Supplementary Information

Polymerase θ inhibition activates the cGAS-STING pathway and cooperates with immune checkpoint blockade in models of BRCA-deficient cancer

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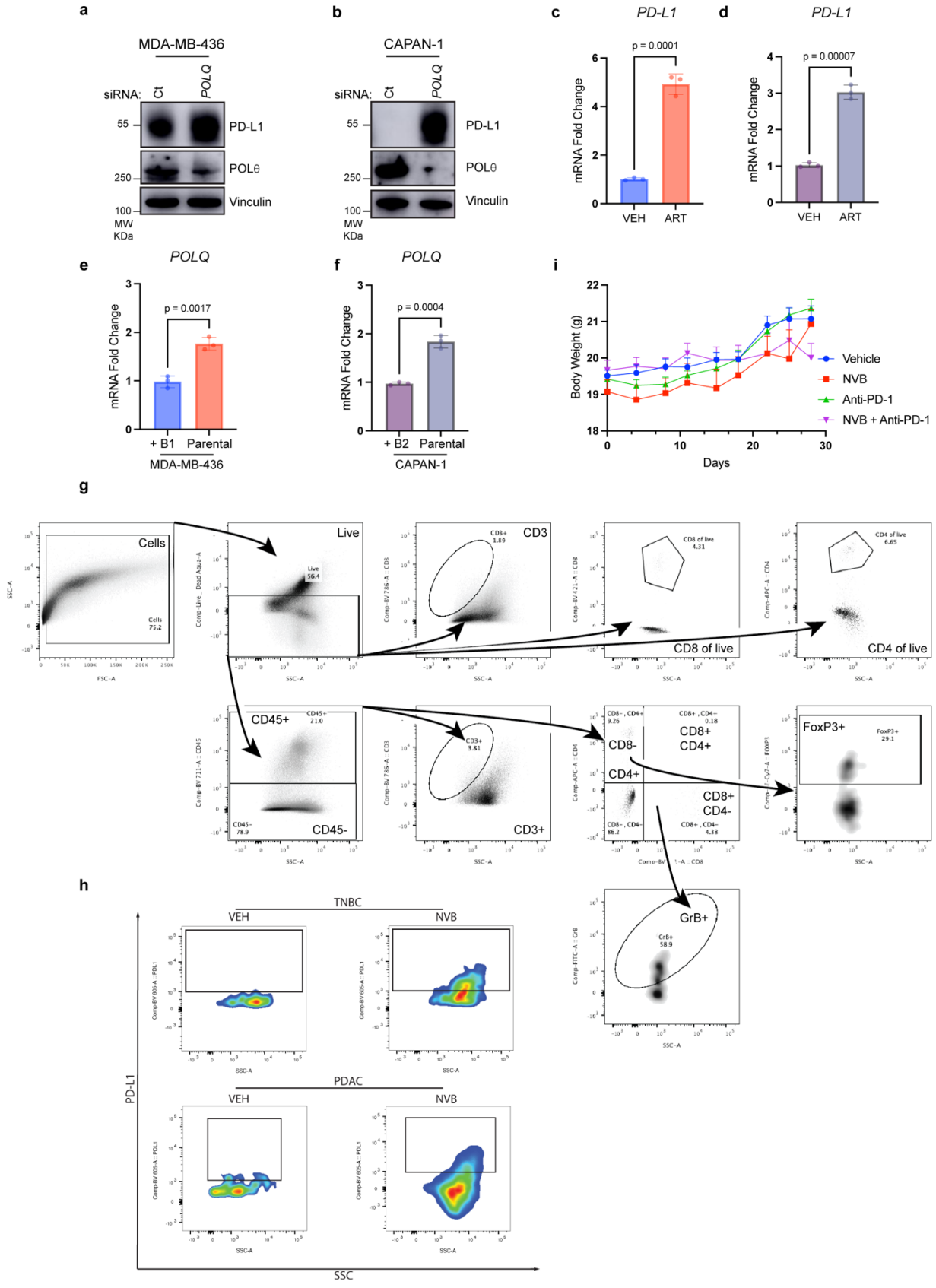
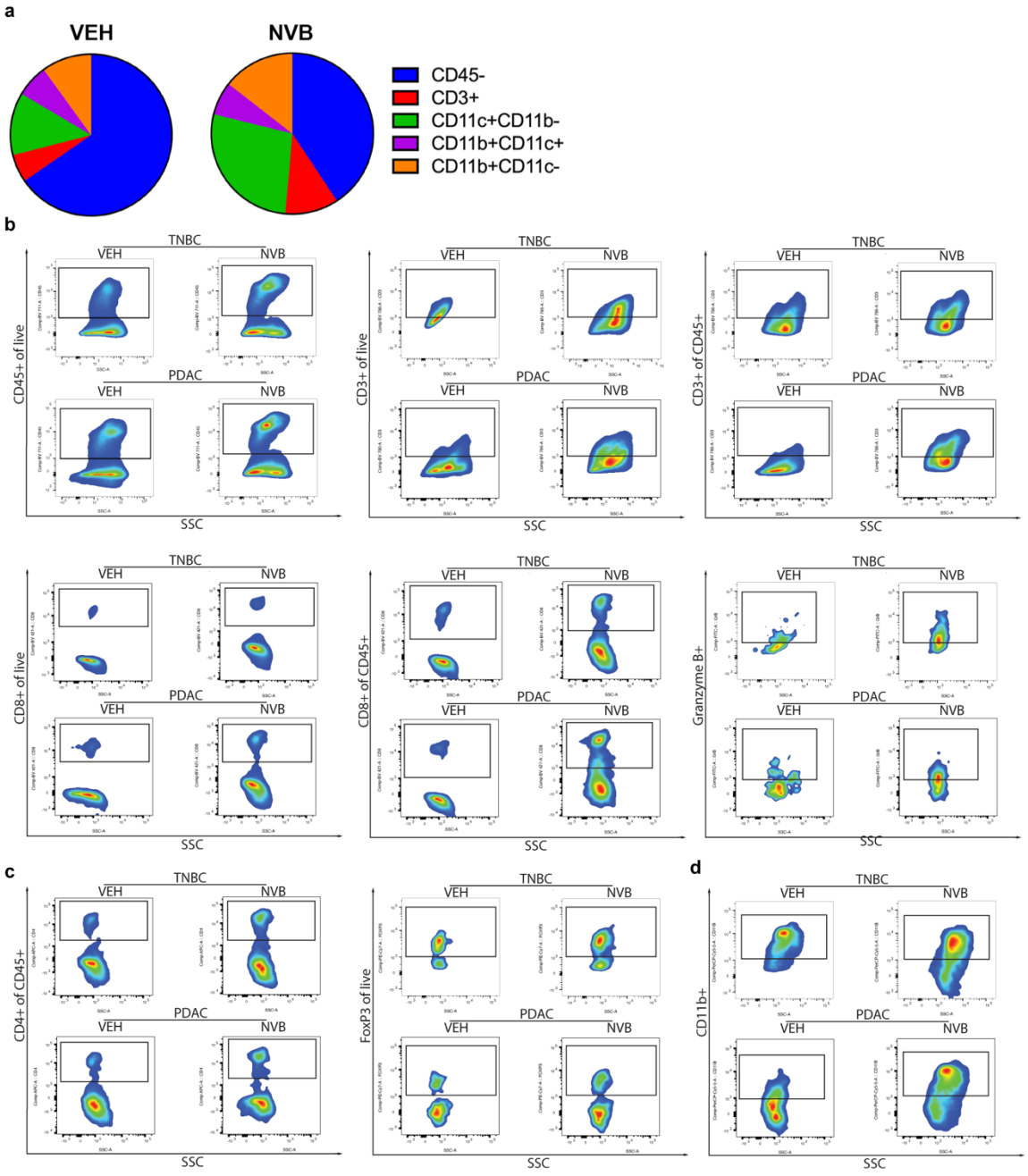
Supplementary figures and legends 1 to 7

