Supplemental Information

BET-Bromodomain Inhibitors Engage
the Host Immune System and Regulate Expression
of the Immune Checkpoint Ligand PD-L1

Simon J. Hogg, Stephin J. Vervoort, Sumit Deswal, Christopher J. Ott, Jason Li, Leonie A. Cluse, Paul A. Beavis, Phillip K. Darcy, Benjamin P. Martin, Andrew Spencer, Anna K. Traunbauer, Irina Sadovnik, Karin Bauer, Peter Valent, James E. Bradner, Johannes Zuber, Jake Shortt, and Ricky W. Johnstone
SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Retroviral transduction

Retroviral transduction of freshly isolated Eμ-Myc lymphomas with murine stem-cell virus-internal ribosomal entry site-green fluorescence protein (MSCV-IRES-GFP) and Bcl2 (MSCV-IRES-GFP/Bcl-2) constructs were performed as previously described (Shortt et al., 2013). For Myc overexpression experiments, wild-type human Myc cDNA was subcloned into MSCV-IRES-GFP. For Pd-l1 rescue experiments, wild-type murine Cd274 cDNA was subcloned into MSCV-IRES-BFP.

For inducible Myc knockdown experiments, shRNAs were expressed from the TRMPV-ns vector (27991, addgene) which has been previously described (Zuber et al., 2011). shRNAs targeting murine Myc (1891 and 2105) or Renilla (REN) were subcloned into the TRMPV vector and transfected into HEK293T Phoenix packaging cells using standard calcium phosphate transfection protocols. Viral supernatant was used to transduce E μ-Myc lymphoma cells (3) or AT3 cells in RetroNectin (TaKaRa, Shiga, Japan)-pre-coated 6-well plates (Becton Dickinson, Franklin Lakes, NJ). After 72 hours, GFP-positive cells were sorted by flow cytometry. To express an rtTA in the TRMPV-transduced cells, HEK293T cells were then transfected with the pRetroX-Tet-On Advanced vector using the same protocol. Viral supernatant was used to transduce the GFP-sorted TRMPV-transduced Eμ-Myc and AT3 cells which were then selected for 5 days using 1 mg/mL G418 (Invitrogen).

For inducible BRD4 knockdown, shRNAs targeting BRD4 were subcloned into the TRMPVIR Tet-shRNA expression vectors (27995, addgene) and transfected into HEK293T Phoenix packaging cells, as described above. The viral supernatant was used to transduce Eμ-Myc lymphoma cells (1). After 72 hours, GFP-positive cells were sorted by flow cytometry and expanded in vitro. GFP-positive cells were treated in vitro with 1 μg/mL doxycycline (DOX, Sigma-Aldrich) to induce shRNA/DsRed expression. Cell sorting was performed on a FACSAria Fusion flow sorter (BD Biosciences). The following shRNA sequences were used: Brd4 (#498), 5'-ACTATGTTTACAAATTGTT-3', Brd4 (#500), 5'-AGCAGAAACAAACCAAAGAA-3', Myc (#1891) 5'-TGTTCCTAATTCTGTCGTCG-3', Myc (#2105) 3'-TACTATTTAAGTTTGGCCAG-5'.
Flow cytometry

Flow cytometry antibodies used in this study were: anti-mouse CD3 (pacific blue, 1:400, clone 17A2), anti-mouse CD4 (APC, 1:400, clone RM4-5), anti- anti-mouse CD8 (PE-Cy7, 1:400, clone 53-6.7), anti-mouse MHC class I (H-2Kb; Biotin, 1:200, clone AF6-88.5, anti-mouse PD-1 (FITC, 1:400, clone J43), anti-mouse Pd-l1 (PE, 1:100, clone MIH5), or anti-human PD-L1 (PE, 1:200, clone 29E.2A3. Biotinylated antibodies were subsequently incubated with Streptavidin-PE-Cy7 (1:1000, catalogue number 25-4317-82) or Streptavidin-Pacific Blue (1:1000, catalogue number 48-4317-82) for 30 minutes on ice. Anti-Armenian Hampster IgG (FITC, 1:400, catalogue number 554011), Mouse IgG2ak (PE, 1:100, clone RTK2758), Mouse IgG2bx (PE, 1:200, catalogue number 559529), and Mouse IgG2ax (Biotin, 1:200, clone eBM2a) were used as isotype control antibodies, respectively. All antibodies were purchased from BioLegend (San Diego, CA), eBiosciences (San Diego, CA), or BD Bioscience (San Diego, CA).

