Supplementary Materials for

UBR5 promotes tumor immune evasion through enhancing IFN-γ-induced *PDL1* transcription in triple negative breast cancer

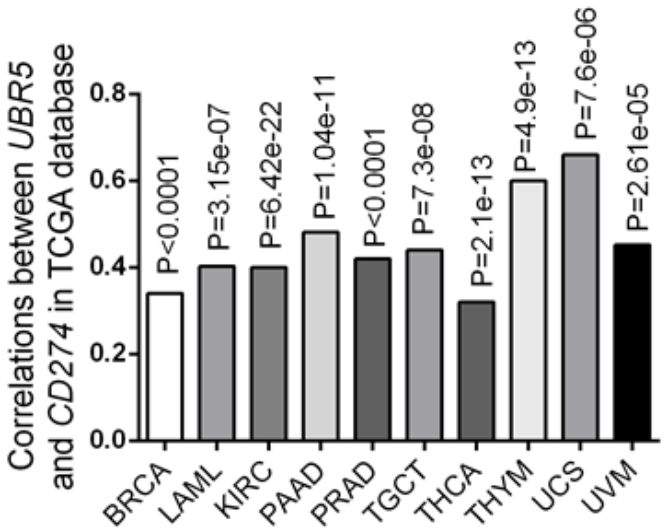


Figure S1. *UBR5* and *PDL1* are positively correlated in multiple cancer types.

The correlation of mRNA expression between *UBR5* and *PDL1* was assessed in TCGA database, normalized by GAPDH. Breast Invasive Carcinoma (BRCA), Acute Myeloid Leukemia (LAML), Kidney Renal Clear Cell Carcinoma (KIRC), Pancreatic Adenocarcinoma (PAAD), Prostate Adenocarcinoma (PRAD), Testicular Germ Cell Tumors (TGCT), Thyroid Carcinoma (THCA), Thymoma (THYM), Uterine Carcinosarcoma (UCS) and Uveal Melanoma (UVM)

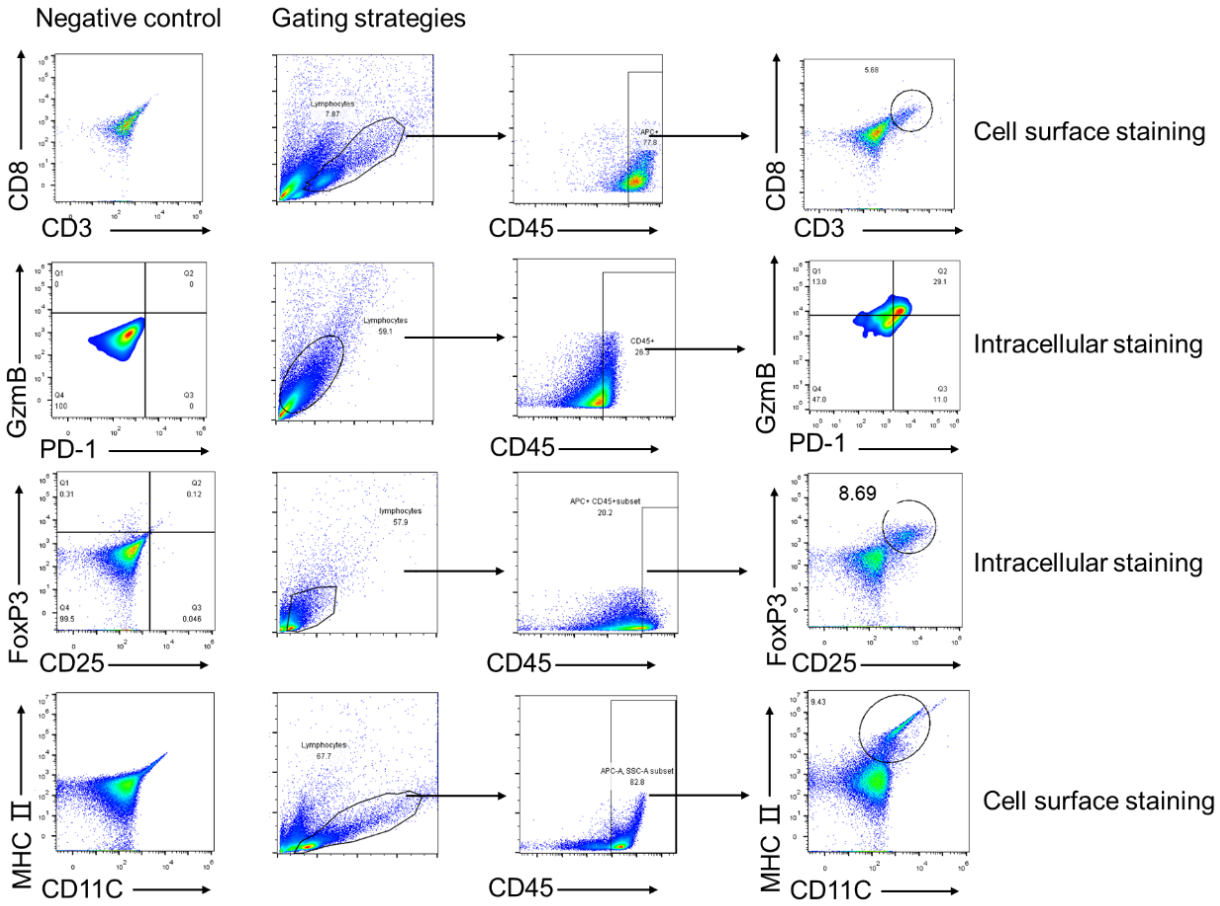


Figure S2. The negative control and gating strategies of FACS analysis.

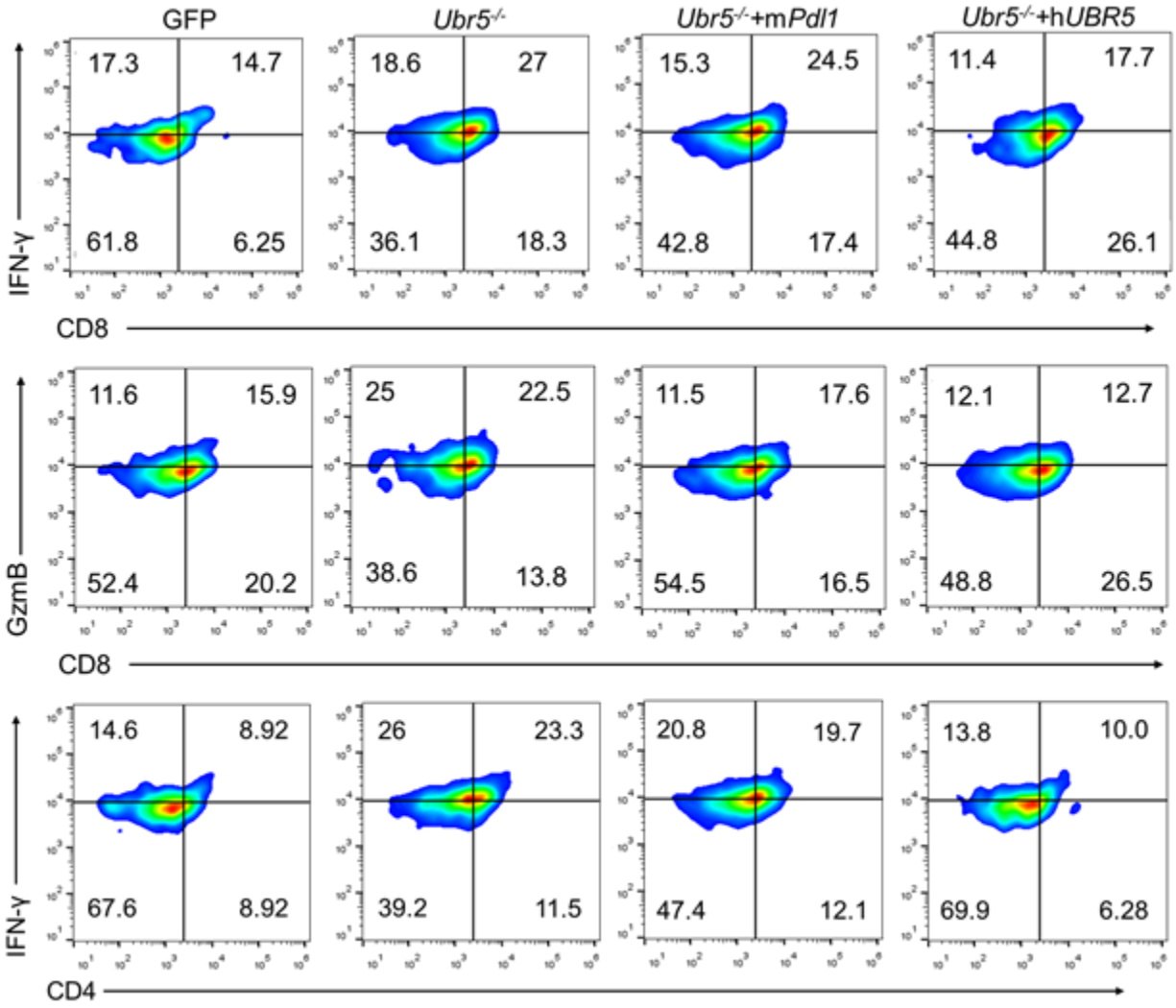


Figure S3. Lower levels of IFN-γ and GzmB were produced by infiltration T cells in tumors of mice bearing h*UBR5* and m*Pdl1*-reconstituted *Ubr5*$^{-/-}$ 4T1 tumor than in *Ubr5*$^{-/-}$ 4T1 tumor.