Supplementary tables 1 and 2

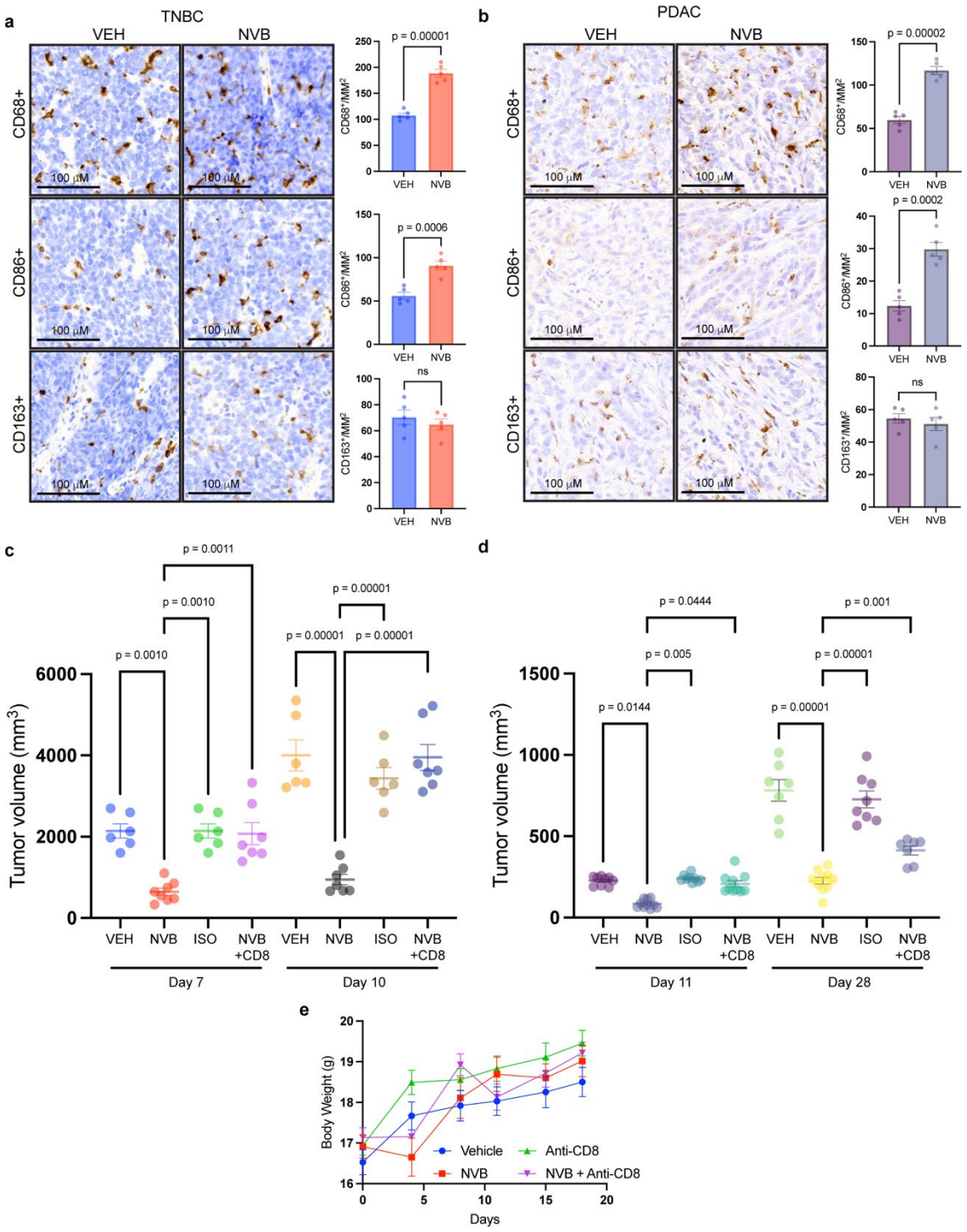
Source data for immunoblots presented in Supplementary figures 1 and 6