Quantitative real-time PCR

qRT-PCR primer sequences were:

**Mus musculus Cd274**
F: TTCGTACGGGCGTTTACTAC  
R: TCCCGTTTCTACAGGGAATCT,

**Mus musculus Gapdh**
F: CCTTCATTGACCTCAACTAC  
R: GGAAGGCCATGCCAGTGAGC,

**Mus musculus Actin**
F: AGCTTCTTTGTCAGCTCTCGTGTTG  
R: TTCTGACCCATTCCCACCACACACA,

**Mus musculus** endogenous **Myc**
F: CAGCTCCTCCTCAGAGTAG  
R: TGAGGAAACGACGAGAACAG,

**Mus musculus** transgenic **Myc**
F: TCGAACAGCTTTCCAAGCTCTGTGTG  
R: TTTAATCGAAGTGGACTGCTGCG,

**Homo sapiens** **MYC**
F: GGACGACGACCTCATCAA  
R: CCAGCTCTCCTGAGACGAGCTTT,

**Homo sapiens** **CD274**
F: TATGGTGGTGCCGACTCAAA  
R: TGGCTCCAGAATTACAAG,

**Homo sapiens** **L32**
F: TTCTGGTCCACAATGTCAAG  
R: TTGTGAGCGATCTCGGCAC
4-thiouridine (4sU) labeling of newly transcribed RNA

Cell pellets were collected and total RNA was collected using TRIzol (Thermo Fisher). 4sU-labelled RNA was then biotinylated with 2μg EZ-link biotin-HPDP (Pierce) per μg of RNA in biotinylation buffer for 1.5 hours at room temperature. The biotinylated RNA was extracted using an equal volume of chloroform, precipitated in ethanol, and resuspended in water. The biotinylated RNA fraction was then purified using μMacs Streptavidin kit (Miltenyi) and washed three times with washing buffer (5mM Tris-HCl [pH7.5], 0.5mM EDTA, 1mM NaCl in H2O). The biotinylated RNA was eluted from the magnetic beads using 100mM DTT and purified using the RNeasy MinElute cleanup kit (Qiagen). cDNA was synthesized according to the manufacturer’s instructions (Promega, Sydney, NSW) and quantitative PCR analysis of samples was performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems, Mulgrave, VIC, Australia) with SYBR-green ROX mix (Agilent, Mulgrave, VIC, Australia).

RNA sequencing and analysis

Early passage Eμ-Myc lymphoma #1 variants (#1.1 and #1.2) were cultured in the presence or absence of 250nM JQ1 for 2 hours in duplicate and cell pellets collected. Total RNA was extracted using the Nucleospin® RNA extraction kit (Macherey-Nagel, Bethlehem, PA) as per the manufacturer’s instructions. cDNA libraries were prepared and then processed for using TruSeq RNA library Prep Kit (Illumina, San Diego, CA, USA) and paired-end RNA-sequencing was performed on the HiSeq 2000. Short-read data was processed using Seqliner RNA-seq pipeline (seqliner.org). Sample QC was first performed and overrepresented sequences identified using FastQC (Babraham Bioinformatics, Babraham Institute). The RNA sequencing reads were trimmed using cutadapt(v1.7) (Martin, 2011) to remove poor quality ends. Read data was then aligned using TopHat/bowtie2 (Kim et al., 2013) to the mouse reference genome (GRCm38/mm10). Read counting was performed with HTSeq-Count from HTSeq (Anders S, 2014) using transcript annotation from Ensembl Release 73 (Flicek et al., 2014). The Voom-LIMMA workflow (Law et al., 2014) was used to normalise data and compute statistical significance of differential gene expression. GSEA2-2.2.2 was used for identification of enriched signatures obtained from the MSigDB Hallmarks datasets (Liberzon et al., 2015).
Chromatin Immunoprecipitation library preparation and sequencing analysis