The percentage of IFN-γ and GzmB producing by CD8^{+} T cells and IFN-γ producing by CD4^{+} T cells were analyzed by flow cytometry in tumors of mice bearing WT, *Ubr5*$^{-/-}$, h*UBR5* or m*Pdl1*-reconstituted *Ubr5*$^{-/-}$ 4T1 tumors.

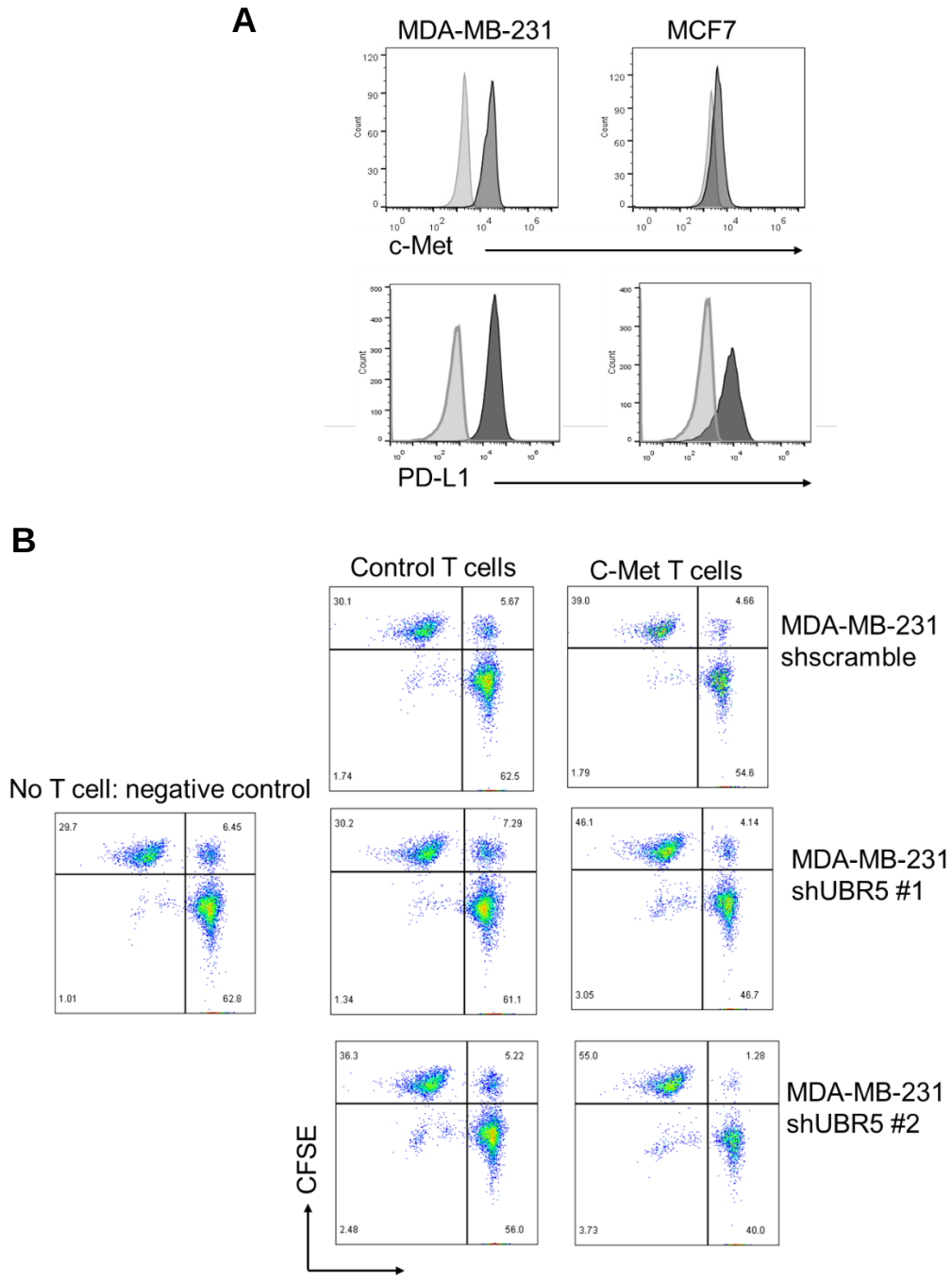


Figure S4. UBR5 deficient decreased PD-L1 expression levels in MDA-MB-231 cells and could promote c-Met specific chimeric antigen receptor T cells-mediated killing efficiency.

(A) The expression of c-Met and PD-L1 in MDA-MB-231 and MCF cells were evaluated by flow cytometry. (B) T cells Cytotoxicity difference toward BT549 cells with different UBR5 expression levels. dt-Tomato Red stably expressed MDA-MB-231 cells (target cells) were mixed with CFSE labeled MCF7 cells (non-target cells) at a ratio of 1:1, and then co-cultured for 18 h with either control or c-Met specific chimeric antigen receptor T cells at a ratio of 1:2 separately. Cells were harvested and analyzed by flow cytometry. The data are presented as the mean ± SEM (error bar) from three replicates. *P< 0.05.