a
MDA-MB-436
siRNA:
Ct
POLQ
55
PD-L1
250
POLθ
Vinculin
100
MW
KDa
b
CAPAN-1
c
PD-L1
p = 0.0001
mRNA Fold Change
VEH
ART
d
PD-L1
p = 0.00007
e
POLQ
p = 0.0017
+ B1
Parental
f
POLQ
p = 0.0004
+ B2
i
Body Weight (g)
Days
Vehicle
NVB
Anti-PD-1
NVB + Anti-PD-1
g
Cells
Live
CD3
CD8 of live
CD4 of live
CD45+
CD45-
CD3+
CD8-
CD8+
CD4+
CD4-
FoxP3+
h
TNBC
PDAC
PD-L1
SSC
GrB+

Supplementary Fig. 1. *POLQ* depletion or POLθ inhibition enhances PD-L1 expression and is well tolerated in combination with anti-PD-1 blockade. a and **b,** Detection of PD-L1 protein by immunoblot in homologous recombination deficient *BRCA1*-deficient MDA-MB-436 triple negative breast cancer cells (**a**), or homologous recombination deficient *BRCA2*-deficient CAPAN-1 pancreatic cells (**b**) treated with siRNA to deplete *POLQ*. Vinculin serves as an internal loading control. Immunoblot analysis **c** and **d,** Level of *PD-L1* mRNA in MDA-MB-436 cells (**c**), or CAPAN-1 cells (**d**) treated with 1 μM ART558 (ART) for 48 hours. Transcript levels are measured relative to *ACTB* by qPCR. Data, mean+/- SD of 3 independent experiments, unpaired two-tailed *t* test. **e** and **f**, *POLQ* mRNA expression in MDA-MB-436 +/- BRCA1 WT (**e**), or CAPAN-1 +/- BRC A2 WT (**f**). Transcript levels are measured relative to *ACTB* by qPCR. Data, mean+/- SD of 3 independent experiments, unpaired two-tailed *t* test. **g**, Representative gating strategy for flow cytometry analysis of TNBC and PDAC tumors. **h**, Representative PD-L1+ flow cytometry dotplots of TNBC and PDAC tumors from **1h,** and **j**. **i**, Bodyweight measurements of mice (VEH, *n* = 11; NVB, *n* = 10; isotype, *n* = 11; anti-PD-1, *n* = 11) from **1l**. Data, mean +/- SEM.