DNA was quantified using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and indexed libraries were prepared using KAPA Hyper Prep Kit for Illumina platforms (Kapa Biosystems) and the SeqCap Adapter Kit (Roche) following vendor’s instructions. For library preparation of DNA immunoprecipitated with an anti-Myc antibody, libraries were prepared with the Nugen Ovation Ultralow system (v2, using Nugen protocol M01379v3). Library quality and quantity was evaluated with a Bioanalyser D1000 chip (Agilent Technologies) and size selected between 200bp and 500bp using a Pippin Prep System (Sage Science). Final QC was done using a Tape Station High Sensitivity DNA Analysis Kit (Agilent Technologies). For sequencing analysis of immunoprecipitated DNA with anti-Myc antibody, libraries were then pooled and sequenced on a HiSeq1500 (Illumina) and 13 to 18 million single-end 50 bp reads were generated per sample. For sequencing analysis of DNA immunoprecipitated with anti-BRD4 and RNA Polymerase II antibodies, libraries were then pooled and sequenced on a HiSeq2500 (Illumina) and 13 to 18 million single-end 50 bp reads were generated per sample. CASAVA (v1.8.2) was used for demultiplexing. The Fastq files generated by sequencing were aligned to the mouse reference genome (GRCm38/mm10) using bowtie (v2.2.3). Samtools (v1.3) was used for manipulation of SAM and BAM files, after which MACS (v2.0.10) was used for peak calling. Browser viewable TDF files were generated using IGVTools (v2.3.72) and ChIP-Seq tracks were visualized using IGV (v2.3.55). HOMER (v4.8.3) was used for quantification and annotation of the ChIP-Seq datasets after which R was used for visualization.

QuantSeq 3’-mRNA library preparation, sequencing and analysis

3’-mRNA sequencing reads were demultiplexed using CASAVA v1.8.2 and low-quality reads Q<30 were removed. The RNA sequencing reads were trimmed at the 5’end using cutadapt(v1.9)(Martin, 2011) to remove bias introduced by random primers used in the library preparation and 3’end trimming was performed to eliminate poly-A-tail derived reads. Reads were mapped to the reference genomes (hg19 for human and mm10 for mouse) using HISAT2. Read counting was performed using featureCounts part of the subread v1.5.0 software package (Liao et al., 2014). Differential gene expression analysis was performed using Voom-LIMMA. R was used for general figure preparation and HOMER was used for metagene analysis.
Generation and analyses of a PDAC mouse model harboring Tet-repressible (Tet-off) Myc

