.11**). These findings demonstrate that increased expression of *CSNK1A1* specifically rescues del(5q) cells from lenalidomide treatment.

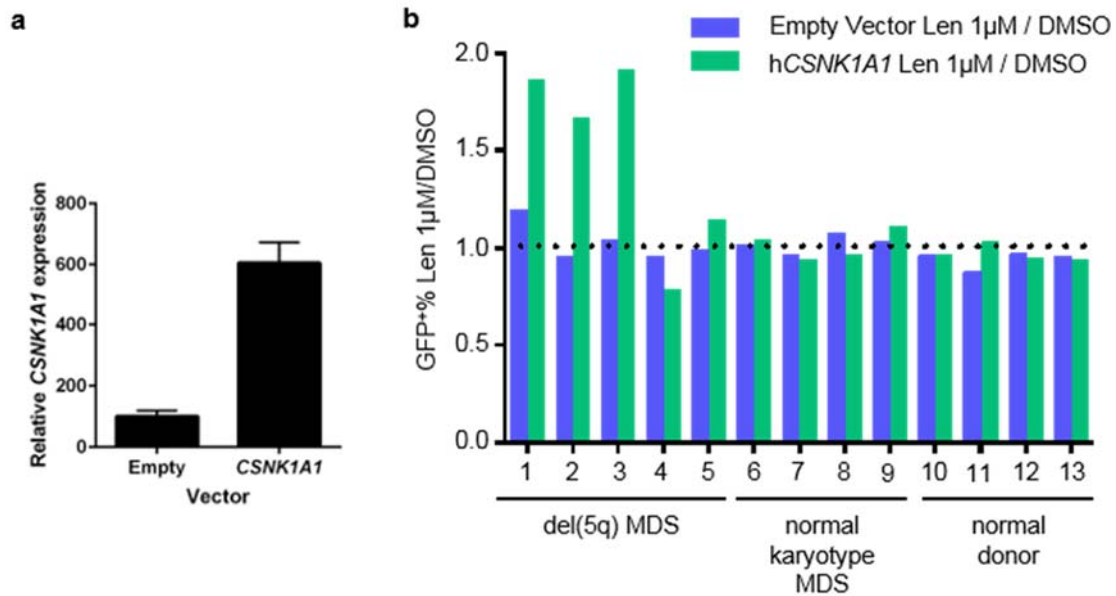


Figure 2.10: Ectopic *CSNK1A1* overexpression reduces lenalidomide sensitivity in primary del(5q) MDS cells. **a**, mRNA expression of *CSNK1A1* in cord blood CD34⁺ cells infected with lentivirus expressing *CSNK1A1* and *GFP* or control vector expressing *GFP* alone. CD34⁺ cells were infected with lentivirus and GFP⁺ cells were sorted three days later. Values are mean $\pm$ s.d. **b**, CD34⁺ cells derived from patient or control bone marrow were transduced with the vectors used in **a** and treated with DMSO or 1 μ M lenalidomide. Results are reported as a ratio of the percentage of GFP⁺ cells in the lenalidomide condition to the percentage of GFP⁺ cells in the DMSO condition after 5 days of treatment. A ratio greater than 1 for the *CSNK1A1* vector but not for the empty vector indicates that *CSNK1A1* expression reduces lenalidomide sensitivity. More information about the source of the patient samples used in this experiment is in Table 2.1.

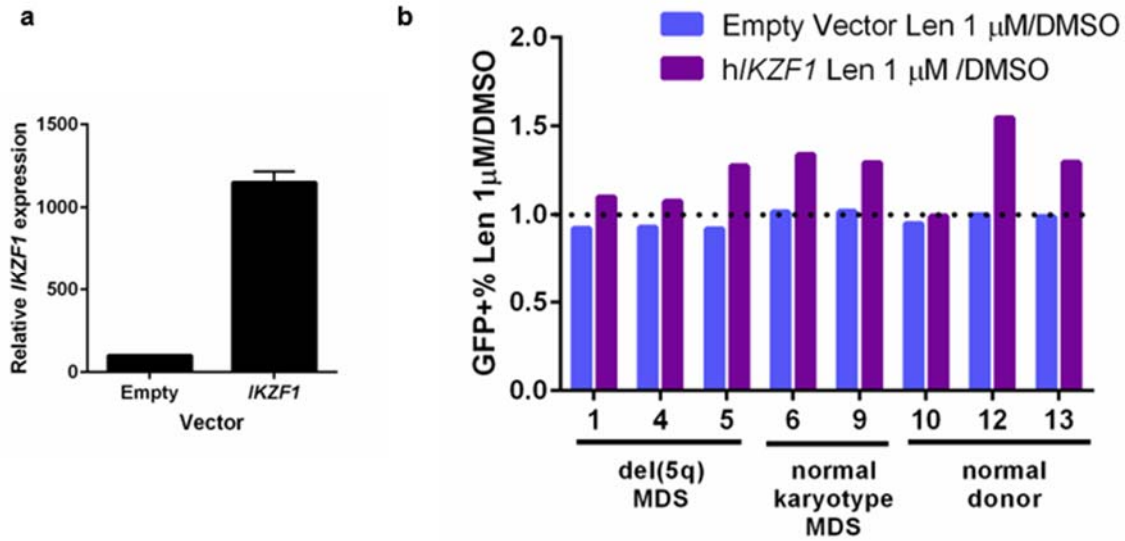


Figure 2.11: Expression of *IKZF1* in patient samples. **a**, mRNA expression of *IKZF1* in cord blood CD34⁺ cells infected with lentivirus expressing *IKZF1* and *GFP* or *GFP* only as in Figure 2.10a. Values are mean $\pm$ s.d. **b**, CD34⁺ cells derived from patient or control bone marrow were transduced with a lentivirus expressing *IKZF1* and *GFP* or a *GFP*-only control vector and treated with DMSO or 1 μ M lenalidomide. The percentage of GFP⁺ cells was assessed by flow cytometry after five days for each vector-drug combination. Results are reported as a ratio of the percentage of GFP⁺ cells in the lenalidomide condition to the percentage of GFP⁺ cells in the DMSO condition for each vector. More information about the source of the patient samples used in these experiments is in Table 2.1.

Table 2.1: Characteristics of patient samples used for *CSNK1A1* and *IKZF1* expression

experiments. Results of *TP53* sequencing, including exons with adequate coverage, is given in the rightmost column. All samples sequenced had wild-type *TP53*. ND, not done due to limited patient material. WT, *TP53* exon sequence has only known benign polymorphisms.

Sample #	Karyotype	Category	<i>TP53</i> Status (exons sequenced)
1	46,XX,del(5)(q15q33)[14] / 46,XX,del(5)(q15q33),del(12)(p11p13.3)[6] / 47,XX,del(5)(q15q33),+8[1] / 46,XX[3]	Lenalidomide	WT (2-8, 10-11)
2	46,XX,del(5)(q13q33)[17] / 46,XX[3]	Lenalidomide	ND
3	46,XY,del(5)(q15q35) [23] / 46,XY[2]	Lenalidomide	ND
4	46,XX,del(5)(q13q33),t(12;13)(q15;q11) [16]/46,XX,+8[1] / 46,XX [3]	Lenalidomide	WT (2-11)
5	46,XX,del(5)(q13q33) [14] / 46,XX [6]	Lenalidomide	WT (2-11)
6	Normal	Normal Karyotype MDS	WT (4-7, 9-10)
7	Normal	Normal Karyotype MDS	WT (2-11)
8	Normal	Normal Karyotype MDS	WT (2-11)
9	Normal	Normal Karyotype MDS	WT (3-4, 6-11)
10	Normal	Normal Adult Bone Marrow CD34 ⁺	ND
11	Normal	Normal Adult Bone Marrow CD34 ⁺	ND
12	Normal	Normal Adult Bone Marrow CD34 ⁺	ND
13	Normal	Cord Blood CD34 ⁺	ND
ND:	<i>TP53</i> sequencing not done due to limited sample size or normal donor sample		
WT:	<i>TP53</i> sequence is wild-type (known benign polymorphisms only) in the exons sequenced		

Species-specific effects of lenalidomide

We next sought to use a conditional knockout mouse model to determine whether haploinsufficiency for *Csnk1a1* sensitizes cells to lenalidomide treatment [139]. In initial experiments, we found that lenalidomide did not decrease Ck1 α protein levels in mouse Ba/F3

cells, primary murine leukemia cells, or mice treated *in vivo* (**Figure 2.12**), suggesting that mouse cells are intrinsically resistant to IMiD compounds. Consistent with these findings, mice do not develop the limb malformations observed in human embryos exposed to thalidomide [21, 24, 27] and murine multiple myeloma cells do not respond to lenalidomide [117]. Since CRBN is the direct protein target of lenalidomide, we examined whether expression of human CRBN could confer lenalidomide sensitivity to mouse cells. Overexpression of human, but not mouse, CRBN in mouse Ba/F3 cells resulted in a lenalidomide-dependent decrease of Ck1 α protein levels, implying that amino acid differences between mouse CRBN (mCrbn) and human CRBN (hCRBN) are responsible for the species-specific response to lenalidomide (**Figure 2.13**).

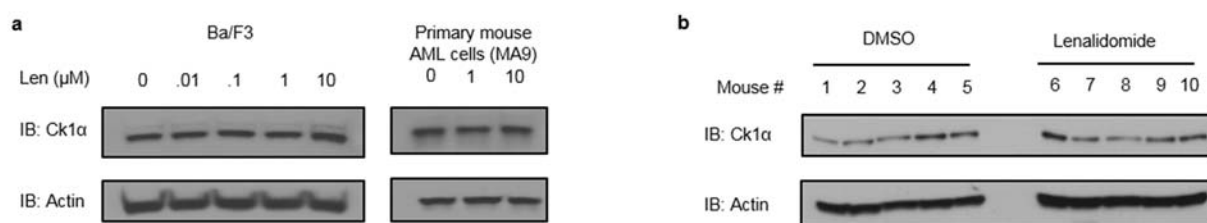


Figure 2.12: Lenalidomide does not induce degradation of Ck1 α in mouse cells. **a**, Ck1 α protein levels are unaffected in mouse Ba/F3 cells and primary mouse AML cells (MA9) treated with a range of lenalidomide (Len) doses. **b**, Ck1 α expression in bone marrow cells of mice treated with DMSO or lenalidomide. Part **a** is from Jan Krönke and Slater Hurst. Part **b** is from Rebekka Schneider and Jan Krönke.

To identify the amino acids responsible for this difference, we tested human/mouse CRBN chimeric proteins for their ability to confer lenalidomide-induced Ck1 α degradation in mouse Ba/F3 cells (**Figure 2.13a**). Lenalidomide sensitivity was determined by the C-terminal half of CRBN, which contains only 5 non-conserved amino acids between human and mouse. When these non-conserved positions in human CRBN were substituted with the corresponding

amino acid in mouse Crbn, only one substitution, V387I (human CRBN isoform 2, which is equivalent to V388 in human CRBN isoform 1), disrupted the lenalidomide-responsiveness of human CRBN (**Figure 2.13a**). Substitution of the isoleucine at this position in mouse Crbn for the human valine (mCrbn^{I391V}) was sufficient to confer lenalidomide-induced CK1 α degradation in mouse cells (**Figure 2.13b**), and there was no effect of similar substitutions at nearby residues (**Figure 2.13c**). Similar effects of the hCRBN^{V387I} and mCrbn^{I391V} point mutations were observed on lenalidomide-induced degradation of IKZF1 and IKZF3 in human 293T cells (**Figure 2.13d**), suggesting that a single amino acid change in CRBN determines lenalidomide-responsiveness for multiple substrates. Consistent with previous reports [63], we found that mCrbn binds lenalidomide and we did not observe increased lenalidomide binding of the mCrbn^{I391V} mutant (**Figure 2.14**).

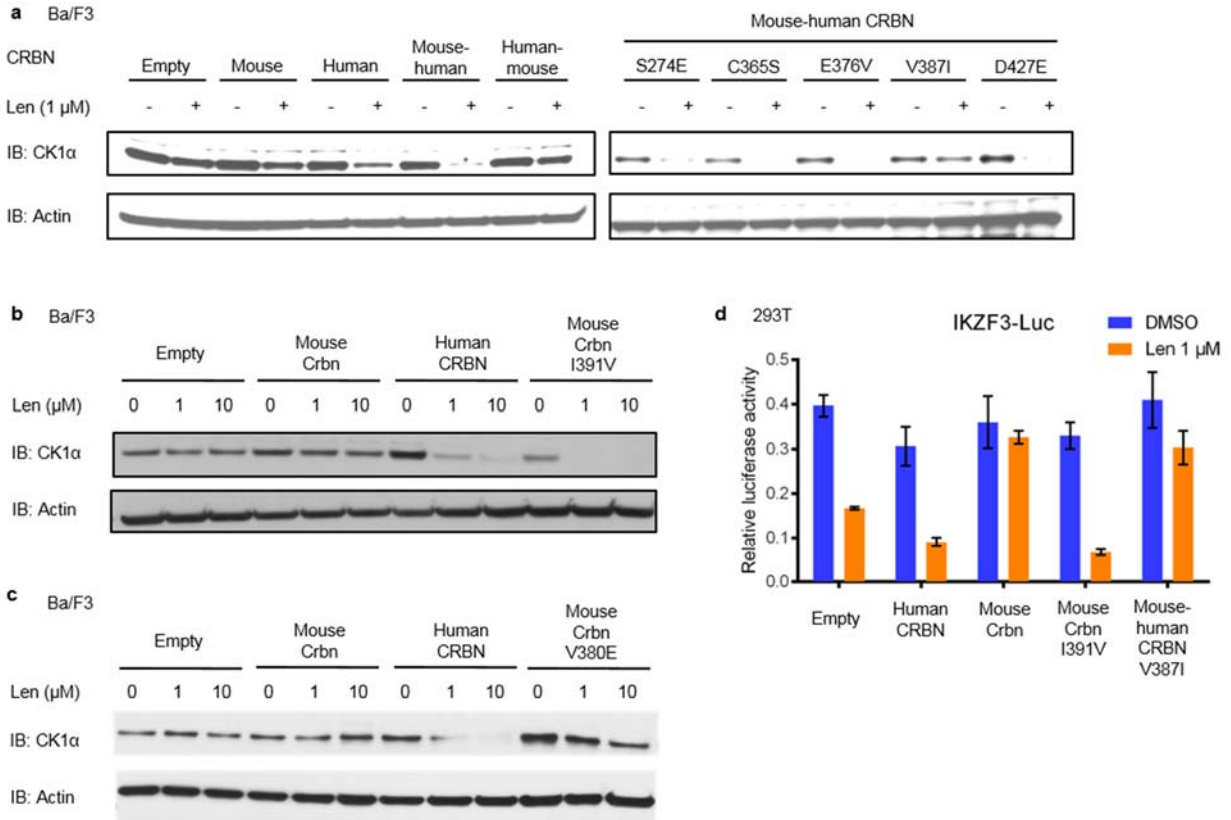


Figure 2.13: Amino acid changes in CRBN explain species-specific lenalidomide effects. **a**, Effect of the expression of human CRBN (h), mouse Crbn (m), chimeras of human and mouse CRBN (m-h and h-m, breakpoint at residue 221 (human) / 225 (mouse)) and variants of the m-h chimera where single amino acids in the C-terminus were mutated to their corresponding mouse residue on lenalidomide-dependent CK1 α degradation in mouse Ba/F3 cells. **b**, Expression of mouse Crbn^{I391V} restores lenalidomide-dependent CK1 α degradation in mouse Ba/F3 cells. **c**, Expression of mouse Crbn^{V380E} does not alter lenalidomide-dependent CK1 α degradation in mouse Ba/F3 cells. **d**, Effect of hCRBN, hCRBN^{V387I}, mCrbn, and mCrbn^{I391V} on lenalidomide sensitivity of an IKZF1-luciferase fusion protein expressed in human 293T cells. Data are mean $\pm$ s.e.m. **a**, **b** and **d** are from Jan Krönke and Slater Hurst.

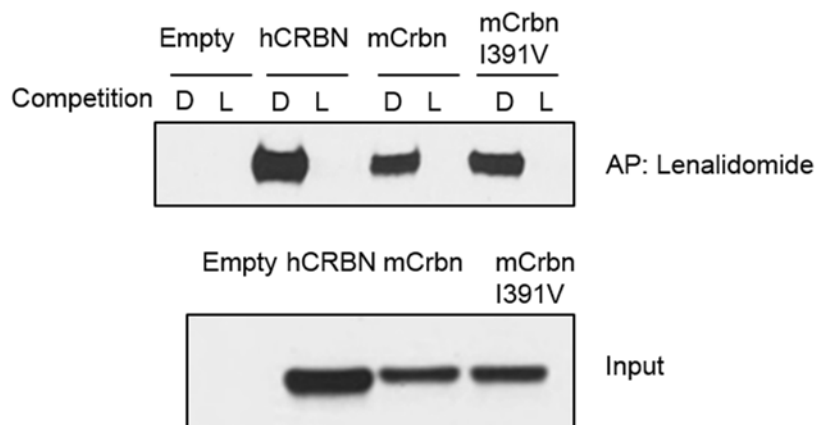


Figure 2.14: Lenalidomide binds mCrbn and mCrbn^{I391V}. Lenalidomide-bead affinity purification of various forms of FLAG-tagged CRBN. Human CRBN, mouse Crbn, and mouse Crbn^{I391V} are all equally pulled down with lenalidomide beads in the presence of DMSO (D), but these interactions are inhibited by pre-incubation with 100-fold free lenalidomide (L). Input CRBN expression is shown below.

We modeled the effects of this mouse-human amino acid substitution based on recently published crystal structures of the CRBN-DDB1-IMiD drug complex [68, 69]. V387 of human CRBN (equivalent to I391 of mouse Crbn) is located in the IMiD drug binding region of CRBN but does not directly interact with lenalidomide (**Figure 2.15a**). To investigate how the substitution of isoleucine for valine in mouse Crbn confers lenalidomide-responsiveness, we superimposed the structures for the mouse and human IMiD-binding regions bound to lenalidomide as solved in Chamberlain et al. [68]. No backbone changes are present at the site of the valine-isoleucine species differences (**Figure 2.15b**), but the isoleucine residue is well-defined in the electron density with the long arm of the side chain oriented towards the indole NH moiety of W403 in the mouse structure. The increase in steric bulk of the isoleucine side chain, relative to valine, results in a bulge in the solvent accessible surface of the mouse protein

adjacent to both W403 and lenalidomide (**Figure 2.15c, d**). It has been proposed that IMiD binding produces a hotspot for substrate interactions by placement of the hydrophobic phthalimide or isoindolinone ring in an environment of potential hydrogen bond donors and acceptors from the surface of CRBN [68]. In this case, the larger side chain of the isoleucine residue found in rodents may sterically clash with substrate proteins such as IKZF1 and CK1 α , blocking access to key hydrogen bonds from CRBN, such as from the indole NH from tryptophan 403 (mouse numbering). Steric clashes and occlusion of key bonds with substrate proteins thereby provide a potential explanation of why IMiD compounds bind mouse Crbn (**Figure 2.14**) but do not promote degradation of Ikbzf1 and Ck1 α .

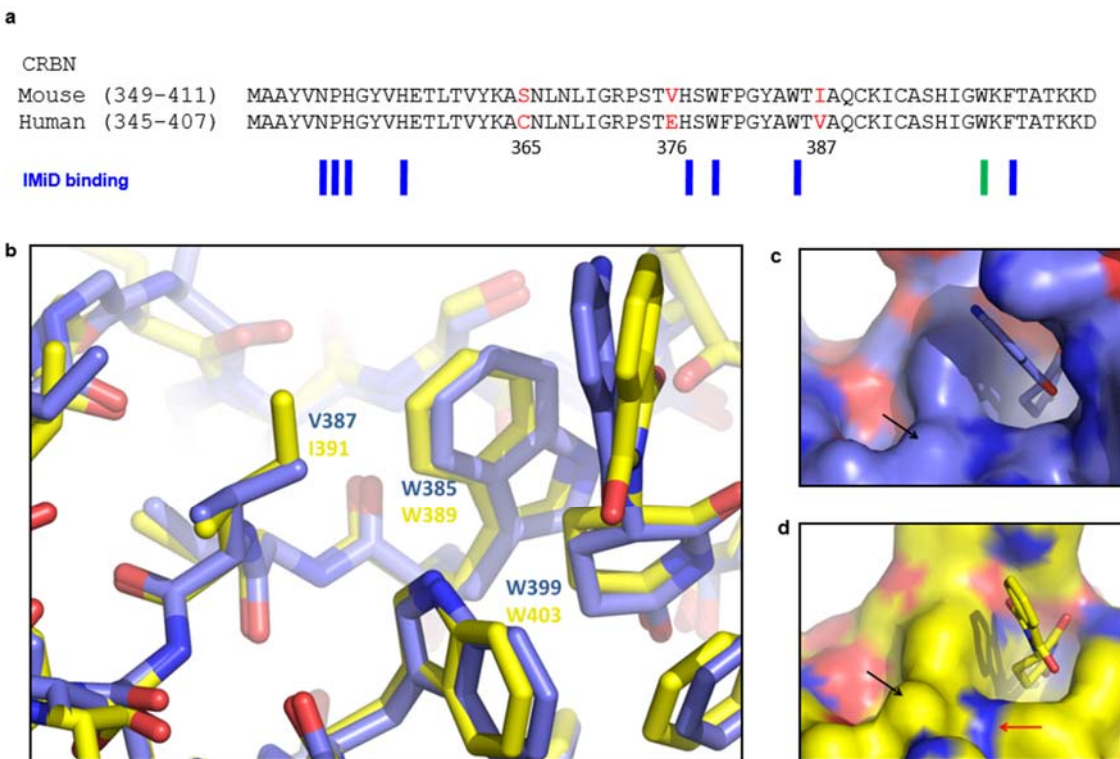


Figure 2.15: Comparison of the IMiD-binding regions of human and mouse CRBN. **a**, Alignment of human and mouse CRBN IMiD binding region. Non-conserved amino acids are red. Amino acids involved in IMiD binding [68, 69] are indicated by blue bars. Mouse W403 is indicated with a green bar. **b**, Superposition of the IMiD binding domains of human CRBN (blue, PDB accession 4TZ4) and mouse Crbn (yellow, PDB accession 4TZC). Residues are labeled according to human isoform 2 (blue numbers) and mouse isoform 2 (yellow numbers). **c**, The V388 residue is indicated on the surface of human CRBN with a black arrow. **d**, The corresponding mouse residue, I391, is indicated on the surface of mouse Crbn with a black arrow. Mouse W403 is indicated by a red arrow. Philip Chamberlain provided the graphics in this figure.

Having determined the mechanism of lenalidomide resistance in mouse cells, we expressed the mCrbn^{I391V} cDNA in hematopoietic cells from *Csnk1a1* conditional knockout mice to determine the effects of *Csnk1a1* haploinsufficiency on lenalidomide sensitivity. We isolated CD45.2⁺ c-Kit⁺ hematopoietic stem and progenitor cells from *Csnk1a1*^{+/-}*Mx1Cre*⁺ and *Mx1Cre*⁺ control littermates treated with poly (I:C) to induce gene excision in hematopoietic cells. We transduced these cells with a retroviral vector expressing mCrbn^{I391V} and cultured them in competition with similarly transduced isogenic CD45.1 c-Kit⁺ cells in the presence or absence of lenalidomide (**Figure 2.16a**). Lenalidomide had no effect on control cells, but *Csnk1a1*^{+/-}*Mx1Cre*⁺ cells were significantly depleted in the presence of lenalidomide (**Figure 2.16b**). The enhanced sensitivity of *Csnk1a1*^{+/-}*Mx1Cre*⁺ cells to lenalidomide was associated with induction of the p53 target gene *p21* (**Figure 2.16c**) and increased levels of apoptosis (**Figure 2.16d**) and was rescued by heterozygous deletion of *Trp53* (**Figure 2.16b**), demonstrating a critical downstream role for the p53 pathway. These results are consistent with the clinical observation that *TP53* mutations confer lenalidomide resistance in MDS with del(5q) [108].

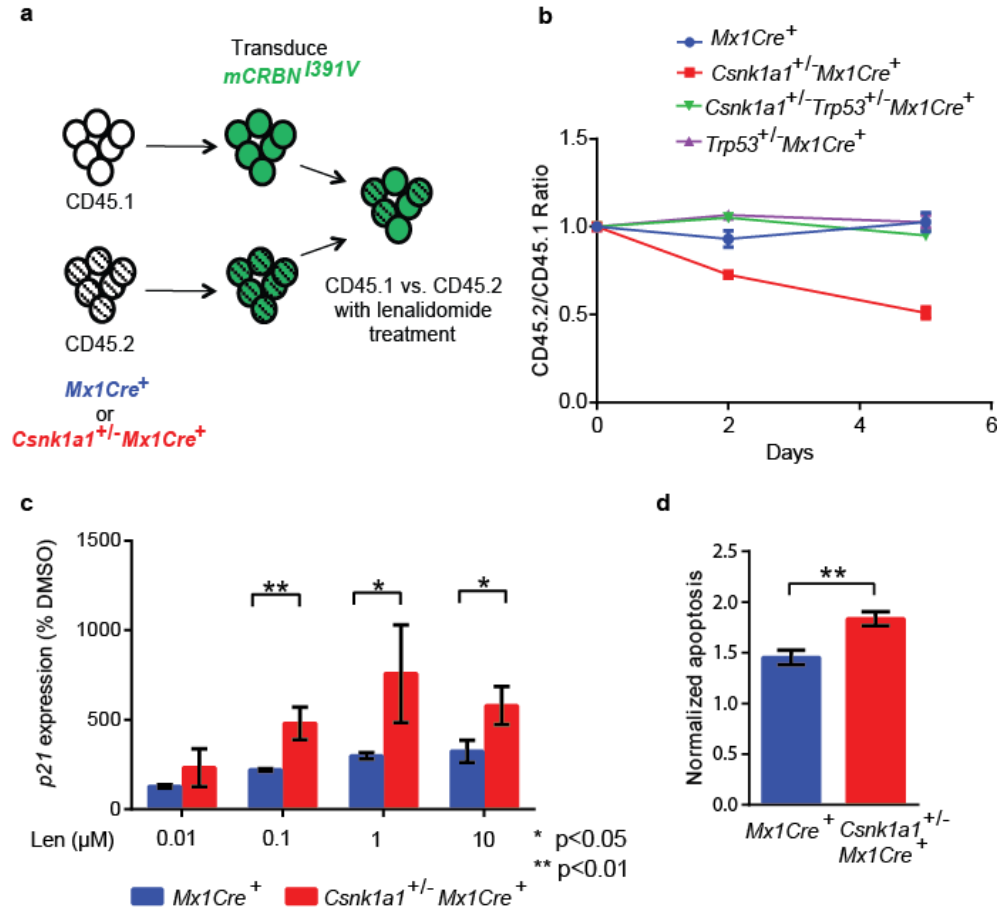


Figure 2.16: Effects of lenalidomide treatment on *Csnk1a1^{+/-}* mouse hematopoietic cells. **a**, *Csnk1a1^{+/-}Mx1Cre⁺* or *Mx1Cre⁺* c-Kit⁺ hematopoietic stem and progenitor cells (CD45.2⁺) and competitor cells (CD45.1⁺) were transduced with mouse *Crbn^{I391V}*, mixed in equal ratios, and treated with lenalidomide or DMSO. The relative percentage of CD45.1⁺ and CD45.2⁺ cells was followed by flow cytometry over 5 days. **b**, Effects of 0.1 μM lenalidomide on the chimerism of *Csnk1a1^{+/-}Mx1Cre⁺*, *Csnk1a1^{+/-}Trp53^{+/-}Mx1Cre⁺*, *Trp53^{+/-}Mx1Cre⁺*, or *Mx1Cre⁺* cells (CD45.2⁺) transduced with mouse *Crbn^{I391V}* in comparison to CD45.1⁺ competitor cells. **c**, Quantitative RT-PCR analysis of *p21* expression in *Csnk1a1^{+/-}* or control cells transduced with Mouse *Crbn^{I391V}* and treated with DMSO or lenalidomide. Data are normalized to DMSO and shown as mean ± s.d. **d**, Ratio of CD45.2⁺ cells and CD45.1⁺ cells in late apoptosis (Annexin V⁺

Figure 2.16 (Continued)

DAPI⁺) after transduction with mouse *Crbn*^{I391V} and four-day treatment with 0.1 μ M lenalidomide. CD45.2⁺ cells are either *Csnk1a1*^{+/-} *Mx1Cre*⁺ or *Mx1Cre*⁺. Data are normalized to DMSO treatment. Data are mean $\pm$ s.e.m.

