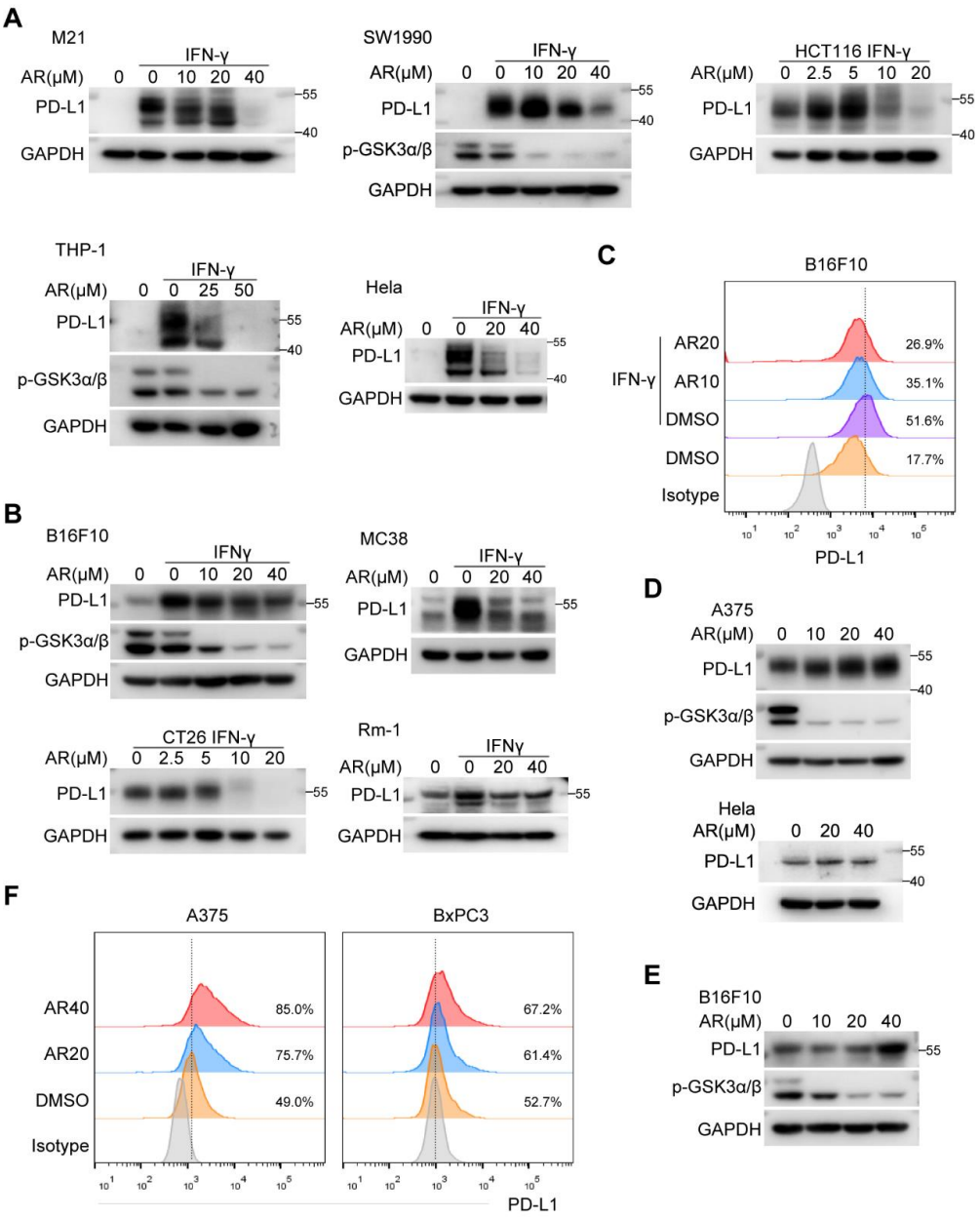


Figure S1. AR-A014418 blocks full-length PD-L1 induction in multiple tumor cell lines.

A-B. Human (A) and murine (B) cancer cells were treated with IFN-γ and AR-A014418 for 24 h and subjected to western blot. C. Flow cytometry analysis of cell surface PD-L1 expression in B16F10 cells treated with IFN-γ and AR-A014418 for 24 h. D-F. Western blot (D, E) and flow cytometry (F) analysis of basal PD-L1 expression in cancer cells treated with AR-A014418.