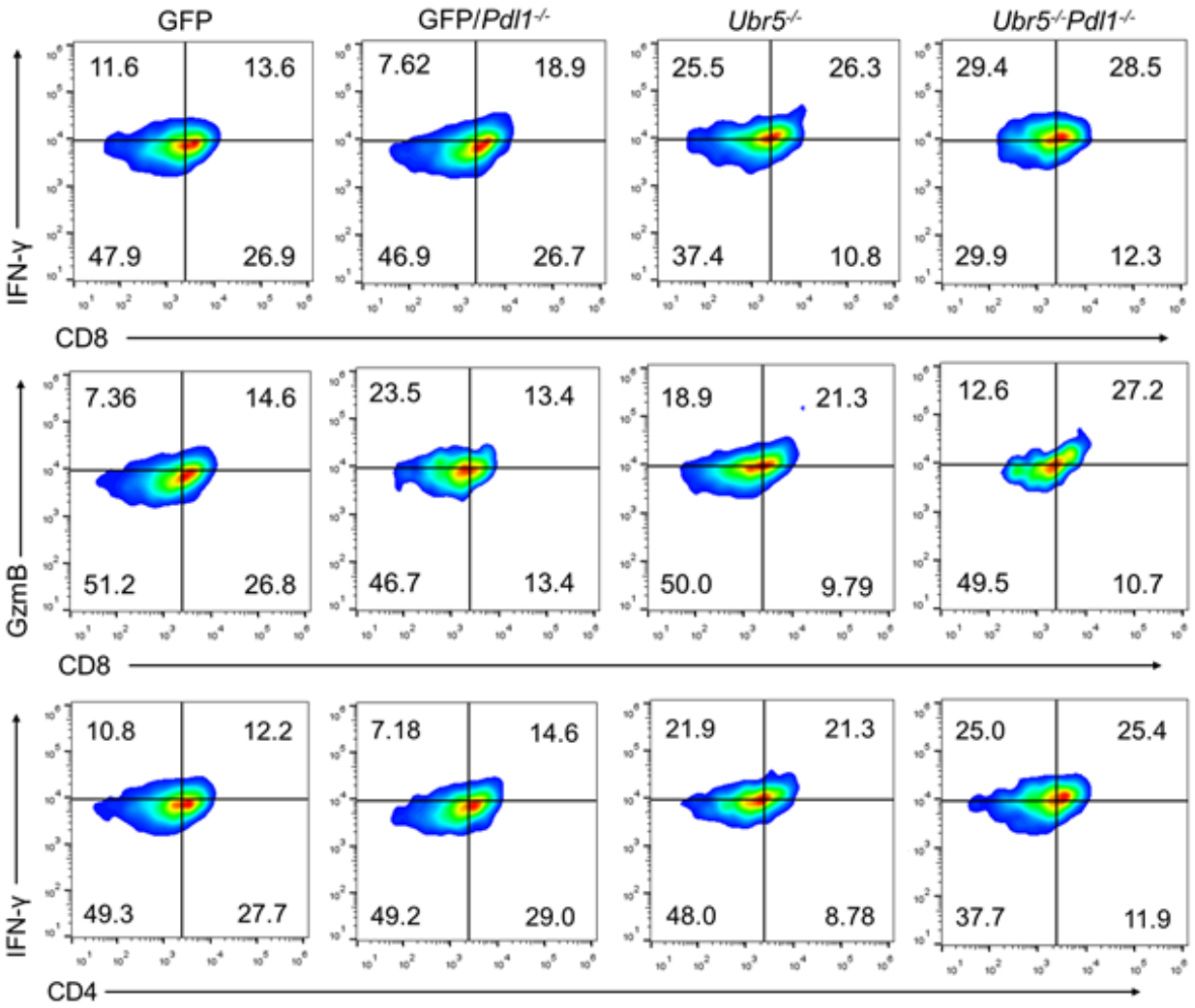


Figure S5. More IFN-γ and GzmB were produced by infiltration T cells in tumors of mice bearing *Ubr5*$^{-/-}$*Pdl1*$^{-/-}$ 4T1 tumor than in WT, *Pdl1*$^{-/-}$, *Ubr5*$^{-/-}$ 4T1 tumor.

The percentage of IFN-γ and GzmB producing by CD8^{+} T cells and IFN-γ producing by CD4^{+} T cells were analyzed by flow cytometry in tumors of mice bearing WT, *Ubr5*$^{-/-}$, *Pdl1*$^{-/-}$, *Ubr5*$^{-/-}$ *Pdl1*$^{-/-}$ 4T1 tumors.

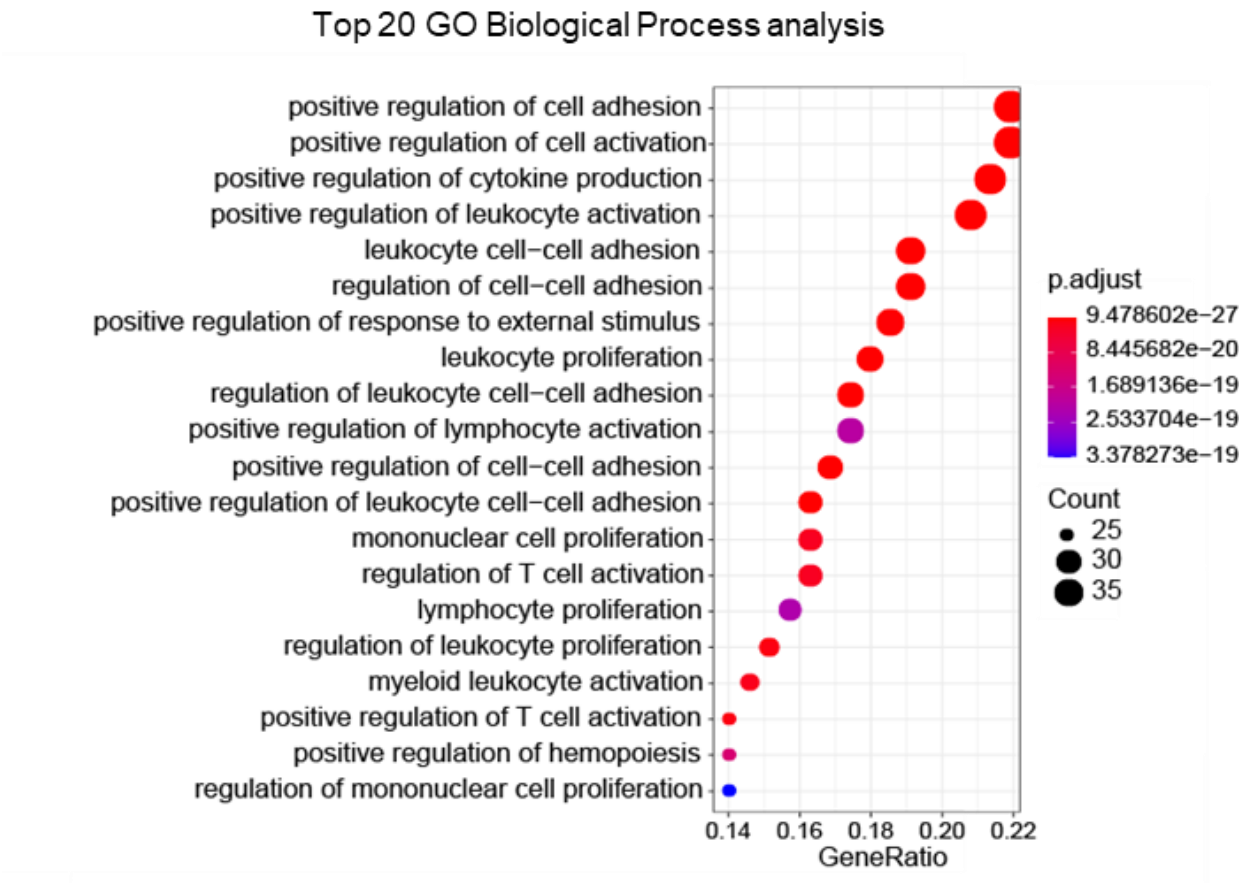


Figure S6. Gene Ontology (GO) analysis of the biological process between GFP and *Ubr5*$^{-/-}$ 4T1 cells treated with IFN-γ.