Differential substrate specificity

Thalidomide, lenalidomide, and pomalidomide target IKZF1 and IKZF3 for ubiquitination and degradation and are active in multiple myeloma, but only lenalidomide has been shown to be clinically effective in del(5q) MDS [53, 54, 65, 107]. We therefore asked whether different thalidomide analogs have different relative substrate specificities. Treatment of KG-1 cells with 0.1 μ M lenalidomide resulted in a greater decrease in CK1 α protein levels than treatment with 10 μ M pomalidomide or 100 μ M thalidomide, in contrast to the relative effects of these treatments on IKZF1 (**Figure 2.17**). Consistent with the role of CK1 α as a negative regulator of Wnt signaling, we observed a larger increase in protein levels of β -catenin after treatment with lenalidomide than pomalidomide or thalidomide (**Figure 2.17**).

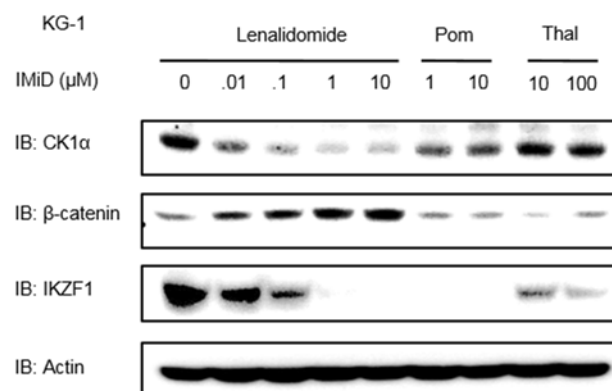


Figure 2.17: Lenalidomide has a stronger effect on CK1α than thalidomide or pomalidomide. Effect of lenalidomide, pomalidomide (Pom), and thalidomide (Thal) on protein levels of CK1α, β-catenin, and IKZF1 in KG-1 cells treated for 24 hours with the indicated drug concentrations. Data are from Jan Krönke and Slater Hurst.

We next investigated the substrate specificity of CC-122, a novel CRBN-binding agent that shares the glutarimide ring but has significant structural differences from the other IMiDs and has recently entered clinical trials (**Figure 2.18**). We used tandem mass tag (TMT) quantitative proteomics [140] in the MDS-L cell line to compare the activities of lenalidomide and CC-122. As expected, treatment with lenalidomide significantly decreased protein levels of both IKZF1 and CK1α. In striking contrast, treatment with 1 μM CC-122 caused an even greater decrease in IKZF1 ($P=2.77 \times 10^{-10}$, 72 hours) than 10 μM lenalidomide ($P=2.10 \times 10^{-8}$, 72 hours), but had no effect on CK1α protein levels ($P>0.05$)(**Figure 2.19**).

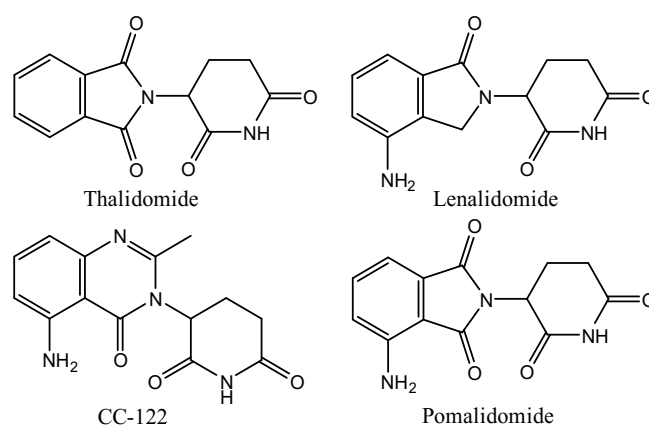


Figure 2.18: Structures of thalidomide, lenalidomide, pomalidomide, and CC-122.

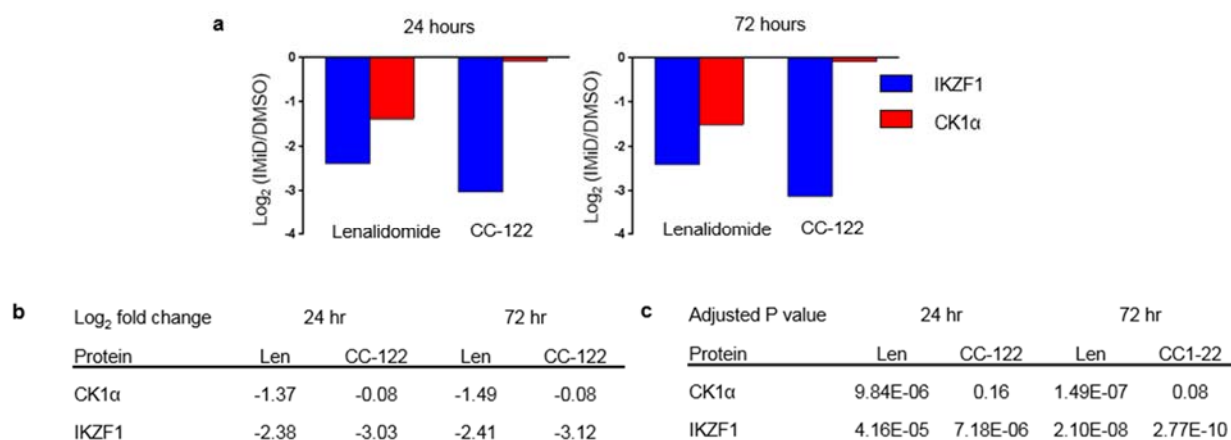


Figure 2.19: Global proteomics with lenalidomide and CC-122. **a**, Protein levels of IKZF1 and CK1α assessed by tandem mass tag quantitative proteomics in MDS-L cells treated with DMSO, 10 μM lenalidomide, or 1 μM CC-122 for 24 hours (left panel) or 72 hours (right panel). **b**, Comparison of Log₂ ratios for CK1α and IKZF1 in MDS-L cells after treatment with lenalidomide or CC-122 for 24 or 72 hours assessed by tandem mass tag (TMT) quantitative proteomics. **c**, Adjusted *P* values for CK1α and IKZF1 proteomic data in MDS-L cells. Data are from Kyle MacBeth.

We confirmed the TMT mass spectrometry findings by western blot for IKZF1 and CK1 α in MDS-L and KG-1 cells (**Figure 2.20a**). While all compounds induced degradation of IKZF1, thalidomide and CC-122 did not affect CK1 α protein levels, even at high concentrations. Although CC-122 has a greater potency than lenalidomide for degradation of IKZF1 and IKZF3, it was ineffective in decreasing CK1 α protein levels compared to lenalidomide, suggesting that subtle chemical modifications can affect substrate preference (**Figure 2.19, Figure 2.20a**). Furthermore, treatment with excess CC-122 abrogated the lenalidomide-induced degradation of CK1 α , demonstrating that lenalidomide and CC-122 compete for the same glutarimide binding site on CRBN (**Figure 2.20b**). These experiments demonstrate that despite structural similarity, the substrate specificities of thalidomide analogs differ. Notably, only lenalidomide has a strong effect on CK1 α , suggesting that it may indeed be the most appropriate modulator of CRL4^{CRBN} for the treatment of del(5q) MDS (**Figure 2.21**).

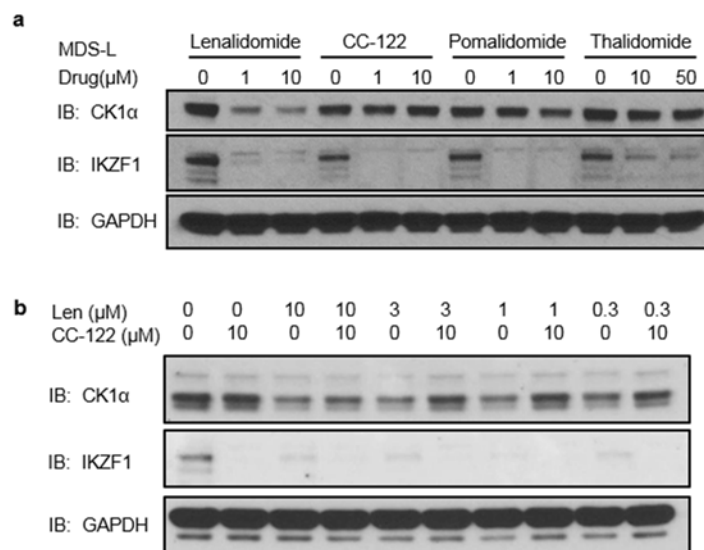


Figure 2.20: Western Blot verification of the effects of different IMiDs on CK1 α protein levels. Western blot analysis of CK1 α and IKZF1 protein levels in MDS-L cells treated with DMSO or different concentrations of thalidomide, lenalidomide, pomalidomide, or CC-122. **d**, KG-1 cells were treated with DMSO or lenalidomide (Len) in the absence or presence of different concentrations of CC-122. Data are from Kyle MacBeth.

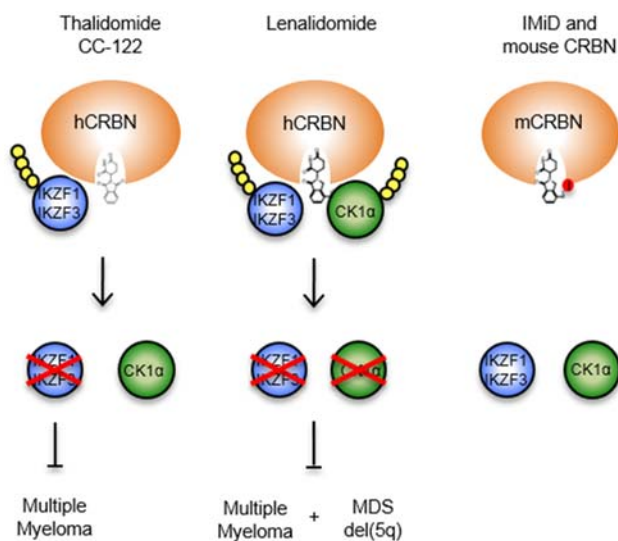


Figure 2.21: Schematic presentation of the interaction of different thalidomide analogs with CRBN, substrates, and therapeutic indications.

Intriguingly, lenalidomide, but not thalidomide or pomalidomide, has been reported to induce the formation of two β -strands composed of CRBN residues 346-363 [68]. Although conformational differences are difficult to interpret in the absence of a substrate-bound structure, the formation of these β -strands is expected to make significant changes in the surface of CRBN near the IMiD binding site [68] and thus it may contribute to the differential recruitment of IKZF1 and CK1 α . The interaction of specific thalidomide analogs with particular substrates may therefore be governed by unique structural determinants, revealing the biological and clinical potential for members of this class of drugs to induce degradation of distinct sets of proteins.

Discussion

We demonstrated that lenalidomide targets CK1 α for degradation and that heterozygous deletion of *CSNK1A1* in del(5q) MDS provides a therapeutic window for selective targeting of the malignant cells by lenalidomide. The concept that genes within heterozygous deletions could

cause vulnerabilities in cancer cells was first proposed 20 years ago [141] and has been more recently demonstrated as CYCLOPS genes (cancer vulnerabilities unveiled by genomic loss) in cell lines [142]. Our data demonstrate that in del(5q) MDS, lenalidomide-induced degradation of CK1 α below haploinsufficient levels induces p53 activity and growth inhibition, as CK1 α is a negative regulator of p53. Deletion of contiguous genes on chromosome 5q, such as *RPS14*, may further sensitize del(5q) cells to p53 activation [101, 110, 143]. This mechanism of activity is consistent with the acquisition of *TP53* mutations in del(5q) MDS patients who develop resistance to lenalidomide. Degradation of CK1 α may also contribute to other clinical effects of lenalidomide such as activity in the activated B-cell (ABC) subtype of diffuse large B-cell lymphoma [77] and lenalidomide-induced myelosuppression. Further investigation is required to determine the complete biological effects from degradation of each substrate.

Lenalidomide, like thalidomide and pomalidomide, binds CRBN and induces degradation of specific substrates. We found that a single amino acid difference between mouse and human CRBN renders mouse cells insensitive to IMiD compounds. This discovery enabled us to demonstrate, using a genetically engineered mouse model, that *Csnk1a1* haploinsufficiency sensitizes cells to lenalidomide. Non-conserved amino acid changes in CRBN may also explain why thalidomide does not cause teratogenicity in mice and was approved for use in pregnant women, leading to the birth of more than 10,000 newborns with limb malformations and other disabilities.

Thalidomide, lenalidomide, and pomalidomide all induce CRL4^{CRBN}-mediated degradation of IKZF1 and IKZF3, but the subtle differences in chemical structure between these molecules cause dramatic changes in potency. We now find that thalidomide and a novel compound, CC-122, induce the degradation of IKZF1 but not CK1 α . CC-122 may have a greater

therapeutic window for the treatment of B cell malignancies and other diseases that depend on IKZF1 and IKZF3, but would not be predicted to have activity in del(5q) MDS.

CC-122, like thalidomide and its analogs, has a glutarimide ring that anchors the molecule in CRBN, and structural variation in the remainder of the molecule is thought to determine substrate specificity [68, 69]. These findings provide evidence that thalidomide-related molecules have distinct biological activities, mediated by degradation of distinct sets of substrates, and that these compounds will be the first in a larger class of drugs with therapeutic utility through the targeting of specific proteins for degradation.

Methods

Reagents

Lenalidomide (Toronto Research Chemicals, Selleck Chemicals, and Celgene), Thalidomide (Millipore and Celgene), Pomalidomide (Selleck Chemicals and Celgene), MG-132 (Selleck Chemicals), CC-122 (Celgene), PR619 (Lifesensors), MLN4924 (Active Biochem), and Leptomycin B (Santa Cruz) were dissolved in DMSO at 10 to 100 mM and stored at -20°C for up to 6 months. For cell culture experiments drugs were diluted at least by 1:1000 so that the final DMSO concentration was 0.1% or lower.

Cell lines

KG-1, Ba/F3, K562, MM1S, Jurkat, HEL, and 293T cells were obtained from American Type Culture Collection (ATCC) and their identity was not further authenticated. MDS-L cells were provided by Kaoru Tohyama, Kawasaki Medical School (Japan). Cells were cultured in RPMI 1640 (Mediatech) or DMEM (Mediatech) supplemented with 10-20% heat-inactivated fetal bovine serum (FBS) (Omega Scientific) and 1% penicillin, streptomycin, and L-glutamine

(Mediatech). Cells were grown at 37°C in a humidified incubator under 5% CO₂. Ba/F3 cells were cultured in the presence of 10 ng/mL mouse IL-3 (Miltenyi) and MDS-L cells were cultured with 10 ng/mL human GM-CSF. 293T cells were transfected using TransIT-LT1 (Mirus Bio) according to the manufacturer's protocol. Cell lines were intermittently tested for mycoplasma.

Cell culture and treatment for K-ε-GG and proteome profiling

KG-1 cells were cultured for 2 weeks (~6 cell doublings) in RPMI depleted of L-arginine and L-lysine (Caisson Labs Inc.) and supplemented with 10% dialyzed FBS (Sigma) and L-arginine (Arg0) and L-lysine (Lys0) (light), ¹³C₆¹⁴N₄-L-arginine (Arg6) and 4,4,5,5-D₄-L-lysine (Lys4) (medium) or ¹³C₆¹⁵N₄-L-arginine (Arg10) and ¹³C₆¹⁵N₂-L-Lysine (Lys8) (heavy) to generate light-, medium- and heavy-labeled cells. Media was exchanged every 3rd day. On day 14 cells were treated with 1 μM lenalidomide, 10 μM lenalidomide or DMSO for 4 hours for ubiquitination profiling and 24 hours for protein level assessment. Experiments were performed in two biological replicates with flipped SILAC labeling: Replicate 1: DMSO/light, lenalidomide 1 μM/medium; lenalidomide 10 μM/ heavy; Replicate 2: lenalidomide 1 μM/light; lenalidomide 10 μM / medium; DMSO/heavy.

SILAC based K-ε-GG and proteome profiling of KG-1 cells

Cell lysis and trypsin digestion, basic pH reversed phase fractionation, K-ε-GG enrichment, and LC-MS/MS analysis for KG-1 cells were performed as recently described [65] with minor changes. Cell pellets used for K-ε-GG profiling were lysed in 8 M urea, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 μg/mL aprotinin (Sigma-Aldrich), 10 μg/mL leupeptin (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 μM PR-619, and 1 mM chloroacetamide at 4°C. Pellets used for proteome profiling were lysed in 8 M

urea, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 µg/mL aprotinin (Sigma-Aldrich), 10 µg/mL leupeptin (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride (PMSF), at 4 °C [65]. For this work, 10 mg of protein was input per SILAC state for the ubiquitin workflow. For proteome profiling, 1.5 mg of protein was input per SILAC state. Proteins were reduced with 5 mM dithiothreitol for 45 minutes at room temperature and subsequently carbamidomethylated with 10 mM iodoacetamide for 30 min at RT in the dark. Samples were diluted to 2 M urea with 50 mM Tris-HCl, pH 7.5, and digested with sequencing grade trypsin (Promega) at 25 °C overnight using an enzyme-to-substrate ratio of 1:50. Digested samples were acidified to 1% formic acid (Sigma-Aldrich). Tryptic peptides were centrifuged for 5 min at 3000 x g to remove the precipitate. Peptides were desalted exactly as previously described [65].

Samples were fractionated by basic pH reversed phase (bRP) fractionation using an Agilent 1100 Series HPLC and Zorbax 300 Å Extend-C18 columns as previously described [129, 144]. A 9.4 mm × 250 mm column (Agilent, 5 µm bead size) was used to fractionate samples intended for K-ε-GG enrichment, whereas a 4.6 mm × 250 mm column (Agilent, 3.5 µm bead size) was used to fractionate samples intended for proteome analysis [144]. For K-ε-GG samples, approximately 15 mg of peptide sample was resuspended in 1.8 mL of basic RP solvent A (2% MeCN, 5 mM ammonium formate, pH 10), separated into 2 HPLC vials and injected with Solvent A at a flow rate of 3 mL/min. A 64-min method was used for fractionation exactly as previously described [65, 129]. A total of 96 2 mL fractions were collected every 0.66 min at a flow rate of 3 mL/min. After separation, bRP fractions were pooled in a serpentine, non-contiguous manner to generate 8 final fractions (final fraction 1 = 1, 9, 17, 25, 33, 41, 49, 57, 65; final fraction 2 = 2, 10, 18, 26, 34, 42, 50, 58, 66; ...). Since 10 mg of protein per SILAC state

was used for K- ϵ -GG samples and the maximum loading capacity on the 9.4 mm $\times$ 250 mm column bRP column is 15 mg, two rounds of fractionation were completed per replicate sample.

Proteome samples were brought up in 0.9 mL of basic RP solvent A and injected with solvent A at a flow rate of 1 mL/min. Peptides were separated using the bRP method previously described for the 4.6 mm $\times$ 250 mm column [144]. Approximately 5% of each proteome fraction was taken and pooled to generate 24 final fractions (final fraction 1 = 1, 25, 49; final fraction 2 = 2, 26, 50; ...) plus a fraction “A” that contains early eluting peptides. All bRP pooled fractions were dried using a SpeedVac concentrator.

K- ϵ -GG enrichment was completed using the anti-K- ϵ -GG antibody obtained from the PTMScan® ubiquitin remnant motif (K- ϵ -GG) kit (Cell Signaling Technology) as previously described [65, 129]. Briefly, bRP fractions were reconstituted in 1.5 mL of immunoaffinity purification buffer and each fraction was incubated with 31 μ g of cross-linked anti-K- ϵ -GG antibody for 1 hour at 4 °C with end-over-end rotation. Following incubation, samples were spun down and the supernatant was removed. Antibody-bound beads were washed 4x with 1.5 mL of ice-cold phosphate buffered saline (PBS). Peptides were eluted with 2 $\times$ 50 μ L of 0.15% TFA. Eluted peptides were desalted using C18 StageTips.

K- ϵ -GG and proteome fractions were reconstituted in 9 μ L and 20 μ L of 3% MeCN/1% FA, respectively, and analyzed using a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled on-line to a Proxeon Easy-nLC 1000 system. For analysis of each fraction, 4 μ L and 1 μ L of K- ϵ -GG and global proteome samples were injected, respectively. Samples were injected onto a microcapillary column (360 μ m OD $\times$ 75 μ m ID) packed with 24 cm of ReproSil-Pur C18-AQ 1.9 μ m beads (Dr. Maisch GmbH) that was heated to 50 °C and equipped with an integrated electrospray emitter tip (10 μ m). For online LC separation, solvent A was 0.1 % FA/3 % MeCN

and solvent B was 90% MeCN/0.1% FA. Peptides were eluted into the mass spectrometer using the LC-MS method previously described [65]. The Q Exactive instrument was operated in the data-dependent mode acquiring 12 HCD MS/MS scans ($R=17,500$) after each MS1 scan ($R=70,000$) using an MS1 ion target of 3×10^6 ions and an MS2 target of 5×10^4 ions. The maximum ion time for the MS/MS scans was set to 120 ms, the collision energy was set to 25, the dynamic exclusion time was set to 20 s, and the peptide match setting was set to on.

The MaxQuant software version 1.3.0.5 was used to analyze MS data. Data were searched against the human UniProt database as well as a database provided by MaxQuant containing common laboratory contaminants. The search parameters were as follows: enzyme specificity was set to trypsin, maximum number of mixed cleavages set to 2, precursor mass tolerance was set to 20 ppm for the first search and set to 6 ppm for the main search. Oxidized methionine and N-terminal protein acetylation were searched as variable modifications and carbamidomethylation of cysteine was searched as a fixed modification. Data files from K- ϵ -GG enriched samples were also searched with Gly-Gly addition to lysine as a variable modification. The minimum peptide length was set to 6, and false discovery rate for peptide, protein, and site identification was set to 1%. Reverse and contaminant hits were removed from datasets. Normalized ratios were utilized for quantification. For proteome data, proteins identified by 2 or more razor/unique peptides and quantified by 2 or more ratio counts in both biological replicates were considered for the final dataset. For the K- ϵ -GG data, K- ϵ -GG sites were considered if they were quantified in both biological replicates.

For data analysis, normalized SILAC ratios for the 2 biological replicates were filtered to retain only those deemed reproducible. Reproducibility was based on replicates being confined within the 95% limits of agreement of a Bland-Altman plot [145]. In the Bland-Altman plot,

differences of the replicates are plotted against the average values and the limits of agreement correspond to the prediction confidence interval for a regression line with unit slope.

Reproducible replicates were then subjected to a moderated t -test to assess statistical significance [146]. This statistic is similar to the ordinary t -statistic, with the exception that the standard errors are calculated using an empirical Bayes method utilizing information across all proteins, thereby making inference about each individual protein more robust. The nominal P values arising from the moderated t -statistic are corrected for multiple testing by controlling the false discovery rate (FDR), as proposed by Benjamini and Hochberg [147]. Proteins with an FDR adjusted P value of less than 0.05 were deemed to be reproducibly regulated. Figures containing scatter plots of SILAC data show all points regardless of the reproducibility measure. Statistical significance was assessed using only reproducible data points.

The original mass spectra may be downloaded from MassIVE (<http://massive.ucsd.edu>) using the identifier: MSV000079014. The data are accessible at <ftp://MSV000079014:a@massive.ucsd.edu>.

Plasmids and Virus constructs

The following cDNAs were cloned in the pRSF91 retrovirus backbone (kind gift of Christopher Baum, Hanover Medical School) or pEF1a-IRES-GFP lentiviral backbone: *CSNK1A1* Isoform 2 (ccsbBroadEN_06055), *CSNK1A1* Isoform 1 (kind gift from William G. Kaelin), *CSNK1E* (ccsbBroadEN_00379), mouse *Crbn* Isoform 2 (Thermo Scientific), and human *CRBN* Isoform 2 (ccsbBroadEn_08244). Human *IKZF1* isoform 1 was synthesized using gBlocks (IDT) with internal BstXI and BsrGI sites removed using synonymous substitutions. For certain experiments, GFP was replaced by GFP-T2A-Puro to allow for drug selection of positively transduced cells. Chimeric cDNAs and point mutations were cloned with overlapping

PCR primers. Point mutations in mouse-human CRBN chimeric proteins are annotated according to their position in human *CRBN* isoform 2. Lentivirus was concentrated by ultracentrifugation for transduction of primary cells.

Lentiviral vectors (TRC005 backbone) expressing shRNAs targeting *luciferase* (TRCN0000072254: ATGTTTACTACACTCGGATAT) and *CSNK1A1* (#1: TRCN0000342505, CATCTATTTGGCGATCAACAT; #2: TRCN0000342507, GCAGAATTTGCGATGTACTTA) were obtained from The RNAi Consortium (TRC) of the Broad Institute. For certain experiments, the puromycin resistance gene was replaced by *GFP*.

The luciferase reporter plasmid pCMV-IRES-RenillaLUC-IRES-Gateway-FireflyLUC was a kind gift from William G. Kaelin (Dana-Farber Cancer Institute). Cloning of cDNAs was performed using Gateway LR reaction (Invitrogen).

CRISPR-mediated genetic deletion was performed with the pSgRNA-CAS9-T2A-Puro plasmid [148] using *CRBN* exon 1-specific guide RNAs (*CRBN* targeting sequence: #1 TCCTGCTGATCTCCTTCGC, #2 AACCACCTGCCGCTCCTGCC). 1×10^5 293T cells were transfected in a 12-well with 500 ng of each plasmid using TransLTI (Mirus). After 24 hours, transfected cells were selected with 2 μ g/mL puromycin for 4 days. Then 293T cells were diluted to single cell and plated in 96-well. Colonies were tested by western blot and Sanger sequencing of the endogenous *CRBN* exon 1 locus for inactivating biallelic out-of-frame mutations.

Western blot and antibodies

Protein lysates were run on Tris-HCl, 1mm Criterion Precast gels (Bio-Rad) or NuPAGE Bis-Tris gels (Novex) gels at a constant voltage. Proteins were transferred onto Immobilon-P transfer membranes (Millipore) at a constant amperage. Before staining with primary antibodies,

blots were blocked in 5% non-fat dry milk (Santa Cruz) or 5% BSA in TBS-T 0.1% for 30 minutes.

For protein detection primary antibodies detecting CK1 α (C-19, Santa Cruz or Abcam ab108296), β -catenin (Cell Signaling #9587 and #8480), HA (HRP-conjugate, Miltenyi, GG8-1F3.3), FLAG (M2, HRP-conjugate Sigma Aldrich), ubiquitin conjugates (FK2, HRP-conjugate Enzo Life Sciences), Actin (HRP-conjugate, Abcam), β -tubulin (Cell Signaling #2146) and GAPDH (Santa Cruz sc-47724) were used. Secondary antibodies were HRP conjugated Bovine anti-Goat (Jackson ImmunoResearch) and HRP conjugated donkey anti-rabbit (GE Healthcare). SuperSignal (Thermo Scientific) chemiluminescent substrate was used for detection. For re-probing, blots were stripped in Restore Western Blot Stripping Buffer (Thermo Scientific), activated in methanol, and re-blocked.

Flow cytometry

Flow cytometry was performed on a FACS Canto II (BD Bioscience) using the PE and FITC channels for the detection of dTomato and GFP, respectively. DAPI staining was performed to exclude dead cells. A High-Throughput Sampler (BD) was used for some experiments.