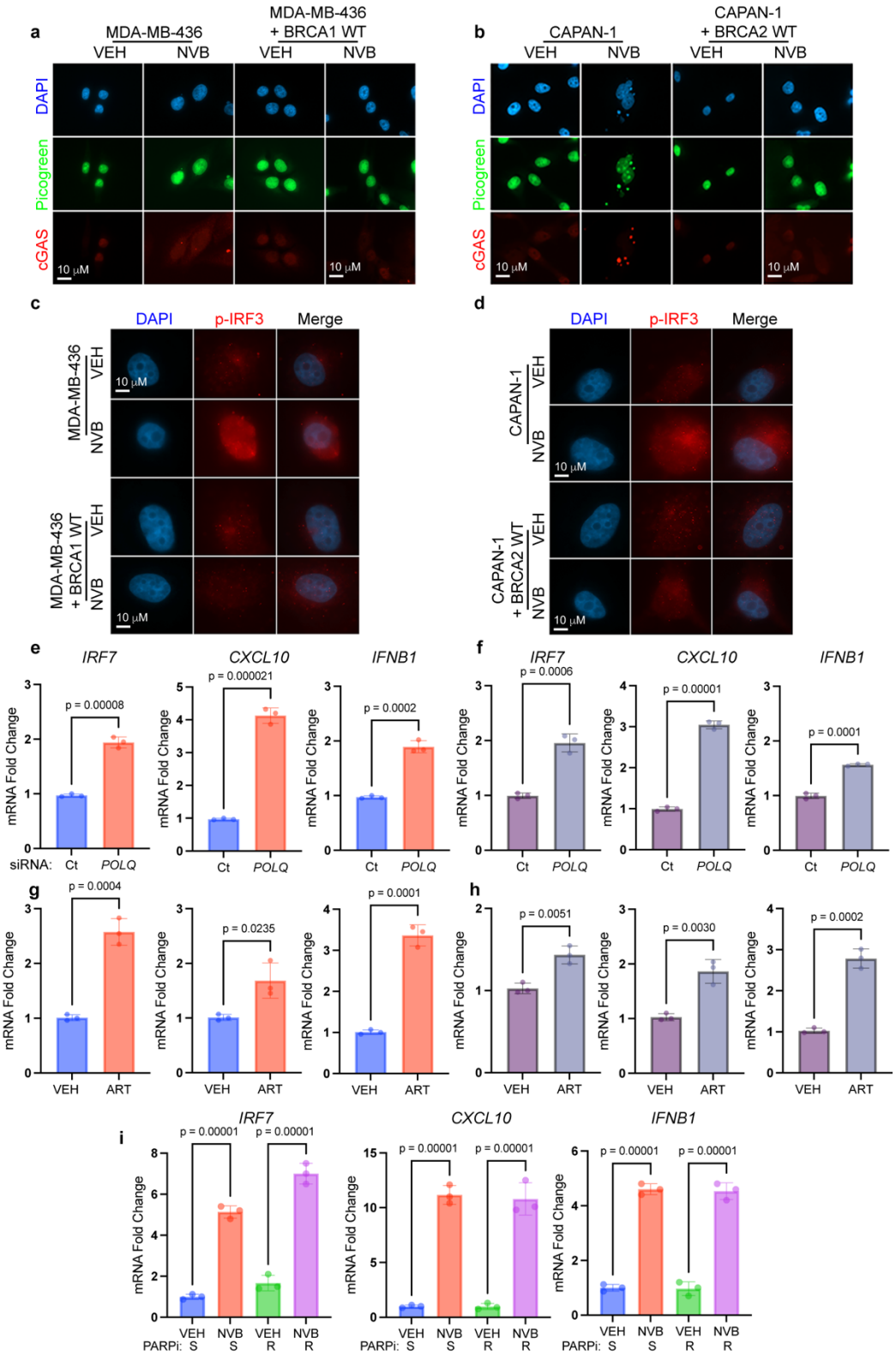


Supplementary Fig. 2. Diverse immune cell recruitment secondary to POLθ inhibition in HR-deficient cancers. a, Pie chart from vehicle or NVB-treated mice from **1e** showing the proportions of different cell types (as a percentage of total live events) in the tumor microenvironment. **b**, **c**, and **d**, Representative flow cytometry dotplots of TNBC and PDAC tumors from **2c-h**.