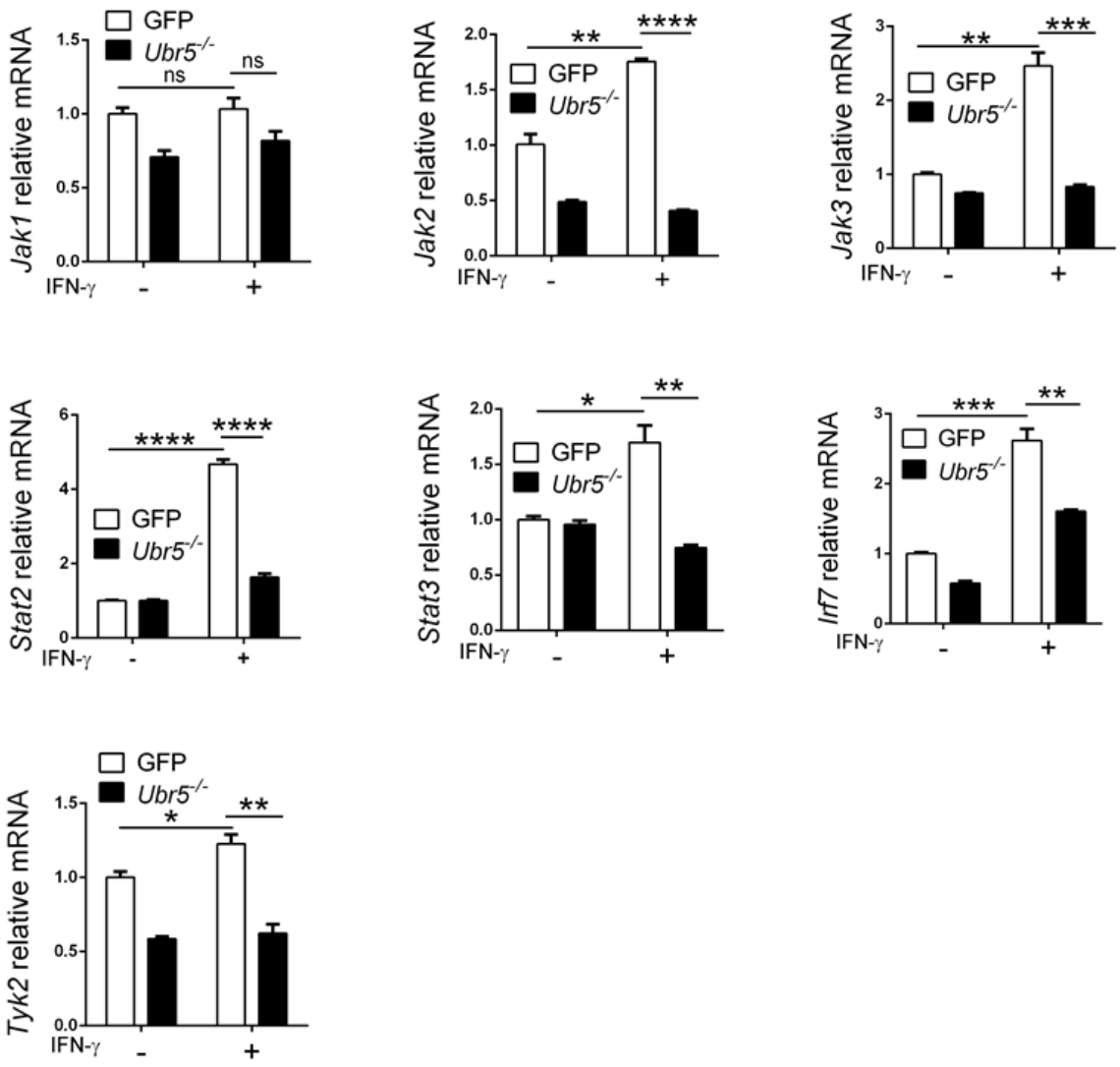


Figure S7. Relative mRNA levels of *Jak1/2/3*, *Stat2/3*, *Irf7* and *Tyk2* in GFP and *Ubr5*$^{-/-}$ cells treated with or without IFN-γ.

The mRNA levels of *Jak1/2/3*, *Stat2/3*, *Irf7* and *Tyk2* in GFP and *Ubr5*$^{-/-}$ 4T1 cells (treated with or without IFN-γ) were detected. GAPDH was used for normalization. Results are presented as mean ± SEM of three individual experiments. ns, no significance, *P<0.05, **P<0.01, ***P< 0.001, ****P < 0.0001.

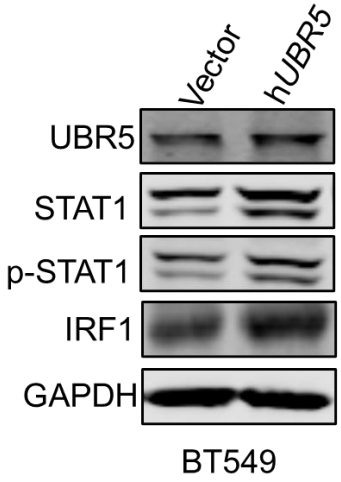


Figure S8. Overexpression UBR5 increased the protein levels of STAT1, pSTAT1 and IRF1 in BT549 cells.

BT549 cells were transfected with either an empty vector or UBR5 plasmids. 24 hours later, the cells were treated with IFN-γ for 24 h. Then, the protein levels of STAT1, pSTAT1 and IRF1 were measured by western blot.

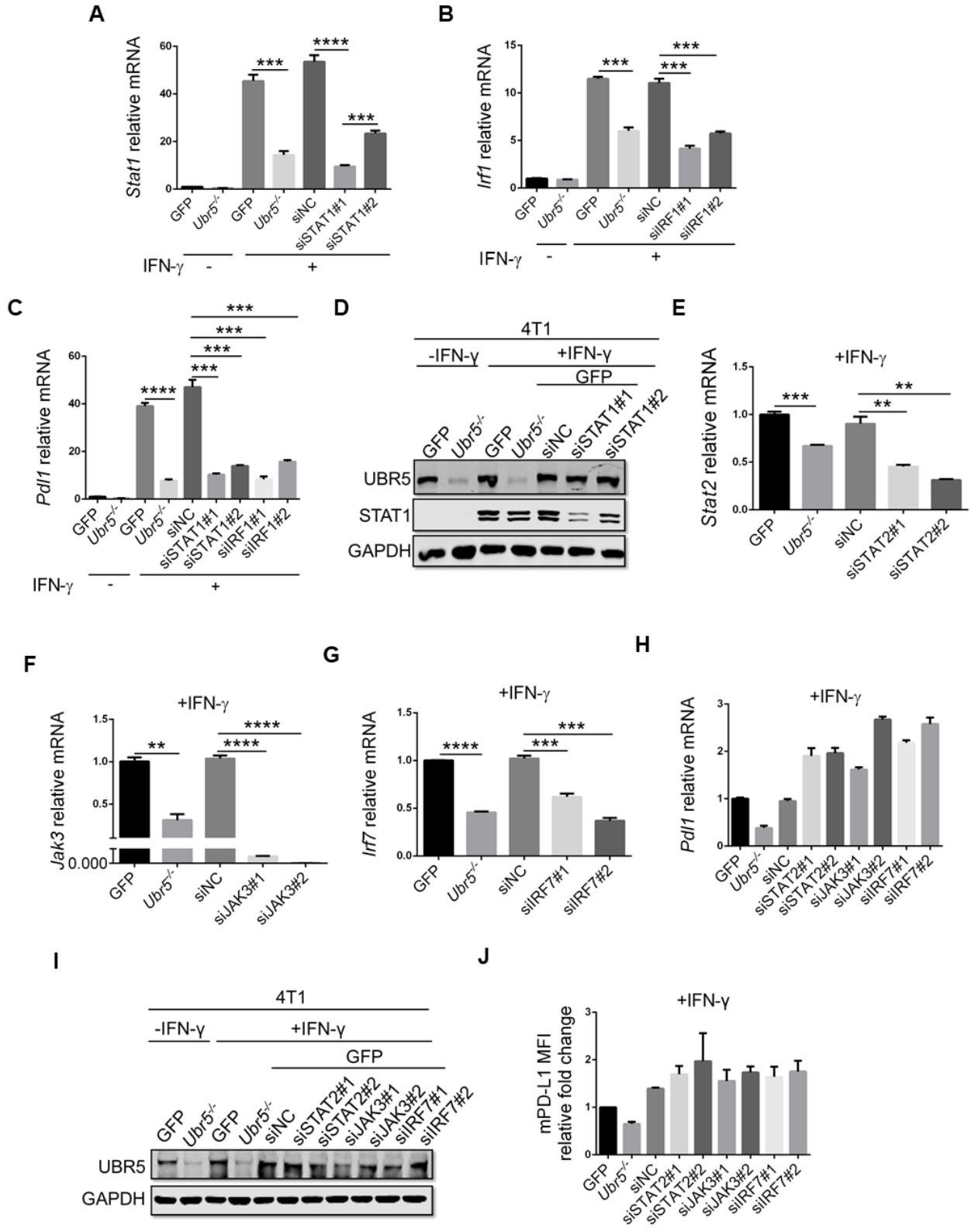


Figure S9. The regulation of PD-L1 by UBR5 is mediated through STAT1 and IRF1 rather than JAK3, STAT2 or IRF7.

(A-D) The GAPDH normalized mRNA levels of *Stat1* (A), *Irf1* (B) and *Pdl1* (C), protein levels of UBR5 and STAT1 (D) were detected separately in siSTAT1 (A) or siIRF1 (B) transiently transfected 4T1/GFP cells. GFP and *Ubr5*$^{-/-}$ 4T1 treated with or without IFN-γ were used for positive and negative controls. siNC transfection served as silencing control. (E-J) The mRNA levels of *Stat2* (E), *Jak3* (F), *Irf7* (G) and *Pdl1* (H), protein of UBR5 (I), and surface PD-L1 level (J) were detected in separately in siSTAT2, siJAK3, siIRF7 transiently transfected 4T1/GFP cells. GFP, *Ubr5*$^{-/-}$ 4T1 cells with IFN-γ stimulation were used for positive and negative controls. GAPDH was used for normalization. Results are presented as mean ± SEM of three individual experiments. ***P< 0.001, ****P < 0.0001.

