Supplemental Data

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CK1α and IRF4 are Essential and Independent Effectors of Immunomodulatory Drugs in Primary Effusion Lymphoma

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Short Title: CK1α and IRF4 are IMiD effectors in PEL

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Supplementary Methods

Vector design and cloning procedures

For sgRNA sequences and primers see Table S1, other primers and synthetic DNAs Table S2, and shRNA target and hairpin sequences Table S3. sgRNAs were designed using published parameters[1] and inserted into BsmBI sites of pLenti-guide puro[2] (Addgene # 52963) or pLenti SpBsmBI sgRNA Hygro[3] (Addgene # 62205). sgAAVS1, sgIRF4 or sgPSMD1 are published controls[4-6].

Lentiviral miR-30-embedded shRNA expression cassettes were designed using shERWOOD[7], synthesized as dsDNA fragments by Epoch Life Sciences, TX, and inserted between NotI and MluI sites of pZIP-hCMV-ZsGreen-P2A-Puro-NT4 using Gibson Assembly. pZIP-hCMV-ZsGreen-P2A-Puro-NT4 is derived from pZIP-hCMV-ZsGreen-Puro-NT control #4 (Transomic Technologies, AL), but was modified to replace the internal ribosomal entry site (IRES) between ZsGreen and the puromycin resistance gene with a P2A peptide. The P2A-Puro^R sequence was amplified from pLenti-CRISPRv2 (Addgene # 52961) with primers p2apuro_F and p2apuro_R and inserted between the EcoRI and BamHI sites of pZIP-hCMV-ZSGreen-Puro-NT control #4 using Gibson Assembly.

Lentiviral CK1α expression vectors were based on the plasmids pLCE-P2A-Puro or pLC/ZsGreen-P2A-Blast. To construct pLCE-P2A-Puro, a P2A-Puro^R fragment was PCR amplified from lentiCRISPRv2[2] with primers 3164 and 3158 and inserted into XhoI-digested pLCE[8] using Gibson assembly. This strategy created a continuous eGFP-P2A-Puro^R coding sequence. To construct pLC/ZsGreen-P2A-Blast, PCR fragments generated using primers 3883/3885 (CMV promoter), 3888/3887 (ZsGreen-p2A) and 3910/3911 (P2A-Blast^R) were Gibson assembled with the large BamHI/EcoRI fragment of pLCE. This strategy created a continuous ZsGreen-P2A-Blast^R coding sequence. To insert the CK1α coding sequence into pLC-P2A-Puro, the CMV-eGFP cassette of pLCE-P2A-Puro was excised using BamHI and replaced with fragments containing the CMV promoter (amplified using primers 3592 and 3660) and the WT CK1α coding sequence (amplified using primers 3661 and 3734, which also introduce an N-terminal

Flag tag), using Gibson Assembly. G40N mutant CK1α was made by overlap PCR using mutant primers 3595 and 3596. To generate pLC-CK1α/G40N-P2A-Blast, pLC/ZsGreen-P2A-Blast was digested with AgeI and BamHI to remove ZsGreen and subjected to Gibson Assembly with a PCR product encoding CK1α (amplified using primers 3971 and 3734).

A newly constructed Dox-inducible lentiviral vector called pCW-MCS-puro was used for insertion of the IRF4 coding sequence. This vector was derived from pCW-Cas9[5] (Addgene plasmid # 50661) in several steps. First, a cloning linker and the human PGK promoter were amplified from gBlock 3035_MCS_hPGK (IDT, Coralville, IA) using primers 3224 and 3225, cut using NheI and XbaI and used to replace the equivalent sequence (containing Cas9-PGK promoter-Puro^R) of pCW-Cas9. Primer 3225 also introduces a P2A peptide sequence. The resulting vector (pCW-MCS-PGK-P2A) was linearized using BamHI and used for insertion of a puro resistance marker, amplified from pLenti-guide puro[2] using primers 2402 and 3309, by Gibson Assembly. The resulting vector was called pCW-MCS-puro and contains the following components from 5' to 3': Dox-inducible minimal CMV promoter, cloning linker, human PGK promoter, Puro^R-P2A-rTTA3 cassette. Finally, an IRF4 coding sequence containing several silent mutations, introduced as part of another study, was PCR amplified from IRF4_gBLOCK using primers 3672 and 3673 and inserted into the NheI site contained in the cloning linker of pCW-MCS-PGK-P2A-puro using Gibson Assembly. All plasmids and PCR amplified inserts were confirmed by Sanger sequencing.

