



Supplementary Information for

Genetic analysis of cancer drivers reveals cohesin and CTCF as suppressors of PD-L1

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This PDF file includes:

Supplementary text
Figures S1 to S4
Tables S1 to S7

Supplementary Information Text

Generation of U937 and K562 cell lines

The U937 and K562 cell lines used in this manuscript were previously published(1). In brief, Cas9-expressing U937 and K562 cell lines were generated with transduction of a lentiviral vector (pLX-311 Cas9, Addgene 96942). Two different sgRNAs were used targeting *Stag2* (STAG2-KO1, STAG2-KO2). Resulting single cell clones had full deletion of STAG2. One sgRNA each targeting *Rad21* and *Smc3* resulting in heterozygous mutations. Independent single cell clones were isolated (3 clones for STAG2-KO1, 1 clone for U937 SMC3-het, and 2 clones for all other cell lines) and used for biological replicates of each genotype. Genotypes of the single cell clones are provided in Supplementary Table 3 as previously published(1).

TSG CRISPR-Cas9 library construction

DNA oligonucleotides encoding sgRNA sequences designed to target the top 500 TUSON-predicted TSGs were synthesized on a custom microarray (Agilent). These oligonucleotides were PCR-amplified separately with specific sets of primers in two batches: the top 100 sgRNAs were PCR-amplified separately from the bottom 400 sgRNAs in two pools. PCR-amplified gRNA libraries were digested with BbsI and purified on a 10% TBE PAGE gel. Purified, digested fragments were cloned into BsmBI-digested pLentiCRISPRv2 (Addgene Plasmid #52961). A negative control non-cutting gRNA library comprised of 500 gRNAs targeting the *E. coli* genome was designed and cloned in parallel.

Lentivirus production and titering

HEK293T cells were transfected with plasmid DNA diluted into serum-free medium with a lentiviral packaging plasmid mixture of SV40 VSVg, Gag/Pol, Tat, and Rev, and PolyJet (SignaGen). Cell culture media was changed 24 hours later. After 48 h, the supernatant was harvested, filtered through a PES membrane filter with 0.45 μm pores (Westnet

#430514), and stored at -80°C . Lentiviral titer was determined by transducing U2-OS cells plated at clonogenic density with serial dilutions of virus in the presence of $4\ \mu\text{g/mL}$ polybrene. Colonies were then stained with methylene blue and counted to determine viral titer.

Supplementary Figures

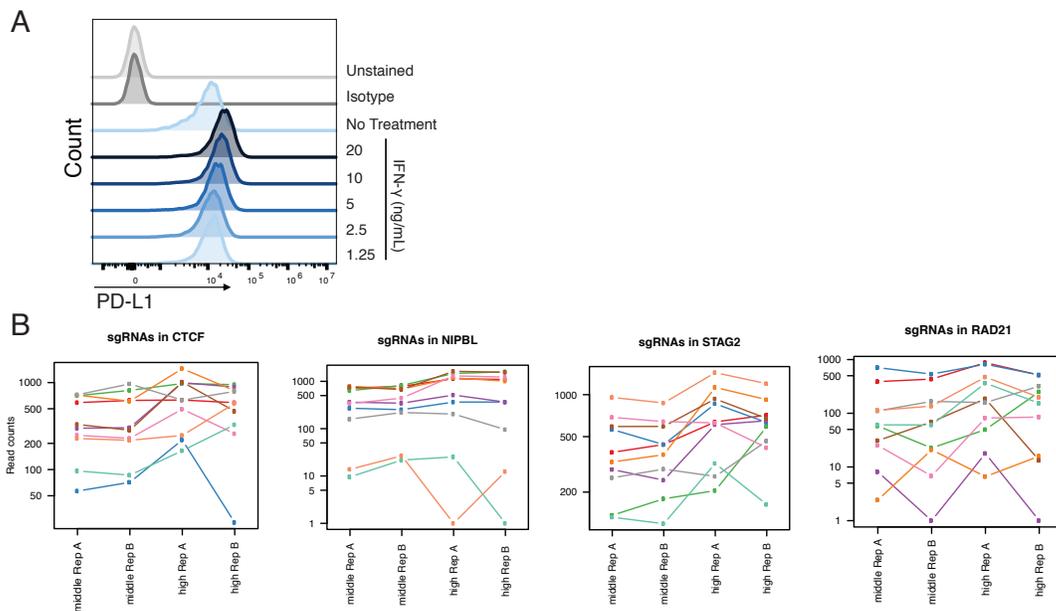


Fig. S1.