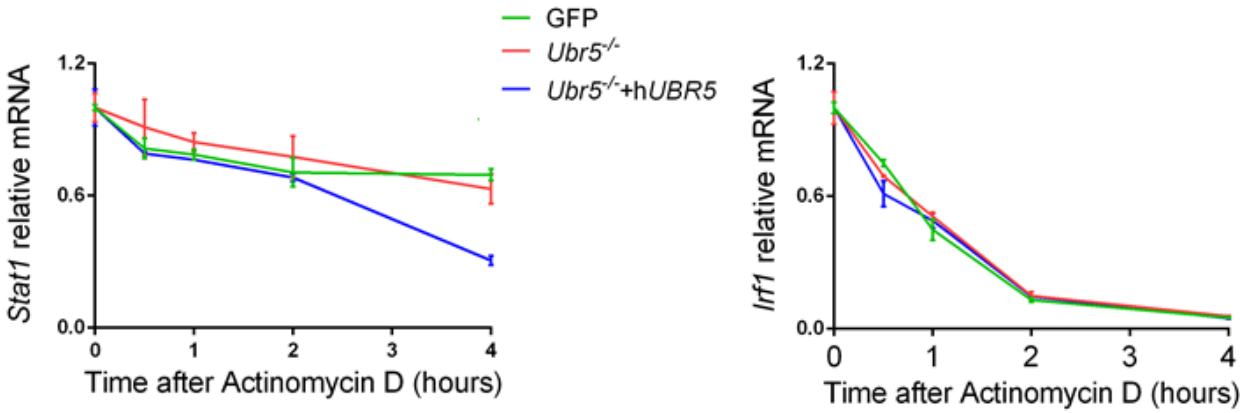
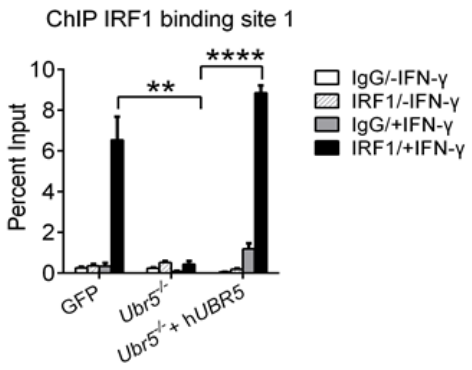


Figure S10.The regulation of *STAT1* and *IRF1* by UBR5 does not depend on mRNA stability.

IFN-γ-treated WT, *Ubr5*$^{-/-}$, and h*UBR5*-reconstituted *Ubr5*$^{-/-}$ 4T1 cells were treated with the transcription inhibitor actinomycin D (1 μg/mL). *Stat1 and Irf1* mRNA levels were quantified using qPCR (±SEM, n = 3). GAPDH was used for normalization. The results are presented as the mean ± SEM from three repliations.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6
4
2
0
**
****
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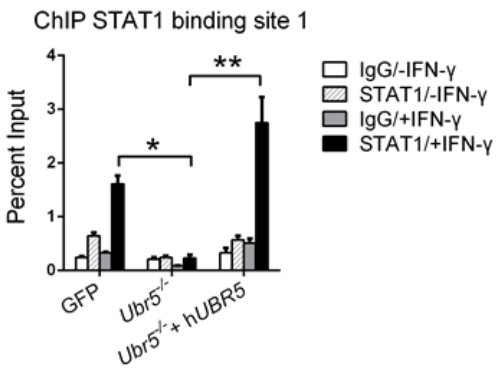
IRF1/-IFN-γ
IgG/+IFN-γ
IRF1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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Percent Input
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2
1
0
*
**
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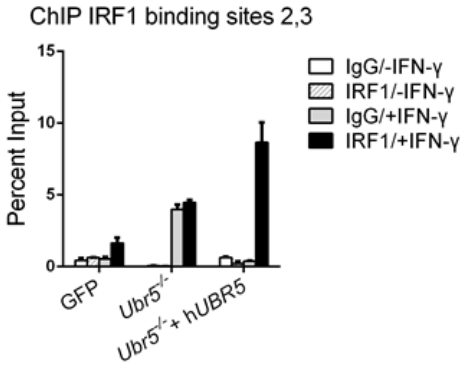
STAT1/-IFN-γ
IgG/+IFN-γ
STAT1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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Percent Input
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0
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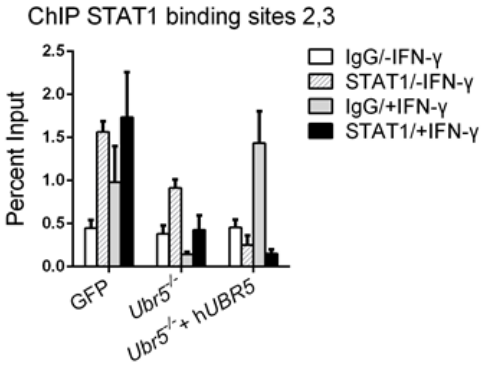
IRF1/-IFN-γ
IgG/+IFN-γ
IRF1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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0.5
0.0
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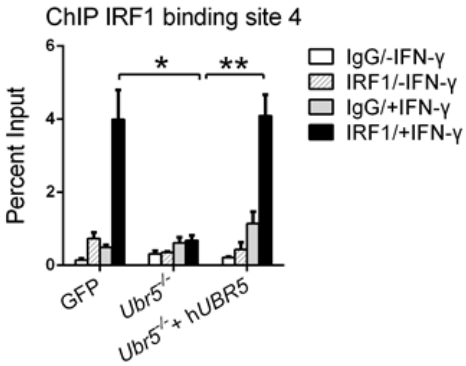
STAT1/-IFN-γ
IgG/+IFN-γ
STAT1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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Percent Input
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2
0
*
**
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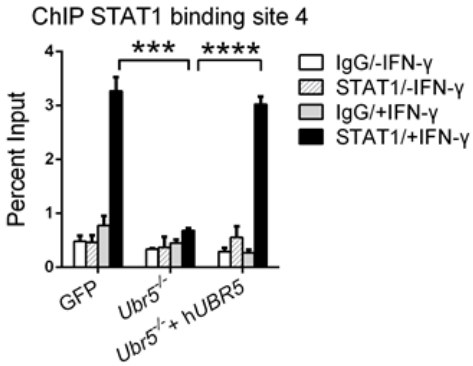
IRF1/-IFN-γ
IgG/+IFN-γ
IRF1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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Percent Input
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2
1
0
***
****
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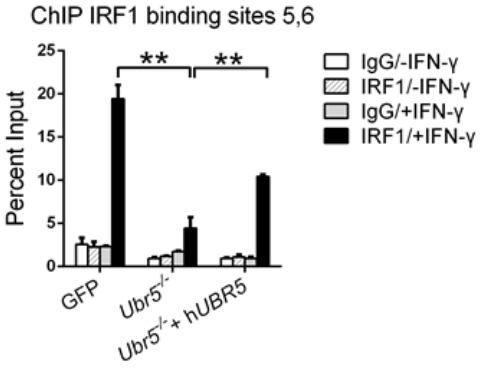
STAT1/-IFN-γ
IgG/+IFN-γ
STAT1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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Percent Input
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15
10
5
0
**
**
IgG/-IFN-γ
IRF1/-IFN-γ
IgG/+IFN-γ
IRF1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5

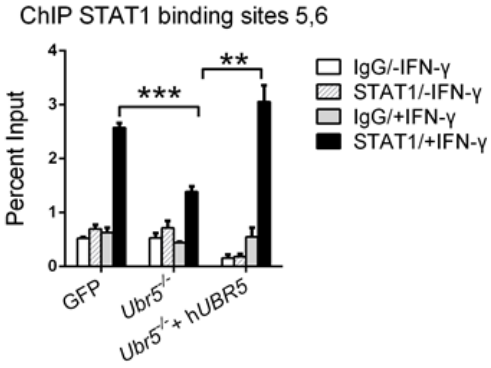
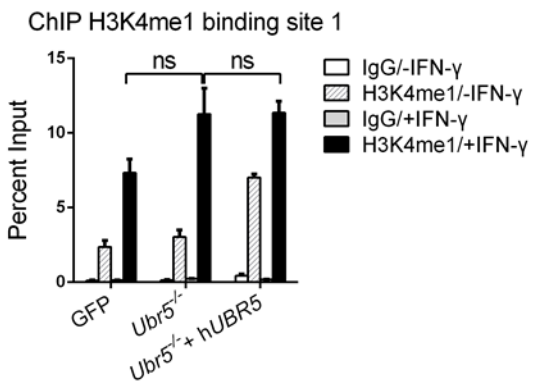

ChIP STAT1 binding sites 5,6
Percent Input
4
3
2
1
0
***
**
IgG/-IFN-γ
STAT1/-IFN-γ
IgG/+IFN-γ
STAT1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5

Figure S11. The enrichment of STAT1 and IRF1 in the *Pdl1* promoter region is affected by the expression of UBR5.