Supplementary References

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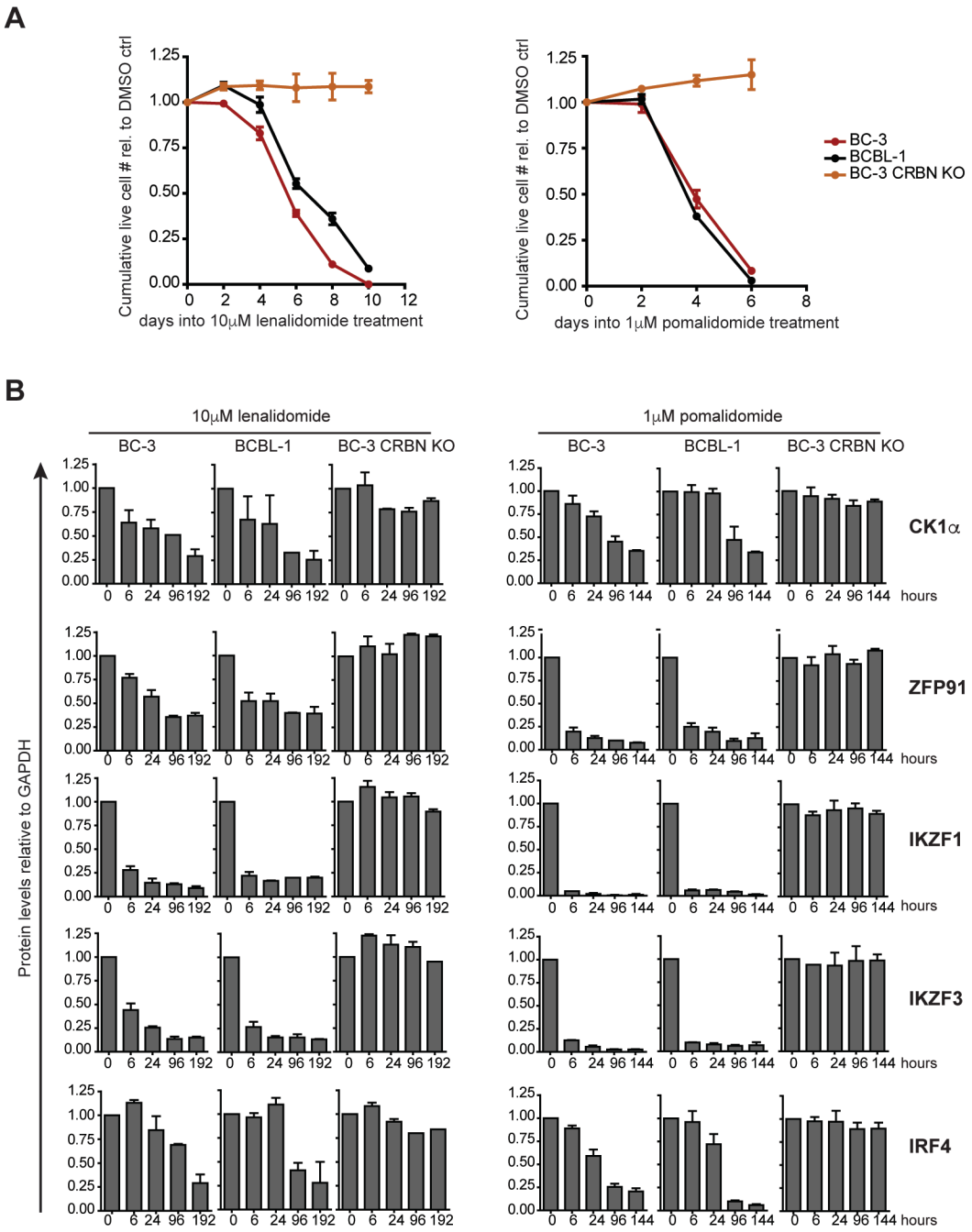