(A) FACS staining of PD-L1 cell surface expression in HMEC cells after treatment with increasing concentrations of IFN- γ .

(B) Raw read counts of sgRNAs targeting components of the cohesin ring (RAD21 and STAG2), NIPBL and CTCF in middle sort and high sort bins across two replicates of the screen (replicate A and replicate B).

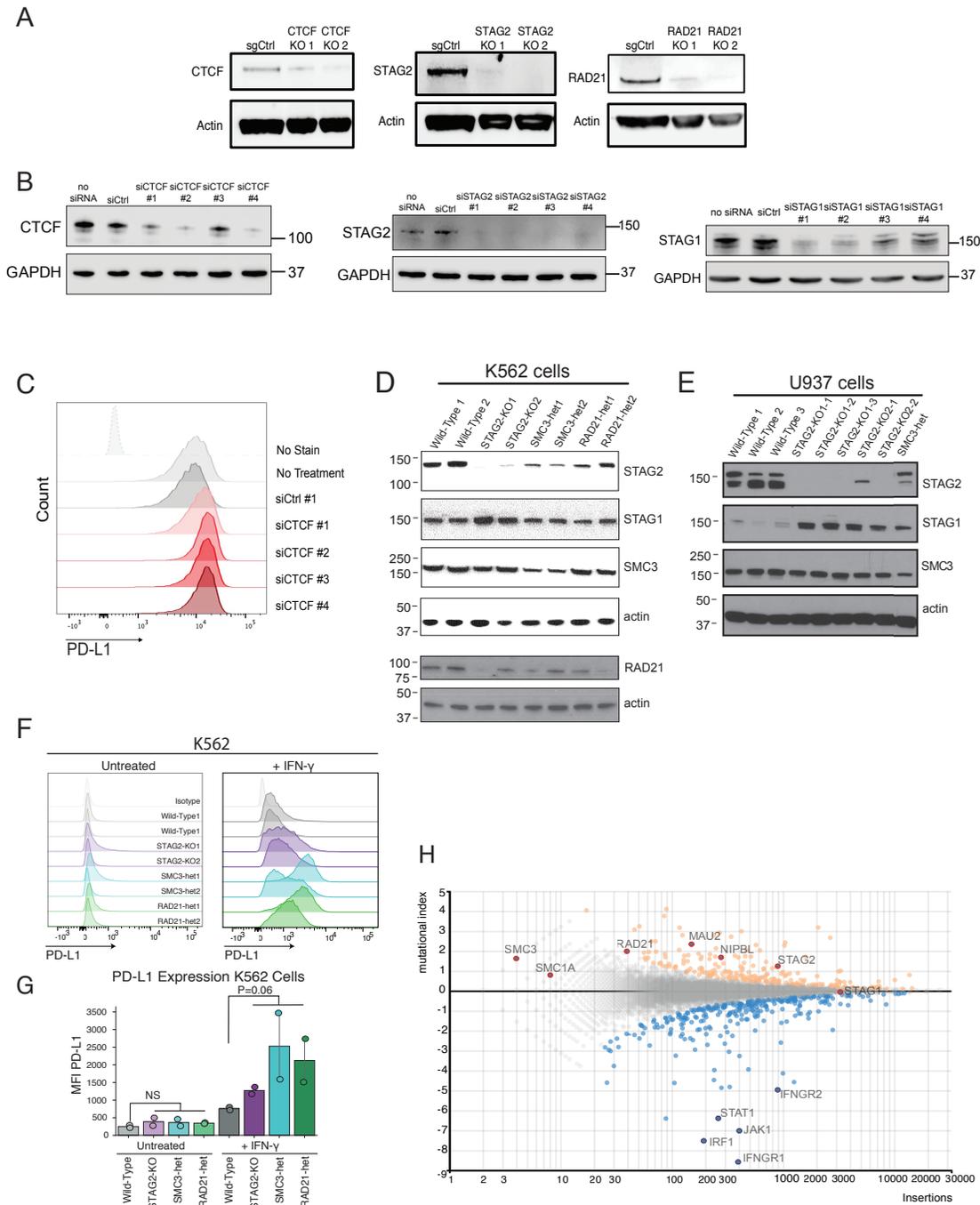


Fig. S2.

(A) Western blot analysis of RAD21, STAG2 and CTCF in *RAD21*, *STAG2* and *CTCF*-mutant cell populations generated using two different sgRNAs in HMEC cells. Mutant cells were compared to cells infected with negative control sgRNA targeting the AAVS1 sequence.

(B) Western blot analysis of CTCF, STAG2 and STAG1 in CTCF, STAG2 and STAG1 siRNA-treated HMEC cells using four different siRNAs. siRNA-treated cells were compared to cells infected with negative control scrambled siRNA and no siRNA control.

(C) FACS analysis of PD-L1 expression in control and CTCF siRNA-treated HMEC cells. Cells were cultured in the presence and absence of 20 ng/mL IFN- γ for 24 h prior to FACS analysis.

(D) Western blot analysis of STAG2, SMC3, STAG1 and RAD21 protein levels in *STAG2*, *SMC3* and *RAD21*-mutant K562 cell lines.

(E) Western blot analysis of STAG2, STAG1 and SMC3 protein levels in *STAG2* and *SMC3*-mutant U937 cell lines.