ChIP assay was performed using anti-IRF1 and STAT1 antibodies in WT, *Ubr5*$^{-/-}$ or h*UBR5*-reconstituted *Ubr5*$^{-/-}$ 4T1 cells after treatment with or without IFN-γ.
Results are presented as mean ± SEM of three replications. *P< 0.05, **P < 0.01, ***P< 0.001, ****P < 0.0001.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Percent Input
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10
5
0
ns
ns
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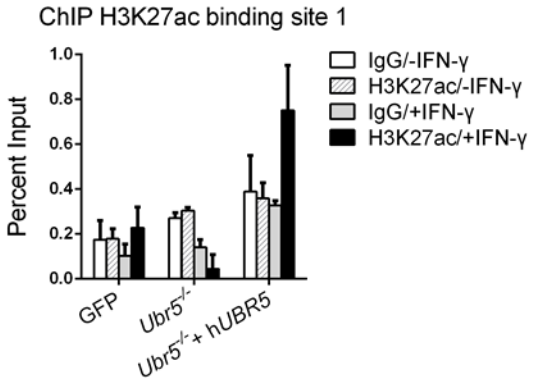
H3K4me1/-IFN-γ
IgG/+IFN-γ
H3K4me1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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0.0
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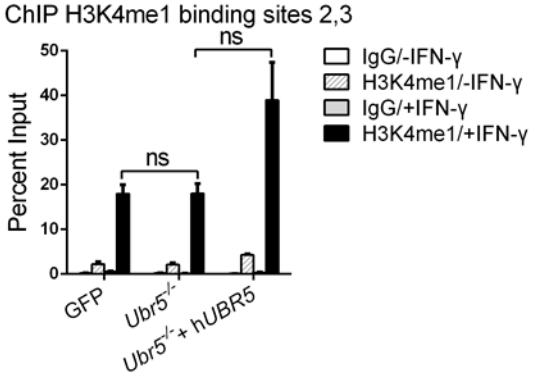
IgG/+IFN-γ
H3K27ac/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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Percent Input
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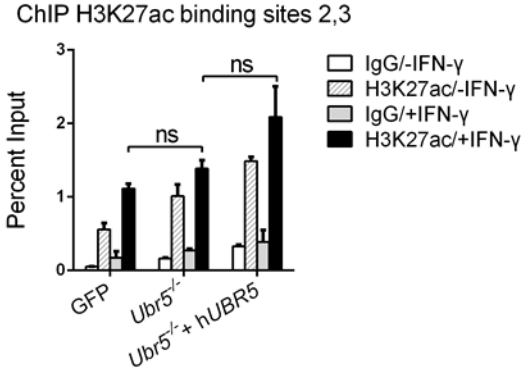
40
30
20
10
0
ns
ns
IgG/-IFN-γ
H3K4me1/-IFN-γ
IgG/+IFN-γ
H3K4me1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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Percent Input
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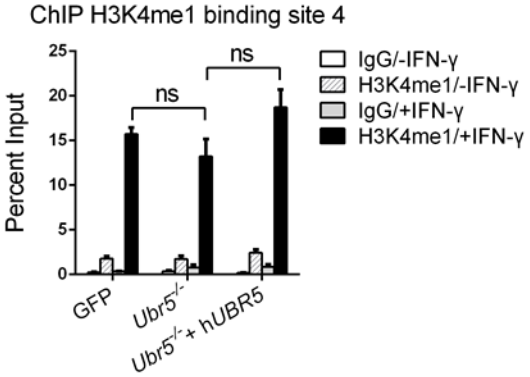
2
1
0
ns
ns
IgG/-IFN-γ
H3K27ac/-IFN-γ
IgG/+IFN-γ
H3K27ac/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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Percent Input
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ns
ns
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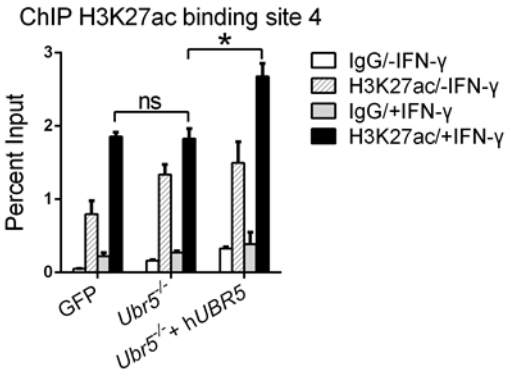
GFP
Ubr5-/-
Ubr5-/- + hUBR5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0
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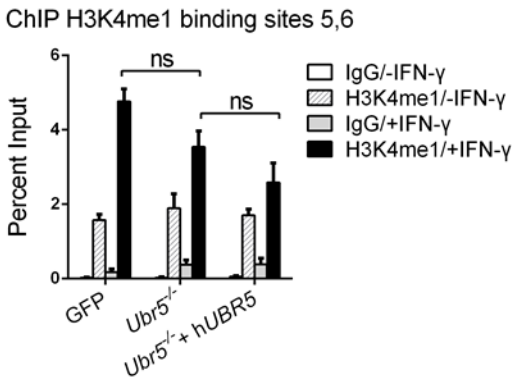
H3K27ac/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5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Percent Input
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4
2
0
ns
ns
IgG/-IFN-γ
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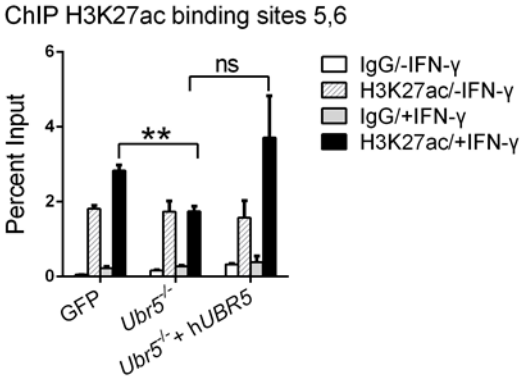
IgG/+IFN-γ
H3K4me1/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5


ChIP H3K27ac binding sites 5,6
Percent Input
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4
2
0
ns
**
IgG/-IFN-γ
H3K27ac/-IFN-γ
IgG/+IFN-γ
H3K27ac/+IFN-γ
GFP
Ubr5-/-
Ubr5-/- + hUBR5

Figure S12. The methylation of H3K4 and acetylation of H3K27 are not involved in *PDL1* transcription regulation by UBR5.

ChIP assay was performed using anti-H3K4me1 and H3K27ac antibodies in WT, *Ubr5*$^{-/-}$ or h*UBR5*-reconstituted-*Ubr5*$^{-/-}$ 4T1 cells after treated with or without IFN-γ.
Results are presented as mean ± SEM of three replications. ns, no significance, *P<0.05, **P<0.01.

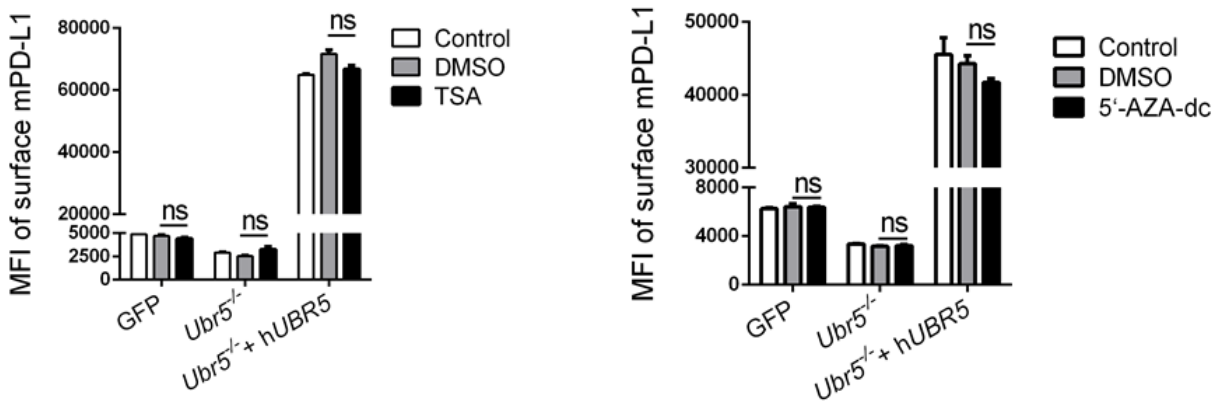

Figure S13. Histone acetylation and methylation are not involved in *PDL1* transcription regulation by UBR5.