Fig. S1 Treatment of PEL cell lines with lenalidomide and pomalidomide leads to toxicity and degradation of neosubstrates IKZF1, IKZF3, ZFP91 and CK1α in a CRBN dependent manner. (A) Growth curve analyses of samples described in Fig. 2B, C (n=3, error bars SEM). (B) Quantitative analysis of Western Blots shown in Figs. 2B and C (n=2, error bars SEM).

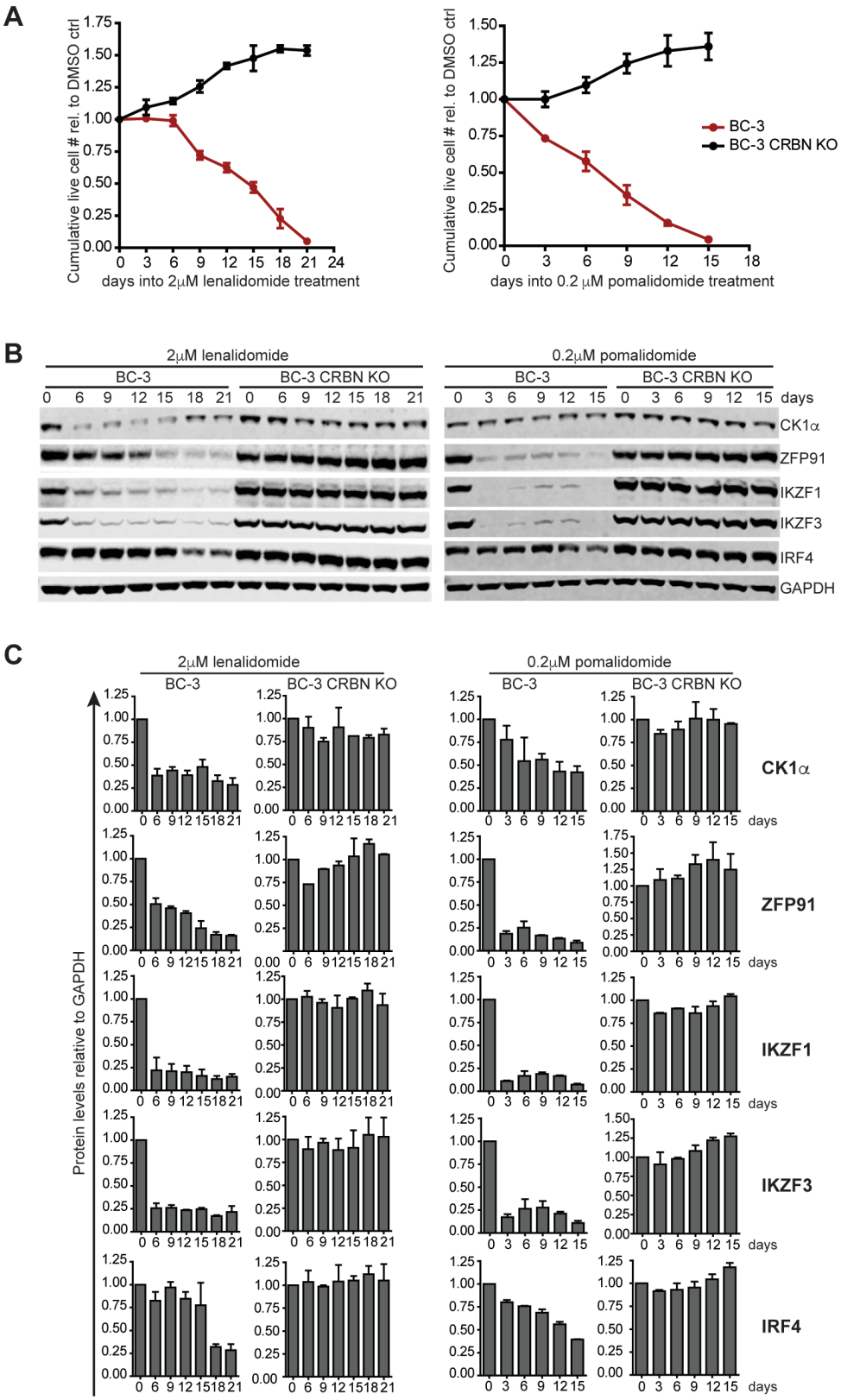
A
Cumulative live cell # rel. to DMSO ctrl
days into 2μM lenalidomide treatment
days into 0.2 μM pomalidomide treatment
BC-3
BC-3 CRBN KO
B
2μM lenalidomide
0.2μM pomalidomide
BC-3
BC-3 CRBN KO
days
CK1α
ZFP91
IKZF1
IKZF3
IRF4
GAPDH
C
Protein levels relative to GAPDH
CK1α
ZFP91
IKZF1
IKZF3
IRF4