(F) FACS analysis of PD-L1 expression in wild-type, *STAG2* null, *SMC3* heterozygous (*SMC3*-het) and *RAD21* heterozygous (*RAD21*-het) K562 cells cultured with 2000 IU/mL IFN- γ for 72 h. Cohesin-mutant K562 cells upregulate PD-L1 cell surface levels compared to WT controls. Results shown are from one representative example of three independent experiments.

(G) Mean fluorescence intensity (MFI) of PD-L1 staining in *STAG2* null, *SMC3*-het, *RAD21*-het and wild-type K562 control cells replicates shown in panel (F). All three cohesin-mutant genotypes were grouped per condition (untreated, + IFN- γ) for statistical analysis. Kruskal-Wallis H test was used ($p=0.01$), results of post-hoc analysis comparing wild type to cohesin mutant cells in each condition are shown on the plot. Results shown are from one representative example of three independent experiments.

(H) Fish plot of whole genome CRISPR screen of regulators of cell surface PD-L1 in HAP1 cells performed by Mezzadra et al. (2). HAP1 cells were mutagenized using gene-trap mutagenesis. Cells were stained and FACS sorted into PD-L1 high and low bins. Y-axis (mutational index) represents \log_2 value of frequency of mutations in the high channel divided by the frequency of mutations in the low channel for each gene. X-axis represents total number of insertion mutations assigned to each gene. Colored in orange and blue are genes with p -value <0.007 . Cohesin subunits and CTCF (red) as well as components of JAK-STAT signaling known to regulate PD-L1 (blue) are highlighted.