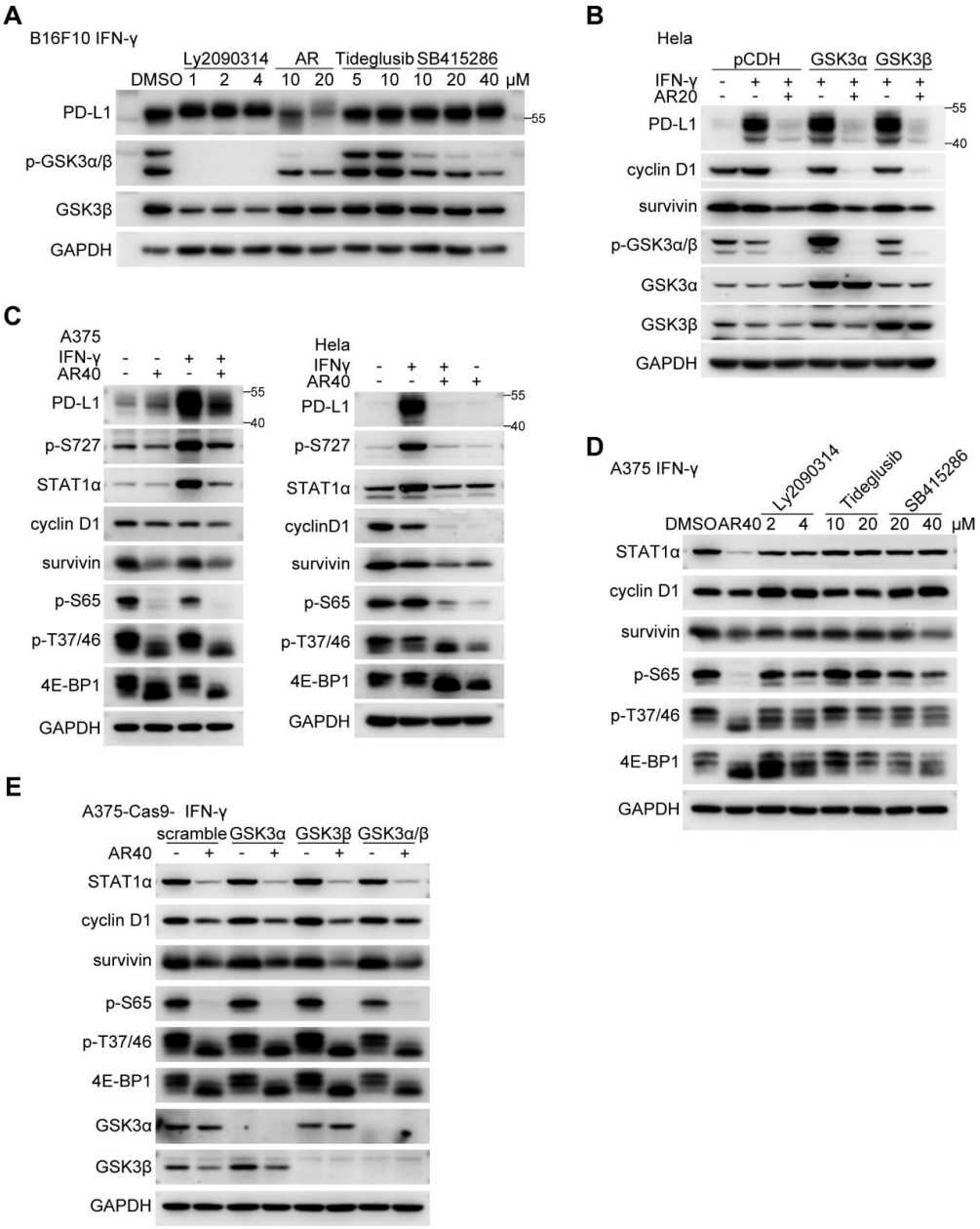


Figure S2. The influence of AR-A014418 on the expression or phosphorylation of some proteins are unrelated to GSK3 pathway.