Quantitative RT-PCR

Gene expression was measured by reverse transcription quantitative PCR (RQ-PCR). For RNA isolation and reverse transcription, a cDNA Synthesis Kit for MultiMacs (Miltenyi) was used according to the manufacturer's protocol. The following primer-probe sets from Life Technologies were used with TaqMan Gene Expression Master Mix (Life Technologies): human *GAPDH* (402869), human *CSNK1A1* (Hs00793391_m1), human *IKZF1* (Hs00958474_m1), mouse *Gapdh* (Mm99999915_g1), mouse *p21* (Mm04205640_g1). Analysis was performed on a

7900HT Fast Real-Time PCR System (Applied Biosystems) in a 384-well plate. Relative expression levels were calculated using the $\Delta\Delta CT$ method.

Immunoprecipitation of CRBN

For immunoprecipitation of FLAG-CRBN, 3×10^6 293T cells were plated in a 10 cm dish and transfected with 10 μ g FLAG-hCRBN or empty vector. Cells were treated with DMSO or 1 μ M lenalidomide in the presence of 10 μ M MG132 for 3 hours. Cells were lysed in Pierce IP Lysis Buffer and lysates were cleared by centrifugation. FLAG-CRBN was immunoprecipitated overnight using anti-FLAG M2 Affinity Gel (Sigma-Aldrich) in the presence of 10 μ M MG132 and DMSO or 1 μ M lenalidomide. The beads were washed 3 times with IP lysis buffer (Pierce) and protein was eluted from the affinity gel with 250 μ g/mL FLAG peptide (Sigma) after incubation for 30 min at 4°C. Protein lysates were then analyzed as described above.

For immunoprecipitation of endogenous CRBN 5×10^6 293T cells were treated with DMSO or 10 μ M lenalidomide and 10 μ M MG132 for 4 hours. Protein lysates were incubated overnight at 4 °C with 1 μ g of a polyclonal mouse anti-CRBN antibody (abcam) in the presence of lenalidomide or DMSO and MG132, Protein G sepharose beads were added for one hour. The beads were washed once with IP lysis buffer (Pierce) and protein was eluted from the beads by incubation with LDS loading buffer (Life Technologies) at 70 °C for 10 minutes.

In vivo ubiquitination

For assessment of endogenous ubiquitination of CK1 α 2×10^7 KG-1 cells were treated with DMSO, 1 or 10 μ M lenalidomide for 4 hours and then lysed in IP lysis buffer containing 10 mM NEM and 10 μ M MG132. Ubiquitinated proteins were pulled down by Ubiquitin 1 Tandem UBA (TUBE2) Agarose (Boston Biochem) for 4 hours at 4 °C and washed 3x with IP lysis

buffer. Protein was eluted by incubation with Laemmli buffer (Biorad) at 95 °C for 5 minutes, separated by SDS-PAGE, transferred to PVDF membrane and probed with anti-CK1 α .

In vitro ubiquitination

293T cells were transfected with either HA-CK1 α or FLAG-CRBN expressing vectors. After 48 hours, cells were lysed in Pierce IP lysis buffer (Thermo Scientific) and immunoprecipitated overnight with FLAG-Sepharose beads (Anti-FLAG M2 Affinity Gel, Sigma) or HA-Sepharose beads (EZView Red anti-HA affinity gel, Sigma). The beads were washed 3x in IP lysis buffer and 2x in E3 Ligase Reaction buffer (Boston Biochem) and eluted with 250 μ g/mL FLAG peptide (Sigma) or 100 μ g/mL HA peptide for 30 min at 4 °C. The eluates were mixed in a 1:1 ratio and added to a ubiquitination reaction mixture containing 200 nM E1 (UBE1), 2 μ M UbcH5a, 1 μ M UbcH5c, 1 μ g/ μ L K₀ ubiquitin, 1 μ M ubiquitin aldehyde, 1x Mg-ATP, 1x E3 Ligase Reaction Buffer (all Boston Biochem), 10 μ M MG132, 100 nM MG101 and 1 μ M lenalidomide, 10 μ M lenalidomide, or DMSO (1:1000) as appropriate in a total volume of 25 μ l.

Negative controls did not include E1 and E2 enzymes. After a 90-minute incubation at 30 °C, the reaction was denatured by adding 5x SDS containing loading buffer (Boston Biochem), boiled at 95 °C for 5 minutes, separated by SDS-PAGE and transferred to a PVDF membrane in order to detect HA-CK1 α and its ubiquitinated forms with a CK1 α -specific antibody. The membrane was then stripped and re-probed with anti-FLAG antibody.

Immunofluorescence

50,000 293T cells were grown on Lab-Tek 8 well chamber slides (Nunc) for 24 hours and then treated with DMSO or 10 μ M lenalidomide for various durations. At the conclusion of treatment, the media was decanted and the wells were washed 1x with PBS. Cells were fixed in

4% formaldehyde in PBS for 15 minutes, washed 3x 5 min in PBS and blocked for 1 hour at room temperature in PBS with 0.3% Tween-20 and 5% BSA. Primary antibody was anti-CK1 α (C-19, Santa Cruz), which was diluted 1:100 in PBS with 0.3% Tween-20 and 1% BSA (antibody dilution buffer) and incubated for 2 hours at room temperature. After 3x 5 min washes in PBS, Alexa Fluor 488 donkey anti-Goat (Life Technologies) was added at 1:200 in antibody dilution buffer and incubated for 1 hour at room temperature. After 3x 5 min washes in PBS slides were coverslipped with Vectashield mounting media with DAPI (Vector Laboratories). Slides were analyzed by fluorescence microscopy at 100x using a Nikon Eclipse 90i and NIS Elements. Channels were merged using ImageJ.

Purification, culture, and lentiviral infection of human CD34⁺ cells for shRNA experiments

Research cord blood units were obtained from The New York Blood Center according to an Institutional Review Board-approved protocol. Cord blood CD34⁺ hematopoietic cells were isolated from Ficoll purified PBMCs with an Indirect CD34 MicroBead kit (Miltenyi) and an Auto MACS Pro (Miltenyi) according to the manufacturer's protocol. Cells were cultured in serum-free media (SFEM, StemSpan) containing 50 ng/mL recombinant human TPO (Miltenyi), 40 ng/mL human FLT3 ligand (Miltenyi), 25 ng/mL recombinant human SCF (Miltenyi), and 10 ng/mL IL-3 (Miltenyi). For shRNA experiments, CD34⁺ cells were transduced with a VSV-G pseudotyped TRC pLKO.005 lentiviral vector expressing GFP instead of the puromycin resistance gene. Infection was performed after 24 hours in culture in a 96-well using spinfection in the presence of 2 μ g/mL polybrene (hexadimethrine bromide, Sigma). Forty-eight hours after transduction the number of transduced cells was analyzed by flow cytometry and was used as the baseline. Then cells were cultured in 1 μ M lenalidomide or DMSO and the relative number of infected cells was assessed by flow cytometry for 3 weeks.

Purification, culture, and lentiviral infection of patient samples

Viably frozen bone marrow mononuclear cells were obtained from healthy donors or patients with del(5q) MDS according to IRB approved protocols at the University of Pennsylvania and Roswell Park Cancer Institute. Informed consent was obtained from all subjects. Samples were thawed and CD34⁺ hematopoietic cells were isolated 20-24 hours later using an Indirect CD34 MicroBead kit (Miltenyi) and an Auto MACS Pro (Miltenyi). Cells were grown in serum-free media (SFEM, StemSpan) supplemented with 25 ng/mL SCF, 40 ng/mL FLT3 ligand, 50 ng/mL thrombopoietin, 40 µg/mL lipids, 100 U/mL Pen/Strep and 2 mM glutamine. 6-8 hours after CD34⁺ isolation, cells were transduced with concentrated VSV-G pseudotyped pEF1a-GFP-IRES-hCSNK1A1, pEF1a-GFP-IRES-hIKZF1 or empty vector control virus via spinfection in the presence of 4 µg/mL polybrene (Sigma, diluted to 2 µg/mL after spinfection). After 3 days, the initial percentage of transduced cells was determined by flow cytometry and remaining cells were split to treatment with either DMSO or 1 µM lenalidomide. The relative abundance of transduced cells in each condition was assessed after 5 days by flow cytometry. Control cord-blood CD34⁺ cells were isolated as above. Adult bone marrow CD34⁺ cells were purchased as single-donor lots from AllCells (Alameda, CA). The number of replicates for each patient sample, vector, and treatment was limited by the number of cells available and was as follows: one for samples 1-5, 8, 9 and 12; two for samples 6, 7 and 10; three for sample 11; four for sample 13. Samples were combined from three experiments.

For qPCR validation of *CSNK1A1* or *IKZF1* mRNA expression, cord blood CD34⁺ cells were transduced with lentivirus expressing *GFP* and the cDNA of interest or empty vector. After 3 days, transduced GFP⁺ cells were FACS sorted and RNA extraction and qPCR were performed as above.

TP53 Sequencing of patient samples

Genomic DNA was extracted from the CD34⁺ fraction of the patient bone marrow samples using a DNA Blood Mini Kit (Qiagen). PCR and sequencing were performed as described in the International Agency for Research on Cancer's Direct Sequencing Protocol (http://p53.iarc.fr/download/tp53_directsequencing_iarc.pdf). Mutations were identified using Mutation Surveyor (Softgenetics). Benign polymorphisms were identified using the International Agency for Research on Cancer's Portal (<http://p53.iarc.fr/TP53GeneVariations.aspx>).

Expressing different CRBN proteins in Ba/F3 cells

Variants of human and mouse CRBN were cloned into a modified pRSF91 backbone to generate pRSF91-CRBN-IRES-GFP-T2A-Puro retroviral constructs. 200,000 Ba/F3 cells were infected with ecotropic retrovirus in the presence of 2 µg/mL polybrene. After 24 hours, 1 µg/mL puromycin (Gibco) was added and cells were selected for 3-4 days. Cells were confirmed to be >90% GFP⁺ by flow cytometry and 1,000,000 cells were plated per 6-well and treated with DMSO or lenalidomide for 24 hours. Protein lysates were harvested and immunoblotted for CK1α as described above.

IKZF1 and IKZF3 luciferase reporter assay

10,000 293T cells were transfected with 50 ng of pCMV-IRES-RenillaLUC-IRES-IKZF1/IKZF3-FireflyLUC reporter plasmid together with 100 ng of a vector expressing hCRBN, mCrbn, different chimeric or mutant CRBN forms or empty control. After 48 hours, cells were treated with DMSO and lenalidomide for 4 hours. Firefly and Renilla luciferase activity were measured using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol.

Lenalidomide affinity purification

A lenalidomide derivative was conjugated to affigel beads as described previously [65]. FLAG-tagged cDNAs encoding hCRBN, mCrbn, and mCrbn^{I391V} were expressed from the pRSF91 retroviral backbone in 293T cells. Cell lysates were pre-mixed with DMSO or a 100-fold excess of lenalidomide and lenalidomide-conjugated affigel beads were added. After incubation, the lenalidomide-bound affigel bead fraction was isolated by centrifugation and washed. Bead-associated proteins were eluted in hot Laemmli buffer and run on a denaturing protein gel. Various forms of CRBN were detected with an anti-FLAG antibody.

Mouse experiments

Mouse experiments were performed according to an IACUC approved protocol at Children's Hospital Boston. Generation and characterization of the conditional *Csnk1a1* knockout mouse has been described previously [130]. *Csnk1a1*^{flox/+} mice were crossed with *Mx1Cre*⁺ mice on a C57BL/6NTac background to obtain *Csnk1a1*^{flox/+} *Mx1Cre*⁺ mice. *Csnk1a1*^{flox/+} *Mx1Cre*⁺ or control *Csnk1a1*^{+/+} *Mx1Cre*⁺ mice were treated with 3 doses of 200 µg poly(I:C) (Invivogen HMW) at 8-10 weeks of age and gene excision was confirmed where applicable. At least 2 weeks following poly(I:C) treatment, the long bones and spines were harvested and crushed, and red blood cells were lysed. Bone marrow from age-matched mice of the same genotype was pooled to create sex-balanced groups. C-Kit⁺ cells were isolated with a CD117 MicroBead Kit (Miltenyi) and an AutoMacs Pro and grown in SFEM (StemSpan) supplemented with antibiotics and 50 ng/mL mTPO (Peprotech) and 50 ng/mL mSCF (Peprotech) for 24 hours. Ecotropic pseudotyped retrovirus was spun onto Retronectin (Clontech) coated 6-well plates and cells were added in 1 mL of media with 2 µg/mL polybrene. An additional 1 mL of media was added after 24 hours. After 48 hours, GFP⁺ cells were isolated

by FACS sorting (BD FACS Aria II) and CD45.1⁺ and CD45.2⁺ cells were mixed. Cells were treated with various doses of lenalidomide and the percent CD45.1⁺ and CD45.2⁺ cells expressing the fluorescent marker was followed by flow cytometry over time following cell surface staining. Antibodies for flow cytometry were as follows: CD45.1 APC/Cy7 (A20, BioLegend), CD45.2 PE (104, eBioscience), and CD45.2 FITC (104, eBioscience). The number of mice needed for each experiment was calculated by assuming a yield of 5 million c-Kit⁺ cells per mouse (long bones and spine), a transduction rate of 40%, and a target of 50,000 GFP⁺ cells per experimental well, which was found to reduce variability in pilot experiments. Due to the experimental design, the genotypes of the mice could not be blinded or randomized.

For *in vivo* treatment, female C57BL/6 mice were treated with once-daily intraperitoneal injection of 10 mg/kg lenalidomide dissolved in DMSO and diluted in 100 μ l PBS or with DMSO in 100 μ l PBS. Bone marrow was harvested after 14 days and lysed with IP lysis buffer (Pierce). Due to experimental design, the treatment groups could not be blinded.

Quantitative Mass Spectrometry in MDS-L cells

MDS-L multiplexed quantitative mass spectrometry samples were processed and analyzed by the Thermo Fisher Scientific Center for Multiplexed Proteomics at Harvard Medical School. Samples were prepared as previously described [149] with the following modification. All solutions are reported as final concentrations. Lysis buffer (8 M Urea, 1% SDS, 50 mM Tris pH 8.5, Protease and Phosphatase inhibitors from Roche) was added to the cell pellets to achieve a cell lysate with a protein concentration between 2-8 mg/mL. A micro-BCA assay (Pierce) was used to determine the final protein concentration in the cell lysate. Proteins were reduced and alkylated as previously described. Proteins were precipitated using methanol/chloroform. In brief, four volumes of methanol were added to the cell lysate, followed by one volume of

chloroform, and finally three volumes of water. The mixture was vortexed and centrifuged to separate the chloroform phase from the aqueous phase. The precipitated protein was washed with one volume of ice-cold methanol. The washed precipitated protein was allowed to air dry. Precipitated protein was resuspended in 4 M Urea, 50 mM Tris pH 8.5. Proteins were first digested with LysC (1:50; enzyme:protein) for 12 hours at 25 °C. The LysC digestion is diluted down to 1 M Urea, 50 mM Tris pH8.5 and then digested with trypsin (1:100; enzyme:protein) for another 8 hours at 25 °C. Peptides were desalted using a C₁₈ solid phase extraction cartridges as previously described. Dried peptides were resuspended in 200 mM EPPS, pH 8.0. Peptide quantification was performed using the micro-BCA assay (Pierce). The same amount of peptide from each condition was labeled with tandem mass tag (TMT) reagent (1:3; peptide:TMT label) (Pierce). The 6-plex and 10-plex labeling reactions were performed for 2 hours at 25 °C. Modification of tyrosine residue with TMT was reversed by the addition of 5% hydroxyl amine for 15 minutes at 25 °C. The reaction was quenched with 0.5% TFA and samples were combined at a 1:1:1:1:1:1 ratio for 6-plex experiments or 1:1:1:1:1:1:1:1:1:1 for 10-plex experiments. Combined samples were desalted and offline fractionated into 24 fractions as previously described.

Liquid chromatography-MS3 spectrometry (LC-MS/MS) in MDS-L cells

12 of the 24 peptide fractions from the basic reverse phase step (every other fraction) were analyzed with an LC-MS3 data collection strategy on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) equipped with a Proxeon Easy nLC 1000 for online sample handling and peptide separations. Approximately 5 µg of peptide resuspended in 5% formic acid + 5% acetonitrile was loaded onto a 100 µm inner diameter fused-silica microcapillary with a needle tip pulled to an internal diameter less than 5 µm. The column was

packed in-house to a length of 35 cm with a C₁₈ reverse phase resin (GP118 resin 1.8 μ m, 120 Å, Sepax Technologies). The peptides were separated using a 120 min linear gradient from 3% to 25% buffer B (100% ACN + 0.125% formic acid) equilibrated with buffer A (3% ACN + 0.125% formic acid) at a flow rate of 600 nL/min across the column. The scan sequence for the Fusion Orbitrap began with an MS1 spectrum (Orbitrap analysis, resolution 120,000, 400–1400 m/z scan range, AGC target 2×10^5 , maximum injection time 100 ms, dynamic exclusion of 75 seconds). ‘Top speed’ (1 second) was selected for MS2 analysis, which consisted of CID (quadrupole isolation set at 0.5 Da and ion trap analysis, AGC 4×10^3 , NCE 35, maximum injection time 150 ms). The top ten precursors from each MS2 scan were selected for MS3 analysis (synchronous precursor selection), in which precursors were fragmented by HCD prior to Orbitrap analysis (NCE 55, max AGC 5×10^4 , maximum injection time 150 ms, isolation window 2.5 Da, resolution 60,000 (10-plex experiments) or 15,000 (6-plex experiments)).

LC-MS3 data analysis for MDS-L cells

A suite of in-house software tools were used for .RAW file processing and controlling peptide and protein level false discovery rates, assembling proteins from peptides, and protein quantification from peptides as previously described [149]. MS/MS spectra were searched against a UniProt human database (February 2014) with both the forward and reverse sequences. Database search criteria are as follows: tryptic with two missed cleavages, a precursor mass tolerance of 50 ppm, fragment ion mass tolerance of 1.0 Da, static alkylation of cysteine (57.02146 Da), static TMT labeling of lysine residues and N-termini of peptides (229.162932 Da), and variable oxidation of methionine (15.99491 Da). TMT reporter ion intensities were measured using a 0.03 Da window (6-plex) or 0.003 Da window (10-plex) around the theoretical m/z for each reporter ion in the MS3 scan. Peptide spectral matches with poor quality MS3

spectra were excluded from quantitation (<100 summed signal-to-noise across 6 channels and <0.5 precursor isolation specificity for 6-plexes or (<200 summed signal-to-noise across 10 channels and <0.5 precursor isolation specificity for 10-plexes).

A moderated *t*-test was applied across all proteins to assess statistical significance [146]. This statistic is similar to the ordinary *t*-statistic, with the exception that the standard errors are calculated using an empirical Bayes method utilizing information across all proteins, thereby making inference about each individual protein more robust. This test assumes normality and uses an estimated standard deviation intended to handle relatively few replicates per condition. Posterior residual standard deviations are used in place of ordinary standard deviation in the moderated *t*-test applied. This shrinkage of protein-wise sample variances to a pooled estimate provides more stable inference when sample numbers are reduced. The nominal *P* values arising from the moderated *t*-statistic are corrected for multiple testing by controlling the false discovery rate (FDR), as proposed by Benjamini and Hochberg [147]. Proteins with an FDR adjusted *P* value of less than 0.05 were deemed to be reproducibly regulated.

IMiD compound substrate selectivity in KG-1 and MDS-L cells

Cells ($2-4 \times 10^6$) were plated in 10cm dishes and incubated overnight (18-24 h). Cells were treated with DMSO, lenalidomide (1-10 μ M), CC-122 (1-10 μ M), pomalidomide (1-10 μ M), or thalidomide (10-100 μ M) for 6 hours. Drug- treated cells were collected, washed with PBS and cell pellets were lysed in RIPA buffer containing protease and phosphatase inhibitors for 30-45 minutes followed by sonication and centrifugation. Protein lysates were quantified using BCA protein assay kit and 10-15 μ g of protein was used for western analysis. CC-122 and lenalidomide competition experiment was conducted as above except cells were pre-treated with

DMSO or 10 μ M CC-122 for 90 minutes followed by treatment with lenalidomide (0.3-10 μ M) or DMSO for 6 hours.

The synthesis and characterization of CC-122, which was carried out by Celgene, is described in the Supplementary Methods section of the publication describing this work [82].

**Chapter 3 Development of a thalidomide-sensitive mouse model via a single amino acid
change in Cereblon**

Contributions

I designed and performed the experiments in this chapter including the characterization of known IMiD-target degradation, competitive transplants, peripheral blood analysis, hematopoietic stem and progenitor analysis, and fetal loss experiments except as noted below.

The *Crbn*^{I391V} mouse model was generated by Ingenious Targeting Labs, who also provided the Southern Blot design and data. James Kennedy and Andrew Nguyen generated the Hoxb8 cell lines. Andrew Guirguis performed the reporter Hoxb8 experiments. Proteomics were performed by the Carr group at the Broad Institute, including Namrata Udeshi, Tanya Svinkina, and D.R. Mani. Daniel Haldar performed the Western Blots for the Zfp91 and the T cell time course that are shown. Dylan Adams helped generate the c-Kit⁺ reporter data.

I had significant help in establishing, maintaining, breeding and organizing the *Crbn*^{I391V} mouse colony from Marie McConkey and Dylan Adams. Marie McConkey, Dylan Adams, Daniel Haldar, and Michelle Chen provided help with treating and harvesting mice, establishing timed matings, weighing mice, and other of the myriad of technical tasks that were required to complete these experiments.

Rebekka Schneider and Ronald Mathieu provided helpful guidance on the development of flow cytometry antibody panels. Brian Liddicoat provided guidance on recognizing different mouse embryonic stages. Quinn Sievers kindly provided reporter constructs for IKZF3, ZFP91, and their associated mutations.

Note: We are currently in the process of submitting this work for publication.

Summary

Thalidomide, once infamous for its teratogenic effects, and derivatives lenalidomide and pomalidomide have found new clinical utility as treatments for multiple myeloma and myelodysplastic syndrome with del(5q). Despite their widespread clinical use, studies of these drugs have been limited by their lack of effect in rodent models. Here, we report the development of a knock-in mouse which is sensitive to thalidomide derivatives. Thalidomide analogs bind to CRBN and recruit protein targets to the CRL4^{CRBN} E3 ubiquitin ligase, resulting in their degradation by the proteasome. Mice with a single amino acid change in *Crbn*, *Crbn*^{I391V}, show thalidomide-induced degradation of drug targets identified in human cells including *Ikzf1*, *Ikzf3*, zinc finger protein 91 (Zfp91), and *Ck1α*. Previous work suggested that haploinsufficient expression of *CK1α* could explain the efficacy of lenalidomide in del(5q) myelodysplastic syndrome. Using the *Crbn*^{I391V} model, we demonstrate that haploinsufficiency for *Ck1α* confers lenalidomide sensitivity *in vivo* and that both lenalidomide-induced selection and *Trp53*-mediated resistance occur at the level of hematopoietic stem cells. We also demonstrate that *Crbn*^{I391V} is sufficient to confer thalidomide-induced fetal loss in mice. Further study of the *Crbn*^{I391V} model will provide valuable insights into the *in vivo* efficacy and toxicity of this class of drugs.

Introduction

The use of thalidomide as a treatment for morning sickness in the 1950s resulted in the birth of more than 10,000 children with phocomelia and other characteristic malformations, as well as an untold but likely high number of miscarriages [1, 150]. Since that time IMiDs have demonstrated clinical efficacy in multiple myeloma [71-75], other B cell neoplasms including mantle cell lymphoma [57] and chronic lymphocytic leukemia [58, 59], and myelodysplastic syndrome (MDS) with del(5q) [53, 54, 107]. Despite our rapidly advancing understanding of the molecular mechanism of these drugs, *in vivo* studies of thalidomide derivatives have been limited by their lack of effect in rodent models [20, 21, 24, 26-28, 115]. In the case of thalidomide, this species difference may have prevented the recognition of teratogenicity, as pre-clinical studies in rodent models are reported to have been performed [4, 7, 24]. Predictive *in vivo* models are particularly important for understanding the clinical mechanism of thalidomide derivatives, as they are thought to have effects on the immune system, stroma-tissue interactions, and organismal development that are not well recapitulated *in vitro* [46, 151-153].

In Chapter 2, we found that *Ikzf1*, *Ikzf3*, and *Ckl1 α* are not degraded in mouse cells treated with thalidomide analogs. We showed that expression of human CRBN restored the thalidomide responsiveness of mouse cells, implicating differences in the amino acid sequence of CRBN in the lack of IMiD effects in mouse cells. Here, we report the development of a knock-in mouse model that is sensitive to thalidomide analogs.

Design considerations

In Chapter 2, we showed that retroviral overexpression of human CRBN or mouse *Crbn* with substitution of the isoleucine at position 391 with the corresponding human valine residue

(*Crbn*^{I391V}) results in a mouse cell that degrades Ikaros1, Ikaros3, and C/EBP α in response to thalidomide derivatives. This suggests several potential ways to make a lenalidomide-sensitive mouse. First, human or humanized *CRBN* could be introduced into the *Rosa26* or endogenous *Crbn* locus. Although *Rosa26* transgenic mice are generally faster and easier to make, they express the transgene ubiquitously, without endogenous patterns of regulation, and often at high levels [154, 155]. Conversely, it is more difficult to knock-in a gene at the endogenous locus, but it is more likely to be expressed at physiologic levels with tissue-specific expression and endogenous mechanisms of control. Since knock-in mice can be used as heterozygotes or homozygotes, they also provide a way to eliminate endogenous mouse *Crbn* that might compete with the transgenic *CRBN* for lenalidomide binding. Importantly, *Crbn* knock-out mice are viable and have no significant hematologic defects [124, 125], so expression of *Crbn* is not required for normal development. Additionally, *CRBN* is expressed widely in humans, with detectable expression in testis, spleen, prostate, liver, pancreas, placenta, kidney, lung, skeletal muscle, ovary, small intestine, peripheral blood leukocytes, colon and brain, so expression of *CRBN* in a variety of tissues will likely be fairly well tolerated [156].

However, in earlier experiments (data not shown), we observed selection against *CRBN* overexpression both *in vitro* and in bone marrow transplant models, raising concerns that similar negative selection might occur during embryonic development or in other organ systems when *CRBN* is expressed at high levels without endogenous regulatory mechanisms. Thus, we opted to create a knock-in at the endogenous mouse *Crbn* locus under the control of the endogenous promoter.

Either full-length human *CRBN* or the single point mutation mouse *Crbn*^{I391V} could be knocked-in to the endogenous *Crbn* locus to create a lenalidomide-sensitive mouse. Expression

of mouse *Crbn*^{I391V} recapitulates the effects of human CRBN on Ikaros and Cdk1α degradation in lenalidomide-treated mouse cell lines, as well as the relative depletion of *Csnk1a1*^{+/-} primary mouse bone marrow cells treated with lenalidomide. However, we do not understand all aspects of the mechanism of lenalidomide, most notably its teratogenic targets. The fact that Ikaros and Cdk1α degradation depend on the same species-difference in CRBN argues that I391V plays a crucial role in lenalidomide's central mechanism. However, this single point mutant change may not be sufficient to affect lenalidomide's other unknown targets. Conversely, full-length human *CRBN* may not be well tolerated by mouse cells due to novel targets or the disruption of endogenous interactions. Since mouse *Crbn*^{I391V} only differs by a single amino acid from endogenous mouse *Crbn*, it is less likely to significantly disrupt mouse cells or development. Additionally, it is technically easier to knock-in a single point mutant than a cDNA and the resulting transgene is more likely to express well. For these reasons, we decided to make a mouse *Crbn*^{I391V} knock-in mutant in order to study the effect of thalidomide analogs *in vivo*. Specifically, we sought to understand the effects of lenalidomide on hematopoiesis, to demonstrate that haploinsufficiency for Cdk1α can explain lenalidomide's therapeutic window *in vivo*, and to determine whether *Crbn*^{I391V} is sufficient to confer thalidomide-induced teratogenicity.