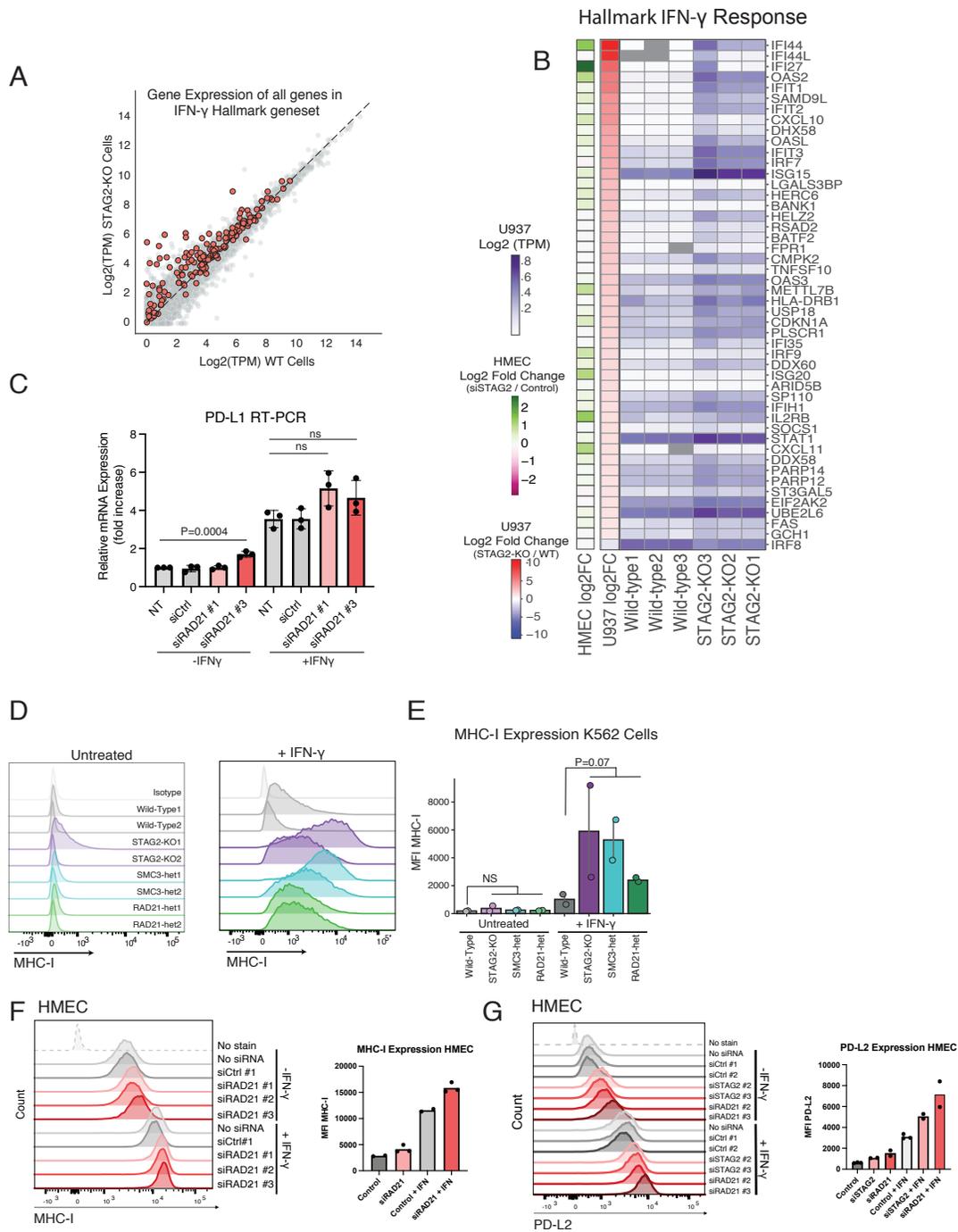


Fig. S3.

(A) Gene expression (Average Log₂(TPM)) of *STAG2*-KO and WT control cells with genes present in IFN- γ gene set highlighted in red and all other genes colored in grey.

(B) Heatmap of gene expression (TPM values in purple and log₂FC values in red-blue) for genes in the IFN- γ expression signature with absolute value of log₂ fold change >1

between the *STAG2* KO and WT U937 control cells. Log₂FC changes in siSTAG2 HMEC cells compared to controls depicted in green-pink.

(C) RT-PCR analysis of PD-L1 expression levels in the presence or absence of 20 ng/mL IFN- γ in control and RAD21 siRNA-depleted cells from three biological replicates. PD-L1 expression levels were normalized to β -Actin and shown relative to no siRNA control. One-way ANOVA with Dunnett correction analysis was used to calculate differences between means of siRAD21 groups and no siRNA group for each condition (-IFN- γ , +IFN- γ). Data is represented as the means \pm SD.