A. B16F10 cells were treated with Ly2090314, AR-A014418, Tideglusib or SB415286 together with IFN-γ for 24 h and analyzed by western blot. B. Western blot analysis of indicated protein expression or phosphorylation in Hela cells transiently overexpressing GSK3α or GSK3β. C. A375 and Hela cells were treated with IFN-γ and AR-A014418 (40 μM) for 24 h, the whole-cell lysates were subjected to western blot analysis. D. A375 cells were treated with AR-A014418, Ly2090314, Tideglusib or SB415286 together with IFN-γ

for 24 h and analyzed by western blot. E. Western blot analysis of GSK3α and/or GSK3β knockout A375 cells.

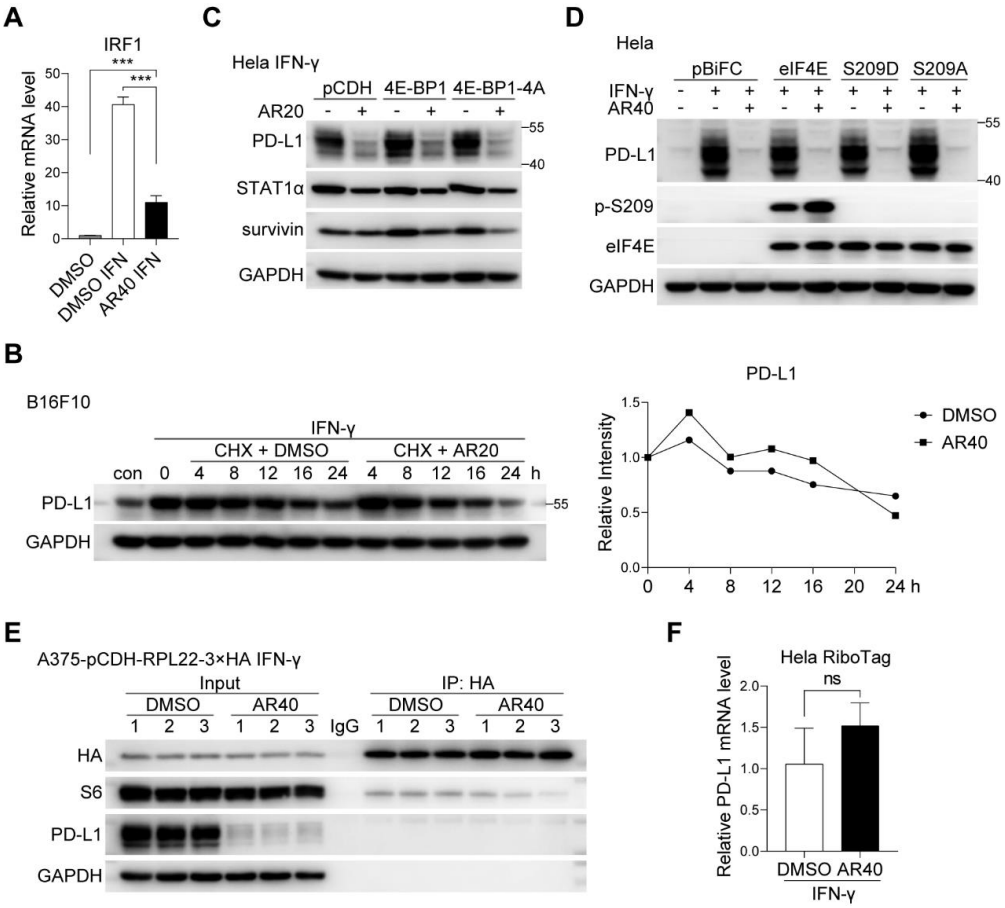
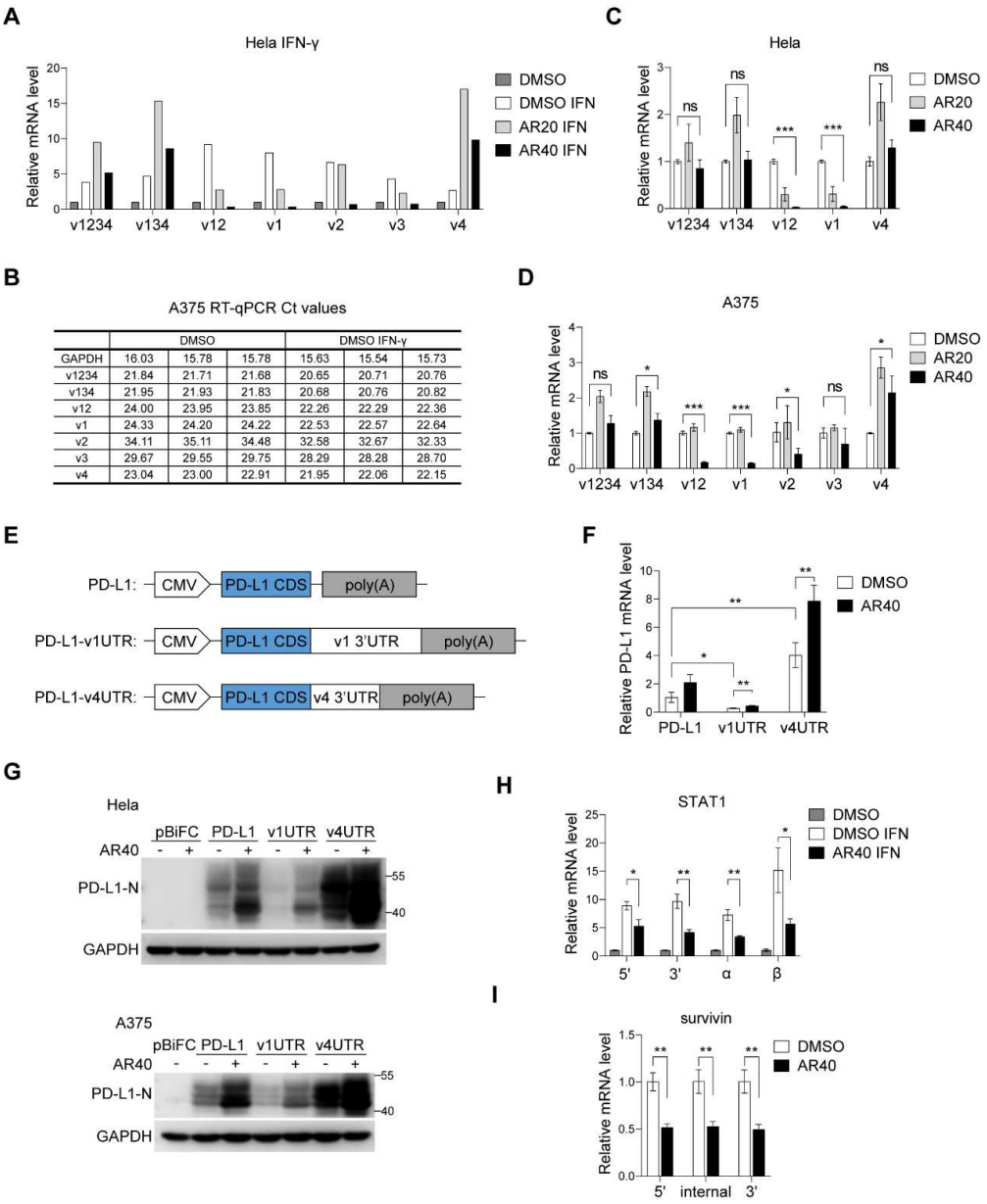