Results

Thalidomide derivatives induce degradation of substrates in Crbn^{I391V} knock-in mice

To enable the study of thalidomide derivatives *in vivo*, we generated a knock-in mouse model in which isoleucine was substituted with valine at position 391 in murine *Crbn* by homologous recombination (**Figure 3.1a**). Integration of the targeting vector was assessed by Southern Blot and PCR at the *Crbn* locus (**Figure 3.1b-d**). Pups homozygous for the I391V

mutation were born at the expected Mendelian frequency (**Figure 3.2a**). The I391V amino acid change was confirmed in adult tissues by DNA sequencing (**Figure 3.2b**) and did not impair the expression of *Crbn* in hematopoietic cells (**Figure 3.2c**).

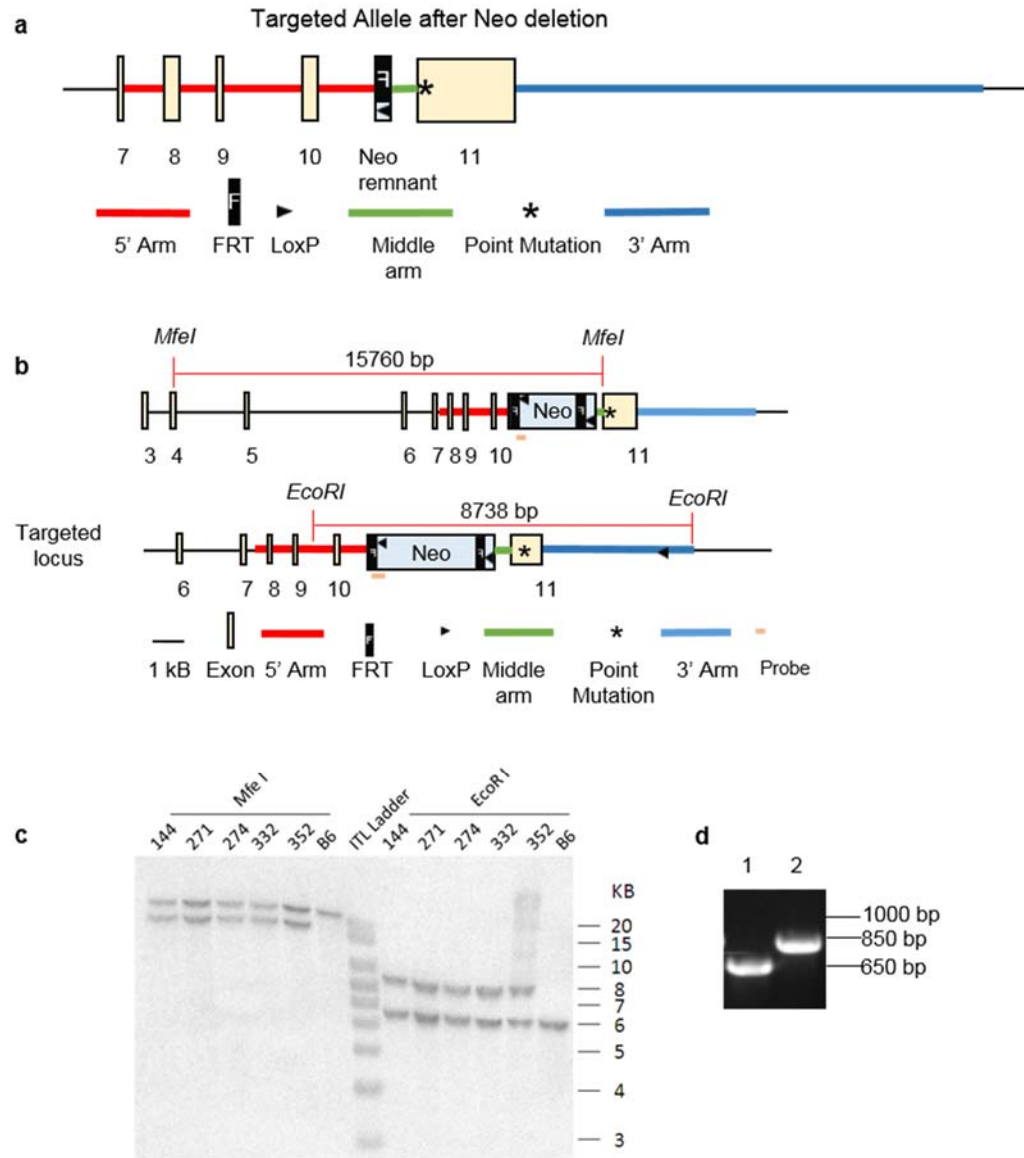


Figure 3.1: Design and generation of *Crbn*^{I391V} knock-in mice. **a**, Schema of *Crbn*^{I391V} allele after FLP-excision of the Neo cassette in the knock-in mice used in these studies. **b**, Design of Southern blot experiment showing the location of restriction enzyme sites and probe. **c**, Southern blot showing insertion of targeting cassette at *Crbn* locus in targeted ES cells. 144, 271, 274, 332, and 352 are successfully targeted mouse embryonic stem cell clones. B6 is wild-type control mouse embryonic stem cells. **d**, Results of genotyping PCR. Lane 1 is tissue from a wild-type mouse. Lane 2 is tissue from a *Crbn*^{I391V/I391V} mouse wild-type mouse.

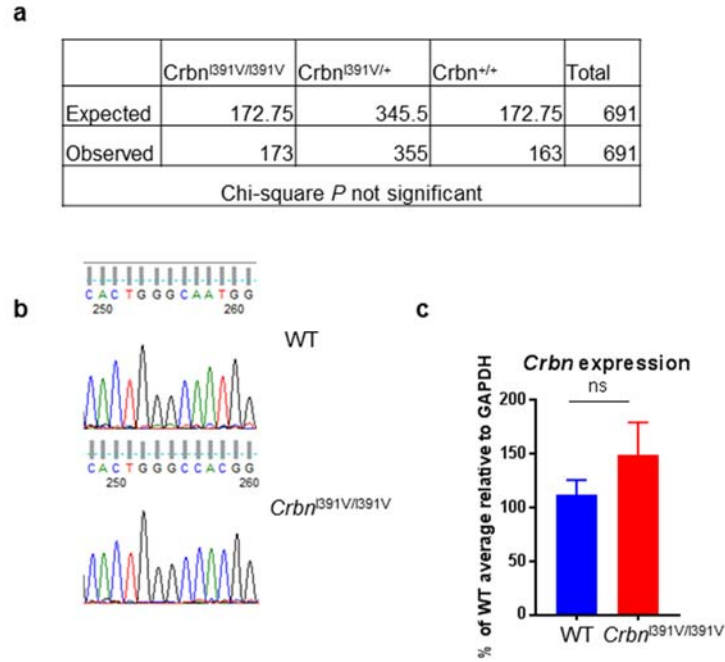


Figure 3.2: Genotype ratios and *Crbn* expression in *Crbn*^{I391V} mice. **a**, Genotype ratios in pups derived from *Crbn*^{I391V/+} x *Crbn*^{I391V/+} crosses do not show a statistically significant deviation from Mendelian ratios. **b**, Sanger sequencing results of *Crbn* from peripheral blood of wild-type or *Crbn*^{I391V/I391V} mice. **c**, Presence of the *Crbn*^{I391V} allele does not decrease expression of *Crbn* in T cells, as assessed by qPCR. ns is not significant.

We first assessed whether cells from *Crbn*^{I391V/I391V} mice degrade substrates when treated with thalidomide derivatives *in vitro*. We used tandem mass tag (TMT) mass spectrometry to assess changes in the global proteome of c-Kit⁺ hematopoietic stem and progenitor cells from knock-in and wild-type mice treated with 1 μ M lenalidomide. In *Crbn*^{I391V/I391V} cells, lenalidomide treatment resulted in decreased protein abundance of *Ikzf1* (Log₂ fold change=-0.7785, adjusted *P* value= 0.02 for isoform CRA_a and Log₂ fold change=-0.727 , adjusted *P* value=0.03 isoform CRA_b) and *Ck1 α* (Log₂ fold change = -1.044 , adjusted *P* value=0.02)) relative to vehicle-treated cells (**Figure 3.3, Figure 3.4a**). In contrast, no statically-significant

changes in protein abundance were seen following lenalidomide treatment of wild-type cells (**Figure 3.3**).

Since lenalidomide and pomalidomide differ in their substrate specificities (Chapter 2), we also evaluated the effect of 1 μ M pomalidomide treatment on *Crbn*^{I391V/I391V} c-Kit⁺ cells (**Figure 3.3c, Figure 3.4b**). We found that pomalidomide treatment decreased protein abundance of *Ikzf1* (Log₂ fold change= -1.3245, adjusted *P* value= 0.003 for isoform CRA_a and Log₂ fold change= -1.2715, adjusted *P* value=0.003 for CRA_b) relative to vehicle-treated cells. There was no change in the protein levels of *Ckl1 α* . Pomalidomide treatment also decreased protein levels of the zinc finger protein *Zfp91* (Log₂ fold change= -1.315, adjusted *P* value= 0.01), which was recently shown to be degraded by lenalidomide in human cells [157].

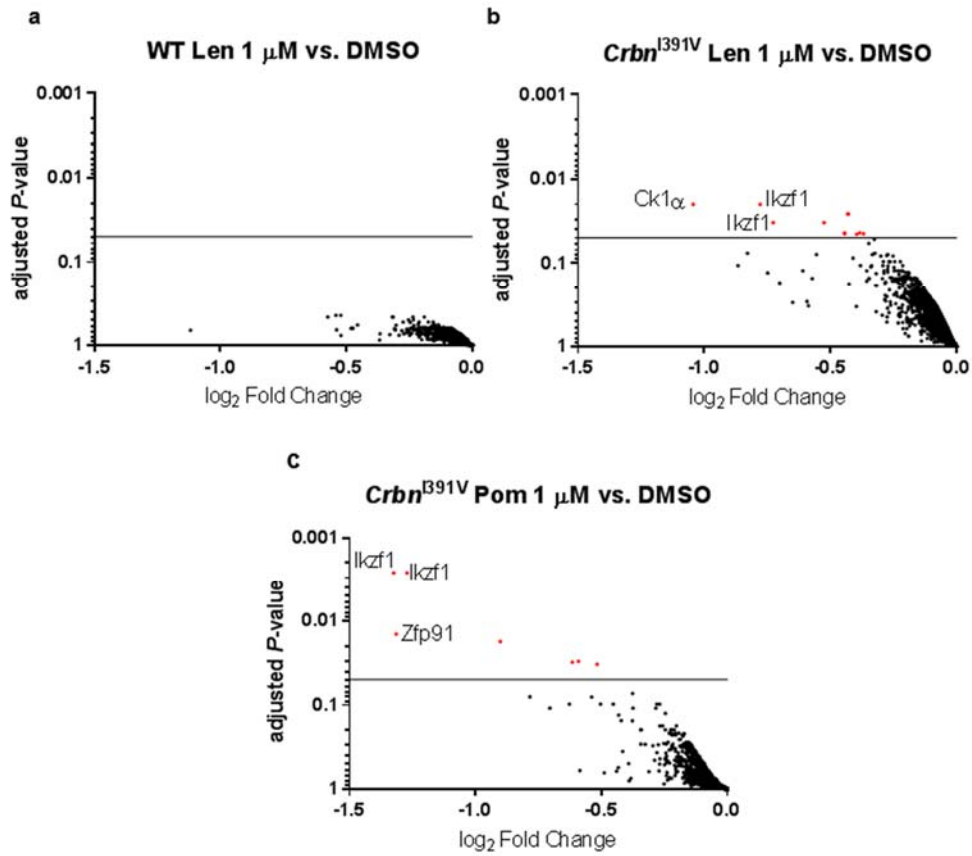


Figure 3.3: Global proteome profiling identifies downregulated proteins in wild-type and knock-in cells treated with thalidomide derivatives *in vitro*. c-Kit⁺ hematopoietic stem and progenitor cells were isolated from $Crbn^{I391V/I391V}$ mice or wild-type mice and treated *in vitro* with 1 μ M lenalidomide, 1 μ M pomalidomide, or DMSO vehicle for 12 hours. Protein abundance was assessed by tandem mass tag mass spectrometry and downregulated proteins are shown here. Statistical significance was calculated with a two-sample modified t -test and P values were adjusted for multiple comparisons according to Benjamini and Hochberg [147]. The horizontal line indicates an adjusted P value of 0.05 and proteins with a P value < 0.05 are colored red. Log₂ fold change is the average of two biological replicates.

a				b			
Protein	Average Log ₂ Fold Change	Adjusted P-value	Direction of change	Protein	Average Log ₂ Fold Change	Adjusted P-value	Direction of change
Csnk1a1	-1.04	0.019938	down	Ikzf1	-1.32	0.002607	down
Ikzf1	-0.78	0.019938	down	Zfp91	-1.32	0.014282	down
Ikzf1	-0.73	0.032828	down	Ikzf1	-1.27	0.002607	down
Ca1	-0.53	0.032828	down	Pde6d	-0.90	0.017569	down
Hemgn	-0.44	0.043492	down	Rnf166	-0.62	0.030850	down
Pklr	-0.44	0.044624	down	Ppil4	-0.59	0.030067	down
Ca2	-0.43	0.025918	down	Serinc5	-0.52	0.032607	down
Rnf166	-0.43	0.025918	down	Apoe	0.38	0.042404	up
Add2	-0.40	0.044997	down	Stard5	0.40	0.030850	up
Suca	-0.38	0.043492	down	Serpinh1	0.44	0.030067	up
Casp3	-0.37	0.044624	down	Aif1	0.46	0.040362	up
Comp	0.33	0.044997	up	Ankrd63	0.47	0.040362	up
Aif1	0.34	0.043492	up	Wdr65	0.48	0.030850	up
Tlr4	0.40	0.042160	up	Igfbp4	0.52	0.030067	up
Cep70	0.43	0.034730	up	Pepd	0.84	0.002607	up
Agbl1	0.45	0.033331	up	Ctss	0.89	0.002607	up
Plk4	0.45	0.032828	up	Scarf1	1.02	0.002607	up
Ctss	0.45	0.025918	up	Ctsk	1.08	0.003486	up
Ccdc136	0.52	0.043492	up	Mrc1	1.29	0.030850	up
Scarf1	0.58	0.019938	up	Gpx1	1.84	0.002607	up
Wdr35	0.64	0.019938	up				
Bnc1	0.65	0.022098	up				
Rev3l	0.72	0.019938	up				
Ttc39b	0.73	0.019938	up				
Mrc1	0.78	0.019938	up				
Lingo4	0.85	0.043492	up				

Figure 3.4: Proteins significantly altered by IMiD treatment in *Crbn*^{I391V/I391V} cells. These tables provide additional data from the tandem mass tag global proteome experiment shown in Figure 3.3. **a**, List of significantly regulated proteins in *Crbn*^{I391V/I391V} c-Kit⁺ cells treated with 1 μ M lenalidomide vs. DMSO. **b**, List of significantly regulated proteins in *Crbn*^{I391V/I391V} c-Kit⁺ cells treated with 1 μ M pomalidomide vs. DMSO. There were no significantly regulated proteins in wild-type c-Kit⁺ cells treated with 1 μ M lenalidomide. Average Log₂ fold change is of two biological replicates.

To validate these results, we established an immortalized myeloid progenitor cell line by expressing the transcription factor Hoxb8 in c-Kit⁺ cells from knock-in and wild-type mice and culturing the cells in stem cell factor [158]. Using flow cytometry, we measured the post-

translational stability of an IKZF3 degron reporter in which a 60 amino-acid region of IKZF3 is fused in frame with GFP (**Figure 3.5a**) [65, 126]. As shown in **Figure 3.5b**, treatment with low doses of lenalidomide, pomalidomide, and thalidomide induced degradation of the IKZF3 reporter in Hoxb8 cells from *Crbn*^{I391V/I391V} mice. In contrast, only minimal degradation of the IKZF3 reporter was observed in Hoxb8 cells from wild-type mice. Comparison of the dose-response curves from knock-in and wild-type Hoxb8 cells suggests that wild-type cells are approximately 100-fold less sensitive to treatment with various thalidomide derivatives. Additionally, degradation was not seen in knock-in Hoxb8 cells expressing a mutant version of the IKZF3 degron which is resistant to thalidomide derivatives (**Figure 3.5c**).

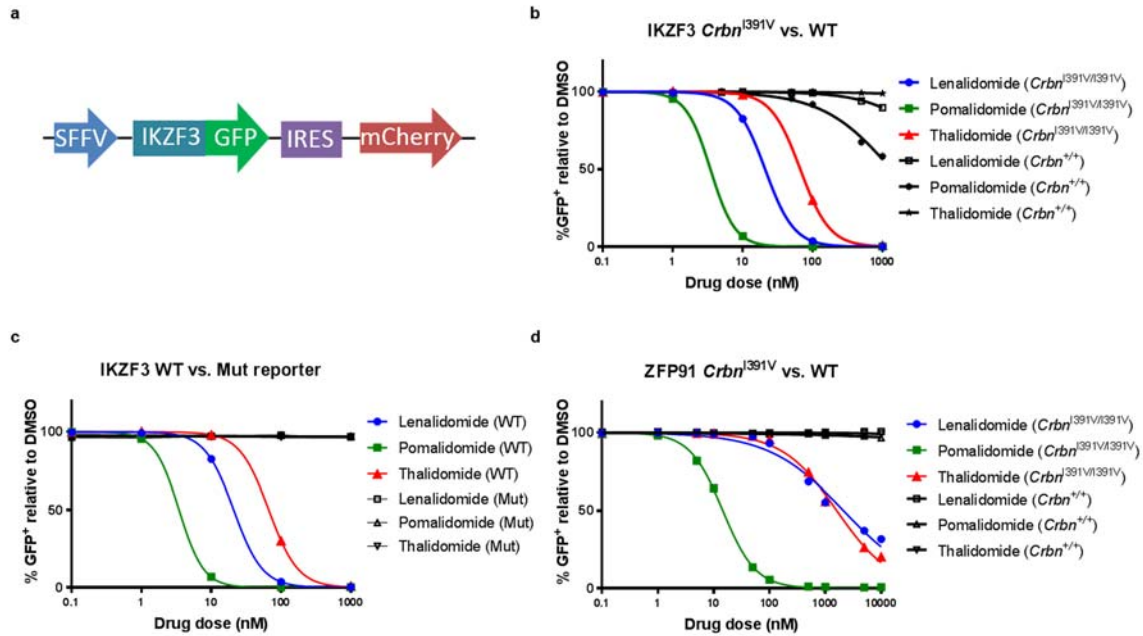


Figure 3.5: IKZF3 and ZFP91 reporter assays in Hoxb8 cells. Hoxb8 cells derived from wild-type or *Crbn*^{I391V/I391V} mice were infected with a fluorescent reporter (**a**) containing a 60 amino-acid region of IKZF3 or the fourth zinc finger of ZFP91 fused in-frame to GFP. This construct also contains a copy of *mCherry*, separated from the GFP-fusion protein by an internal ribosome entry sequence, as a transcriptional control. Comparison of the percentage of mCherry⁺ cells that are also GFP⁺ before and after treatment with IMiDs provides a measure of post-translational degradation. **b**, Post-translational degradation of the 60 amino-acid IKZF3 reporter in *Crbn*^{I391V/I391V} or wild-type Hoxb8-transformed cells treated with lenalidomide, thalidomide, or pomalidomide *in vitro* for 6 hours. **c**, Post-translational degradation of a wild-type or a thalidomide-resistant C148A mutant IKZF3 reporter in *Crbn*^{I391V/I391V} Hoxb8-transformed cells treated *in vitro* for 6 hours. **d**, Post-translational degradation of a ZFP91 flow reporter in knock-in or wild-type Hoxb8 cells treated *in vitro* for 12 hours.

We used a similar GFP fluorescent reporter containing a thalidomide-sensitive region of human ZFP91 to assess the post-translational stability of this protein in knock-in and wild-type Hoxb8 cells (**Figure 3.5d**). Treatment with pomalidomide, lenalidomide, and thalidomide induced degradation of a ZFP91 reporter in *Crbn*^{I391V/I391V} Hoxb8 cells but not wild-type Hoxb8 cells.

We next asked whether treatment with thalidomide derivatives induces the degradation of endogenously expressed proteins. We found that lenalidomide treatment resulted in degradation of endogenously expressed Ikzf3 and Ck1 α in cultured, c-Kit⁺ bone marrow cells and splenic T cells from *Crbn*^{I391V/I391V} mice (**Figure 3.6**). No degradation of these proteins was observed with high dose treatment of wild-type mouse cells, while *Crbn*^{I391V/+} cells had an intermediate phenotype.

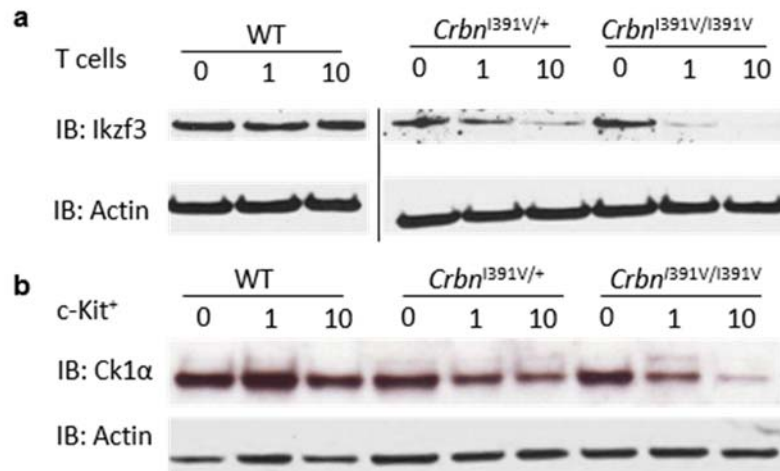


Figure 3.6: Degradation of IKZF3 and CK1 α in T and c-Kit cells from *Crbn*^{I391V} mice treated *in vitro*. Degradation of Ikzf3 and Ck1 α as assessed by western blot in T cells (a) and c-Kit⁺ cells (b) from *Crbn*^{I391V/I391V}, *Crbn*^{I391V/+}, or wild-type mice after 18-hour *in vitro* treatment with the drug indicated. All samples on the T cell blot were run on adjacent lanes on the same gel, but the order of lanes has been changed for clarity.

Similar results were observed with *in vivo* administration of thalidomide analogs (**Figure 3.7**). Single dose treatment with thalidomide or lenalidomide decreased protein levels of Ikzf1, Ck1 α , and Zfp91 in T cells from *Crbn*^{I391V/I391V} mice. Maximum degradation of these proteins was seen approximately 6 hours after treatment with 50 mg/kg lenalidomide, with recovery of baseline protein levels of Ikzf1, Ck1 α , and Zfp91 by 24 hours (**Figure 3.7a,b**). While higher and more frequent doses of thalidomide derivatives are required to achieve a similar effect in mice as in humans, this is consistent with the higher rate of thalidomide metabolism in mice [120, 159].

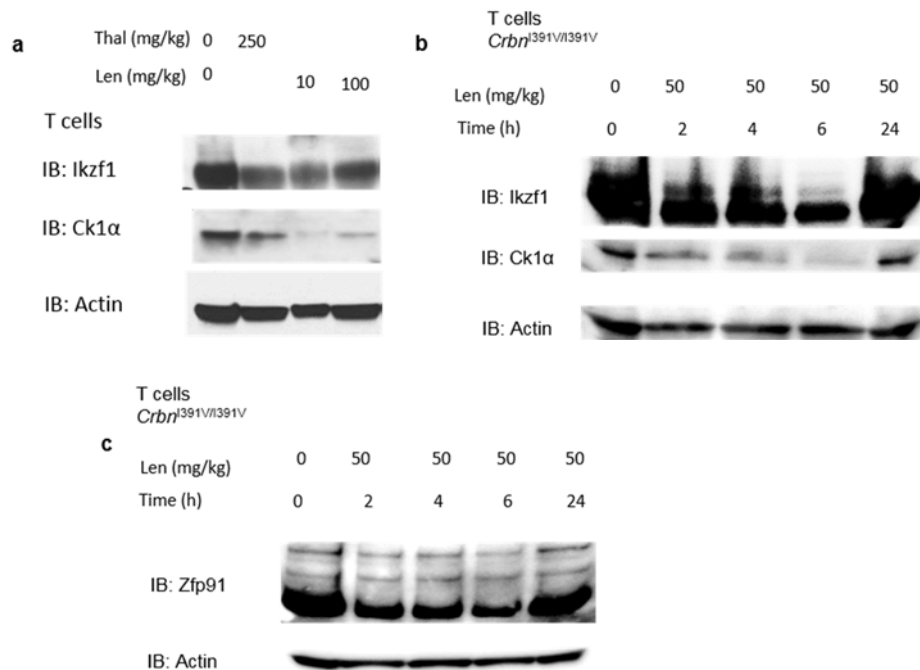


Figure 3.7: Effects of *in vivo* IMiD treatment on cells from *Crbn*^{I391V/I391V} mice. **a**, *In vivo* treatment of *Crbn*^{I391V/I391V} mice with lenalidomide or thalidomide results in degradation of Ikzf1 and Ck1 α in T cells after 12 hours. **b**, Western Blot time course of effects of *in vivo* lenalidomide treatment in *Crbn*^{I391V/I391V} mice shows maximum degradation of Ikzf1 and Ck1 α in T cells at 6 hours, with recovery of baseline protein levels by 24 hours. **c**, Zfp91 protein levels are reduced in T cells from *Crbn*^{I391V/I391V} mice treated with lenalidomide with similar kinetics.

Thalidomide derivatives have immunomodulatory effects which are observed in humans but not mice, including increased production of the cytokine IL-2 by T cells [160], which contributes to lenalidomide's efficacy in leprosy [161]. IKZF3 is a transcriptional repressor of the *IL2* gene, and degradation of IKZF3 increases *IL2* mRNA and protein [162]. In *Crbn*^{I391V} T cells cultured *in vitro* and treated with lenalidomide or pomalidomide, we observed a genotype-dependent increase in the transcription and secretion of IL-2 (**Figure 3.8**). This confirms the downstream functional effects of thalidomide target degradation in *Crbn*^{I391V} mice and supports the use of this model to further study the *in vivo* immunomodulatory effects of thalidomide derivatives. In aggregate, these findings demonstrate that thalidomide analog induced degradation of Ikzf1, Ikzf3, Ck1 α , and Zfp91 can be restored with a single isoleucine to valine change in mouse Crbn.

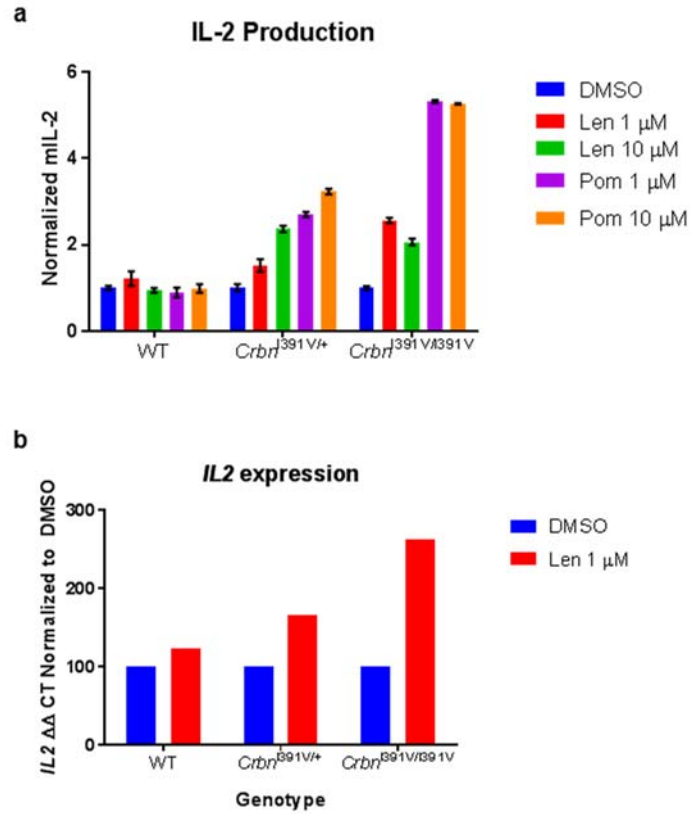


Figure 3.8: Treatment with lenalidomide or pomalidomide increases IL-2 production by *Crbn*^{I391V} T cells. **a**, T cells from *Crbn*^{I391V/I391V} and *Crbn*^{I391V/+} mice have increased production of IL-2 by ELISA when treated with lenalidomide (Len) or pomalidomide (Pom) for 24 hours. For wild-type cells, P value >0.05 for all comparisons with DMSO. For *Crbn*^{I391V/+} and *Crbn*^{I391V/I391V}, all comparisons with DMSO have P value <0.01 . **b**, T cells from *Crbn*^{I391V/I391V} and *Crbn*^{I391V/+} mice have increased expression of *IL2* by qPCR after 24-hour treatment with 1 μ M lenalidomide. *IL2* expression for the vehicle treated samples in each genotype is normalized to 100%.