IFN-γ-pretreated WT, *Ubr5*$^{-/-}$ and hUBR5-reconstituted *Ubr5*$^{-/-}$ 4T1 cells were treated with 150 nmol/L trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, for 24 hours (left panel) or 2 mmol/L 5-aza-2'-deoxycytidine (5'-AZA-dC), a DNA methylation inhibitor, for 48 hours (right panel). Surface PD-L1 levels were measured by FACS. The results are presented as the mean ± SEM from three individual experiments.

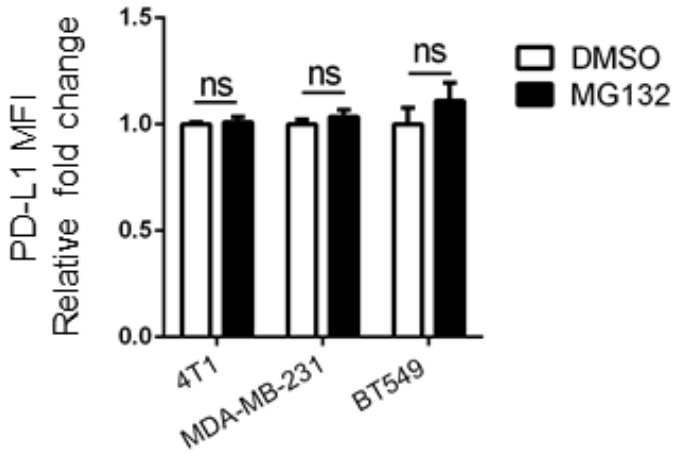

Figure S14. The expression of PD-L1 surface protein is not influenced by UBR5 in the degradation stage.

IFN-γ pre-treated 4T1, MDA-MB-231 and BT549 cells were treated with DMSO or 10 μmol/L MG132 for 6 h. Surface PD-L1 level was measured by FACS.
Results are presented as mean ± SEM of three individual experiments. ns, no significance.

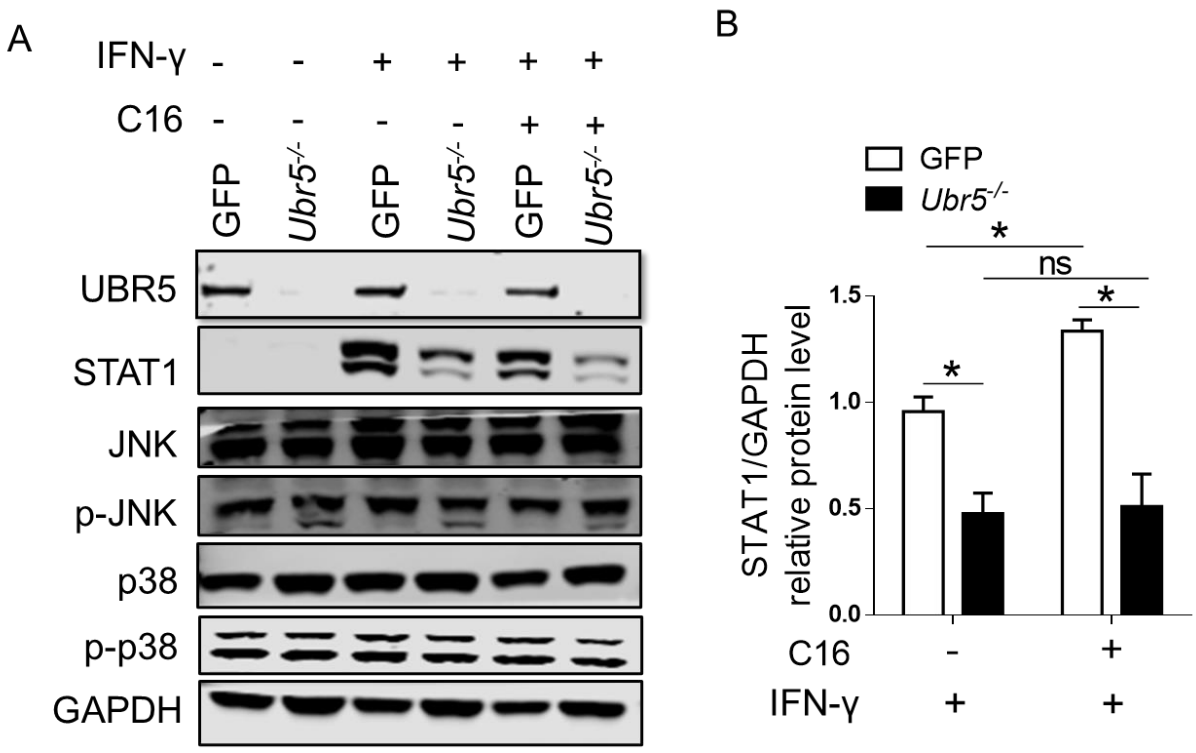

Figure S15. The regulatory activity of UBR5 on STAT1 is no difference when the kinase activity of PKR is inhibited.

(A)The protein levels of STAT1, p38 and JNK (and their phosphorylation form) were detected in GFP and *Ubr5*$^{-/-}$ 4T1 cells that were pre-treated with 0.5 μM PKR inhibitor C16 for 4 h, followed with IFN-γ stimulation 24 h. (B) The quantitative results of STAT1 protein levels are relative to GAPDH with or without C16 treatment in GFP and *Ubr5*$^{-/-}$ 4T1 cells in the presence of IFN-γ.

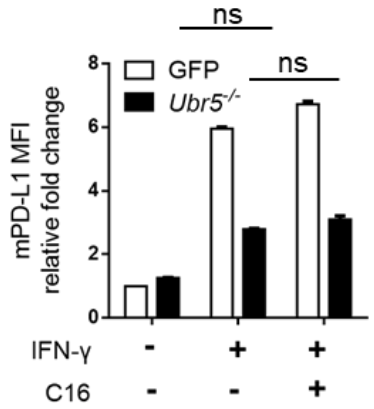

Figure S16. PKR participates in UBR5-mediated *PDL1* transcription activation in a kinase-independent manner.

Surface PD-L1 levels were detected in GFP and *Ubr5*$^{-/-}$ 4T1 cells pretreated with 0.5 μM C16 (PKR inhibitor) for 4 h and then stimulated or not stimulated with IFN-γ for 24 h.
The results are presented as the mean ± SEM from three individual experiments. ns, no significant.

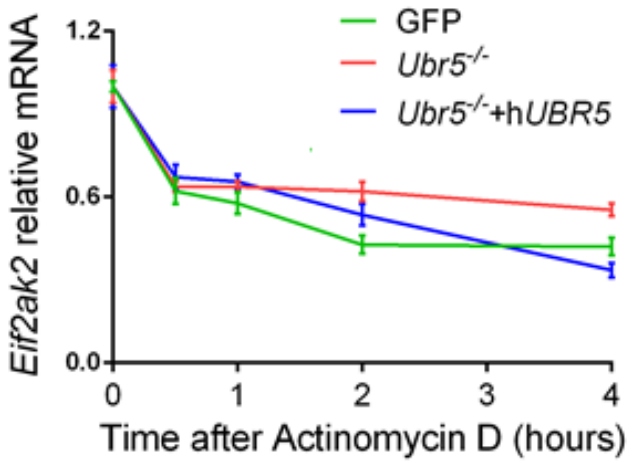

Figure S17. The stability of *Eif2ak2* mRNA is not influenced by UBR5.

IFN-γ-pretreated WT, *Ubr5*$^{-/-}$, and h*UBR5*-reconstituted *Ubr5*$^{-/-}$ 4T1 cells were treated with the transcription inhibitor actinomycin D (1 μg/mL). *Eif2ak2* mRNA levels were quantified using qPCR. GAPDH was used for normalization. The results are presented as the mean ± SEM from three replications.