Fig. S2 Lower concentrations of lenalidomide and pomalidomide are toxic and reduce neosubstrate and IRF4 expression. (A) Growth curve analyses of BC-3 or BC3 CRBN KO cells treated with 2μM lenalidomide or 0.2 μM pomalidomide (n=3, error bars SEM). (B) Representative quantitative Western blot analysis of CK1α, ZFP91, IKZF1, IKZF3, and IRF4 expression at the indicated time points in hours (hrs) following treatment of BC-3, or BC-3 CRBN KO cells with 2μM lenalidomide 0.2μM pomalidomide GAPDH served as loading control. (C) Quantitative analysis of the data shown in Fig. S2B over two replicates (n=2, error bars SEM).

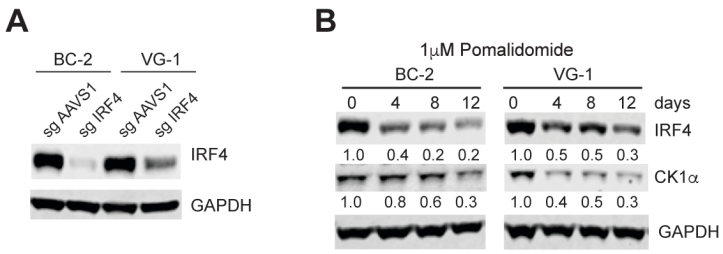

Fig. S3 Pomalidomide treatment of BC-2 and VG-1 leads to loss of IRF4 expression. (A) Quantitative Western Blotting confirms loss of IRF4 expression following delivery of sgIRF4 into BC-2 or VG-1 (see Fig. 3A, where sgIRF4 served as a positive control). (B) Treatment of BC-2 and VG-1 cells with 1μM pomalidomide leads reduced IRF4 and CK1α expression. Numbers below the panels indicate relative expression.

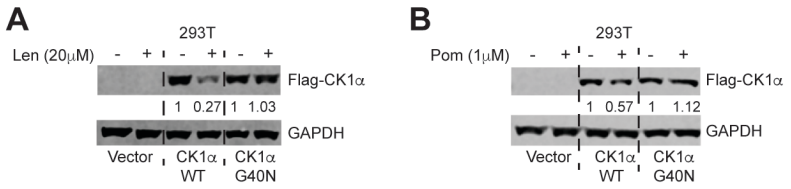

Fig. S4 G40N mutant CK1α is resistant to IMiD-induced degradation. 293T cells were transfected with either empty vector or lentiviral vectors expressing Flag-tagged WT or G40N mutant CK1α. Cells were treated for 4 days with the indicated amounts of lenalidomide (A) or pomalidomide (B) before analysis. GAPDH served as loading control.