Tet-off Myc murine pancreatic cancer cells were generated and characterized as previously described (Lito et al., 2014). In brief, pancreatic progenitor cells isolated from a murine fetus (ED17.5–18.5) harboring a conditional endogenous KrasG12D allele (lox-STOP-lox-KrasG12D) (Jackson et al., 2001) and a conditional Trp53 deletion (Trp53-fl/fl) (Jonkers et al., 2001) were co-transduced with two retroviral constructs (pSIN-TRE3G-Myc-IRES-GFP and MSCV-Luc2-PGK-CreERT2-IRES-tTA; cloning details available upon request). After 4 days of 4-hydroxy-tamoxifen treatment, cells were orthotopically injected into the pancreas of syngeneic recipient mice, which were monitored using bioluminescence imaging (Caliper Life Sciences). Emerging tumors were characterized to display histological features of human pancreatic ductal adenocarcinoma (PDAC) and used to derive a cell line, which was cultured in DMEM (Gibco-Invitrogen) with 10% FBS, 20mM glutamate, 10mM sodium pyruvate, 100U/ml penicillin and 100 µg/ml streptomycin. For Pd-l1 expression studies, cells were treated with 250 nM JQ1, 1 µg/ml Doxycycline (D9891, Sigma Aldrich) and/or 50 ng/ml mouse recombinant IFN-γ (14-8311-63, Affymetrix) in various combinations / time courses. For flow cytometry analysis, cells were incubated with mouse TruStain fcX Fc receptor blocking agent (Biolegend) for 15 min at room temperature, stained with PE-conjugated anti-mouse Cd274 (124307, BioLegend) diluted 1:400 in FACS buffer (10% FCS in PBS), washed twice using FACS buffer, and analyzed on an LSR Fortessa (BD) flow cytometer. Data analysis was performed using FlowJo software (Treestar). For immunoblotting of Myc, about 20 µg of whole-cell lysate (RIPA lysis buffer + 1x protease inhibitor cocktail, 4693159001, Roche Diagnostics) were loaded onto each lane (NuPAGE Novex 4-12% Bis-Tris Gels, NP0322BOX, Life Technologies). Protein extracts were resolved by SDS polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membrane (Thermo Fisher Scientific) for blotting (70 V, 90 min). The antibodies used for WB were: Myc (56058, Cell Signaling), β-actin (A3854, Sigma Aldrich) and secondary antibody anti-rabbit IgG, HRP-linked (7074, Cell Signaling).
Hi-C
Bedtools2.21 was used to identify Brd4 peaks interacting with 3D chromatin-loops identified in CH12-LX mouse B-lymphoblasts using HiCCUPS (GSE63525). The Hi-C contact map and indicated histone marks for the genomic locus encompassing Cd274 was visualized using Juicebox software (Durand et al., 2016; Rao et al., 2014).

Immunoblot
Cell lysate preparation, SDS polyacrylamide gel electrophoresis and immunoblotting were performed according to standard protocols (Shortt et al., 2013) with the following primary antibodies: anti-Brd4 (Abcam), anti-phospho-STAT1 [Tyr701] (Cell Signalling Technologies), anti-STAT1 (BD Biosciences), and α-tubulin (Merck KGaA, Darmstadt, Germany).

Supplementary References
Durand, N.C., Robinson, J.T., Shamim, M.S., Machol, I., Mesirov, J.P., Lander, E.S., and Aiden, E.L. (2016). Juicebox Provides a Visualization System for Hi-C Contact Maps with Unlimited Zoom. Cell systems 3, 99-101.
Flicek, P., Amode, M.R., Barrell, D., Beal, K., Billis, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G., Fitzgerald, S., et al. (2014). Ensembl 2014. Nucleic Acids Res 42, D749-755.
Jackson, E.L., Willis, N., Mercer, K., Bronson, R.T., Crowley, D., Montoya, R., Jacks, T., and Tuveson, D.A. (2001). Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. Genes Dev 15, 3243-3248.
Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M., and Berns, A. (2001). Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet 29, 418-425.
Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 14, R36.
Kress, T.R., Pellanda, P., Pellegrinet, L., Bianchi, V., Nicoli, P., Doni, M., Recordati, C., Bianchi, S., Rotta, L., Capra, T., et al. (2016). Identification of MYC-Dependent Transcriptional Programs in Oncogene-Addicted Liver Tumors. Cancer Res 76, 3463-3472.
Law, C.W., Chen, Y., Shi, W., and Smyth, G.K. (2014). voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol 15, R29.
Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923-930.

Liberzon, A., Birger, C., Thorvaldsdottir, H., Ghandi, M., Mesirov, J.P., and Tamayo, P. (2015). The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell systems 1, 417-425.

Lito, P., Saborowski, A., Yue, J., Solomon, M., Joseph, E., Gadal, S., Saborowski, M., Kastenhuber, E., Fellmann, C., Ohara, K., et al. (2014). Disruption of CRAF-mediated MEK activation is required for effective MEK inhibition in KRAS mutant tumors. Cancer Cell 25, 697-710.

Martin, M.B. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMNet 17, 10-12.

Rao, S.S., Huntley, M.H., Durand, N.C., Stamenova, E.K., Bochkov, I.D., Robinson, J.T., Sanborn, A.L., Machol, I., Omer, A.D., Lander, E.S., et al. (2014). A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. Cell 159, 1665-1680.