Effect of lenalidomide treatment on hematopoiesis

Much of the clinical efficacy and toxicity of thalidomide analogs is due to effects on hematopoietic cells that reside and differentiate within a complex microenvironment *in vivo*. We therefore examined the effects of thalidomide derivatives on *in vivo* hematopoiesis. We focused further work on lenalidomide given its widespread clinical use in both multiple myeloma and del(5q) MDS. Administration of lenalidomide for 21 days resulted in a decline in total white blood cell count in *Crbn*^{I391V} mice, but not wild-type mice, without a significant change in red blood cell number (**Figure 3.9**). The leukopenia was driven primarily by a decrease in the number of B220⁺ B cells, but there was also a statistically significant decrease in the number of CD3⁺ T cells and CD11b⁺ Gr1⁺ granulocytes. These findings are consistent with the role of IKZF1 and IKZF3 in lymphocyte development [163-165]. We also observed a statistically significant decrease in platelet count of knock-in mice treated with lenalidomide, without a significant change in the platelet counts of wild-type mice. This finding is consistent with the thrombocytopenia commonly seen in patients treated with thalidomide derivatives, which is often a dose-limiting toxicity [53, 54, 73, 166, 167].

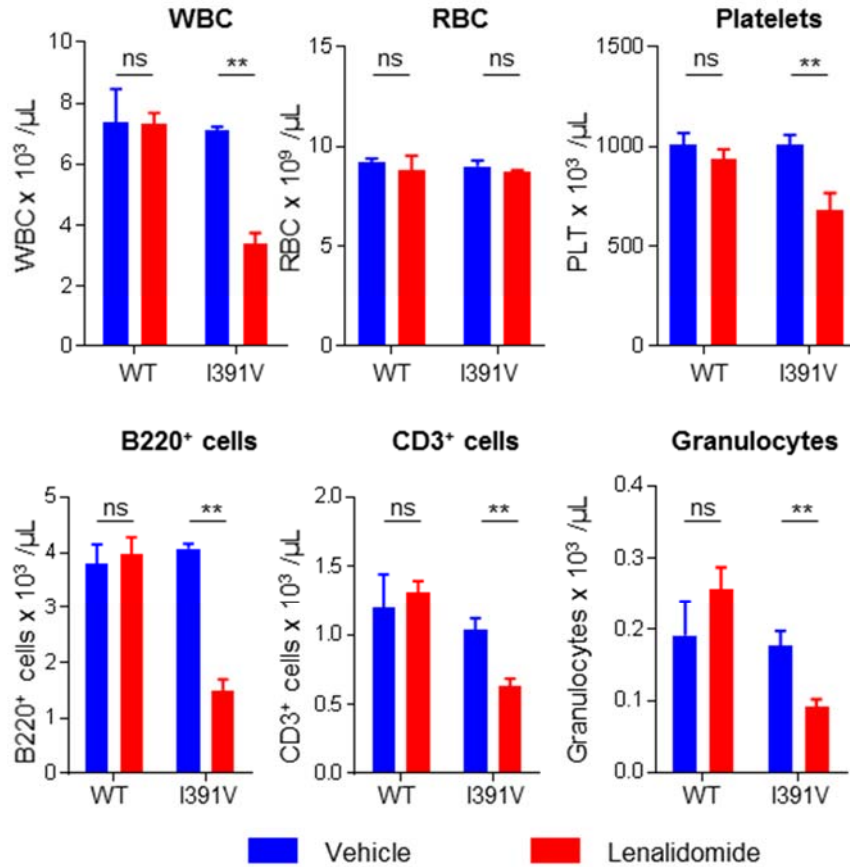


Figure 3.9: Effect of lenalidomide treatment on peripheral blood counts. Peripheral blood counts of wild-type and *Crbn*^{I391V/I391V} mice after 21 days of treatment with 50 mg/kg lenalidomide or vehicle. CD11b⁺ Gr1⁺ granulocyte and platelet data are pooled from two cohorts of mice treated for 21-24 days. ** $P < 0.005$. P values calculated using unpaired Students t -test. ns is not significant.

We next examined the effects of lenalidomide on normal hematopoietic stem and progenitor cells, which has been difficult to examine in patients with hematologic malignancies due to the presence of neoplastic hematopoiesis as well as therapy-induced alterations of the bone marrow microenvironment. Lenalidomide treatment resulted in a decrease in the proportion of Lineage⁻ c-Kit⁺ (LK) and Lineage⁻ c-Kit⁺ Sca1⁺ (LSK) cells as well as phenotypically-defined

Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁻ CD150⁺ long-term hematopoietic stem cells (LT-HSC) and Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁻ CD150⁻ short-term hematopoietic stem cell (ST-HSC) in the bone marrow (**Figure 3.10, Figure 3.11**).

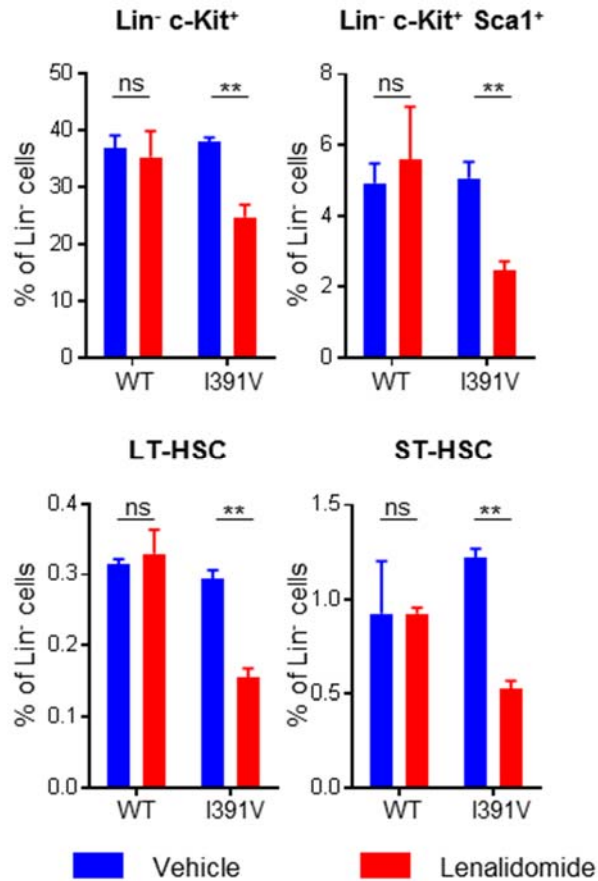


Figure 3.10: Effect of lenalidomide treatment on hematopoietic stem and progenitor cells.

Hematopoietic stem and progenitor cell compartments in wild-type and *Crbn*^{I391V/I391V} mice after 21 days of treatment with 50 mg/kg lenalidomide or vehicle. ST-HSC are Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁻ CD150⁻. LT-HSC are Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁻ CD150⁺. Lineage markers are CD3, B220, CD11b, Gr1, and Ter119. ** indicates *P* value < 0.005. ns is not significant. *P* values calculated using unpaired Students *t*-test.

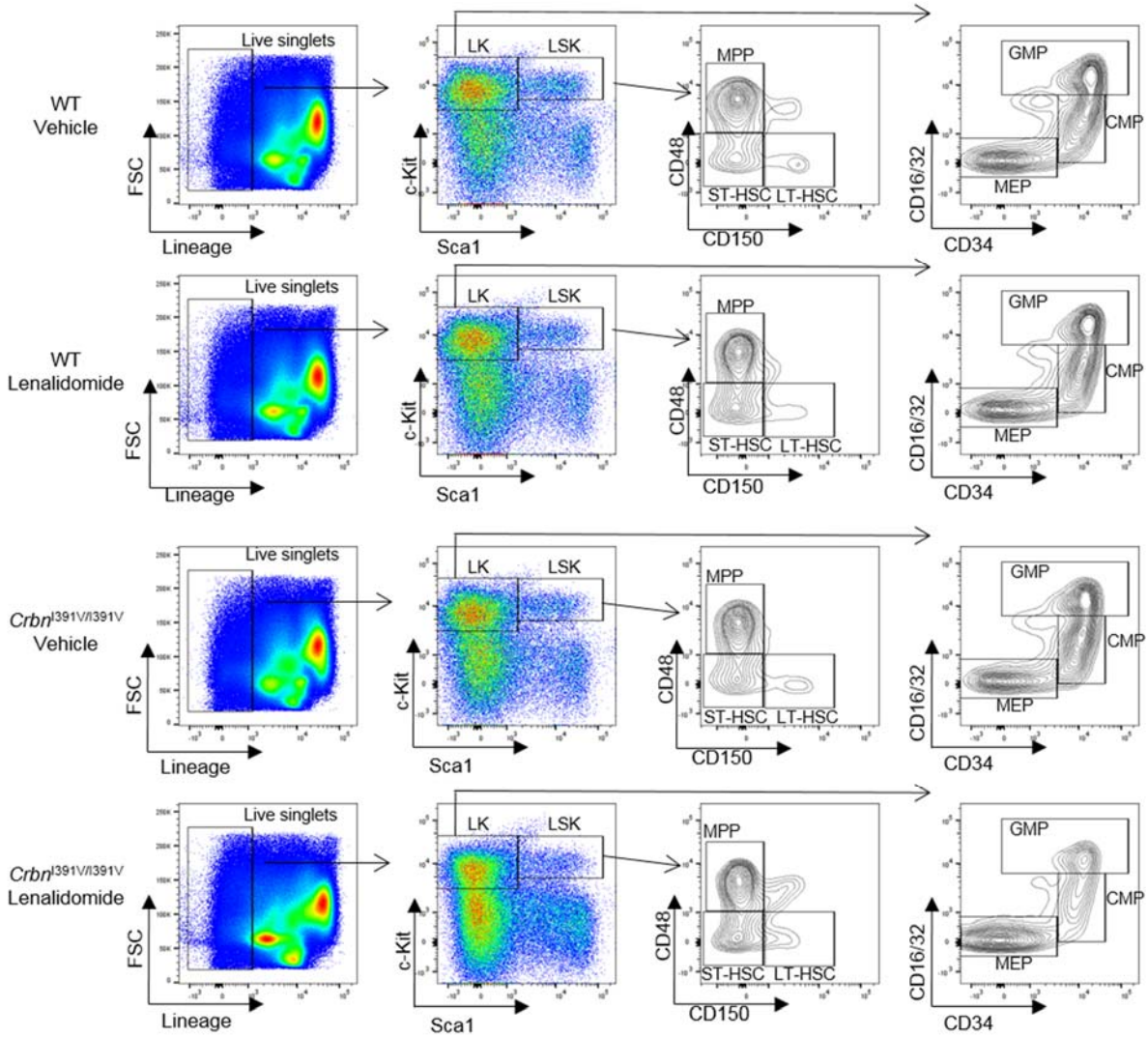


Figure 3.11: Flow cytometry gating strategy for hematopoietic stem and progenitor compartments. Representative flow plot showing effect of lenalidomide on hematopoietic stem and progenitor cells in *Crbn*^{I391V/I391V} mice. LK are Lineage⁻ c-Kit⁺ Sca1⁻. LSK are Lineage⁻ c-Kit⁺ Sca1⁺. ST-HSC are Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁻ CD150⁻. LT-HSC are Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁻ CD150⁺. MPP are Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁺ CD150⁻. CMP are Lineage⁻ c-Kit⁺ Sca1⁻ CD34⁺ CD16/32^{mid}. GMP are Lineage⁻ c-Kit⁺ Sca1⁻ CD34⁺ CD16/32^{hi}. MEP are Lineage⁻ c-Kit⁺ Sca1⁻ CD34⁺ CD16/32^{low}. Lineage markers are CD3, B220, CD11b, Gr1, and Ter119.

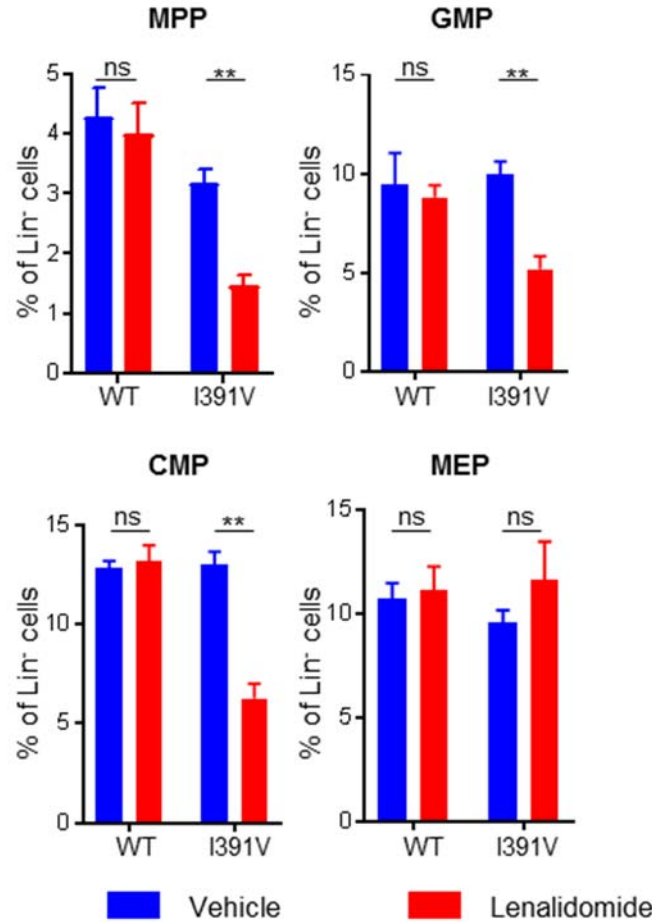


Figure 3.12: Effect of lenalidomide on hematopoietic progenitor compartments in *Crbn*^{I391V} mice. Hematopoietic progenitor compartments in wild-type and *Crbn*^{I391V/I391V} mice after 28 or 35 days of treatment with 50 mg/kg lenalidomide or vehicle. MPP are Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁺ CD150⁻. CMP are Lineage⁻ c-Kit⁺ Sca1⁻ CD34⁺ CD16/32^{mid}. GMP are Lineage⁻ c-Kit⁺ Sca1⁻ CD34⁺ CD16/32^{hi}. MEP are Lineage⁻ c-Kit⁺ Sca1⁻ CD34⁺ CD16/32^{low}. Lineage markers are CD3, B220, CD11b, Ter119, and Gr1. Data are combined from two independent experiments. ** $P < 0.005$. P values calculated using unpaired Students t -test. ns is not significant.

There was also a decrease in the proportion of multipotent progenitors (MPP; Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁺ CD150⁻), common myeloid progenitors (CMP; Lineage⁻ c-Kit⁺ Sca1⁻ CD34⁺

CD16/32^{intermediate}), and granulocyte-macrophage progenitors (GMP; Lineage⁻ c-Kit⁺ Sca1⁻ CD34⁺ CD16/32^{hi}) in knock-in mice treated with lenalidomide, but not wild-type mice (**Figure 3.11, Figure 3.12**). This finding indicates that long-term, high dose treatment with lenalidomide will impair the hematopoietic stem and progenitor cell compartment, consistent with the known myeloablative effects of high dose lenalidomide [53, 54, 73, 166, 167].

In contrast to its effects on other hematopoietic stem and progenitor cells, lenalidomide treatment did not significantly alter the percentage of megakaryocyte-erythroid progenitors (MEP; Lineage⁻ c-Kit⁺ Sca1⁻ CD34⁺ CD16/32^{low}, **Figure 3.12**) in the bone marrow of *Crbn*^{I391V/I391V} mice. Since the HSC, MPP, and CMP that give rise to the MEP lineage are all decreased in lenalidomide-treated *Crbn*^{I391V/I391V} mice, this suggests that lenalidomide treatment enhances the development of megakaryocyte-erythroid lineage, which is consistent with the normal RBC counts seen in these mice. We also found that there was an expansion of the erythoblast II erythroid progenitor (Ter119⁺ CD71^{intermediate}, see **Figure 3.13**) compartment in the bone marrow of lenalidomide-treated *Crbn*^{I391V/I391V} mice, but not wild-type mice. While the effects of lenalidomide or related analogs on normal erythropoiesis have not been examined previously *in vivo*, these effects are consistent with studies of lenalidomide on human erythroid progenitor cells cultured *in vitro* [168, 169].

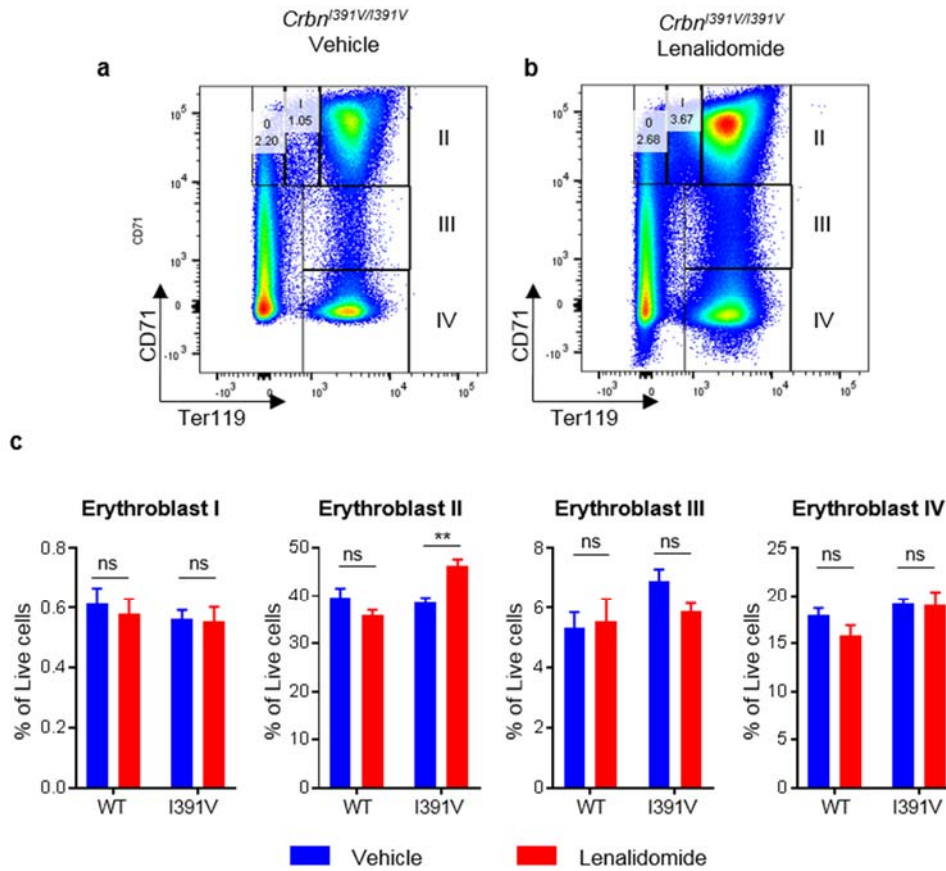


Figure 3.13: Effect of lenalidomide treatment on erythroid differentiation in *Crbn*^{I391V} mice.

Erythroid differentiation subsets were identified by staining for CD71 and TER119 in the bone marrow of wild-type and *Crbn*^{I391V/I391V} mice after 35 days of treatment with vehicle or 50 mg/kg lenalidomide. Flow plots with labeled erythroid differentiation subsets are shown for *Crbn*^{I391V/I391V} mice treated with vehicle (a) or lenalidomide (b). c, Quantitation of erythroid differentiation subsets in all treated mice. ** indicates $P < 0.005$, P values calculated using unpaired Students t -test. ns is not significant.

In vivo modeling of efficacy of lenalidomide in cells haploinsufficient for *Csnk1a1*

In Chapter 2, we found that lenalidomide induces ubiquitination of CK1 α by CRL4^{CRBN}, ultimately resulting in its degradation by the proteasome. Since CK1 α is encoded within the

del(5q) common deleted region [97] and is expressed at haploinsufficient levels in del(5q) MDS cells [99], this degradation could provide a mechanistic basis for lenalidomide's therapeutic window in del(5q) MDS. In Chapter 2, we used retroviral overexpression of *Crbn*^{I391V} to demonstrate that *Csnk1a1*^{+/-} cells are more sensitive to *in vitro* lenalidomide treatment than wild-type cells and preferentially undergo apoptosis through activation of Tp53. Here, we sought to use the *Crbn*^{I391V} model to demonstrate that haploinsufficiency for *Csnk1a1* is sufficient to explain lenalidomide's therapeutic window *in vivo*.

We first examined whether physiologic expression of *Crbn*^{I391V} is sufficient to confer lenalidomide sensitivity to *Csnk1a1*^{+/-} cells *in vitro*. We bred *Crbn*^{I391V} mice to *Csnk1a1*^{+/-} *MxCre*⁺ mice, in which exon 5 of *Csnk1a1* is floxed and Cre recombinase can be induced in hematopoietic cells [130]. We harvested c-Kit⁺ cells from these mice for an *in vitro* competition experiment (**Figure 3.14**). After inducing excision of the floxed *Csnk1a1* allele, we saw no effect of *in vitro* lenalidomide treatment on *Crbn*^{+/+} cells (**Figure 3.14**). In contrast, *Crbn*^{I391V} *Csnk1a1*^{+/-} *MxCre*⁺ cells were significantly more sensitive to lenalidomide than *Crbn*^{I391V} *MxCre*⁺ cells and were depleted from the culture over time (**Figure 3.14**). Their depletion correlated with increased expression of the Tp53 transcriptional target *p21* and an increased percentage of Annexin V⁺ cells, consistent with apoptotic cell death (**Figure 3.15**). This effect was seen both in *Crbn*^{I391V/I391V} and *Crbn*^{I391V/+} cells, confirming that heterozygous expression of *Crbn*^{I391V} is sufficient to confer response.

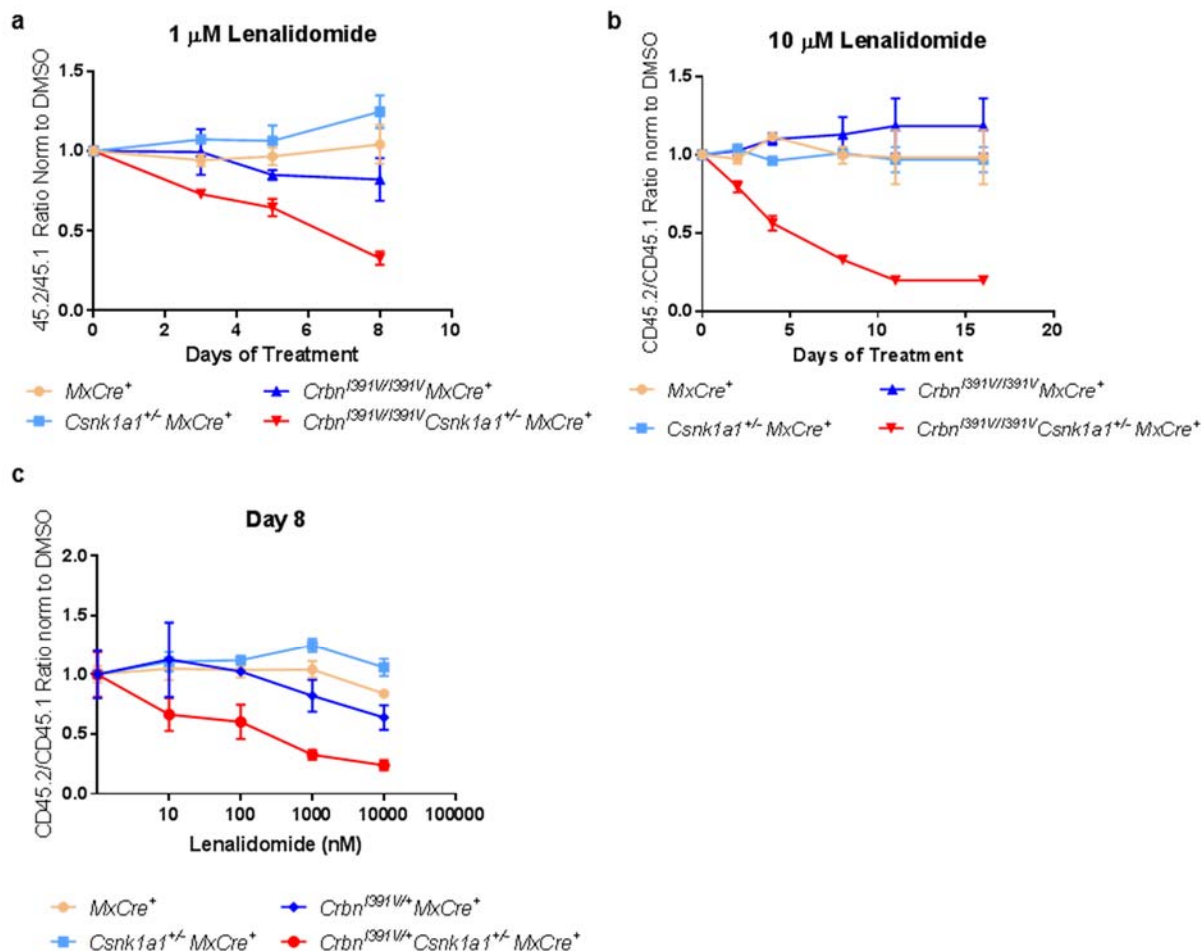


Figure 3.14: $Crbn^{I391V}$ cells heterozygous for $Csnk1a1$ are more sensitive to lenalidomide *in vitro*. An *in vitro* competition experiment was performed by isolating CD45.2⁺ c-Kit⁺ cells of the listed genotypes, mixing them in a 1:1 ratio with CD45.1⁺ c-Kit⁺ cells of the same $Crbn$ genotype, treating the cells with vehicle, 1 μ M lenalidomide (**a**) or 10 μ M lenalidomide (**b**), and following the percentage of CD45.1⁺ and CD45.2⁺ cells in the cultures over time by flow cytometry. The data are given as the ratio of the percentage of CD45.2⁺ cells to the percentage of CD45.1⁺ cells at each time point, normalized to the value of the DMSO treated sample at that time point. Part **c** shows the results of different doses of lenalidomide on the relative proportion of CD45.1⁺ and CD45.2⁺ cells in the cultures after 8 days of treatment.