sgRNA	Sequence	Vector	Forward Primer	Reverse Primer
AAVS1	GGGCCACTAGGGACAGGAT	pLenti-guide puro	CACCGGGGCCACTAGGGACAGGAT	AAACATCCTGTCCCTAGTGGCCCC
CRBN sg1	ACCAATGTTCATATAAATGG	pLenti SpBsmBI sgRNA Hygro	ACACCGACCAATGTTCATATAAATGGG	AAAACCCATTTATATGAACATTGGTCG
CK1α sg1	TTGCTCTCGTACAGCAACTG	pLenti-guide puro	CAACGTTGCTCTCGTACAGCAACTG	AAACCAGTTGCTGTACGAGAGCAAC
CK1α sg2	CGCCAAATAGATGTCCCCGA	pLenti-guide puro	CAACGCGCCAAATAGATGTCCCCGA	AAACTCGGGGACATCTATTTGGCGC
IKZF1 sg1	TCCAAGAGTGACAGAGTCGT	pLenti SpBsmBI sgRNA Hygro	ACACCGTCCAAGAGTGACAGAGTCGTG	AAAACACGACTCTGTCACTCTTGGACG
IKZF1 sg1	TCCAAGAGTGACAGAGTCGT	pLenti-guide puro	CACCGTCCAAGAGTGACAGAGTCGT	AAACACGACTCTGTCACTCTTGGAC
IKZF1 sg2	GAAAATGAATGGCTCCCACA	pLenti-guide puro	CACCGAAAATGAATGGCTCCCACA	AAACTGTGGGAGCCATTCATTTTC
IKZF3 sg1	AAGATGAACTGCGATGTGTG	pLenti-guide puro	CACCGAAGATGAACTGCGATGTGTG	AAACCACACATCGCAGTTCATCTTC
IKZF3 sg2	CAAGCAGAGAAGTTCCCTTG	pLenti-guide puro	CACCGCAAGCAGAGAAGTTCCCTTG	AAACCAAGGGAACTTCTCTGCTTGC
IRF4 sg1	CTGATCGACCAGATCGACAG	pLenti-guide puro	CACCGCTGATCGACCAGATCGACAG	AAACCTGTCGATCTGGTCGATCAGC
ZFP91 sg1	GCTGCATCTAGACCTAGCCG	pLenti-guide puro	CACCGGCTGCATCTAGACCTAGCCG	AAACCGGCTAGGTCTAGATGCAGCC
ZFP91 sg2	AGGCCGAGTATCCCCGCCGG	pLenti-guide puro	CACCGAGGCCGAGTATCCCCGCCGG	AAACCCGGCGGGGATACTCGGCCTC

Table S1. sgRNA sequences and sgRNA primers used in this study.