Shortt, J., Martin, B.P., Newbold, A., Hannan, K.M., Devlin, J.R., Baker, A.J., Ralli, R., Cullinane, C., Schmitt, C.A., Reimann, M., et al. (2013). Combined inhibition of PI3K-related DNA damage response kinases and mTORC1 induces apoptosis in MYC-driven B-cell lymphomas. Blood 121, 2964-2974.

Tasdemir, N., Banito, A., Roe, J.S., Alonso-Curbelo, D., Camiolo, M., Tschaharganeh, D.F., Huang, C.H., Aksoy, O., Bolden, J.E., Chen, C.C., et al. (2016). BRD4 Connects Enhancer Remodeling to Senescence Immune Surveillance. Cancer discovery 6, 612-629.

Zuber, J., Shi, J., Wang, E., Rappaport, A.R., Herrmann, H., Sison, E.A., Magoon, D., Qi, J., Blatt, K., Wunderlich, M., et al. (2011). RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. Nature 478, 524-528.
**SUPPLEMENTARY FIGURES**

(A) **Eu-Myc**

- CD3^+CD4^+
- CD3^+CD8^+

(B) **Myc-bound genes (<5kb TSS)**

- DMSO: 341
- JQ1: 9517
- JQ1: 1180

(C) **Myc peak distribution**

- DMSO
- JQ1

(D) **MYC ChIP-seq; DMSO-treated**

| Transcription Factor | Motif          | Log P-value | Rank |
|----------------------|----------------|-------------|------|
| c-Myc                | CACGGTGC       | -4.742e3    | 1    |
| Elk1                 | AAGCGGGAGT     | -1.08e3     | 3    |
| KLF5                 | CGGCGTGCC      | -4.93e2     | 4    |

(E) **MYC and CD274 expression in IGCG datasets**

- Pearson's Correlation
- P-value
Figure S1, related to figure 1 and 3. Tumor-infiltrating lymphocytes express Pd-1
(A) Spleens from C57BL/6 mice bearing Eμ-Myc lymphomas were harvested and
stained by FACS for cell surface Cd3, Cd4 and Cd8. Expression of Pd-1 is shown
using an anti-Pd1 Ab (blue histogram) with isotype control staining shown in grey histo-
grams. Analysis of Myc ChIP-seq datasets in Eμ-Myc cells; (B) Venn-diagram repre-
senting the number of Myc-bound genes (within 5kb of the TSS) in DMSO and JQ1
treated conditions. (C) Genomic distribution of Myc peaks across annotated regions of
the genome in DMSO and JQ1 treated conditions. The contribution of annotated
regions is presented in the bottom panel. (D) De novo motif discovery on Myc bound
sites in DMSO and JQ1 treated conditions. (E) Correlation of MYC and CD274 expres-
sion was measured in publically available datasets from the International Cancer
Genomics Consortium (IGCG) using Pearson’s correlation coefficient.
**A**

Fetal pancreas LSL-Kras\textsuperscript{G12D} p53\textsuperscript{−/−} → Retroviral transduction

Pancreatic progenitors → in situ transplantation

Bioluminescence imaging → PDAC Cell line

Week 2 6 8 9

**B**

![Histological image](image)

**C**

IFN-γ pre-treated

\[
\begin{align*}
\text{Pd-l1 MFI} & \quad \text{Myc ON IFN-OFF} \\
& \quad \text{Myc OFF IFN-OFF} \\
& \quad \text{Myc ON IFN-ON} \\
& \quad \text{Myc OFF IFN-ON}
\end{align*}
\]

**D**

-IFN-γ pre-treatment +IFN-γ pre-treatment

\[
\begin{align*}
\text{Pd-l1 MFI} & \quad \text{DOX} \\
& \quad \text{+DOX}
\end{align*}
\]

**E**

![Western blot image](image)

**F**

DOX pre-treated

\[
\begin{align*}
\text{Pd-l1 MFI} & \quad \text{Myc ON IFN-OFF} \\
& \quad \text{Myc OFF IFN-OFF} \\
& \quad \text{Myc ON IFN-ON} \\
& \quad \text{Myc OFF IFN-ON}
\end{align*}
\]