Table S1. Primers used in plasmids construction

Gene name	Primer Sequences
mPD-L1-promoter (-2000-+11bp)	Forward (KpnI) : GCCGGTACCCCAAACCACCTCACCTCTTC Reverse (NheI): GCCGCTAGCTTGGGGACCACGATTTCCTG
mIRF1 promoter (-2000-+100 bp)	Forward (KpnI): GGGTACCCCCAGCATTAGGAAATAGAAGC Reverse (NheI): CTAGCTAGCTCAGCTGAAGCCCAGGCAGA
mSTAT1 promoter (-970-+16bp)	Forward (KpnI): GGGGTACCAAAAGAAAACTATTCTTTAAAATGTG Reverse (NheI): CTAGCTAGCCATCCTCTGCAGAAAGAACATGCG
mEif2ak2	Forward (AvrII): CCTAGGATGGCCAGTGATACCCCAGG Reverse (SalI): GTCGACCTAACATGTGTTTCTTTTCTT
mPD-L1	Forward (XbaI): GCTCTAGAATGAGGATATTTGCTGGCAT Reverse (EcoRI): CCGGAATTCTTACGTCTCCTCGAATTGTG
hPDL1 promoter (-2000-0 bp)	Forward (KpnI): GGGGTACCTTTCTCTTTTTCTAAACACAGCCTG Reverse (NheI): CTAGCTAGCGGGGCCGCGCGGGACGCGCCAG
hIRF1 promoter (-820-+138bp)	Forward (KpnI): CGGGTACCCGACCTTGAAAACTACTCAGC Reverse (NheI): CTAGCTAGCAAGAGGGAAGAAGGCAGAG
hSTAT1 promoter (-972-+884bp)	Forward (KpnI): GAGGTACCTGTCATGGGAGGAAACTGGTGG Reverse (NheI): CTAGCTAGCCCTTATCTATACAAACAACATTC
UBR5-ΔPABC overlap PCR upstream	Forward (BstBI): GTTCGAAATGATGACGTCCATCCATTTCGT Reverse: ATCAGCTCCATTTTCTCTAAAGGGCCTAGT
UBR5-ΔPABC overlap PCR downstream	Forward: ACTAGGCCCTTTAGAGAAAATGGAGCTGAT Reverse (NotI): AAGCGGCCGCTTACTACACAAAACCAAAATTCTTG
hPD-L1 promoter ΔIRF1 overlap PCR upstream	Forward (SacI): CGAGCTCTAGAAGTTCAGCGCGGGATA Reverse: GTGTATAGAAATGAAGTCCAGTTTTCTTGT

hPD-L1 promoter ΔIRF1- overlap PCR downstream	Forward: ACAAGAAAACTGGACTTCATTTCTATACAC Reverse (BglII): GAAGATCTCAGCGAGCTAGCCAGAGATACT
hPD-L1 promoter ΔSTAT1/3 overlap PCR upstream	Forward (SacI): CGAGCTCTAGAAGTTCAGCGCGGGATA Reverse: TTATCAGAAAGGCGTCTTCAAGGTGACTGA
hPD-L1 promoter ΔSTAT1/3 overlap PCR downstream	Forward: TCAGTCACCTTGAAGACGCCTTTCTGATAA Reverse (BglII): GAAGATCTCAGCGAGCTAGCCAGAGATACT

Table S2. Targeting sequences for gene knockdown or knockout

Gene name	Primer Sequences
shEIF2AK2-1-mus	Forward: TCGAGTGCTGTTGACAGTGAGCGACGCCAGGTTTAACAGCG ATTTTAGTGAAGCCACAGATGTAAAATCGCTGTTAAACCTG GCG GTGCCTACTGCCTCGGAA Reverse: CGCGTTCCGAGGCAGTAGGCACCGCCAGGTTTAACAGCGAT TTTACATCTGTGGCTTCACTAAAATCGCTGTTAAACCTGGCG TCGCTCACTGTCAACAGCAC
shEIF2AK2-2-mus	Forward: TCGAGTGCTGTTGACAGTGAGCGAGGAGTAGCCATTACGTA TAAATAGTGAAGCCACAGATGTATTTATACGTAATGGCTAC TCCGTGCCTACTGCCTCGGAA Reverse: CGCGTTCCGAGGCAGTAGGCACGGAGTAGCCATTACGTATA AATACATCTGTGGCTTCACTATTTATACGTAATGGCTACTCC TCGCTCACTGTCAACAGCAC
shScramble-homo	Forward: TCGAGTGCTGTTGACAGTGAGCGACCGCAGGTATGCACGCG TTAGTGAAGCCACAGATGTAACGCGTGCATACCTGCGGGTG CCTACTGCCTCGGAA Reverse: CGCGTTCCGAGGCAGTAGGCACCCGCAGGTATGCACGCGTT ACATCTGTGGCTTCACTAACGCGTGCATACCTGCGGTCGCT CACTGTCAACAGCAC
shUBR5-1-homo	Forward: TCGAGTGCTGTTGACAGTGAGCGATTGGAACAGGCTACTAT TAAATAGTGAAGCCACAGATGTATTTAATAGTAGCCTGTTC CAAGTGCCTACTGCCTCGGAA

	Reverse: CGCGTTCCGAGGCAGTAGGCACTTGGAACAGGCTACTATTA AATACATCTGTGGCTTCACTATTTAATAGTAGCCTGTTCCAA TCGCTCACTGTCAACAGCAC
shUBR5-2-homo	Forward: TCGAGTGCTGTTGACAGTGAGCGACAACTTAGATCTCCTGA AATAGTGAAGCCACAGATGTATTTCAGGAGATCTAAGTTGG TGCCTACTGCCTCGGAA Reverse: CGCGTTCCGAGGCAGTAGGCACCAACTTAGATCTCCTGAAA TACATCTGTGGCTTCACTATTTCAGGAGATCTAAGTTGTCGC TCACTGTCAACAGCAC
CD274-sgRNA#1	AGGTTTCCTGAACATGCACC
CD274-sgRNA#2	CATACCGTCGTTGCAGTGCT
CD274-sgRNA#3	GACCTAGGTGCCTTTAAGAG

Table S3. Primers used in quantitative RT-PCR (RT-qPCR) assays

Gene name	Primer Sequences
GAPDH (Mus)	Forward: AGGTCGGTGTGAACGGATTTG Reverse: TGTAGACCATGTAGTTGAGGTCA
PD-L1 (Mus)	Forward: AGTATGGCAGCAACGTCACG Reverse: TCCTTTTCCCAGTACACCACTA
UBR5 (Mus)	Forward: GTCCATCCATTTCGTGGTCCA Reverse: GGGTGGCTGTTCAAATTGTACTT
IRF1 (Mus)	Forward: GTTGTGCCATGAACTCCCTG Reverse: GTGTCCGGGCTAACATCTCC
STAT1 (Mus)	Forward: GCTGCCTATGATGTCTCGTTT Reverse: TGCTTTTCCGTATGTTGTGCT
Eif2ak2 (Mus)	Forward: ATGCACGGAGTAGCCATTACG Reverse: TGACAATCCACCTTGTTTTCGT
CD40 (Mus)	Forward: TGTCATCTGTGAAAAGGTGGTC Reverse: ACTGGAGCAGCGGTGTTATG

Siglec15 (Mus)	Forward: ACACCGCTGGCTACTTGG Reverse: GTGTGCTGTGACAAAGGCAG
Isg15 (Mus)	Forward: GGTGTCCGTGACTAACTCCAT Reverse: TGGAAAGGGTAAGACCGTCCT
GAPDH (Homo)	Forward: AATGGACAACTGGTCGTGGAC Reverse: CCCTCCAGGGGATCTGTTTG
UBR5 (Homo)	Forward: CCAGACAGATTGGAATTGGGTAA Reverse: CATGGAGAGTCGCTTGTCCT
PD-L1 (Homo)	Forward: TGGCATTTGCTGAACGCATTT Reverse: TGCAGCCAGGTCTAATTGTTTT
IRF1 (Homo)	Forward: CTGTGCGAGTGTACCGGATG Reverse: ATCCCCACATGACTTCCTCTT
STAT1 (homo)	Forward: CGGCTGAATTTCGGCACCT Reverse: CAGTAACGATGAGAGGACCCT
Eif2ak2 (homo)	Forward: TGGAAAGCGAACAAGGAGTAAG Reverse: CCAAAGCGTAGAGGTCCACTT

Table S4. Primers used in chromatin immunoprecipitation-qPCR assays

Gene name	Primer Sequences
mGAPDH-ChIP	Forward: CCTCTGCGCCCTTGAGCTAGGA Reverse: CACAAGAAGATGCGGCCGTCTC
mPD-L1 promoter binding site-1	Forward: ATTAACTAGAAATATGTTTTGTCT Reverse: AATAGAATAAAGCAATTAAAGT
mPD-L1 promoter binding site-2,3	Forward: AGTGCACTACTTTGGAATAG Reverse: AATGATGTTGAGAAAGACTTTCTGC
mPD-L1 promoter binding site-4	Forward: AACTTTGAGGAAGTCACCAA Reverse: ATATCAAGCAAATGACTCAG
mPD-L1 promoter binding site-5,6	Forward: AATATCCCAAAGCTGACTCT Reverse: TTGGGGACCACGATTTCCTG