Primers used to clone pZIP-CMV-ZsGreen-P2A-Puro	
Name	**Sequence**
p2apuro_F	CACGCCATCGCCTCCGGCTCCGCCTTGCCCGGATCCGGCGCAACAAACTT
p2apuro_R	CCTCATTCAAACAGGATCCATTGCGGCCGCTCAGGCACCGGGCTTGCGG
dsDNA fragments for shRNAs targeting IKZF1 and IKZF3	
Name	**Sequence**
IKZF1 sh1	GCCCGGTGCCTGAGCGGCCGCAATGGATCCTGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCT GGGATTACTTCTTCAGGTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCGCTACGAGAAGGAGAACGAAATAGT GAAGCCACAGATGTATTTCGTTCTCCTTCTCGTAGCTTGCCTACTGCCTCGGACTTCAAGGGGCTACTTTAGGAGCAATTATCTTGTTTACTA AAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAATGGTATAAATTAAATCACTTTAGCTTGCAGATCTACGCGTGG CGCGCCTGGAACAATCAACCTCT
IKZF1 sh2	GCCCGGTGCCTGAGCGGCCGCAATGGATCCTGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCT GGGATTACTTCTTCAGGTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGAGCAGCGGTCTCATCTACCTGATAGT GAAGCCACAGATGTATCAGGTAGATGAGACCGCTGCGTGCCTACTGCCTCGGACTTCAAGGGGCTACTTTAGGAGCAATTATCTTGTTTACT AAAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAATGGTATAAATTAAATCACTTTAGCTTGCAGATCTACGCGTG GCGCGCCTGGAACAATCAACCTCT
IKZF3 sh1	GCCCGGTGCCTGAGCGGCCGCAATGGATCCTGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCT GGGATTACTTCTTCAGGTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGAAAATCCCTTACAGCTATTCAATAGTG AAGCCACAGATGTATTGAATAGCTGTAAGGGATTTCTGCCTACTGCCTCGGACTTCAAGGGGCTACTTTAGGAGCAATTATCTTGTTTACTAA AACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAATGGTATAAATTAAATCACTTTAGCTTGCAGATCTACGCGTGGC GCGCCTGGAACAATCAACCTCT
IKZF3 sh2	GCCCGGTGCCTGAGCGGCCGCAATGGATCCTGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCT GGGATTACTTCTTCAGGTTAACCCAACAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGCGAACGCCAGAATCACATCTAATAGT GAAGCCACAGATGTATTAGATGTGATTCTGGCGTTCTTGCCTACTGCCTCGGACTTCAAGGGGCTACTTTAGGAGCAATTATCTTGTTTACTA AAACTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAAATGGTATAAATTAAATCACTTTAGCTTGCAGATCTACGCGTGG CGCGCCTGGAACAATCAACCTCT
Primers used to clone pLCE-P2A-Puro	
Name	**Sequence**
3164	ATCACTCTCGGCATGGACGAGCTGTACAAGGGATCCGGCGCAACAAACTT
3158	CCTCGACGAATTCTCTGGCGGCCGCATATTTCAGGCACCGGGCTTGCGG

Primers used to clone pLC-ZsGreen-P2A-Blast	
Name	**Sequence**
3883	ATCCAGTTTGGTTAGTACCGGGCCCGAGAGTTAATTAATTAGTTATTAATAGTAATCA
3885	CTTGGTCAGGCCGTGCTTGGACTGGGCCATGGTGGCGACCGGTAGCGCTA
3887	CGACATCTCCGGCTTGTTTCAGCAGAGAGAAGTTTGTTGCGCCGGATCCGGGCAAGGCGGAGCCGGAGG
3888	TCAGATCCGCTAGCGCTACCGGTCGCCACCATGGCCCAGTCCAAGCACGG
3910	TCTCTCTGCTGAAACAAGCCGGAGATGTCGAAGAGAATCCTGGACCGGCCAAGCCTTTGTCTCAA
3911	TACGAAGTTATTAGGTCCCTCGACGAATTCTTAGCCCTCCCACACATAAC
Primers used to clone pLC-CK1α-WT/G40N-P2A-Puro and pLC-CK1α-WT/G40N-P2A-Blast constructs	
Name	**Sequence**
3592	GTTTGGTTAGTACCGGGCCCGAGAGGATCCTTAGTTATTAATAGTAATCAA
3660	GGAGCCGCTGCTACTCGCCTTATCGTCATCGTCTTTGTAATCCATGGTGGCCGGATCTGA
3661	TCAGATCCGGCCACCATGGATTACAAAGACGATGACGATAAGGCGAGTAGCAGCGGCTCC
3734	GAGAGAAGTTTGTTGCGCCGGATCCGAAACCTTTCATGTTACTCTTG
3595	GCTTCACTGCCACTTCCTCGTTGTTGGTGATGTTGATCGCC
3596	GGCGATCAACATCACCAACAACGAGGAAGTGGCAGTGAAGC
3971	CGTCAGATCCGCTAGCGCTACCGGTCGCCACCATGGATTACAAAGAC
Primers used to clone pCW-MCS-Puro	
Name	**Sequence**
3035 **gBlock**	ACCGTCAGATCGCCTGGAGAATTGGCTAGCAGCGGATCCGACACCGGTGCAGTCGACACCGGAATTCAACCTTAATTAACGACGCGTAGCG GATCCGGGGTTGGGGTTGCGCCTTTTCCAAGGCAGCCCTGGGTTTGCGCAGGGACGCGGCTGCTCTGGGCGTGGTTCCGGGAAACGCAG CGGCGCCGACCCTGGGTCTCGCACATTCTTCACGTCCGTTCGCAGCGTCACCCGGATCTTCGCCGCTACCCTTGTGGGCCCCCCGGCGAC GCTTCCTGCTCCGCCCCTAAGTCGGGAAGGTTCCTTGCGGTTCGCGGCGTGCCGGACGTGACAAACGGAAGCCGCACGTCTCACTAGTAC CCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGCCGACCGCGATGGGCTGTGGCCAATAGCGGCTGCTCAGCAGGGCGCGCC GAGAGCAGCGGCCGGGAAGGGGCGGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCCCTGTTCCTGCCCGCGCGGTGTTCCGCAT TCTGCAAGCCTCCGGAGCGCACGTCGGCAGTCGGCTCCCTCGTTGACCGAATCACCGACCTCTCTCCCCAGCAATTCACC
3224	TTGGCTAGCGACACCGGTGCAGTCGACACCGGAATTCAACCTTAATTAACGACGCGTCGGGGTTGGGGTTGCGCC
3225	CCAGTCTAGACATCGGTCCAGGATTCTCTTCGACATCTCCGGCTTGTTTCAGCAGAGAGAAGTTTGTTGCGCCGGATCCGGTGGTCCTGCA GGGAATTGCTGGGGAGAGAG
2402	CGACCTCTCTCCCCAGCAATTCCCTGCAGGGCCACCATGACCGAGTACAAGCCCAC
3309	CAGCAGAGAGAAGTTTGTTGCGCCGGATCCGGCACCGGGCTTGCGGGTC