Figure S3. AR-A014418 has no effect on PD-L1 translation and protein degradation. A. A375 cells were incubated with DMSO/AR-A014418 (40 μM) in the presence of IFN-γ for 12 h, IRF1 mRNA levels were measured by RT-qPCR. B. PD-L1 expression was induced by IFN-γ for 24 h, then the culture medium was replaced with fresh medium containing DMSO/AR-A014418 (20 μM) and cycloheximide (100 μg/mL) to stop protein synthesis. B16F10 Cells were harvested at 0, 4, 8, 12, 16 and 24 h and used for western blot analysis (shown on the left). The band intensities of PD-L1 (normalized to GAPDH) were quantified with ImageJ (shown on the right). The experiment was repeated three times independently with similar results. C-D. 30 h after transfection with the indicated plasmids, Hela cells were induced by IFN-γ in the presence or absence AR-A014418 for 12 h and subjected to western blot analysis. E. Western blot analysis of A375-pCDH-RPL22-3×HA samples in RiboTag coimmunoprecipitation assays. F. RT-qPCR analysis of ribosome-bound total PD-L1 mRNA in stable Hela cells expressing RPL22-3×HA (Hela-pCDH-RPL22-3×HA). In A and F, the values are presented as mean ± SD (n =3 independent experiments); ***p<0.001.