**G**

DOX pre-treated

\[
\begin{align*}
\text{Pd-l1 MFI} & \quad \text{IFN-γ} \\
& \quad \text{+IFN-γ}
\end{align*}
\]

**H**

DMSO JQ1 IFN-γ JQ1+IFN-γ

**I**

-IFN-γ pre-treatment +IFN-γ pre-treatment

\[
\begin{align*}
\text{Pd-l1 MFI} & \quad \text{DMSO} \\
& \quad \text{JQ1} \\
& \quad \text{IFN-γ} \\
& \quad \text{JQ1+IFN-γ}
\end{align*}
\]
Figure S2, related to figure 3. Development and characterization of tet-OFF Myc PDAC mouse model. (A) Schematic and in vivo imaging data for the tet-OFF Myc mouse model. Pancreatic progenitor cells from E17.5-E18.5 embryos from LSL-KrasG12D; p53fl/fl mice were harvested and retrovirally transduced with the indicated plasmids after two days of culture. After 4 days of 4-hydroxy-tamoxifen treatment, cells were in situ transplanted into C57BL/6 mice and assessed for luciferase expression using in vivo imaging system. Tumor cells were harvested and cultured in normal DMEM medium containing fetal bovine serum. (B) H&E staining shows intermediate to high grade pancreatic ductal adenocarcinoma with less conspicuous (1) and well differentiated ductal structures (2). D = ductal differentiation, P = remnants of pre-existent pancreatic parenchyma (including islets). Genetic depletion of Myc does not affect Pd-l1 expression; (C, D) PDAC cells expressing dox-inducible Myc were either pre-treated with 50 ng/mL IFN-γ (for 24 hr) or left untreated, followed by treatment with Dox or DMSO for 48 hr. Cell surface expression of Pd-l1 was assessed by FACS. MFI (mean ± SD from 4 experiments) is shown. (E) Western blot analysis for expression of Myc and β-actin was performed using lysates from PDAC cells cultured in the presence and absence of Dox for 24 and 48 hr. (F, G) PDAC cells expressing dox-inducible Myc were either pre-treated with Dox for 24 hr or left untreated before 50 ng/mL IFN-γ or vehicle treatment for 48 hr (for Dox pre-treated samples, Dox treatment was continued during these 48 h). (H, I) PDAC cells expressing dox-inducible Myc were either pre-treated with 50 ng/mL IFN-γ (for 24 hr) or left untreated, followed by treatment with 250 nM JQ1 or DMSO for 48 hr. Cell surface expression of Pd-l1 was assessed by FACs. MFI (mean ± SD from 4 experiments) is shown.
(A) Log2 RPKM for Myc and CD24