Codon Altered IRF4 sequence	
Name	**Sequence**
IRF4_gBLOCK	ATGAACCTGGAGGGCGGCGGCCGAGGCGGAGAGTTCGGCATGAGCGCGGTGAGCTGCGGCAACGGGAAGCTCCGCCAGTGGCTGATCG ACCAGATCGACAGCGGCAAGTACCCCGGGCTGGTGTGGGAGAACGAGGAGAAGAGCATCTTCCGCATCCCCTGGAAGCACGCGGGTAAAC AAGATTATAATCGTGAAGAGGACGCCGCGCTCTTCAAGGCTTGGGCACTGTTTAAAGGAAAGTTCCGAGAAGGCATCGACAAGCCGGACCC TCCCACCTGGAAGACGCGCCTGCGGTGCGCTTTGAACAAGAGCAATGACTTTGAGGAACTGGTTGAGCGGAGCCAGCTGGACATCTCAGAC CCGTACAAAGTGTACAGGATTGTTCCTGAGGGAGCCAAAAAAGGAGCCAAGCAGCTCACCCTGGAGGACCCGCAGATGTCCATGAGCCACC CCTACACCATGACAACGCCTTACCCTTCGCTCCCAGCCCAGCAGGTTCACAACTACATGATGCCACCCCTCGACCGAAGCTGGAGGGACTA CGTCCCGGATCAGCCACACCCGGAAATCCCGTACCAATGTCCCATGACGTTTGGACCCCGCGGCCACCACTGGCAAGGCCCAGCTTGTGA AAATGGTTGCCAGGTGACAGGAACCTTTTATGCTTGTGCCCCACCTGAGTCCCAGGCTCCCGGAGTCCCCACAGAGCCAAGCATAAGGTCT GCCGAAGCCTTGGCGTTCTCAGACTGCCGGCTGCACATCTGCCTGTACTACCGGGAAATCCTCGTGAAGGAGCTGACCACGTCCAGCCCC GAGGGCTGCCGGATCTCCCATGGACATACGTATGACGCCAGCAACCTGGACCAGGTCCTGTTCCCCTACCCAGAGGACAATGGCCAGAGG AAAAACATTGAGAAGCTGCTGAGCCACCTGGAGAGGGGCGTGGTCCTCTGGATGGCCCCCGACGGGCTCTATGCGAAAAGACTGTGCCAG AGCAGGATCTACTGGGACGGGCCCCTGGCGCTGTGCAACGACCGGCCCAACAAACTGGAGAGAGACCAGACCTGCAAGCTCTTTGACACA CAGCAGTTCTTGTCAGAGCTGCAAGCGTTTGCTCACCACGGCCGCTCCCTGCCAAGATTCCAGGTGACTCTATGCTTTGGAGAGGAGTTTCC AGACCCTCAGAGGCAAAGAAAGCTCATCACAGCTCACGTAGAACCTCTGCTAGCCAGACAACTATATTATTTTGCTCAACAAAACAGTGGACA TTTCCTGAGGGGCTACGATTTACCAGAACACATCAGCAATCCAGAAGATTACCACAGATCTATCCGCCATTCCTCTATTCAAGAATGA
Primers used to clone pCW-IRF4-Puro	
Name	**Sequence**
3672	ACCGTCAGATCGCCTGGAGAATTGGGCCGCCACCATGAACCTGGAGGGCGGCGG
3673	GTGTCGACTGCACCGGTGTCGCTAGTCATTCTTGAATAGAGGAATGGC