	DMSO			DMSO IFN-γ		
GAPDH	16.03	15.78	15.78	15.63	15.54	15.73
v1234	21.84	21.71	21.68	20.65	20.71	20.76
v134	21.95	21.93	21.83	20.68	20.76	20.82
v12	24.00	23.95	23.85	22.26	22.29	22.36
v1	24.33	24.20	24.22	22.53	22.57	22.64
v2	34.11	35.11	34.48	32.58	32.67	32.33
v3	29.67	29.55	29.75	28.29	28.28	28.70
v4	23.04	23.00	22.91	21.95	22.06	22.15

Figure S4. AR-A014418 downregulates the expression of v1, but upregulates v4.
A. RT-qPCR analysis of PD-L1 variants in Hela cells cultured with IFN-γ and AR-A014418 (0, 20, 40 μM) for 12 h. The experiment was repeated three times independently with similar results. B. Ct values of the indicated PD-L1 variants by RT-qPCR. C-D. Hela or A375 cells were treated with AR-A014418 (0, 20, 40 μM) for 12 h and processed for RT-qPCR. E. Schematic representation of the plasmids composed of PD-L1 coding sequence and original 3' UTR in the vector or 3'UTR from v1 or v4. F-G. Hela or A375 cells were transiently transfected with the corresponding plasmids. DMSO/AR-A014418 (40 μM) treatment was carried out 6 h after transfection. Cells were collected at 12 h (for

RT-qPCR, Hela cells, F) and 18 h (for western blot, Hela and A375 cells, G) post drug treatment. H-I. Examination of STAT1 and survivin expression in A375 cells after the indicated treatment by RT-qPCR. Primer pairs, that recognize different parts of the mRNAs, were used. In C-D, F, and H-I, the values are presented as mean ± SD (n =3 independent experiments); *p<0.05, **p<0.01, ***p<0.001.