(B) Heatmap showing gene expression changes

(C) Diagram illustrating Brd4-bound gene distribution

(D) Venn diagram showing overlap of Brd4-bound genes

(E) Bar charts showing Brd4 peak distribution

(F) Bar charts showing Brd4 transcript levels

(G) Bar charts showing Myc transcript levels

(H) Bar charts showing CD24 transcript levels

(I) Heatmap showing H3K27Ac ranked super-enhancers

(J) Heatmap showing Eu-Myc #1

(K) Heatmap showing Hi-C analysis
Figure S3, related to figure 3 and 4. Reanalysis of RNA-seq data (GSE76062) from Kress and colleagues (Kress et al., 2016). Data from a tet-MYC off model of liver carcinoma demonstrating that upon MYC inactivation, CD274 mRNA is significantly upregulated. Data represents log2RPKM values for (A) MYC and (B) CD274 expression in MYC On (n=16) and MYC Off (n=8) samples (**p<0.01, Student’s t test). Genetic depletion of Brd4 downregulates Pd-l1 expression in Eμ-Myc lymphoma; (C) Eμ-Myc lymphoma #1 were transduced with TRMPVIR.shScrambled, TRMPVIR.shBRD4.498, and TRMPVIR.shBRD4.500 and exposed to doxycycline (DOX) to induce the DsRed-shRNA gene cassette. Representative flow cytometry plots show induction of DsRed following DOX treatment for each construct. (D) Venn-diagram representing the number of genes bound Brd4 (within 5kb of the TSS) in DMSO and JQ1 treated conditions. (E) Genomic distribution of Brd4 peaks across annotated regions of the genome in DMSO and JQ1 treated conditions. The contribution of annotated regions is presented in the bottom panel. Reanalysis of RNA-seq data (GSE74324) from Tasemir and colleagues; Studies using IMR90 HrasV12-transformed human fibroblasts (Tasdemir et al., 2016) cells demonstrate that CD274 is consistently downregulated following genetic depletion of BRD4 or JQ1 treatment in the absence of substantive downregulation of MYC. Data represents log2RPKM values for (F) BRD4 (G) MYC and (H) CD274 expression (**p<0.01, ***p<0.001, Student’s t test). Identification of super-enhancers and 3D chromatin interactions in Eμ-Myc lymphoma; (I) Identification of super-enhancers in Eμ-Myc cells based on H3K27Ac ChIP-Seq data obtained from public dataset (GSE51004). Stitched enhancers (12.5kb distance) were ranked by H3K27Ac signal and normalized to the maximum, resulting in the identification of 429 super-enhancers. (J) H3K27Ac and Brd4 occupancy tracks and super-enhancer peak track across the extended Cd274 genomic locus. (K) Visualization of Hi-C data obtained from CH12-LX mouse B-lymphoblasts (GSE63525) and corresponding H3K4Me3, H3K4Me1, H3K27Ac and H3K36Me3 ChIP-seq data, with Brd4 and H3K27Ac peaks identified in Eμ-Myc indicated underneath. The blue box indicates identified chromatin loops with contact domains indicated by black boxes.
Figure S4, related to figure 5. JQ1 treatment suppresses IFN-γ-induced Pd-l1 expression in a murine mammary tumour cell line (AT3) and a patient-derived plasma cell leukaemia cell line (ALF-1). (A) Gene expression heatmap shows the top 100 IFN-γ-regulated genes by 3’-mRNA-Seq analysis of AT3 or ALF-1 cells treated for 2 hours in the presence of 1μM JQ1, 1ng/mL IFN-γ, combination of JQ1 and IFN-γ, or DMSO vehicle. Row-normalized values are represented. (B) Metagene analysis of strand specific 3’-mRNA-Seq reads indicating accurate mapping to the 3’UTR (C) GSEA enrichment score plots show significant correlation between genes induced by IFN-γ and interferon gamma and inflammatory response signatures. (D) Representative image of 3’-mRNA-Seq reads mapped to the CD274 locus across treatment conditions. (E) AT3 cells were treated as in (A) prior to assessment of phospho-STAT1 (Y701) and total-STAT1 by immunoblot. Tubulin was used as the loading control. (F) Venn diagram representing the overlap between IFN-γ-induced genes in AT3 and ALF1 cells identified a common subset of 16 overlapping genes. IFN-γ does not increase BRD4 expression; (G) 3’-mRNA-Seq reads mapped to the Brd4 and Irf1 loci in ALF-1 and AT3 cells cultured in the presence or absence of 1ng/mL IFN-γ. (H) AT3 cells were treated with a dose titration of recombinant mouse IFN-γ (1ng/mL) prior to assessment of Brd4 protein levels by immunoblot. Actin was used as the loading control.