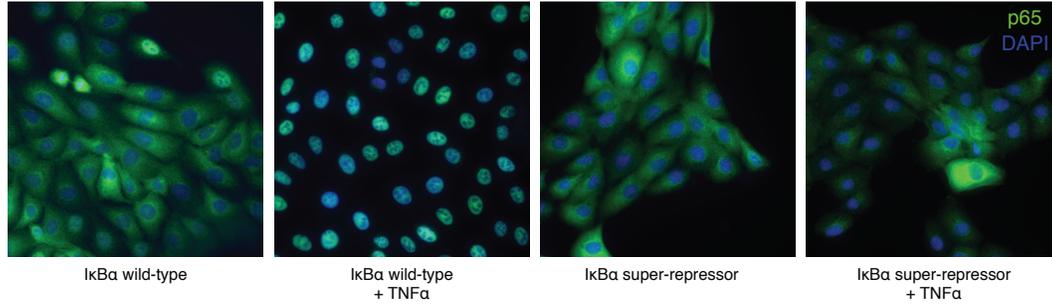
(D) FACS analysis of MHC-I expression in WT, *STAG2* null, *SMC3* heterozygous (*SMC3*-het) and *RAD21* heterozygous (*RAD21*-het) K562 cells cultured with 2000 IU/mL IFN- γ for 72 h. Results shown are from one representative example of three independent experiments.

(E) Mean fluorescence intensity (MFI) of MHC-I staining in *STAG2* null, *SMC3*-het, *RAD21*-het and wild-type K562 control cells from panel (D). All three cohesin-mutant genotypes were grouped per condition (untreated, + IFN- γ) for statistical analysis. Kruskal-Wallis H test was used ($p=0.005$), results of post-hoc analysis comparing wild type to cohesin mutant cells in each condition are shown on the plot. Results shown are from one representative example of three independent experiments.

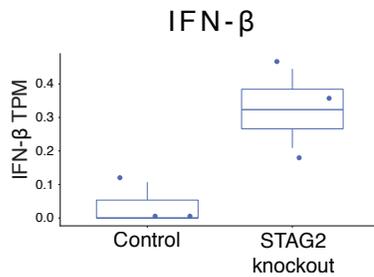
(F) FACS analysis of HLA-A2 in control and RAD21 siRNA-treated HMECs cultured in the presence and absence of 20 ng/mL IFN- γ for 24 h prior to FACS analysis.

(G) FACS analysis of PD-L2 in control and *STAG2*/*RAD21* siRNA-treated HMECs cultured in the presence and absence of 20 ng/mL IFN- γ for 24 h prior to FACS analysis.

A



B



C

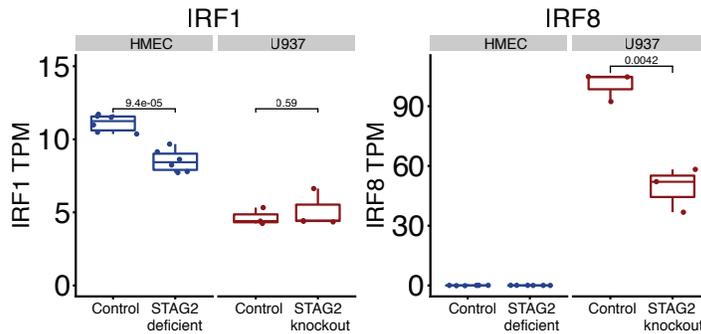


Fig. S4.

(A) Immunofluorescence of p65 and DAPI in cells expression wild-type $I\kappa B\alpha$ or super-repressor $I\kappa B\alpha$. If indicated, the cells were treated with 20ng/mL TNF- α for 20 minutes prior to fixation.

(B) TPM values of IFN- β in RNA-seq analysis of *STAG2* knockout and control U937 cells.

(C) TPM values of IRF1 and IRF8 in RNA-seq analysis of *STAG2* knockout and control U937 and *STAG2* deficient or control HMEC cells. Student's t-test was used to calculate differences between means.