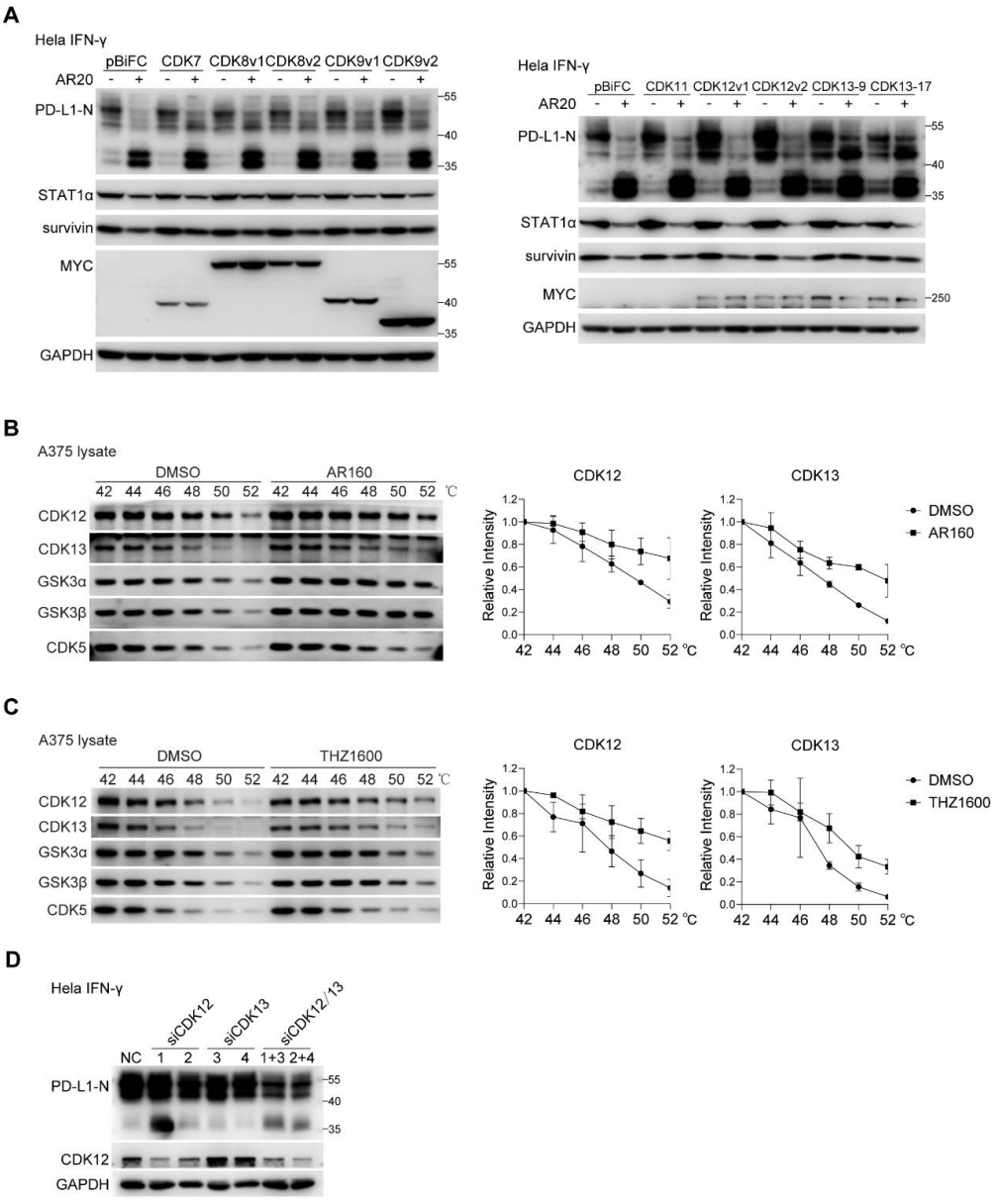


Figure S5. AR-A014418 directly targets CDK12 and CDK13.

A. Hela cells were transiently transfected with the indicated plasmids for 30 h and induced by IFN-γ and AR-A014418 (20 μM) for 16 h. B-C. Western blot analysis following thermal shift assays with DMSO, AR-A014418 (B, 160 μM) or THZ531 (C, 1.6 μM) treatment using

A375 cell lysates. The relative intensity of each sample at different temperatures was quantified with ImageJ (shown on the right). D. Western blot analysis of CDK12 and/or CDK13 knockdown Hela cells.