Table S2. Primers and dsDNA fragments used in this study.

ID	Gene	Target Region	Target Sequence	Hairpin Sequence
sh1	IKZF1	CDS	AGCTACGAGAAGGAGAACGAAA	TGCTGTTGACAGTGAGCGCGCTACGAGAAGGAGAACGAAATAGTGAAGCCACAGATGTATTTCGTTCTCCTTCTCGTAGCTTGCCTACTGCCTCGGA
sh2	IKZF1	CDS	CGCAGCGGTCTCATCTACCTGA	TGCTGTTGACAGTGAGCGAGCAGCGGTCTCATCTACCTGATAGTGAAGCCACAGATGTATCAGGTAGATGAGACCGCTGCGTGCCTACTGCCTCGGA
sh1	IKZF3	CDS	GAAATCCCTTACAGCTATTCAA	TGCTGTTGACAGTGAGCGAAAATCCCTTACAGCTATTCAATAGTGAAGCCACAGATGTATTGAATAGCTGTAAGGGATTTCTGCCTACTGCCTCGGA
sh2	IKZF3	CDS	AGAACGCCAGAATCACATCTA	TGCTGTTGACAGTGAGCGCGAACGCCAGAATCACATCTAATAGTGAAGCCACAGATGTATTAGATGTGATTCTGGCGTTCTTGCCTACTGCCTCGGA

Table S3. shRNA sequences used in this study.

Antibody	Vendor	Catalog #	Dilution used	Blocking agent	Primary Antibody Diluted In
IKZF1	Santa Cruz	sc-13039	1:1000	5% milk in 1X TBS	5% milk in 1X TBST
IKZF3	Cell Signaling	15103	1:1000	5% milk in 1X TBS	5% milk in 1X TBST
IRF4	Cell Signaling	15106	1:1000	5% milk in 1X TBS	5% milk in 1X TBST
MYC	Cell Signaling	13987	1:1000	5% milk in 1X TBS	5% milk in 1X TBST
GAPDH	Cell Signaling	2118	1:5000	5% milk in 1X TBS	5% milk in 1X TBST
Flag	Sigma Aldrich	F1804	1:2000	5% milk in 1X TBS	5% milk in 1X TBST
CRBN	Sigma Aldrich	HPA045910	1:1000	5% milk in 1X TBS	5% milk in 1X TBST
ZFP91	Bethyl	A303-245A	1:1000	5% milk in 1X TBS	5% milk in 1X TBST
CK1α	Bethyl	A301-991A	1:1000	Odyssey Blocking buffer (LICOR Biosciences cat# 927-50100)	Odyssey Blocking buffer (LICOR Biosciences cat# 927-50100)

Table S4. Antibodies used in this study.