Table S1 – siRNAs used for depletion of RAD21, STAG2, STAG1 and CTCF in HMEC cells

Genotypes of U937 and K562 cohesin mutant clones

Clone ID	Full clone name	Genotype
U937 cells		
WT-1	U937-GC1-A1	wild type
WT-2	U937-GC1-A2	wild type
WT-3	U937-GC3-E1	wild type
STAG2-KO1-1	U937-STAG2-A2-F11	STAG2 exon 17, 68bp deletion/54 bp deletion
STAG2-KO1-2	U937-STAG2-A2-G4	STAG2 exon 17, 1bp insertion, homozygous
STAG2-KO1-3	U937-STAG2-A2-H4	STAG2 exon 17, 1bp deletion, homozygous
STAG2-KO2-1	U937-STAG2-A1-C4	STAG2 exon 12, 1bp insertion, homozygous
STAG2-KO2-2	U937-STAG2-A1-D10	STAG2 exon 12, 1bp insertion, homozygous
SMC3 het	U937-Cas9-SMC3-H10	SMC3 exon 9, 17 bp del, 15 bp del, 1bp silent substitution (triploid)
K562 cells		
WT-1	K562-GC1-a1	wild type
WT-2	K562-GC1-a7	wild type
STAG2-KO1	K562-STAG2-A1-a2	STAG2 exon 12, 1bp insertion, homozygous
STAG2-KO2	K562-STAG2-A1-a3	STAG2 exon 12, 1bp deletion, 9bp deletion
SMC3 het 1	K562-SMC3-G1-a5	SMC3 exon 9, 2bp insertion, WT
SMC3 het 2	K562-SMC3-G3-e	SMC3 exon 9, 1bp deletion, WT
RAD21 het1	K562-RAD21-G2-b7	RAD21 exon 2, 1bp deletion, WT
RAD21 het2	K562-RAD21-G2-b8	RAD21 exon 2, 2bp insertion, WT

Table S2 – sgRNA sequences used for construction of CTCF, RAD21 and STAG2 KO HMEC cells

Gene	sgRNA #	Sequence
CTCF	1	CGATCCAAATTTGAACGCCG
CTCF	2	GAGCAAACCTGCGTTATACAG
RAD21	1	AAGTGTTGTTTGATCAGTCA
RAD21	2	ACATACTCTAAGTCAGGCAG
STAG2	1	AATACTAACCTTGAACCGAC
STAG2	2	ATACCTTGTGGATAGCATGT

Table S3 – siRNAs used for depletion of RAD21, STAG2, STAG1 and CTCF in HMEC cells

Gene	siRNA #	Dharmacon catalog #
RAD21	1	J-006832-08-0002
RAD21	2	J-006832-06-0002
RAD21	3	J-006832-07-0002

RAD21	4	J-006832-09-0002
STAG2	1	J-021351-05-0002
STAG2	2	J-021351-07-0002
STAG2	3	J-021351-08-0002
STAG2	4	J-021351-06-0002
STAG1	1	J-010638-09-0002
STAG1	2	J-010638-10-0002
STAG1	3	J-010638-11-0002
STAG1	4	J-010638-12-0002
CTCF	1	J-020165-09-0002
CTCF	2	J-020165-07-0002
CTCF	3	J-020165-10-0002
CTCF	4	J-020165-08-0002

Dataset S1 – MAGeCK analysis of read counts in PD-L1 – IFN γ screen high vs. middle bin

Dataset S2 – MAGeCK analysis of read counts in PD-L1 + IFN γ screen high vs. middle bin

Dataset S3 – GSEA enrichment analysis of RNA-seq analysis in STAG2 KO U937 clones compared to wild-type controls

Dataset S4 – GSEA enrichment analysis of RNA-seq analysis in STAG2 deficient HMEC cells compared to no siRNA and control siRNA controls

References

1. Z. Tothova, *et al.*, Cohesin mutations alter DNA damage repair and chromatin structure and create therapeutic vulnerabilities in MDS/AML. *JCI Insight* **6** (2021).
2. R. Mezzadra, *et al.*, Identification of CMTM6 and CMTM4 as PD-L1 protein regulators. *Nature* *2017* *549:7670* **549**, 106–110 (2017).