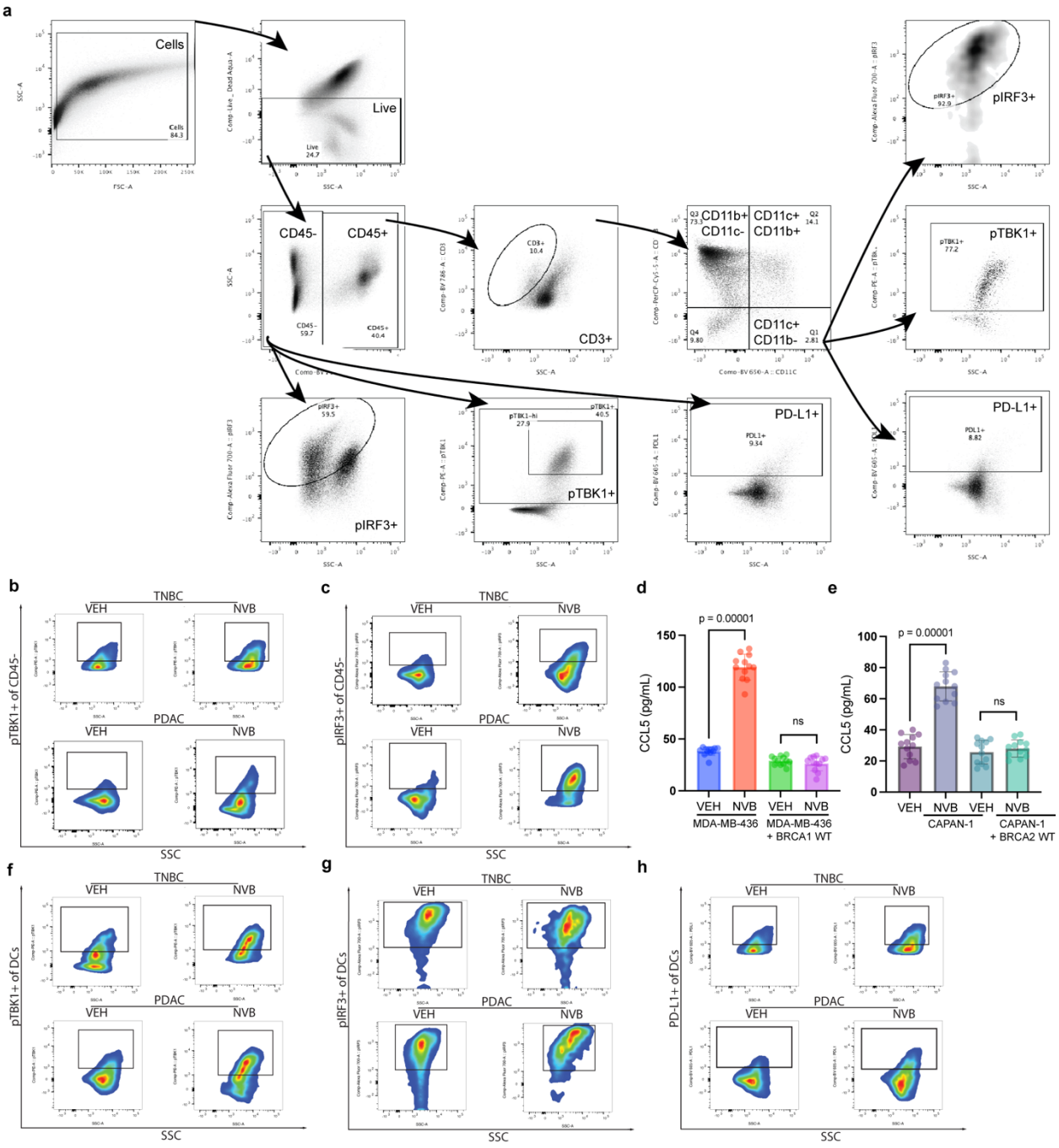


Supplementary Fig. 3. Efficacy of POLθ inhibition in HR-deficient cancers requires CD8+ T-cells. a, Pie chart from vehicle or NVB-treated mice from **1e** showing the proportions of different cell types (as a percentage of total live events) in the tumor microenvironment. **a** and **b**, TNBC (VEH, *n* = 5; NVB, *n* = 5) (**b**) or PDAC (VEH, *n* = 5; NVB, *n* = 5) (**c**) tumor chunks were subjected

to IHC for CD68, CD86, and CD163 (left) expression. CD68 is a marker for all macrophages, CD86 is a marker for M1 macrophages, and CD163 is a marker for M2 macrophages[40]. Representative IHC images at 40x magnification, scale bars, 100 μM. IHC staining was quantified using Aperio algorithms (right). Data, mean +/- SEM, unpaired two-way *t*-tests. **d** and **e**, Individual TNBC tumor volumes (mm^3) of mice treated with vehicle (*n* = 6) or NVB (75 mg/kg twice daily; *n* = 8), along with isotype control (*n* = 6) or an anti-CD8 antibody (200 μg twice weekly; *n* = 8) (**c**) or PDAC tumor volumes (mm^3) of mice treated with vehicle (*n* = 9) or NVB (75 mg/kg twice daily; *n* = 10), along with isotype control (*n* = 9) or an anti-CD8 antibody (200 μg twice weekly; *n* = 10) (**d**) at indicated days from **2i** and **2j** respectively. Error bars, SEM. Statistical analysis was performed using one-way ANOVA. **e**, Bodyweight measurements of mice from **2j** (VEH, *n* = 9; NVB, *n* = 10; isotype, *n* = 9; anti-CD8, *n* = 10). Data, mean +/- SEM.

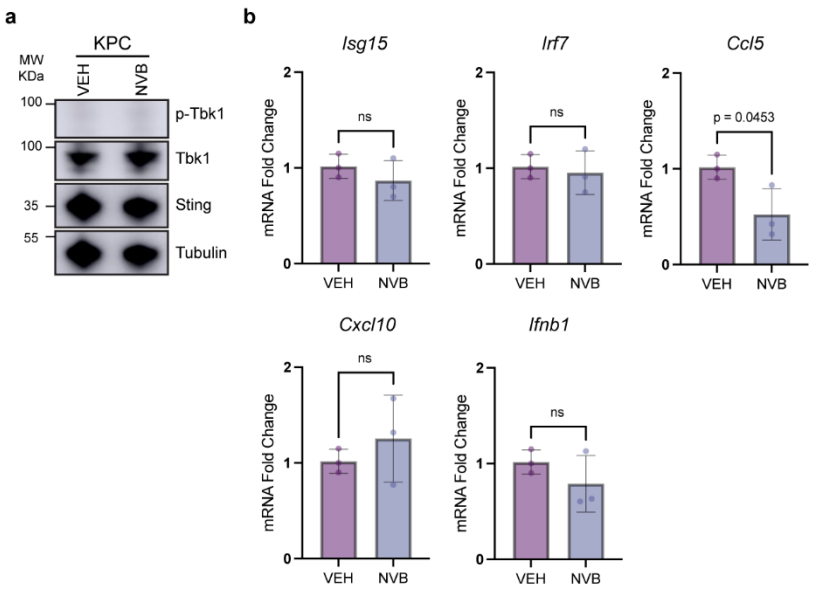

a
MDA-MB-436
MDA-MB-436 + BRCA1 WT
VEH
NVB
DAPI
Picogreen
cGAS
10 μM
b
CAPAN-1
CAPAN-1 + BRCA2 WT
c
DAPI
p-IRF3
Merge
MDA-MB-436
MDA-MB-436 + BRCA1 WT
d
CAPAN-1
CAPAN-1 + BRCA2 WT
e
IRF7
CXCL10
IFNB1
mRNA Fold Change
p = 0.00008
p = 0.000021
p = 0.0002
siRNA: Ct POLQ
f
p = 0.0006
p = 0.00001
p = 0.0001
g
p = 0.0004
p = 0.0235
p = 0.0001
VEH ART
h
p = 0.0051
p = 0.0030
p = 0.0002
i
p = 0.00001
PARPi: S S R R