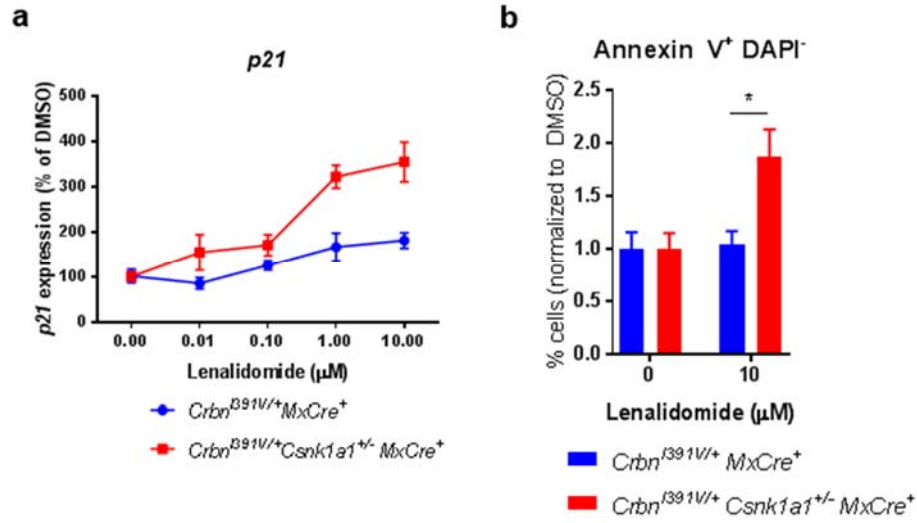


Figure 3.15: Lenalidomide induces *p21* and apoptotic cell death in *Crbn*^{I391V} *Csnk1a1*^{+/-} cells. **a**, Lenalidomide treatment induces expression of *p21* in *Crbn*^{I391V/+} *Csnk1a1*^{+/-} *Mx1Cre*⁺ c-Kit⁺ cells treated for 26 hours. **b**, Lenalidomide treatment increases the proportion of *Crbn*^{I391V/+} *Csnk1a1*^{+/-} *Mx1Cre*⁺ c-Kit⁺ cells in early apoptosis (Annexin V⁺ DAPI⁻) after three days of treatment. Data are normalized to the percentage of cells for each genotype which are Annexin V⁺ DAPI⁻ when treated with DMSO.

To evaluate the selective effect of lenalidomide on *Csnk1a1* haploinsufficient hematopoietic cells *in vivo*, we performed a competitive transplantation assay (**Figure 3.16**). We mixed CD45.2⁺ *Crbn*^{I391V/I391V} cells of various genotypes with CD45.1⁺/CD45.2⁺ *Crbn*^{I391V/I391V} competitor cells and transplanted a 1:1 mixture into lethally-irradiated CD45.1⁺ mice. After an engraftment period, we induced excision of the heterozygous floxed *Csnk1a1* allele and randomized the mice to treatment with lenalidomide or vehicle.

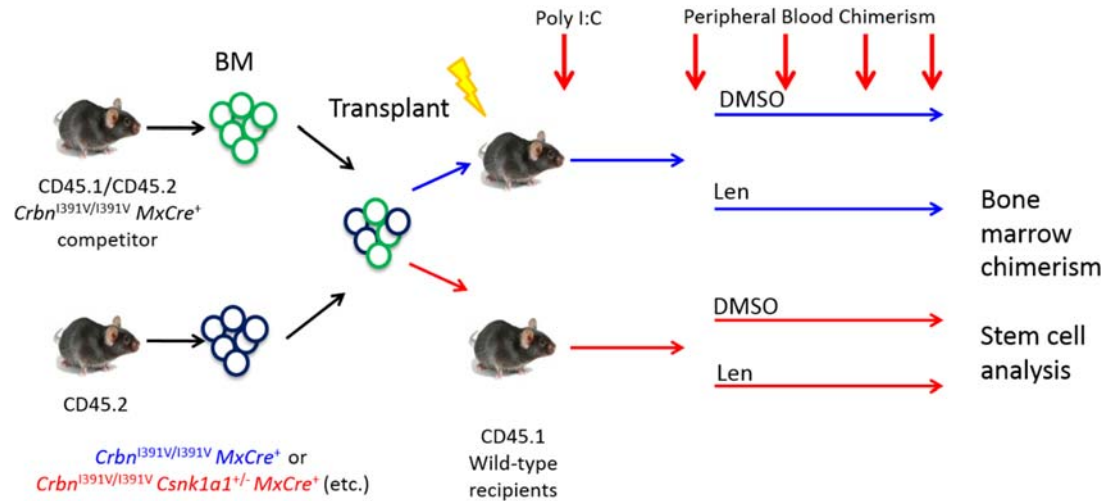


Figure 3.16: Design of *in vivo* transplant experiment.

In the peripheral blood we observed a rapid and sustained depletion of *Csnk1a1*^{+/-} cells, relative to control *Csnk1a1*^{+/+} cells, in mice receiving lenalidomide, but not in those treated with vehicle control (**Figure 3.17**). This enhanced lenalidomide sensitivity of *Csnk1a1*^{+/-} cells was apparent in all hematopoietic stem and progenitor subsets, including LT-HSC and ST-HSC (**Figure 3.18**). This provides the first evidence of therapeutic activity of lenalidomide in hematopoietic stem cells.

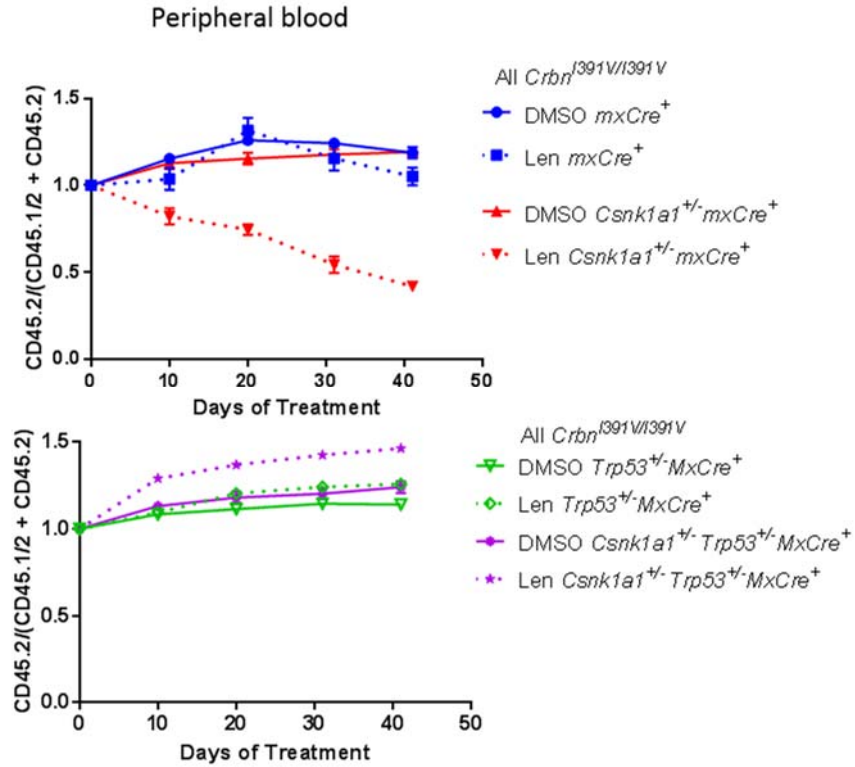


Figure 3.17: *In vivo* lenalidomide treatment results in the depletion of *Csnk1a1*^{+/-} cells but is rescued by deletion of a single allele of *Trp53*. Peripheral blood chimerism over time in transplanted mice treated with vehicle or 50 mg/kg lenalidomide twice per day. Data are given as a ratio of the percentage of CD45.2⁺ cells (test genotype) in the peripheral blood relative to the sum of the percentage of CD45.1⁺/CD45.2⁺ (competitor) and CD45.2⁺ (test genotype) cells. This method of data analysis excludes any CD45.1⁺ cells arising from residual recipient-derived hematopoiesis.

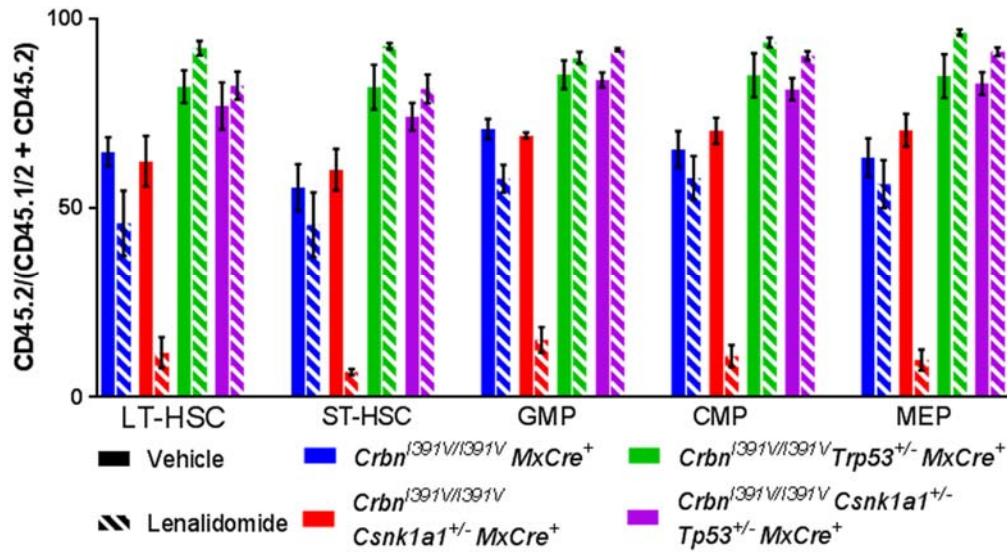


Figure 3.18: Analysis of hematopoietic stem and progenitor cells in *in vivo* competition

experiments. Chimerism in hematopoietic stem and progenitor compartments of transplanted mice after 41 days of treatment with vehicle or 50 mg/kg lenalidomide twice per day. Chimerism is reported as in Figure 3.15. ST-HSC are Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁻ CD150⁻. LT-HSC are Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁻ CD150⁺. Lineage markers are CD3, B220, CD11b, Gr1 and Ter119. GMP are Lineage⁻ c-Kit⁺ Sca-1⁻ CD34⁺ CD16/32⁺, CMP are Lineage⁻ c-Kit⁺ Sca-1⁻ CD34⁺ CD16/32⁻, MEP are Lineage⁻ c-Kit⁺ Sca-1⁻ CD34⁻ CD16/32⁻. Lineage markers are CD3, B220, CD11b, Gr1 and Ter119.

Heterozygous deletion of *Trp53* was sufficient to confer resistance to lenalidomide sensitivity in *Csnk1a1*^{+/-} cells (**Figure 3.17**). This resistance was seen in all hematopoietic stem and progenitor subsets, including LT-HSC and ST-HSC (**Figure 3.18**) and is consistent with the outgrowth of *TP53* mutant cells seen in del(5q) patients who develop lenalidomide resistance [108, 170, 171]. These results demonstrate that *Tp53* plays a key downstream role in the *in vivo* lenalidomide sensitivity of *Csnk1a1*^{+/-} cells.

To further investigate the role of Tp53 levels in lenalidomide sensitivity, we asked whether haploinsufficiency for other del(5q) genes could contribute to lenalidomide response. The ribosomal protein *Rps14* is also encoded with the del(5q) common deleted region and was recently implicated in a p53-dependent erythroid differentiation defect in MDS. Hematopoietic cells heterozygous for *Rps14* have increased baseline levels of Tp53 [172]. To test whether *Csnk1a1*^{+/-} *Rps14*^{+/-} cells are more sensitive to lenalidomide than *Csnk1a1*^{+/-} cells, we bred *Crbn*^{I391V} *Csnk1a1*^{+/-} *MxCre*⁺ mice with *Rps14*^{fl/+} mice in which exons 2-4 of *Rps14* are floxed [172]. As shown in **Figure 3.19a, b**, we observed a minor but statistically significant increase in the lenalidomide sensitivity of *Crbn*^{I391V/I391V} *Csnk1a1*^{+/-} *Rps14*^{+/-} cells relative to *Crbn*^{I391V/I391V} *Csnk1a1*^{+/-} cells treated *in vitro*. No increased lenalidomide sensitivity was seen in *Crbn*^{I391V/I391V} *Rps14*^{+/-} cells relative to *Crbn*^{I391V/I391V} cells.

In contrast to these *in vitro* results, we did not observe a significant difference between *Csnk1a1*^{+/-} *Rps14*^{+/-} cells and *Csnk1a1*^{+/-} cells in an *in vivo* competition experiment (**Figure 3.19c**). The discrepancy between *in vitro* and *in vivo* results could arise from differences in the doses used or the stage of hematopoiesis at which lenalidomide and haploinsufficiency for *Csnk1a1* and *Rps14* exert their effects.

Our results provide the first demonstration that haploinsufficiency for CK1α is sufficient to explain the selective elimination of del(5q) cells in response to lenalidomide *in vivo*. They confirm the essential role of Tp53 as a downstream effector in this mechanism and driver of *in vivo* resistance at the stem cell level. Thus, casein kinase is a unique, mechanistically-based determinant of *in vivo* lenalidomide sensitivity.

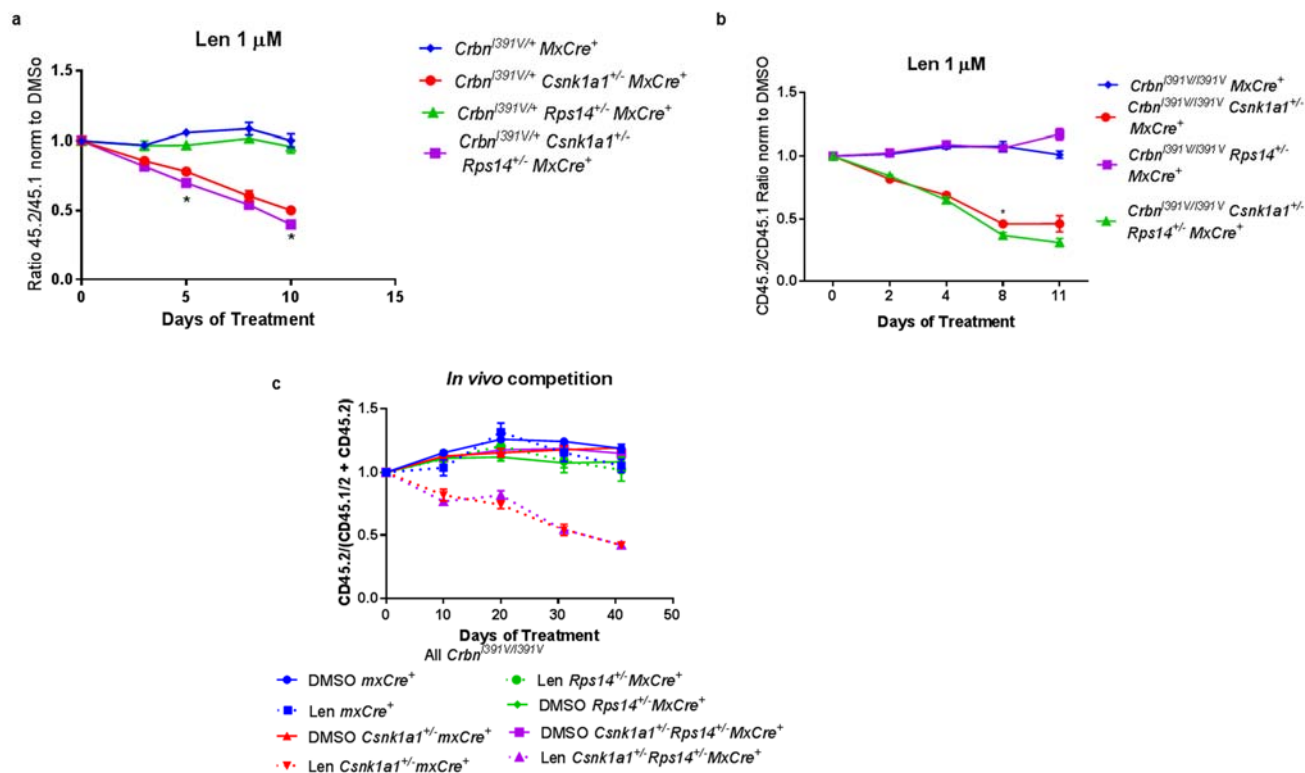


Figure 3.19: Haploinsufficiency for *Rps14* contributes to lenalidomide sensitivity *in vitro* but not *in vivo*. **a**, Heterozygous deletion of *Rps14* further enhances the lenalidomide sensitivity of $Crbn^{I391V/+} Csnk1a1^{+/-} MxCre^{+}$ cells *in vitro* but does not affect lenalidomide sensitivity as a single mutation. **b**, Similar results are seen in $Crbn^{I391V/I391V}$ cells. **c**, Peripheral blood chimerism in an *in vivo* competition experiment shows no difference in lenalidomide sensitivity of $Crbn^{I391V/I391V} Csnk1a1^{+/-}$ and $Crbn^{I391V/I391V} Csnk1a1^{+/-} Rps14^{+/-}$ cells after twice daily treatment with 50 mg/kg lenalidomide. * indicates $P < 0.05$.

Thalidomide-induced teratogenicity in $Crbn^{I391V}$ mice

The differences between species in CRBN sequence and response to thalidomide derivatives suggests a possible basis for the species-specific teratogenicity of thalidomide. While there are several amino acid differences between mouse and human CRBN, several pieces of evidence implicate the V388 residue (hCRBN, the corresponding residue to I391V in mice;

Note: Since the work in Chapter 2 was performed with isoform 2 of CRBN, this residue was called V387 in that chapter) in thalidomide's effects (**Figure 3.20**). In contrast to mice, rabbits carry a valine at this position and have thalidomide embryopathy [23-25]. The only primate known to carry the mouse-like isoleucine residue, the greater bush baby, is also the only primate known to be resistant to thalidomide [173]. Other thalidomide resistant species, including rats, cats, and swine also have a valine at this position.

Human	Y AWT V A Q C	Thalidomide teratogenicity
Orangutan	Y AWT V A Q C	
Rhesus Macaque	Y AWT V A Q C	
Marmoset	Y AWT V A Q C	
Green Monkey	Y AWT V A Q C	
Rabbit	Y AWT V A Q C	
Bushbaby	Y AWT I A Q C	No thalidomide teratogenicity
Mouse	Y AWT I A Q C	
Rat	Y AWT I A Q C	
Cat	Y AWT I A Q C	
Pig	Y AWT I A Q C	

Figure 3.20: Comparison of CRBN sequences in species with and without thalidomide teratogenicity. Humans [1, 3, 5], orangutans [8], rhesus macaques [10-13], marmosets [16], green monkeys [17], and rabbits [18-25] exhibit limb defects, fetal loss, and other malformations following treatment with thalidomide *in utero*. In contrast, such malformations are not seen in the greater bushbaby [173], mouse [21, 24, 27], rat [20, 21, 26, 27], cat [174], or pig [175, 176]. Thalidomide teratogenicity correlates with the presence of a valine at the amino acid position corresponding with 388 in human CRBN (highlighted), while the presence of an isoleucine at this position predicts lack of thalidomide teratogenicity. More details about the sequences included in the alignment are in the Methods section. While Butler [173] reported that he studied *Galago crassiceudatus*, the naming and classification of *Galago* has undergone significant changes since then, including the recognition of several new species and a change in the genus name to *Otolemur* [177]. The UniProt sequence for the only fully sequenced *Otolemur* species, *O. garnettii*, is shown. This could be either the species that Butler studied or a closely related species.

We asked whether altering the isoleucine at this position in mouse Crbn to the corresponding human valine (Crbn^{I391V}) was sufficient to confer thalidomide-induced fetal loss

in mice. Treatment with either lenalidomide or thalidomide during pregnancy resulted in a high rate of fetal loss in *Crbn*^{I391V/I391V} mice without any effect on wild-type mice (**Figure 3.21**). Additionally, when fetuses survived to birth, *Crbn*^{I391V/I391V} litters treated with lenalidomide were significantly smaller than those treated with vehicle (**Figure 3.22**).

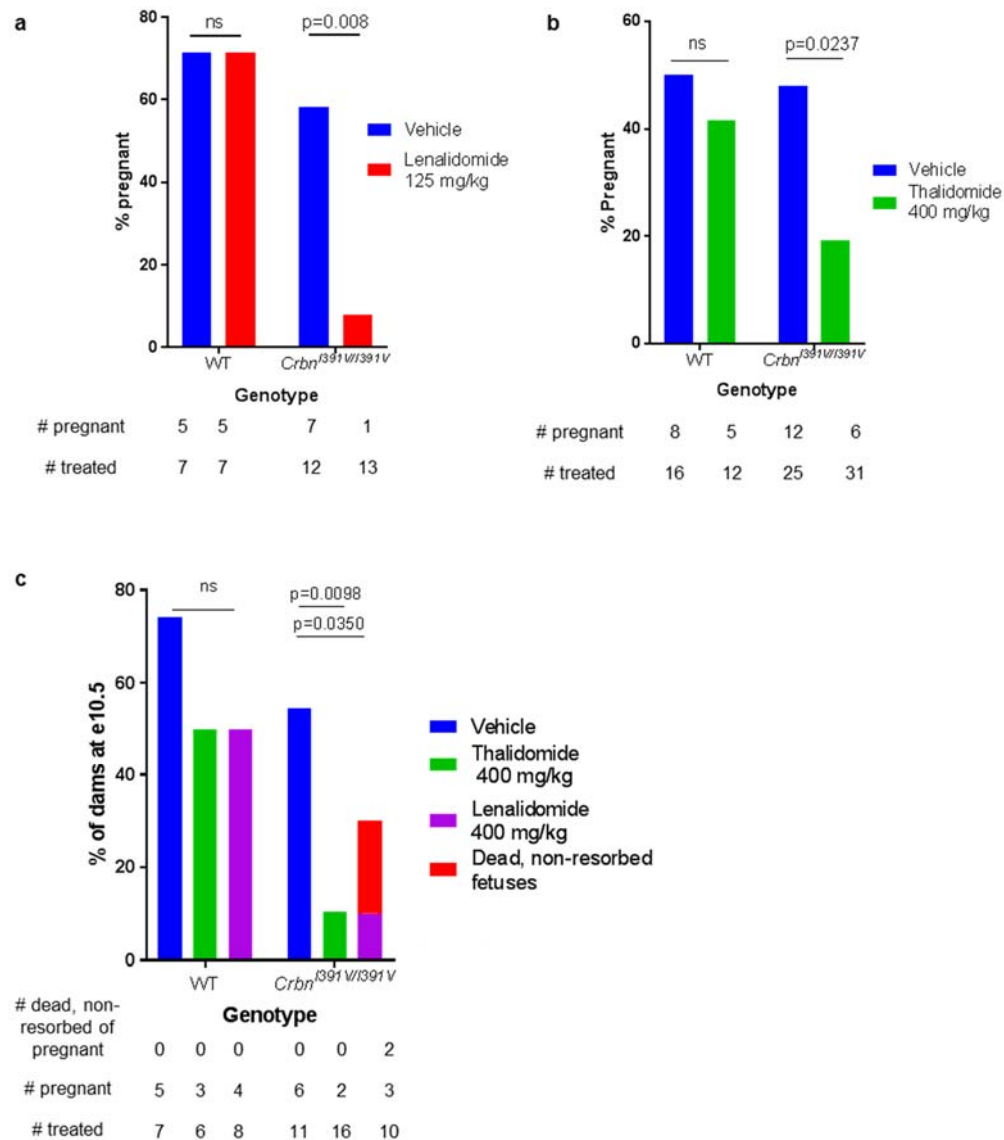


Figure 3.21: Thalidomide-derivative induced fetal loss in *Crbn*^{I391V} mice. **a**, Lenalidomide induces fetal loss of *Crbn*^{I391V/I391V} litters but not wild-type litters. Plugged wild-type or *Crbn*^{I391V/I391V} dams who had been mated with males of the same genotype were treated with 125

Figure 3.21 (Continued)

mg/kg lenalidomide every 12 hours from e4.5-e10.5. Data shown are the percent of dams pregnant at e17.5. The table below lists the total number of dams treated and the number pregnant at e17.5. *P* values are from an *N*-1 Chi-square test. **b**, Thalidomide also induces fetal demise of *Crbn*^{I391V/I391V} litters but not wild-type litters. Plugged wild-type or *Crbn*^{I391V/I391V} dams who had been mated with males of the same genotype were treated with 400 mg/kg thalidomide from e4.5-e10.5. Data shown are the percent of dams pregnant at harvests on e10.5 and e17.5 combined. The table below lists the total number of dams treated and the number pregnant. *P* values are from an *N*-1 Chi-square test. **c**, Lenalidomide and thalidomide treatment cause miscarriage before e10.5. Pregnant dams were treated with lenalidomide or thalidomide from e4.5-e10.5 and the presence of litters was assessed by harvest at e10.5. Red bars indicate the presence of two recently aborted litters with dead fetuses in the lenalidomide treatment group.

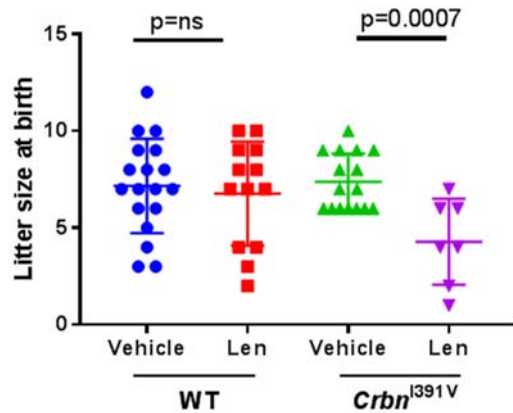


Figure 3.22: Effect of lenalidomide treatment on litter size at birth. Given that lenalidomide treatment of pregnant *Crbn*^{I391V/I391V} dams causes a penetrant fetal loss phenotype, data are a pool of multiple experiments in which dams were treated with 25-125 mg/kg of lenalidomide every 12 hours for e4.5-e9.5 or e4.5-e10.5 presumed days of gestation. Data were analyzed with an unpaired *t*-test. ns is not significant.

Through sequential studies, we narrowed the critical period for this effect to e4.5-e9.5, with a trend towards a less penetrant fetal loss phenotype with treatment from e8.5-e11.5 (**Figure 3.23**). Fetal loss was observed to occur by e10.5, and dead but non-resorbed fetuses were observed at this time point (**Figure 3.21c**, **Figure 3.23**). There was a trend towards an increased rate of anophthalmia in litters treated at e7.5 only (**Figure 3.23f**), but otherwise litters that were carried to term appeared to have normal morphology at birth and at weaning.