Figure S5, related to figure 6. JQ1 suppresses IFN-γ-induced PD-L1 expression in a variety of solid tumour cell lines. Mouse mammary (AT3 and 4T1.2) and mouse colon (MC38) tumour cell lines were cultured for 24 hours in the presence of 1μM JQ1, 10ng/mL mouse IFN-γ, combination of JQ1 and IFN-γ, or DMSO vehicle, prior to assessment of Pd-l1 expression by flow cytometry. Representative histograms and MFI of (A-B) Pd-l1 and (C-D) MHC Class I (H-2Kb) expression are shown for cells cultured. Human mammary (AU565) cell lines were cultured for 24 hours in the presence of 1μM JQ1, 10ng/mL human IFN-γ, combination of JQ1 and IFN-γ, or DMSO vehicle, prior to assessment of PD-L1 expression by flow cytometry. Representative histograms and MFI of (E-F) PD-L1 expression are shown for cells cultured. (G) AT3, AU565, 4T1.2, and MC38 were cultured for 2 hours in the presence of 1μM JQ1, 10ng/mL IFN-γ, combination of JQ1 and IFN-γ, or DMSO vehicle, prior to assessment of CD274 mRNA levels by qRT-PCR. Transcript levels are normalized to GAPDH and presented as fold change compared to DMSO. Data presented as fold-change from 3 separate experiments ± S.E.M. (**p <0.01, ***p<0.001, Student’s t test).
Figure S6, related to figure 6. JQ1 suppresses IFN-γ-induced \textit{Cd274} transcription in Eμ-Myc lymphoma despite sustained protein expression; (A) Eμ-Myc \#3 lymphoma cells were cultured in the presence of JQ1 (1μM), 10ng/mL or 1ng/mL recombinant mouse IFN-γ, or combinations of JQ1+IFN-γ for 2 hours prior to assessment of \textit{Cd274} mRNA levels by qRT-PCR. Transcript levels are normalized to actin and presented as fold change compared to untreated. Data presented as fold-change from 3 separate experiments ± S.E.M. (B) Eμ-Myc \#3 lymphoma cells were cultured in the presence of JQ1 (1μM), 10ng/mL or 1ng/mL recombinant mouse IFN-γ, or combinations of JQ1+IFN-γ for 24 hours prior to assessment of cell surface Pd-l1 expression by flow cytometry. (C) Eμ-Myc \#1 lymphoma cells were cultured in the presence of JQ1 (1μM), 10ng/mL or 1ng/mL recombinant mouse IFN-γ, or combinations of JQ1+IFN-γ for 24 hours prior to assessment of cell surface Pd-l1 expression by flow cytometry. Overexpression or genetic depletion of Myc does not modulate IFN-γ-induced or basal Pd-l1 expression; (D) AT3 cells transduced with MSCV.GFP or MSCV.hsMYC.GFP were cultured for 24 hours in the presence of 1μM JQ1, 1ng/mL IFN-γ, combination of JQ1 and IFN-γ, or DMSO vehicle, prior to assessment of Pd-l1 expression by flow cytometry. Representative data is presented as MFI of cells cultured. (E) Myc and \textit{Cd274} mRNA levels were determined by qRT-PCR in AT3 cells were transduced with tetracycline-inducible shRNAs targeting Myc (\#1891 and \#2105) or Renilla (REN) and exposed to doxycycline for 18 hours. Transcript levels are normalized to Actin and presented as fold change compared to -DOX. Data presented as fold-change from 3 separate experiments ± S.E.M. (*p <0.05, Student’s t test).
**Figure S7**, related to figure 5. Stat1 is recruited to the Irf1 locus following IFN-γ stimulation; (A) Visualization of H3K27Ac, Brd4, RNA Pol II, Irf1, and Stat1 occupancy at the Irf1 locus in AT3 cells cultured for 2 hours in the presence of 1 μM JQ1, 1 ng/mL IFN-γ, combination of JQ1 and IFN-γ, or DMSO vehicle, prior to ChIP-Seq analysis. (B) Visualization of Irf1 and Stat1 recruitment to the Irf1 locus following IFN-γ stimulation for 2 hours, with Stat1 and Irf1 consensus binding motifs indicated. No Irf1 motifs were identified at this locus. (C) Visualization of Irf1 and Stat1 recruitment to the Cd274 locus following IFN-γ stimulation for 2 hours, with Stat1 and Irf1 consensus binding motifs indicated. Ectopic expression of murine Pd-l1 from a retroviral promoter; (D) Eμ-Myc lymphoma #1 cells were retrovirally transduced with murine stem cell virus (MSCV-BFP) containing murine Pd-l1 cDNA (#1/PD-L1) or empty vector control (#1/MSCV). BFP+ cells were isolated by FACS sorting and Pd-l1 expression was assessed by flow cytometry.