Supplementary Fig. 4. POLθ inhibition activates the cGAS/STING pathway in HR-deficient cancers. a-b, The pair of MDA-MB-436 +/- BRCA1 isogenic cell lines (**a**), or the pair of CAPAN-1 +/- BRCA2 isogenic cell lines (**b**) were treated with vehicle (DMSO) or 100 μM NVB. After 48 hours, cells were fixed with paraformaldehyde, stained with picogreen, and subjected to immunofluorescence (IF) for cGAS. Representative IF mages at 100x magnification, scale bars, 10 μM,.. **c** and **d,** Isogenic cell line pairs (MDA-MD-436 +/- BRCA1, **c**) and (CAPAN-1 +/- BRCA2, **d**) were treated with DMSO or 100 μM NVB for 48 hours. Cells were then fixed and subjected to immunofluorescence for pIRF3^{Ser396}. Representative IF mages at 100x magnification, scale bars, 10 μM, **e, f,** Cell lines (MDA-MD-436, **e**; CAPAN-1, **f**) were transfected with siRNA targeting *POLQ*, and RNA was extracted, and qPCR performed. *IRF7, CXCL10*, and *IFBN1* mRNA levels were normalized to *ACTB* internal control. Data, mean +/- SD of 3 independent experiments. Statistical analyses were performed using unpaired two-tailed *t*-tests. **g, h,** Cell lines (MDA-MD-436, **g**; CAPAN-1, **h**) were treated with 1 μM ART558 (ART) for 48 hours, and RNA was extracted, and qPCR performed. *IRF7, CXCL10*, and *IFBN1* mRNA levels were normalized to *ACTB* internal control. Data, mean +/- SD of 3 independent experiments. Statistical analyses were performed using unpaired two-tailed *t*-tests**. i,** PARPi resistant MDA-MB-436 cell lines were treated with DMSO or 100 μM NVB for 48 hours, and RNA was extracted, and qPCR performed for *IRF7, CXCL10* and *IFBN1*, normalized to *ACTB* internal control. Data, mean +/- SD of 3 independent experiments. Statistical analyses were performed using unpaired two-tailed *t*-tests

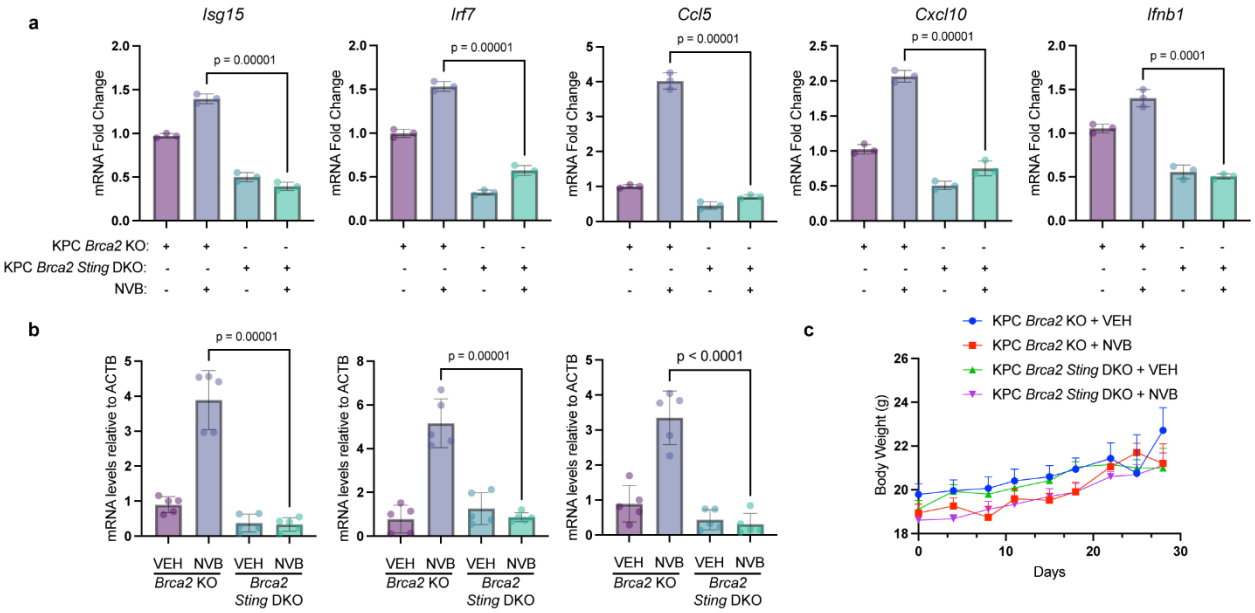


Supplementary Fig. 5. POLθ inhibition in HR-deficient tumors induces the cGAS/STING pathway in dendritic cells. a, Gating strategy for flow cytometry analysis. **b**, and **c**, Representative flow cytometry dotplots of TNBC and PDAC tumors from **5a-d**. **d** and **e**, Analysis of CCL5 levels by ELISA in the isogenic cell line pair MDA-MD-436 +/- BRCA1 (**d**), and the

isogenic cell line pair CAPAN-1 +/- BRCA2 (**d**) treated with DMSO or 100 μM NVB for 48 hours. Data, mean+/- SD of 3 independent experiments, unpaired two-way *t*-tests. **f**, **g**, and **h**, Representative flow cytometry dotplots of TNBC and PDAC tumors from **6a** and **b.**



Supplementary Fig. 6. HR-proficiency abolishes cGAS/STING pathway activation mediated by POLθ inhibition. a, Immunoblot analysis of TBK1 phosphorylation in HR-proficient KPC cells upon 100 μM NVB treatment for 48 hours. NVB-mediated POLθ inhibition did not increase TBK1 phosphorylation. Immunoblot analysis performed in triplicate. **b**, KPC cells were treated with DMSO or 100 μM NVB for 48 hours, RNA was extracted, and qPCR performed. *ISG15*, *IRF7*, *CCL5*, *CXCL10*, and *IFNB1* mRNA levels were normalized to *ACTB* internal control. Data, mean+/-SD of 3 independent experiments, unpaired two-way *t*-tests.