Carnegie developmental stages provide a method to compare the chronology of the development of different vertebrate embryos. By comparison of Carnegie stages, the critical period for fetal loss in lenalidomide-treated *Crbn*^{I391V/I391V} mice precedes the critical period for phocomelia and amelia in humans (**Figure 3.23**), suggesting that the *Crbn*^{I391V} mice may better

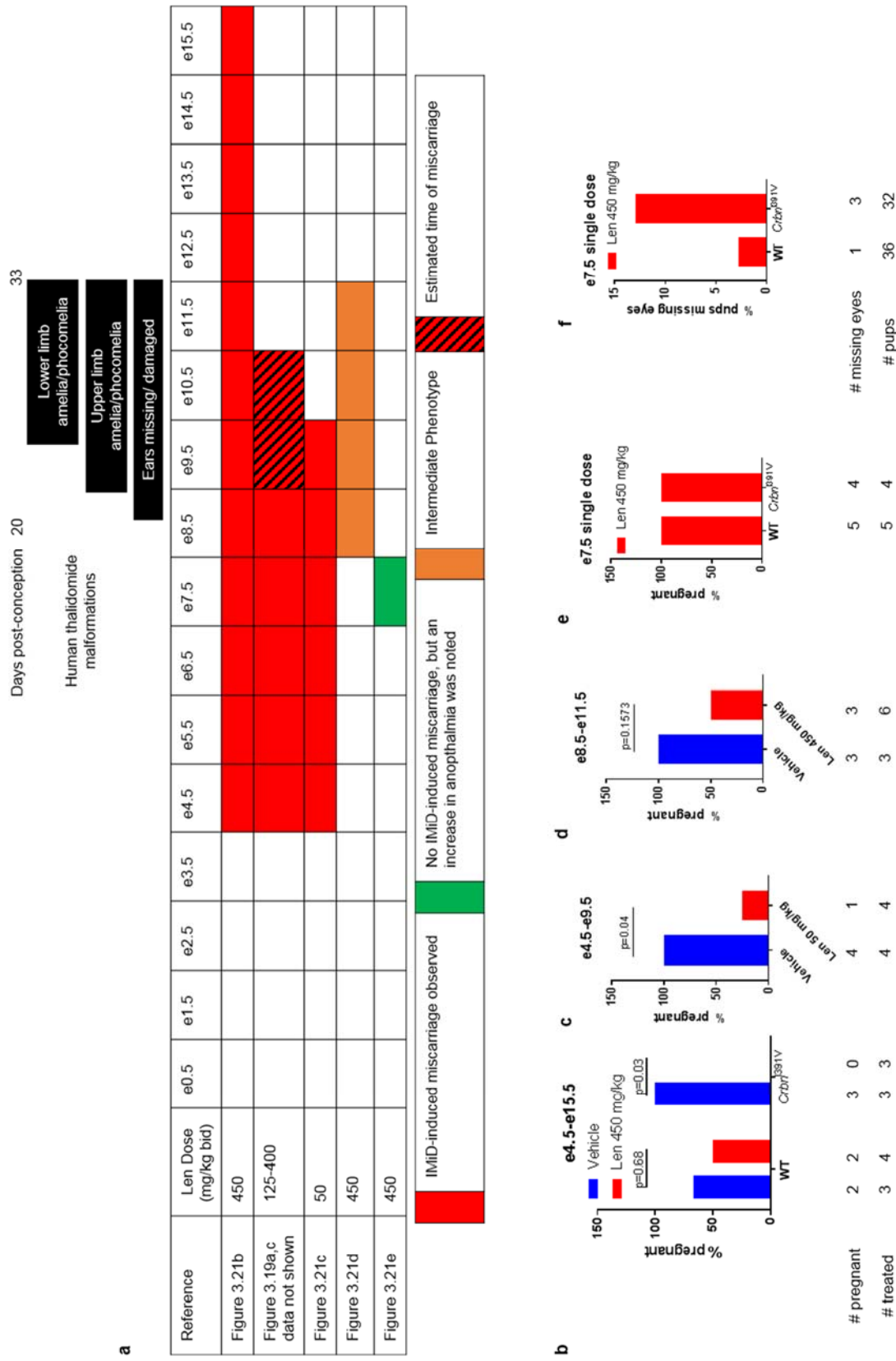
recapitulate thalidomide-induced spontaneous abortion occurring before the critical period for malformations.

In total, these results demonstrate a clear, statistically-significant impact of lenalidomide and thalidomide on *Crbn*^{I391V} mouse embryos. While the causative target of thalidomide embryopathy remains to be identified, our studies demonstrate that at least some of thalidomide's developmental effects can be attributed to a thalidomide-induced target which depends on this single amino acid change in Crbn.

Figure 3.23: Determination of the critical period for IMiD-induced teratogenicity in

***Crbn*^{I391V/I391V} mice** **a**, Each row shows a treatment schedule with the color reflecting whether this dose and treatment schedule produced fetal loss. Black bars above the table represent an approximate comparison with the critical period for various thalidomide-induced malformations in humans, given in days post conception. Human critical periods are from Vargesson [1]. Corresponding mouse embryonic stages were assigned by comparison with Carnegie stages [178, 179]. *P* value is from an unpaired *t*-test. ns, not significant. All treatment doses are calculated based on dam's weight at e4.5. **(b-e)**, Effects of lenalidomide treatment during specified embryonic periods as assessed by the presence of pups at >e10.5. All dams were mated with males of the same genotype. Listed below each graph is the number of dams treated and the number of live litters observed at >e10.5. **f**, Effect of a single dose lenalidomide at e7.5 on the percentage of pups found to be missing eyes. bid indicated twice per day treatment.

Figure 3.23(Continued)



Discussion

Here, we report the development and characterization of a mouse model with a single isoleucine to valine point mutation in *Crbn*. These mice respond to thalidomide derivatives, allowing us to conduct the first *in vivo* studies of these clinically important drugs in mice. We confirmed the degradation of the known targets Ikaros, IKZF3, and Cks1 α , as well as the downstream phenotypic consequences of degradation of these proteins in primary cells. We also demonstrate that the I391V mutation is sufficient to confer degradation of Zfp91 in mouse cells.

We provide the first *in vivo* demonstration that *Csnk1a1*^{+/-} cells are more sensitive to lenalidomide treatment, reinforcing haploinsufficiency for CK1 α as a crucial determinant of lenalidomide's therapeutic window in del(5q) MDS. We show that lenalidomide acts at the level of hematopoietic stem cells to deplete *Csnk1a1*^{+/-} cells, but that heterozygous *Trp53* mutations can provide resistance in the same cell population. This provides a mechanistic explanation for the selection of *TP53* mutant clones seen in lenalidomide-treated patients [108].

In vivo study of the *Crbn*^{I391V} mice offered insights into clinically-relevant toxicities of thalidomide derivatives. Lenalidomide treatment of *Crbn*^{I391V} mice decreased the number of peripheral white blood cells, LT-HSC, and ST-HSC, recapitulating the development of cytopenias in patients receiving these drugs and suggesting that they originate at the stem cell level [53, 54, 73, 166, 167]. Both Ikaros and Cks1 α are known to be important for the survival of hematopoietic stem cells [130, 165]. Further investigation of the effect of lenalidomide treatment on *Trp53* null hematopoietic cells could help to clarify whether activation of Tp53 secondary to Cks1 α degradation mediates this effect.

We also demonstrate that the *Crbn*^{I391V} mutation is sufficient to confer thalidomide-induced fetal loss. Potential mechanistic explanations for thalidomide-induced fetal loss and the absence of thalidomide-induced limb defects in *Crbn*^{I391V} mice are discussed in Chapter 4.

In the future, *Crbn*^{I391V} mice will provide a valuable system in which to identify new biochemical targets of thalidomide derivatives, expand our understanding of their effects in *in vivo* systems, and test new clinical indications. These possibilities are further discussed in Chapter 4.

In the past, thalidomide derivatives were brought into the clinic without a clear understanding of their biochemical mechanism or predictive pre-clinical models. However, the discovery of their mechanism of action has enabled an understanding of the basis for its clinical activity and the generation of a predictive murine model. Since they target otherwise “undruggable” transcription factors, significant effort is currently being devoted to developing new derivatives with unique targets. *Crbn*^{I391V} mice will play an important role in the future development of thalidomide derivatives by promoting the identification of novel substrates and allowing hypothesis-driven pre-clinical testing, as well as studies of biomarkers of response and biological mechanisms of resistance. The *Crbn*^{I391V} mice will also help to promote the identification of the biochemical target of thalidomide teratogenicity, as well as the cause of other observed side effects. Ultimately, biochemical manipulations may be able to eliminate or reduce degradation of these unwanted substrates, resulting in new generations of safer drugs which can be more widely used.

Methods

Animal Studies:

Animal work was carried out according to IACUC-approved protocols at Boston Children's Hospital and Brigham and Women's Hospital. Mice were housed in a barrier facility with a 12 hr: 12 hr light: dark cycle. *Crbn*^{I391V} mice were generated via homologous recombination by Ingenious Targeting Labs (Ronkonkoma, NY) on a C57BL/6 background. After generation of germline chimeras, the remaining FRT sites and Neomycin cassette at the *Crbn* locus were removed by crossing with a C57BL/6 FLP mouse line.

Conditional knockout mice for *Csnk1a1* (B6.129(Cg)-*Csnk1a1*<tm1.1Ybn>/J) and *Rps14* (B6.129(Cg)-RPS14<tm1.1Ybn>/J) have been previously described [130, 172]. *Mx1-Cre* mice (Tg(Mx1-cre)1Cgn, stock number 002527) and *Trp53* conditional knockout mice (B6.129S2-*Trp53*<tm1Tyj>/J, stock number 002101) were obtained from the Jackson Laboratory (Bar Harbor, ME). We induced excision of condition alleles under the control of *Mx1-Cre* with three intraperitoneal injections of 0.2 mg high molecular weight poly(I:C) (Invivogen).

In vivo treatment

For *in vivo* treatment, lenalidomide, thalidomide, and pomalidomide were obtained from Ark Pharm (Arlington Heights, IL). Stock solutions of drugs were prepared in DMSO, stored at -80°C, and diluted to 10-20% DMSO with sterile saline directly before administration. Drugs were dosed by gavage using Instech Lab's (Plymouth Meeting, PA) flexible feeding tubes. Mice were randomized to treatment groups using the rand() function of Excel (Microsoft).

Teratogenicity experiments

For teratogenicity experiments, females were checked each morning for the presence of a mating plug. When a plug was observed, the female was removed from the mating cage. Noon on the day that a plug was discovered was considered to be e0.5. Weight gain over the first four days of the pregnancy was tracked and any females which had lost significant weight from e0.5 to e4.5 were excluded from randomization and treatment. Maternal day e4.5 weights were used to determine the amount of drug given for the whole experiment. When indicated, dams were sacrificed at e10.5 or e16.5 to determine the number of viable pups at that developmental stage. In other cases, the dams were allowed to give birth and the number and phenotype of the pups was determined the day after birth and at the time of weaning.

In vivo transplant

Whole bone marrow was obtained from the long bones and spine of donor mice by crushing, followed by filtration, and erythrocyte lysis in BD Pharm Lyse. Recipients were prepared for transplant with two doses of 475 cGy irradiation, at least 4 hours apart. Immediately following the second dose of irradiation, unselected donor bone marrow white cells were resuspended in Hank's Buffered Salt Solution and 5-10 million cells/recipient were administered by retro-orbital injection. Mice were transferred to autoclaved cages for at least four weeks to allow for engraftment before treatment was started.

Genotyping of mice

Mice were initially genotyped using the NDEL1 and PT4 primers, listed below. In knock-in mice, one set of LoxP-FRT sites remains (143 bp). The NDEL1-PT4 primer pair produces a band of 705 bp in the wild-type mice and 848 bp in the knock-in mice. For routine colony

maintenance, mice were genotyped by Transnetyx (Cordova, TN) using a custom assay which can be shared upon request.

NDEL1: 5'-ACT TTG GAA GTG GCA GAA GAA TTA GGG -3'

PT4: 5'-GCT CTT GAA CTT GGT AGG CAA ATG C -3'

Generation of Hoxb8 cell lines and Hoxb8 cell line experiments

An immortalized myeloid progenitor cell line was generated from homozygous *Crbn*^{I391V/I391V} and wild-type mice via estrogen-regulated expression of *Hoxb8* as previously described [158]. c-Kit⁺ bone marrow cells were infected with an MSCV retrovirus encoding ER-*Hoxb8*, then grown *in vitro* in RPMI supplemented with 10% fetal calf serum, penicillin/streptomycin, murine stem cell factor (SCF) and 1 μ M β -estradiol (Sigma). Following 1 month of culture, single cell cloning was performed, and a representative clone with bilineage (neutrophil and macrophage) differentiation potential upon estrogen withdrawal was selected for all downstream applications. SCF was generated from a Chinese hamster ovary cell line as previously described [180] with conditioned media used at a final concentration of 2% (~100 ng/mL).

Hoxb8 cell lines were infected with lentiviral reports containing regions of IKZF3 or ZFP91 shown to be sufficient for thalidomide-derivative-induced degradation [126]. The IKZF3 reporter is SFFV-driven and consists of aa 130-189 of human IKZF3 fused to a C terminal enhanced GFP via a rigid linker, an internal ribosome entry sequence, and mCherry as a transcriptional control. For experiments requiring a thalidomide-resistant version of this reporter, a C148A mutant was used. The ZFP91 reporter is PGK-driven and consists of aa 400-422 of human ZFP91 fused to a C terminal enhanced GFP via a flexible linker, an internal ribosome entry sequence, and *mCherry* as a transcriptional control.

Western Blots

Western Blots were performed as previously described [65, 82]. Antibodies used were as follows: anti-Ck1 α (C-19, Santa Cruz Biotechnology), anti-Ikzf1 (H-100, Santa Cruz Biotechnology), anti-Ikzf3 (Imgenex), anti-Zfp91 (A303-245A, Bethyl Laboratories), anti-Actin HRP (ab20272, abcam), Goat anti-Rabbit HRP (Prometheus Labs), and Bovine anti-Goat HRP (Jackson Immunolabs).

Isolation of mouse bone marrow and c-Kit⁺ cells

A single cell suspension of mouse bone marrow was obtained by crushing the long bones and spine in a mortar and pestle. Brief resuspension of the cells in BD Pharm Lyse (BD) was used to lyse the erythrocytes, except in the case of Ter119/CD71 staining. For isolation of c-Kit⁺ cells, erythrocyte-lysed bone marrow was stained with CD117 microbeads (Miltenyi) and isolated using an AutoMACS Pro (Miltenyi). C-Kit⁺ cells were grown in Serum-Free Expansion Media (StemCell Technologies) supplemented with 50 ng/mL mTPO (Peprotech) and 50 ng/mL mSCF (Peprotech).

Isolation of mouse T cells

A single cell suspension of mouse splenocytes was obtained by crushing spleens through a 100 μ m filter. The resulting cell pellet was resuspended in BD Pharm Lyse (BD) for lysis of erythrocytes. T cells were isolated using an AutoMACS Pro (Miltenyi) after staining with either a Pan T Cell Kit II (Miltenyi) or CD3 Microbeads (Miltenyi).

Isolation of peripheral blood and blood counts

Peripheral blood was obtained via retroorbital bleeds according to an IACUC-approved protocol. Blood counts were obtained on a Hemavet (Drew Scientific), using the mouse settings. Before flow staining of peripheral blood, red cells were lysed with BD Pharm Lyse (BD).

Proteomics on c-Kit⁺ cells: In Solution Digestion

c-Kit⁺ were harvested and treated *in vitro* for 12 hours. After treatment, cell pellets were harvested by centrifugation, washed twice in cold PBS, and flash frozen in liquid nitrogen. Cell pellets were lysed at 4 °C in 8 M urea, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2 µg/µl Aprotinin (Sigma-Aldrich), 10 µg/µl Leupeptin (Roche), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma). Protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce). Proteins were reduced with 5 mM (DTT) for 45 min at room temperature (RT), followed by alkylation with 10 mM iodoacetamide for 30 min at room temperature in the dark. Urea concentration was reduced to 2 M with 50 mM Tris-HCl, pH 8. Samples were pre-digested for 2 h at 30 °C with Endoproteinase Lys-C (Wako Laboratories) at an enzyme-to-substrate ratio of 1:50. Samples were digested overnight at 37 °C with sequencing grade trypsin (Promega) at an enzyme-to-substrate ratio of 1:50. Following overnight digest, samples were acidified with neat formic acid to a final concentration of 1%. Acidified samples were subsequently desalted on a 100 mg tC18 Sep-Pak SPE cartridge (Waters). Briefly, cartridges were conditioned with 1 mL of 100% MeCN, 1 mL of 50% MeCN/0.1% FA, and 4x with 1 mL of 0.1% TFA. The sample was loaded, and washed 3x with 1 mL of 0.1% TFA, 1x with 1 mL of 1% FA, and eluted 2x with 600 µl of 50% MeCN/0.1% FA. Following desalting, 100 µg of the sample was dried to completion and stored at -80 °C.

Proteomics on c-Kit⁺ cells: TMT labeling of peptides

Desalted peptides were labeled with TMT 10-plex reagents (Thermo Fisher Scientific). Each TMT reagent was resuspended in 41 µL of MeCN. Peptides were resuspended in 100 µL of 50 mM HEPES and combined with TMT reagent. Samples were incubated at room temperature for 1 hour while shaking. The TMT reaction was quenched with 8 µL of 5%

hydroxylamine at room temperature for 15 min with shaking. TMT labeled samples were combined, dried to completion, reconstituted in 100 μ L of 0.1% FA, and desalted on StageTips or 100 mg SepPak columns as described above.

Proteomics on c-Kit⁺ cells: Basic Reverse Phase (bRP) Fractionation

The TMT labeled samples were fractionated using offline high pH reversed-phase chromatography (bRP). Samples were fractionated using Zorbax 300 Extend C18 column (4.6 $\times$ 250 mm, 300 Å, 5 μ m, Agilent) on an Agilent 1100 series high-pressure liquid chromatography (HPLC) system. Samples were reconstituted in 900 μ L of 5 mM ammonium formate (pH 10.0)/2% (vol/vol) acetonitrile (MeCN) (bRP solvent A). Samples were injected with Solvent A at a flow rate of 1 mL/min and separated using a 96 min gradient. The gradient consisted of an initial increase to 16% solvent B (90% MeCN, 5 mM ammonium formate, pH 10), followed by 60 min linear gradient from 16% solvent B to 40% B and successive ramps to 44% and 60% at a flow rate of 1 mL/min. Fractions were collected in a 96-deep well plate (GE Healthcare) and pooled in a non-contiguous manner into final 24 proteome fractions. Pooled fractions were dried to completeness using a SpeedVac concentrator.

Proteomics on c-Kit⁺ cells: Liquid chromatography and mass spectrometry

Desalted peptides were resuspended in 9 μ L of 3% MeCN/0.1% FA and analyzed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) using Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) coupled on-line to a Proxeon Easy-nLC 1200 (Thermo Fisher Scientific) as previously described. Briefly, 4 μ L of each sample was loaded onto a microcapillary column (360 μ m outer diameter $\times$ 75 μ m inner diameter) containing an integrated electrospray emitter tip (10 μ m), packed to approximately 22 cm with ReproSil-Pur C18-AQ 1.9 μ m beads (Dr. Maisch GmbH) and heated to 50 °C. Samples

were analyzed with 110 min method. The 110 min method contained a mobile phase with a flow rate of 200 nL/min, comprised of 3% acetonitrile/0.1% formic acid (Solvent A) and 90% acetonitrile /0.1% formic acid (Solvent B), with the following gradient profile: (min:%B) 0:2; 1:6; 85:30; 94:60; 95:90; 100:90; 101:50; 110:50 (the last two steps at 500 nL/min flow rate). The Lumos was operated in the data-dependent mode acquiring HCD MS/MS scans ($r = 50,000$) after each MS1 scan ($r = 50,000$) on the 2 s loop using an MS1 target of 3×10^6 and an MS2 target of 5×10^4 . The maximum ion time utilized for MS/MS scans was 105 ms; the HCD-normalized collision energy was set to 34; the dynamic exclusion time was set to 45 s, isotope exclusion function was enabled, and peptide match function was disabled. Charge exclusion was enabled for charge states that were unassigned, 1 and >6 .

Proteomics on c-Kit⁺ cells: Data Analysis

All data were analyzed using Spectrum Mill software package v 6.1 pre-release (Agilent Technologies). Similar MS/MS spectra acquired on the same precursor m/z within ± 60 s were merged. MS/MS spectra were excluded from searching if they were not within the precursor MH^+ range of 750-4000 Da or if they failed the quality filter by not having a sequence tag length >0 . MS/MS spectra were searched against UniProt mouse database with the mutant version of Crbn included. All spectra were allowed ± 20 ppm mass tolerance for precursor and product ions, 30% minimum matched peak intensity, and “trypsin allow P” enzyme specificity with up to 2 missed cleavages. The fixed modifications were carbamidomethylation at cysteine, and TMT at N-termini and internal lysine residues. Variable modifications included oxidized methionine and N-terminal protein acetylation. Individual spectra were automatically designated as confidently assigned using the Spectrum Mill autovalidation module. Specifically, a target-decoy based false-discovery rate (FDR) scoring threshold criteria via a two-step auto threshold strategy at the spectral and protein levels was used. First, peptide mode was set to allow automatic variable

range precursor mass filtering with score thresholds optimized to yield a spectral level FDR of <1.2%. A protein polishing autovalidation was applied to further filter the peptide spectrum matches using a target protein-level FDR threshold of 0. Following autovalidation, a protein-protein comparison table was generated, which contained experimental ratios over the median of all TMT channels. For all experiments, contaminants and reverse hits were removed.

Furthermore, data were filtered to only consider proteins with 2 or more unique peptides and was median normalized. For statistical analysis, a two-sample moderated *t*-test was implemented.

Flow cytometry

Flow cytometry was performed on a BS LSR II or BD Canto II using the following antibodies. Lineage markers are CD3, B220, CD11b, Gr1, and Ter119.

LSK = Lineage⁻ c-Kit⁺ Sca1⁺.

ST-HSC = Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁻ CD150⁻

LT-HSC = Lineage⁻ c-Kit⁺ Sca1⁺ CD48⁻ CD150⁺.

Lineage markers are CD3, B220, CD11b, Gr1 and Ter119.

Table 3.1: Flow cytometry antibodies used in this study.

Marker (Clone)	Color	Supplier	Clone
Annexin V	APC	eBioscience	
B220	APC-eFluor780, Pac Blue	eBioscience, Biolegend	RA3-6B2
CD105	PE/Cy7	Biolegend	MJ7/18
CD117	BV605, APC, Alexa Fluor 488	BD Horizon, Biolegend	2B8
CD11b	Pac Blue, APC	Biolegend	M1/70
CD150	PE/Cy7, PE/Cy5	Biolegend	TC15-12F12.2
CD16/32	PE, APC/Cy7	BioLegend	93
CD3	PE	Biolegend	500A2
CD3	APC	eBioscience	OKT3
CD3	Pac Blue	Biolegend	17A2
CD34	AF700	eBioscience	RAM34
CD41	APC	Biolegend	MWReg30
CD45.1	PE, AlexaFluor 647, APC/Cy7	eBioscience, Biolegend	A20
CD45.2	PE, FITC	eBioscience	104
CD48	PerCP/Cy5.5, APC/Cy7	Biolegend	HM48-1
CD71	PE	BD Pharmingen	C2
CD71	PE, FITC	eBioscience, Biolegend	R17217
Gr1	Pac Blue, Alex Fluor 700, APC	Biolegend, eBioscience	RB6-8C5
Sca1	APC-Cy7, FITC	BD Pharmingen, eBioscience	D7
Ter 119	APC-eFluor780, FITC, APCCy7, PacBlue, Alexa Fluor 700	eBioscience, Biolegend	TER-119

Southern Blot

Secondary confirmation of positive clones identified by PCR was performed by Southern Blotting analysis. DNA was digested with MfeI and EcoRI to confirm the integration of the targeting vector. The digested DNA was electrophoretically separated on a 0.8% agarose gel. After transfer to a nylon membrane, the digested DNA was hybridized with a probe (iNeo) targeted against the Neo Cassette. DNA from C57Bl/6 (B6) mouse strain was used as wild-type controls.

Annexin V staining

Annexin V staining was performed according to the manufacturer's recommended protocol for the Annexin V APC kit. (17-8007-74, eBioscience).

ELISA

T cells were harvested from the mice as described, stimulated with Dynabead Mouse T cell activator and treated with lenalidomide, pomalidomide or DMSO. After 48 hours, the amount of IL-2 in the supernatant was measured with a Mouse IL-2 Quantikine ELISA Kit (R&D Technologies) relative to the provided standard curve.

RT-qPCR

For the *IL2* qPCR, cDNA was prepared from T cells using a MultiMACs mRNA Isolation Kit (Miltenyi) and MultiMACS cDNA Synthesis kit (Miltenyi) per the manufacturer's protocol. For analysis of *Crbn* expression, total RNA was isolated in Trizol (Thermo Fisher) and purified using an RNeasy Mini kit (Qiagen). cDNA synthesis was performed with a QuantiTect reverse transcription kit (Qiagen). qPCR was performed on an ABI 7900HT (Applied Biosystems) using TaqMan Gene Expression Master Mix or TaqMan Fast Advanced Master Mix (ThermoFisher Scientific) and TaqMan probes for *Gapdh* (Mm99999915_g1), *Crbn* (Mm01182416_m1), *IL2* (Mm00434256_m1) and *p21* (Mm04205640_g1). Data were analyzed relative to *Gapdh* using the $\Delta\Delta CT$ method.

Alignment of CRBN sequences

Protein sequences of CRBN homologs in different species were obtained from UniProt and aligned with Clustal Omega. A listing of the UniProt identifiers and residues included in the alignment shown in **Figure 3.20** is shown below.

Table 3.2: CRBN sequences used in alignment in Figure 3.20.

Species	Scientific Name	UniProt Identifier	Residues included
Human	<i>Homo sapiens</i>	Q96SW2	384-391
Orangutan	<i>Pongo abelii</i>	Q5R6Y2	371-378
Rhesus Macaque	<i>Macaca mulatta</i>	H9FVB2	384-391
Marmoset	<i>Callithrix jacchus</i>	F7BPB1	384-391
Green Monkey	<i>Chlorocebus sabaeus</i>	A0A0D9RF76	384-391
Rabbit	<i>Oryctolagus cuniculus</i>	G1U0G4	380-387
Bushbaby	<i>Otolemur garnettii</i>	H0WVJ5	386-393
Mouse	<i>Mus musculus</i>	Q8C7D2	387-394
Rat	<i>Rattus norvegicus</i>	Q56AP7	387-394
Cat	<i>Felis catus</i>	M3XFX7	386-393
Pig	<i>Sus scrofa</i>	F1SFL6	321-328

Chapter 4 Discussion

Thalidomide derivatives have captured significant scientific interest because of their clinical efficacy and unique mechanism of action. This thesis sought to understand the mechanistic basis of lenalidomide's therapeutic efficacy in del(5q) MDS, as well as the species-specificity of response. In Chapter 2, we demonstrated that CK1 α is a direct biochemical target of lenalidomide and that haploinsufficiency for *Csnk1a1* provides an explanation for lenalidomide's therapeutic window in del(5q) MDS. We also found that changes in the chemical structure of thalidomide derivatives can alter their substrate specificity, suggesting that additional changes could enhance the efficacy or reduce the side effects of these drugs. We identified a single amino acid change that accounts for species-specific differences in CRBN and prevents the activity of thalidomide derivatives in mouse cells. Remarkably, this single amino acid change restores IMiD response in mouse cells.

In Chapter 3, we extended these findings by generating a *Crbn*^{I391V} knock-in mouse, thereby creating a novel *in vivo* model in which to study these drugs. Using this model, we found that expression of *Crbn*^{I391V} is sufficient to permit IMiD-induced degradation of *Ikzf1*, *Ikzf3*, *Ck1 α* , and *Zfp91*. We demonstrated that haploinsufficiency for *Csnk1a1* explains lenalidomide's therapeutic efficacy *in vivo* and that this effect occurs at the level of hematopoietic stem cells. We also recapitulated the clinically-observed effects of chronic lenalidomide treatment on hematopoiesis and demonstrated that they also arise from lenalidomide's effects on hematopoietic stem cells. *Crbn*^{I391V} was sufficient to confer IMiD-induced teratogenicity in mice, suggesting that this single amino acid difference contributed to the failure of mouse models to recapitulate thalidomide's teratogenicity.

While these findings provide an explanation for many of the observed effects of

thalidomide-derivatives, they also raise new questions for investigation, which will be discussed here.

Drug-induced synthetic haploinsufficiency

Somatic copy number alterations are common features of cancer and can arise either from selective pressure for deletion of a tumor suppressor gene or incidental genomic instability [181]. In both cases, somatic copy number alterations tend to be large, involving several essential genes in addition to any tumor suppressors. Heterozygous deletion of essential genes has been hypothesized to confer cancer-specific vulnerabilities, termed ‘CYCLOPS’ (Copy-number alterations Yielding Cancer Liabilities Owing to Partial losS) [141, 142]. Several CYCLOPS genes have been reported, including subunits of the proteasome [142], spliceosome [182], and RNA polymerase II complex [183]. Inhibition of these targets through various methods selectively inhibits cancer cell growth [142, 182, 183].

Here, we reported the first example of an FDA-approved drug which derives its therapeutic window from inhibition of a haploinsufficient gene. *Csnk1a1* is slightly different than other CYCLOPS genes in that heterozygous deletion confers a selective advantage to MDS cells and contributes to the disease phenotype [130]. However, unlike traditional tumor suppressors, further inhibition of *Csnk1a1* results in cell death.