Supplementary Fig. 7. POLθ inhibition in HR-deficient tumors induces the cGAS/STING pathway and is required for anti-tumor efficacy. a, a, KPC *Brca2* KO CRISPR/Cas9 control or *Brca2 Sting* DKO cells were treated with 100 μM NVB and subjected to qPCR. *Sting* depletion abolishes NVB-mediated upregulation of *Isg15, Irf7, Ccl5*, *Cxcl10,* and *Ifnb1* mRNA levels normalized to *ACTB* control. Data, mean +/- SD of 3 independent experiments. Significance of difference determined by one-way ANOVA. **b**, PDAC tumors *Brca2* KO (VEH, *n* = 5; NVB, *n* = 5), or *Brca2 Sting* DKO (VEH, *n* = 5; NVB, *n* = 6) from vehicle- or twice daily 75 mg/kg NVB-treated mice were harvested 5 days after treatment and analyzed by whole tumor qPCR. Scatter plots show significant increases in *Ccl5, Cxcl10,* and *Ifnb1*. mRNA levels normalized to *ACTB* control from NVB-treated animals harboring *Brca2* KO PDAC tumors but not *Brca2 Sting* DKO PDAC tumors. Error bars, SEM. Statistical analyses were performed using one-way ANOVA. **c,** Bodyweight measurements of KPC *Brca2* KO (VEH, *n* = 10; NVB, *n* = 10) or KPC *Brca2 Sting* DKO (VEH, *n* = 10; NVB, *n* = 10) mice from **6g**. Data, mean+/-SEM.

Supplementary Tables

Supplementary Table 1: Antibodies

Antigen	Conjugate	Catalog #	Manufacturer	Method	Dilution	RRFID
BRCA2	-	10741	Cell Signaling	WB	1:500	RRID:AB_2797730
CD45	BV711	103147	Biolegend	FC	1:500	RRID:AB_2564383
CD3	BV785	100232	Biolegend	FC	1:1000	RRID:AB_2562554
CD3	-	99940	Cell Signaling	IHC	1:150	RRID:AB_2755035
CD4	APC	100412	Biolegend	FC	1:1000	RRID:AB_312697
CD4	-	25229	Cell Signaling	IHC	1:150	RRID:AB_2798898
CD68	-	97778	Cell Sginaling	IHC	1:200	RRID:AB_2928056
CD8	BV421	100737	Biolegend	FC	1:1000	RRID:AB_10897101
CD8	-	98941	Cell Signaling	IHC	1:400	RRID:AB_2756376
CD86	-	Nbp2-25208	Novus Biologicals	IHC	0.5 µg/ml	RRID:AB_2923115
CD163	-	16646-1-ap	Proteintech	IHC	1:150	RRID:AB_2756528
GrnzB	FITC	515403	Biolegend	FC	1:250	RRID:AB_2114575
FoxP3	PE/Cy7	25-5773-82	eBioScience	FC	1:100	RRID:AB_891552
CD11b	Percp/Cy5.5	101228	Biolegend	FC	1:1000	RRID:AB_893232
pTBK1	PE	13498S	Cell Signaling	FC	1:200	RRID:AB_2798237
pIRF3	AF700	10327S	Cell Signaling	FC	1:100	RRID:AB_2797719
PD-L1	BV605	124321	Biolegend	FC	1:500	RRID:AB_2563635
CD11C	BV650	564079	BD Bioscience	FC	1:500	RRID:AB_2725779
dsDNA	Pico-green	12010	Lumiprobe	IF	1:200	
cGAS	-	79978S	Cell Signaling	WB, IF	1:500	RRID:AB_2905508
pTBK1	-	5483S	Cell Signaling	WB	1:500	RRID:AB_10693472
TBK1	-	3504S	Cell Signaling	WB	1:500	RRID:AB_2255663
PD-L1	-	13684S	Cell Signaling	WB	1:500	RRID:AB_2687655
PD-L1	-	64988	Cell Signaling	IHC	1:200	RRID:AB_2799672
pIRF3	-	29047S	Cell Signaling	WB	1:500	RRID:AB_2773013
pSTING	-	50907S	Cell Signaling	WB	1:500	RRID:AB_2827656
STING	-	13647S	Cell Signaling	WB	1:500	RRID:AB_2732796
pSTAT1	-	9167S	Cell Signaling	WB	1:500	RRID:AB_561284
STAT1	-	149945	Cell Signaling	WB	1:500	
Tubulin	-	ab7291	abcam	WB	1:5000	RRID:AB_2241126
Vinculin	-	sc-25336	Santa Cruz	WB	1:200	RRID:AB_628438
POLQ	-	MBS9612322	MyBioSource	WB	1:500	
gH2AX	-	05-636-1	EMD Millipore	IF	1:1000	
gH2AX	-	9718	Cell Signaling	IF	1:1000	RRID:AB_2118009
rabbit IgG	HRP	ab6728	abcam	WB	1:5000	RRID:AB_955440
mouse IgG	HRP	ab97023	abcam	WB	1:5000	RRID:AB_10679675
rabbit IgG	594		Thermofisher	IF	1:5000	
mouse IgG	488		Thermofisher	IF	1:5000	

FC: flow cytometry, WB: Western blotting, IF: immunofluorescence, IHC: immunohistochemistry

Supplementary Table 2: qPCR probe assays

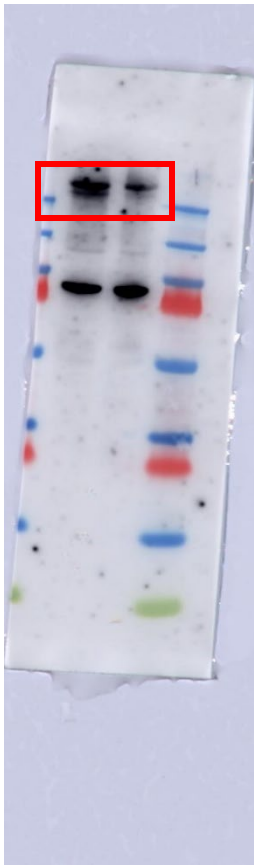
Species	Gene	Assay ID	Primer Sequences
	ACTB	Mm.PT.39a.22214843.g	5'-GATTACTGCTCTGGCTCCTAG 5'-GACTCATCGTACTCCTGCTTG
	Isg15	Mm.PT.58.41476392.g	5'-CACAGTGATCAAGCATTTGCG 5'-CCCCCATCATCTTTTATAACCAAC
	Irf7	Mm.PT.58.32394021.g	5'-GCATCACAGAGTAGTAGCATCT 5'-CCAATAGCCAGTCTCCAAACAG
Mouse	*CCL5*	Mm.PT.58.43548565	5'-GCTCCAATCTTGCAGTCGT 5'-CCTCTATCCTAGCTCATCTCCA
	CXCL10	Mm.PT.58.43575827	5'-ATTTTCTGCCTCATCCTGCT 5'-TGATTTCAAGCTTCCCTATGGC
	Ifnb1	Mm.PT.58.30132453.g	5'-ACTCATGAAGTACAACAGCTACG 5'-GGCATCAACTGACAGGTCTT
	PD-L1	Mm.PT.58.41689106	5'-TCTCAATCAGGTCTCCAGGT 5'-AGCTGATCCACAAACAAGAGG
	POLQ	Mm.PT.58.5536034	5'-GCCAGTCACCCAAATAGTTCT 5'-GATAGTGCGGAGGTAGAGGT
	ACTB	Hs.PT.39a.22214847	5'-ACAGAGCCTCGCCTTTG 5'-CCTTGCACATGCCGGAG
	Isg15	Hs.PT.58.39185901.g	5'-GCCTTCAGCTCTGACACC 5'-CGAACTCATCTTTGCCAGTACA
	Irf7	Hs.PT.58.24613215.g	5'-TCAACACCTGTGACTTCATGT 5'-GTGGACTGAGGGCTTGTAG
Human	*CCL5*	Hs.PT.58.1724551	5'-GCTGTCATCCTCATTGCTACT 5'-TGCCACTGGTGTAGAAATACTC
	CXCL10	Hs.PT.58.3790956.g	5'-GACATATTCTGAGCCTACAGCA 5'-CAGTTCTAGAGAGAGGTACTCCT
	Ifnb1	Hs.PT.58.39481063.g	5'GAAACTGAAGATCTCCTAGCCT 5'GCCATCAGTCACTTAAACAGC
	PD-L1	Hs.PT.56a.21399083.g	5'-CTTTGAGTTTGTATCTTGGATGCC 5'-AGGACTCACTTGGTAATTCTGG
	POLQ	Hs.PT.58.25668129	5'-TCTTCAACTGCTTCCTCTTCC 5'-GAGCAAATATTGTGGAGGTGGA

Source data for immunoblots presented in Supplementary figures 1 and 6

Supplementary figure 1a

PD-L1

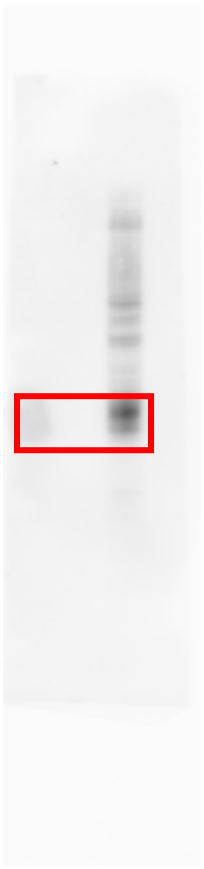
POLQ



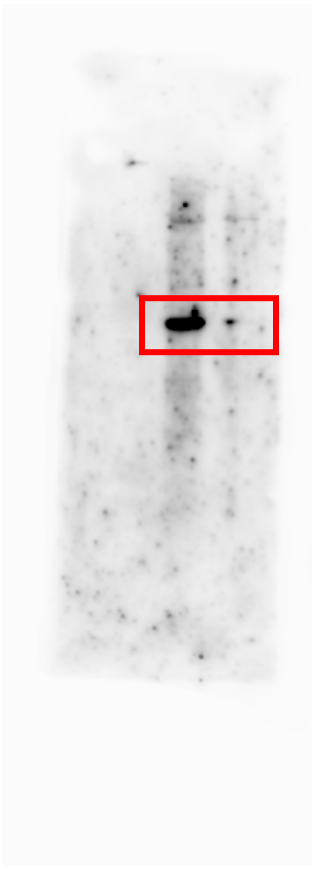
Vinculin

Supplementary figure 1b

PD-L1



POLQ



Vinculin

Supplementary figure 6a

p-Tbk1 Tbk1 Sting Tubulin