Many candidate CYCLOPS genes are not enzymes and are thus not amenable to the traditional pharmacologic strategy of inhibiting an enzymatic active site [182]. Induced protein degradation may be a particularly effective strategy to target CYCLOPS genes, as it can be used to target a wide range of proteins, including transcription factors and other non-enzymes. In addition to our identification of lenalidomide-induced degradation of CK1 α , Paolella et al. [182]

found that cancer cells with copy loss of the splicing factor Sf3b1 could be selectively killed by inhibiting the activity of a deubiquitinase, which resulted in increased degradation of Sf3b1 by the proteasome.

Recently, several groups have reported bifunctional thalidomide-derivative drugs which have the potential to induce the selective degradation of a wide range of protein targets [87-89]. These drugs consist of a small molecule, which binds to the protein of interest, linked to a portion of thalidomide and result in the ubiquitination of the protein of interest by CRBN^{CRL4} and ultimately its degradation by the proteasome. This strategy could be particularly effective in targeting CYCLOPS genes as it can be applied to any protein with a small molecule ligand, regardless of whether the ligand affects protein function. For many non-enzymatic CYCLOPS targets, it may be much simpler to identify a small molecule ligand than a direct inhibitor.

Other therapeutic indications for CK1 α degradation

We demonstrated that degradation of CK1 α plays an important role in the efficacy of lenalidomide in del(5q) MDS both *in vitro* and *in vivo*. However, CK1 α has many other cellular functions and has been implicated in both normal development [137, 184, 185] and disease [132, 133, 136, 186]. Recent studies have reported that *Csnk1a1* is overexpressed in multiple myeloma cells and that malignant plasma cells from these patients are more sensitive to inhibition of CK1 α than normal bone marrow cells [187-189], suggesting that lenalidomide-induced degradation of CK1 α may also contribute to the clinical efficacy of this drug in multiple myeloma. Additionally, inhibition of CK1 α has been shown to reduce the development of erlotinib resistance in EGFR-mutant non-small cell lung cancer [190], kill acute myeloid leukemia stem cells [131], and protect keratinocytes from UV-damage [191]. As it is already FDA-approved, lenalidomide

could be used to test hypotheses regarding CK1 α inhibition in these conditions and is currently being studied for use in acute myeloid leukemia [192].

Contribution of other del(5q) genes to lenalidomide sensitivity in MDS with del(5q)

We demonstrated that CK1 α is a direct biochemical target of lenalidomide and that haploinsufficiency for *Csnk1a1* provides a mechanistic explanation for lenalidomide's therapeutic window in del(5q) MDS both *in vivo* and *in vitro*. However, haploinsufficiency for other genes on 5q could further enhance the susceptibility of del(5q) cells to lenalidomide. IMiD-induced degradation of other proteins encoded with the del(5q) CDR has not been observed in multiple proteomics experiments [65, 82], so they are unlikely to be direct biochemical targets of lenalidomide. However, heterozygous expression of these approximately 40 other genes [97] could change cellular physiology or increase dependence on direct IMiD targets. Since many del(5q) MDS patients have 5q deletions which extend beyond the CDR, reduced expression of other nearby genes on 5q, such as PP2A and CDC26C [110], could also contribute to lenalidomide response.

TP53 activation is a prominent component of del(5q) MDS and is thought to underlie the associated hematopoietic defects [193, 194]. Since CK1 α degradation induces apoptotic cell death through TP53 induction, increased baseline TP53 levels resulting from heterozygous expression of other genes on 5q might act synergistically to increase lenalidomide sensitivity. The ribosomal protein RPS14 is expressed at haploinsufficient levels in del(5q) MDS cells, resulting in the induction of TP53 and the characteristic anemia of del(5q) MDS [101]. We tested the effect of combined *Csnk1a1* and *Rps14* heterozygous loss on lenalidomide sensitivity in the *Crbn*^{I391V} mouse model. While *Csnk1a1*^{+/-} *Rps14*^{+/-} cells showed slightly increased lenalidomide sensitivity *in vitro*, there was no significant difference in the *in vivo* response. This could be due

to the dose level or treatment schedule, but it could also reflect the fact that many genes within the CDR negatively regulate TP53 and collectively contribute to TP53 activation in del(5q) MDS. Other genes encoded within the del(5q) CDR [97] whose knockdown results in TP53 activation include the ribosomal protein RPL7 [195], G3BP [196], and the Treacher Collins syndrome gene TCOF [197].

Heterozygous expression of other genes within the del(5q) CDR could also increase the dependency of del(5q) MDS cells on direct lenalidomide targets. We have characterized lenalidomide-induced degradation of Ikzf1, Ikzf3, Ck1 α , and Zfp91 in the *Crbn*^{I391V} mice. In addition to these proteins, several other putative IMiD-induced targets were seen in the proteomics data (**Figure 3.3, Figure 3.4**). These targets include Rnf166, Znf276, and Pde6d, all of which have also been seen in proteomics experiments in human cell lines and are currently being validated as IMiD-induced targets by our lab [65, 82]. PDE6D, in particular, could be relevant to del(5q) MDS because it is part of a rod phosphodiesterase complex which also contains PDE6A, a protein encoded within the del(5q) CDR [97, 198]. Pde6d knock-out mice are viable, but their hematopoietic system has not been investigated [199]. Additionally, Fang et al. [200] recently reported that IMiD-mediated degradation of IKZF1 de-represses expression of GPR68, a G-protein coupled receptor implicated in calcium metabolism, resulting in increased intracellular calcium and apoptosis in an MDS cell line. They suggest that heterozygous expression of *CAST*, a regulator of calcium signaling, which is not within the CDR but is encoded nearby in 5q15, could further sensitize MDS cells containing larger 5q deletions to lenalidomide treatment.

We could use the *Crbn*^{I391V} mice to investigate these possibilities through a pooled competitive *in vivo* screen. Using bone marrow from *Crbn*^{I391V} *Csnk1a1*^{+/-} Cas9 mice and

lentiviral expression of gRNA, we could generate a pool of *Crbn*^{I391V} *Csnk1a1*^{+/-} Cas9 bone marrow cells with CRISPR-mediated disruption of other genes on 5q. By comparing the relative frequency of each gRNA in the bone marrow of mice before and after *in vivo* treatment with vehicle or lenalidomide, we could assess whether loss of other genes on 5q enhances or diminishes *in vivo* sensitivity to lenalidomide. This screen could also be performed with *Crbn*^{I391V} *Csnk1a1*^{+/-} *Rps14*^{+/-} Cas9 cells to investigate the effect of a third hit on lenalidomide response.

Potential mechanisms of lenalidomide in non-del(5q) MDS

While lenalidomide is most effective in MDS with del(5q), IMiD treatment can also induce an erythroid response in the much larger number of MDS patients without del(5q). Several trials of lenalidomide have been conducted in MDS patients without del(5q). These trials have found that lenalidomide treatment produces an erythroid response in about 40% of patients, with about 25% achieving transfusion independence and another 20% achieving a clinically meaningful reduction in transfusions [53, 201, 202]. In contrast to the effects of lenalidomide in del(5q) MDS, histological and cytogenetic remissions are rare in non-del(5q) MDS. Instead, lenalidomide's therapeutics benefits seem to result from the promotion of erythropoiesis.

Several mechanisms have been proposed to explain lenalidomide-induced erythroid responses in non-del(5q) MDS. Ebert et al. [169] found that non-del(5q) MDS patients who respond to lenalidomide have decreased pre-treatment expression of erythroid genes. *In vitro* lenalidomide treatment promotes erythroid development and the formation of CFU-E in normal human cells, suggesting that lenalidomide may work in MDS patients by stimulating erythroid differentiation [168, 169]. Some investigators have suggested that lenalidomide promotes erythroid differentiation by altering cytokine levels to generate a favorable bone marrow

microenvironment [203]. Others have reported that lenalidomide enhances erythropoietin receptor signaling by inducing the formation of lipid rafts enriched for Jak2 signaling molecules [204] or stabilizing EpoR by inhibiting the E3 ubiquitin ligase RNF41 [205].

Whether lenalidomide's effects in non-del(5q) MDS are mediated by CRBN remains unknown. However, our results in the *Crbn*^{I391V} model provide some evidence that lenalidomide promotes erythropoiesis through CRBN. While we did not specifically study a mouse model of MDS, we observed normal RBC counts, preserved megakaryocyte-erythroid progenitors, and expansion of erythroid progenitor subsets in *Crbn*^{I391V} mice chronically treated with lenalidomide. These erythroid compartments were preserved or expanded despite significantly decreased numbers of hematopoietic stem and progenitor cells, suggesting that lenalidomide may enhance erythropoiesis in more mature cells.

CRBN-dependent, lenalidomide-induced enhanced erythropoiesis could result from degradation of a known or novel target. Degradation of CK1 α is unlikely to explain this effect, as both lenalidomide and pomalidomide promote erythroid differentiation of human cells *in vitro* [206] despite the lack of pomalidomide-induced Ck1 α degradation in the *Crbn*^{I391V} model (**Figure 3.3c**). Additionally, neither the bone marrow expansion of *Csnk1a1*^{+/-} mice or bone marrow failure of *Csnk1a1*^{-/-} mice seems have erythropoiesis-specific features [130]. IKZF3 is not expressed in erythroid cells and an Ikzf3 null mouse has no erythroid phenotype [164], so degradation of IKZF3 is similarly unlikely to explain this phenotype. IKZF1 plays a role in erythroid development, although reports of the effects of *Ikzf1* knock-out on mouse erythropoiesis are mixed. Some investigators report that *Ikzf1* null mice have reduced stem and progenitor function, but that erythropoiesis and the formation of more mature erythroid progenitors are spared [163, 165], a phenotype similar to that observed in lenalidomide-treated

Crbn^{I391V} mice. In contrast, other groups report that *Ikzf1* null mice have anemia, impaired erythropoiesis, and delayed globin switching [207]. However, these findings should be viewed in light of the fact that lenalidomide treatment is unlikely to induce full degradation of IKZF1 analogous to *Ikzf1*^{-/-} null mice, so unique effects of low protein levels of IKZF1 should be considered. One group reported that haploinsufficiency for IKZF1 increases STAT5 signaling [208]. STAT5 is downstream of the EpoR and activation of STAT5 can substitute for Epo in erythroid progenitor expansion and terminal differentiation [209], which would be consistent with effects observed in MDS patients. While their cellular function remains to be elucidated, it is also possible that degradation of ZFP91 or another uncharacterized protein substrate explains lenalidomide's pro-erythropoietic effects in non-del(5q) MDS.

Degradation of IKZF1 and IKZF3 almost certainly alters the expression of cytokines in the bone marrow microenvironment, but which cytokine alterations might promote erythropoiesis in MDS remains uncertain.

Several experiments in *Crbn*^{I391V} mice could help to elucidate the molecular basis of lenalidomide's pro-erythropoietic effects in non-del(5q) MDS. For example, measuring circulating erythropoietin levels, EpoR expression levels, and STAT5 phosphorylation in lenalidomide-treated mice could help to evaluate the aforementioned hypotheses regarding the promotion of erythropoiesis. Similarly, characterizing the bone marrow microenvironment of lenalidomide-treated mice could enhance our understanding of how IKZF1 and IKZF3 degradation alter the *in vivo* hematopoietic milieu.

Teratogenicity in the *Crbn*^{I391V} model

We demonstrated a clear genotype-specific, IMiD-induced fetal loss effect in *Crbn*^{I391V} mice, which is consistent with the effects of thalidomide in humans and primates. While

thalidomide is most infamous for its characteristic malformations, it also caused an increased rate of stillbirths and an approximately 40% rate of infant mortality during the initial tragedy [1, 210, 211]. There is also evidence that thalidomide can also cause spontaneous abortions in humans if received before the critical period for malformations or at high doses [1, 31, 32]. Similar effects are seen in multiple non-human primates, in which thalidomide induces a significant proportion of spontaneous abortions as well as malformations [8-17].

The lack of phocomelia and amelia in our model could result from additional species-specific differences in degraded proteins which prevents their IMiD-induced degradation by *Crbn*^{I391V}, or species-specific differences in the developmental function of these proteins and thus the phenotypic consequences of their degradation. Alternatively, the spectrum of phenotypes that comprise thalidomide embryopathy could result from alteration of multiple downstream targets, only some of which depend on the I391V residue for thalidomide-induced CRBN degradation or retain thalidomide-responsive sequences in mice. It is also possible that the more compressed timescale of development in mice makes it more technically difficult to separate the critical periods for fetal loss from those for malformations.

IMiD-induced fetal loss in the *Crbn*^{I391V} model could result from degradation of a validated or novel IMiD target. Homozygous null *Ikzf1* and *Ikzf3* mice are developmentally normal [163, 164], suggesting that degradation of these targets does not explain the observed fetal loss. Unfortunately, homozygous *Zfp91* knockout mice have not yet been studied. Of the known targets, CK1 α is most likely to explain the fetal loss effect, as homozygous knock-out of CK1 α leads to embryonic death [132]. However, this death occurs before embryonic day e6.5, which is significantly earlier than the death at approximately e10.5 observed with lenalidomide treatment (**Figure 3.21c**). Our observation of thalidomide-induced fetal loss also argues against

degradation of Ck1 α as the causative effect, as thalidomide has a much weaker effect on Ck1 α than lenalidomide *in vitro* (**Figure 2.17**). However, our experiments with thalidomide were conducted with much higher drug doses due to the unfavorable pharmacokinetics of thalidomide in mice, and the degree of *in utero* Ck1 α degradation induced by this dose of thalidomide is unknown. The role of Ck1 α in IMiD-induced fetal loss could be tested by repeating the teratogenicity experiments at a dose-limiting concentration in *Crbn*^{I391V} and *Crbn*^{I391V} *Csnk1a1*^{+/-} mice. If degradation of Ck1 α contributes to the fetal loss phenotype, we would expect to see this effect at lower lenalidomide concentrations in *Crbn*^{I391V} *Csnk1a1*^{+/-} mice due to their lower baseline *Csnk1a1* expression. Alternatively, we could test the effects of IMiD treatment in *Crbn*^{I391V} mice that express a lenalidomide-resistant allele of Ck1 α which retains normal developmental function, although such an allele has not yet been characterized.

If the fetal loss phenotype is instead due to lenalidomide-induced degradation of a novel target, this target may be expressed only in developmental tissues. Thalidomide-derivatives primarily target zinc-finger transcription factors [126], which are often highly restricted in their tissue expression. To identify novel development targets of thalidomide derivatives, we could perform global proteomics studies in mouse embryonic stem cells or whole or dissected tissues from mouse embryos developing *in vitro* or *in utero*.

Future applications of the *Crbn*^{I391V} mouse model

The *Crbn*^{I391V} model represents the first *in vivo* mouse model of thalidomide derivative activity. In the future, this model could be used to advance our understanding of thalidomide derivatives in several ways, including access to unique cell types, the ability to study complex mechanisms not well-modeled *in vitro*, and opportunities for preclinical testing.

Characterization of the *in vivo* response of *Crbn*^{I391V} multiple myeloma mouse models is currently being conducted in the lab of a collaborator.

Crbn^{I391V} mice provide access to a wide variety of tissues in which to identify additional targets that explain the efficacy or toxicity of thalidomide derivatives, including those for which cell lines models are inadequate. As noted above, thalidomide-derivatives mostly target zinc-finger transcription factors [126], whose expression is often lineage-specific. Thus, global proteomics may fail to capture degradation of specific IMiD protein targets because they are only expressed in specific tissue types or under certain physiologic conditions.

Restricted expression of transcription factors is especially important during development. As discussed above, the readily available embryonic and fetal tissue from *Crbn*^{I391V} mice provides a unique opportunity to identify the biochemical targets underlying thalidomide-induced fetal loss.

IMiD-induced sedation, peripheral neuropathy, and thrombosis are also areas in which tissues from the *Crbn*^{I391V} mice could help identify novel targets. Thalidomide was first marketed as a sedative and anti-emetic, although the biochemical bases of these effects remain unknown. If sedation is observed after thalidomide treatment of the *Crbn*^{I391V} mice, brain tissue could be studied by global proteomics and the effects correlated with available genetic knockout models. Similarly, peripheral neuropathy is a commonly reported side effect of thalidomide treatment [212], but studies involving human nerve cells have been limited by tissue availability. This phenotype could be readily studied in *Crbn*^{I391V} mice following chronic IMiD administration, with nerve tissue available as needed. Increased venous and arterial thrombosis is also reported in patients undergoing thalidomide treatment [213-215], but the cellular and mechanistic basis of this effect is unclear. We are currently exploring thrombosis risk in *Crbn*^{I391V} mice treated with

IMiDs in order to recapitulate this effect, and ultimately understand its cellular and biochemical basis.

Many of thalidomide's effects involve complicated interactions which are difficult to dissect *in vitro*. While thalidomide's immunomodulatory effects form the basis of its clinical efficacy in many conditions, the exact mechanism of thalidomide's effect on the immune system is difficult to determine without access to knock-out mice and other well-characterized *in vivo* tools. Similarly, alterations in the bone marrow microenvironment and cytokine production have been hypothesized to play an important role in lenalidomide response in multiple myeloma but studying these effects has thus far been limited to *in vitro* and xenograft systems [46, 151-153]. Our *Crbn*^{I391V} model provides a unique opportunity to dissect the complex *in vivo* interplay of cytokines and immune cell subsets and the relative contributions of cell-autonomous and non-cell autonomous effects.

Thalidomide-derivatives are an active area of drug development and clinical testing. The manufacturer of lenalidomide, Celgene, is currently working to develop thalidomide-derivatives with novel substrate specificities, with the goal of increasing therapeutic indications and reducing unwanted side effects. Currently, thalidomide derivatives are only prescribed through a closely regulated program that requires provider registration and frequent patient follow-up [216]. The development of a thalidomide-derivative that retained clinical efficacy but did not cause teratogenicity would greatly increase ease of prescribing and expand the range of clinical conditions and patient populations for which the potential benefits of treatment would outweigh the risks.

Crbn^{I391V} mice could play an important role in new drug discovery by providing the ability to test the *in vivo* efficacy and side effects of these new derivatives, including some

aspects of reproductive toxicity, in mice, which are easier to work with than primates or rabbits. Various academic and industry groups are also testing IMiDs for additional clinical indications, including inflammatory bowel disease [46] and lupus [46, 217]. Because mouse models of disease are widely used and well-characterized, the *Crbn*^{I391V} mouse could be crossed with disease-specific models to perform pre-clinical testing. If initial pre-clinical results are positive, *Crbn*^{I391V} mice can also be crossed with a multitude of other well-characterized genetically engineered mouse models to investigate the mechanism of IMiDs in these conditions.

Structural basis of the importance of V388 in IMiD-induced degradation of IKZF1, IKZF3, and CK1 α

After we published the work described in Chapter 2, several groups reported crystal structures of CRBN-DDB1-IMiD-substrate complexes which help to explain the biochemical basis of these drugs species-specific effects. Petzold et al. described the first CRBN-DDB1-lenalidomide-CK1 α tertiary crystal structure [70]. This work demonstrated that IMiDs act like molecular glue, binding to CRBN and jointly creating a binding interface with which CK1 α interacts. Despite their limited primary sequence homology, both IKZF1 and CK1 α interact with CRBN through β -loops that are predicted to have striking conformational similarity. The recruitment of CK1 α to CRBN induces a slight repositioning of the phthalimide ring of the lenalidomide relative to the lenalidomide-CRBN-only structure. This repositioning is tolerated when lenalidomide is bound, but the presence of an additional carbonyl group at the C3 phthalimide ring position of pomalidomide and thalidomide is predicted to result in increased steric clash with the backbone of CRBN if CK1 α recruitment were to occur. This provides a structural explanation for lenalidomide's enhanced effects on CK1 α relative to other IMiDs.

In the CRBN-DDB1-lenalidomide-CK1 α structure, V388 (homologous to I391) is in close proximity to Gly40 and Glu41 of CK1 α [70] (Note: This is the same residue referred to in Chapter 2 as V387, as the two isoforms of human CRBN vary by one amino acid in their numbering). The valine to isoleucine mutation found in mice introduces a methyl group which is predicted to disrupt CK1 α -Crbn binding through steric clashes. On the basis of this structural observation, the V388I mutation is predicted to not alter IMiD binding, consistent with our finding that mCrbn, mCrbn^{I391V}, and hCRBN all bind agarose-bead bound lenalidomide with similar affinities (**Figure 2.14**).

Contribution of other Crbn residues to thalidomide derivatives' species-specific effects

In addition to I391, there are several other sequence differences between human and mouse CRBN (**Figure 1.6**) whose significance is not yet determined. While we have demonstrated here that Crbn^{I391V} is sufficient to confer IMiD-induced degradation of Ck1 α , Ikzf1, Ikzf3, and Zfp91, other species-specific differences in CRBN could contribute to the degradation seen in human cells. Given that some of the clinical effects of thalidomide derivatives remain unexplained, new substrates may also have different dependencies on specific CRBN residues. Additionally, several groups are actively developing new thalidomide derivatives which may interact with CRBN in unique ways.

A useful example of these considerations is provided by the case of the novel thalidomide derivative CC-885 [81]. While CC-885 still binds CRBN through a glutarimide ring, the rest of the molecule has a chemical structure distinct from thalidomide, lenalidomide, and pomalidomide. This unique structure confers the ability to recruit and ubiquitinate a novel substrate, GSPT1, in addition to IKZF1 and IKZF3, and induces unique conformational changes in CRBN [81, 218].

Figure 4.1a highlights the amino acid differences between hCRBN and mCrbn on the hCRBN-DDB1-CK1 α crystal structure [70], with species differences colored yellow, green, and red. V388 (which correspond to I391 in the mouse) is buried within the CK1 α -CRBN interface, but there is also the yellow residue, E377, adjacent to CK1 α binding. E377 is instead V380 in the mouse. We did not detect any effects of hCRBN^{E377V} or mCrbn^{V380E} on CK1 α degradation by Western Blot (**Figure 2.13**), as expected since this residue is adjacent to the CK1 α binding site but does not make direct contacts.

In contrast, E377 is essential to CRBN-induced degradation of GSPT1 by CC-885 [81]. **Figure 4.1b** highlights amino acid differences between hCRBN and mCrbn on the hCRBN-DDB1-CC-885-GSPT1 crystal structure [81]. Here, the yellow E377 residue makes close contacts with CC-885 and is buried at the drug-CRBN interface (**Figure 4.1c**). The mouse residue V380 fails to make hydrogen bond contacts with CC-885, reducing drug binding. While CC-885-induced IKZF1 degradation remains dependent on V388, GSPT1 interacts with the CRBN-CC-885 complex differently so that V388I has no effect. Intriguingly, despite some binding of CC-885, mCrbn^{I391V} is not sufficient to confer CC-885-induced degradation of Ikbfl in mouse cells, suggesting that drug binding may introduce conformational changes that make both E377 and V388 essential to the recruitment and ubiquitination of IKZF1. The lab is currently generating a *Crbn*^{I391V V380E} double knock-in mouse to address these questions and generate an *in vivo* model for the activity of CC-885.

In addition to V388 and E377, the crystal structures reveal several other sequence differences between hCRBN and mCrbn which could affect substrate recruitment. In **Figure 4.1a, b**(arrow), several species-dimorphic amino acids are highlighted in red which could play a role in species-specific effects in the case of a larger or differently shaped recruited protein

target. **Figure 4.1c** shows the F150 residue in the DDB1-CRBN-CC-885-GSPT1 structure, which makes clear, tertiary-structure-dependent contacts with GSPT1 and is uniquely required for GSPT1 recruitment. While F150 is identical between mouse and human CRBN, the adjacent residue Asp149 is mutated to a glutamic acid in mouse CRBN, and this change could affect the recruitment of a larger substrate.

Thus, as the number of thalidomide derivatives and their substrates increases, our view of the role of specific CRBN mutations in the species-specific effects of thalidomide derivatives is likely to become more complicated.

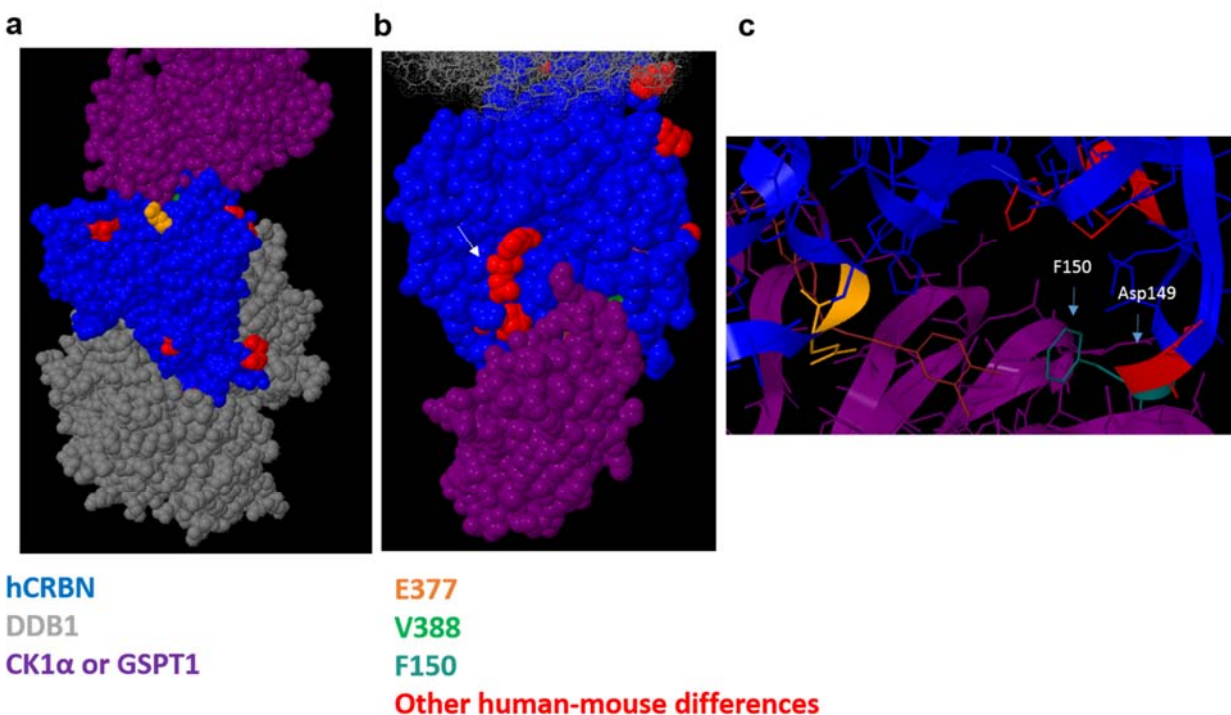


Figure 4.1: Structural visualization of additional human-mouse differences in CRBN. **a**, A rendering of the CRBN-DDB1-Lenalidomide-CK1α crystal structure from PDB 5FQD [70]. CRBN is blue, DDB1 is grey, and CK1α is purple. Human CRBN residue V388 is highlighted in green, and human CRBN residue E377 is shown in yellow. Other residues which differ between human and mouse CRBN are shown in red. **b**, and **c** are renderings of the CRBN-DDB1-CC-885-GSPT1 crystal structure from PDB 5HXB [81]. Residues are colored as in **a**, except GSPT1 is purple and human residue F150 is teal. **b** shows several residues adjacent to the CRBN-GSPT1 binding site which differ between mouse and human CRBN and could contact larger or differently shaped recruited substrates. **c** highlights that CC-885 makes close contact with residue F150. While this residue is the same in mouse and humans, it is adjacent to D149, which is a glutamic acid in mice.

Conclusion

With their novel mechanism and ability to target otherwise “undruggable” protein targets, thalidomide derivatives are likely to form an enlarging and clinically important class of drugs. The work presented here identified CK1 α as a direct target of thalidomide derivatives, providing a mechanistic explanation of the therapeutic window of lenalidomide in del(5q) MDS. By comparing different thalidomide derivatives, we found that changes in chemical structure could alter substrate specificity, providing proof of concept for expansion of this novel drug mechanism to a variety of targets. We also provide a mechanistic explanation for the failure of these drugs in mouse models. By changing a single methyl group, we have generated a novel mouse model which will support the understanding and clinical development of this growing class of drugs.

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