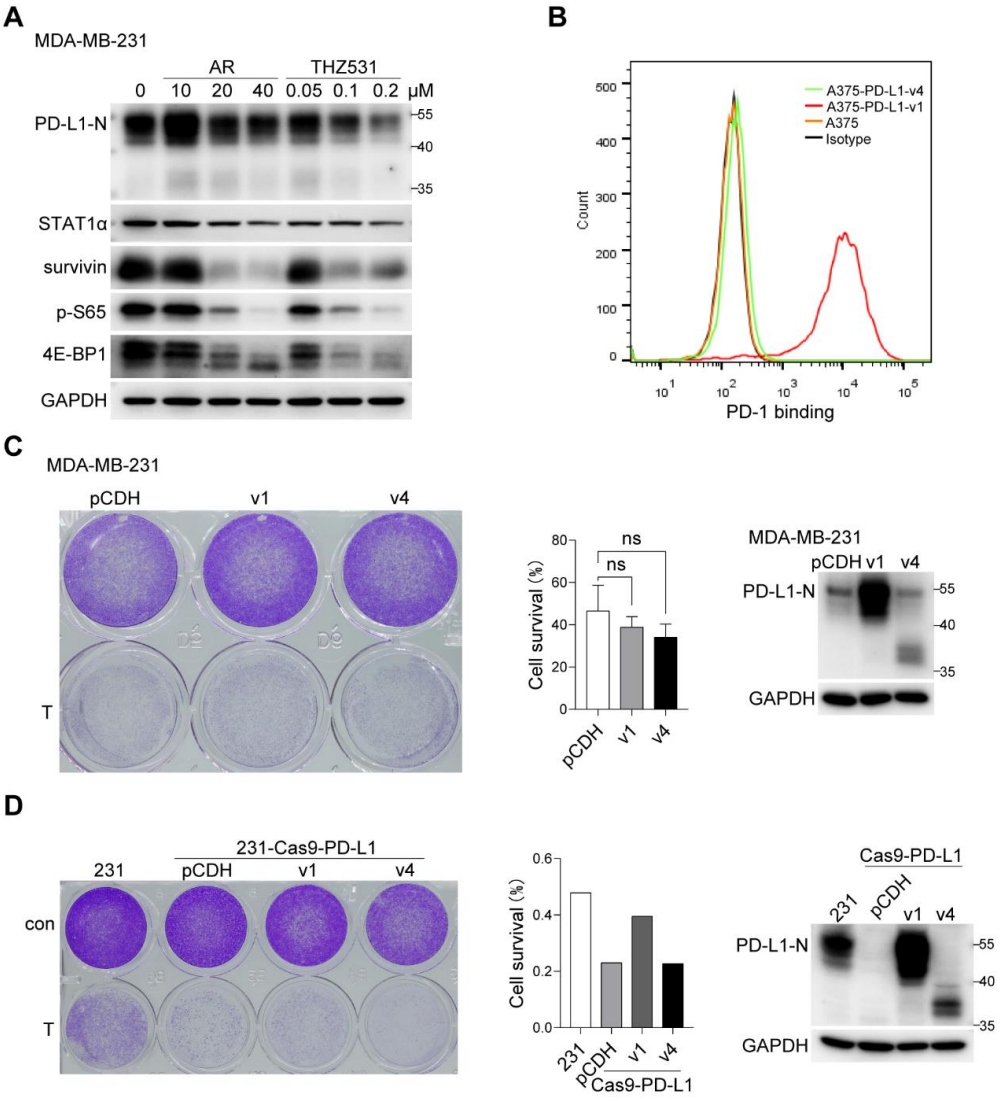


Figure S6. PD-L1-v4 does not significantly attenuate T cell cytotoxicity.
A. Western blot analysis of MDA-MB-231 cells treated with AR-A014418 (10, 20 or 40 μM) or THZ531 (50, 100, 200) for 24 h. B. A375 stable cell lines overexpressing the vector, full-length PD-L1 or PD-L1-v4 were subjected to PD-1 binding assays and analyzed by flow cytometry. C-D. T cell–mediated killing of WT (C) or PD-L1 depleted (D) MDA-MB-231 cells overexpressing vector, full-length PD-L1 or PD-L1-v4. The relative survival rates are presented in the middle panel. The overexpression of PD-L1 isoforms were confirmed by western blot (shown on the right).

Primary Antibodies:

Target	Application	Source	Cat No.
hPD-L1	WB; C-terminal	GeneTex	GTX104763
hPD-L1	WB; N-terminal	ABclonal	A19135
hPD-L1	FC	BioLegend	329702
mPD-L1	WB	R&D	AF1019
mPD-L1	FC; PE conjugated	ThermoFisher	12-5982-81
CDK12	WB	Proteintech	26816-1-AP
CDK13	WB	BOSTER	A05292-1
p-STAT1 (S727)	WB	Abcam	ab278718
STAT1α	WB	Abcam	ab92506
survivin	WB	CST	2808
cyclin D1	WB	Beyotime	AF1183
p-GSK3α/β (Y216+Y279)	WB	BOSTER	BM4836
GSK3α	WB	Sangon	D162911
GSK3β	WB	BOSTER	BM3904
GAPDH	WB	Proteintech	HRP-60004
MYC-tag	WB	Proteintech	60003-2-Ig
HA-tag	IP, WB	Proteintech	66006-2-Ig
GST-tag	WB	Sigma	G7781
p-4E-BP1 (S65)	WB	Beyotime	AF5809
p-4E-BP1 (T37/46)	WB	CST	2855
4E-BP1	WB	CST	9452
p-eIF4E (S209)	WB	BOSTER	BM4282
eIF4E	WB	Beyotime	AF6777
S6	WB	CST	2217
p-RPB1 CTD (S2)	WB	Abcam	ab252855
p-RPB1 CTD (S5)	WB	Abcam	ab5408
p-RPB1 CTD (S7)	WB	Abcam	ab252853
RPB1	WB	Abcam	ab76123

Secondary Antibodies:

	Source	Cat No.
Peroxidase AffiniPure F(ab')$_2$ Fragment Goat Anti-Rabbit IgG (H+L)	Jackson	111-036-045
Peroxidase AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson	715-035-150
Peroxidase AffiniPure Donkey Anti-Rat IgG (H+L)	Jackson	712-035-150
PE Goat anti-Human IgG Fc	ThermoFisher	12-4998-82
PE Goat anti-Mouse IgG	BioLegend	405307

Reagents and recombinant proteins:

	Source	Cat No.
AR-A014418	TargetMol	T1881
THZ531	TargetMol	T4293
Tideglusib	MedChemExpress	HY-14872
LY2090314	MedChemExpress	HY-16294
SB415286	MedChemExpress	HY-15438
Cycloheximide	MedChemExpress	HY-12320
Actinomycin D	J&K Scientific	338112
hIFN-γ	GenScript	Z02986
mIFN-γ	GenScript	Z02916
Recombinant Human PD-1 Fc Chimera Protein	R&D	1086-PD-050

Primers used for RT-qPCR of endogenous genes:

Targets	Forward Primers (5'-3')	Reverse Primers (5'-3')
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
Total PD-L1 (v1234)	TTCTGTCCGCCTGCAGGGCATT	CGTTCAGCAAATGCCAGTAGGTC
v134	GGCATTTGCTGAACGCATTTAC	GTGCAGCCAGGTCTAATTG
v12	GGACAAGCAGTGACCATCAAG	CCCAGAATTACCAAGTGAGTCCT
v1	CATGATCAGCTATGGTGGTG	AGATGGCTCCCAGAATTAC
v2	CATTTGCTGAACGCCCCATAC	CACTCAGGACTTGATGGTC
v3	CTGAGTGGAGATTAGATCCT	CATCCATCATTCTCCCAAG
v4	GGACAAGCAGTGACCATCAAG	GCTAGGGGACAGTGTTAGAC
IRF1	AGCTCAGCTGTGCGAGTGTA	TAGCTGCTGTGGTCATCAGG
STAT1-5'	ATCCTGCGCGCAGAAAAGT	GTGCCAAGACTGTCGAGGT
STAT1-3'	GCGGTTGAACCCTACACGAA	CATTGGCTCTGGTGCTTCCT
STAT1α	CTGAGGAGTTTGACGAGGTGT	GGAAAACTGTCGCCAGAGAA
STAT1β	CCAATGGAACTTGATGGCCC	AAGGCTGGCTTGAGGTTTGT
survivin-5'	AGATTTGAATCGCGGGACC	GAAGGGCCAGTTCTTGAATGT
survivin-internal	AGGACCACCGCATCTCTACAT	AAGTCTGGCTCGTTCTCAGTG
survivin-3'	TGACGACCCCATAGAGGAA	TCAATCCATGGCAGCCAG
WRN-IPA	GCATCTTCCTCATGCTTTCAG	TGCATCAGTCTGTGTGTGTG
WRN-Distal	GACAGCGGACTTCAACCTTC	TTGGCAAACCACACAGGTAA
FANCD2-IPA	ATCTGCAGTGGGAATGAAGC	ACTTGCCCATCATCTCACCT
FANCD2-Distal	CAGGAGAGCACAGCAGATGA	TGCTCCTTTTCTCCAGCACT

Primers used for RT-qPCR of transiently transfected genes:

Targets	Forward Primers (5'-3')	Reverse Primers (5'-3')
MYC-PD-L1	AGCTGCGGAATTGTACCC	CCGTGACAGTAAATGCGTTC

mv1	GTGACCATCAAGTCTTATCG	ATGTGCCAGAGGTAGTTCTGG
mv4	GTGACCATCAAGTCTTATCG	GCTAGGGGACAGTGTTAGAC

sgRNA target sequences:

scramble: GACAGATGCCTTTCCGCCGC
GSK3α: GACAGATGCCTTTCCGCCGC
GSK3β: CTTGCAGCTCTCCGCAAAGG
PD-L1: GTTCCCAAGGACCTATATG

siRNA sequences:

CDK12-1: sense (5'-3')-GGGAACAAGAGACUCUAAATT
antisense (5'-3')-UUUAGAGUCUCUUGUUCCCTT
CDK12-2: sense (5'-3')-CAGCUGACAUGCAGAAUAUTT
antisense (5'-3')-AUAUUCUGCAUGUCAGCUGTT
CDK13-3: sense (5'-3')-GCCAGUGCAUCACAAACAATT
antisense (5'-3')-UUGUUUGUGAUGCACUGGCTT
CDK13-4: sense (5'-3')-GGAGCAACAUGUAGCUUUATT
antisense (5'-3')-UAAAGCUACAUGUUGCUCCTT

Supplemental methods:

Plasmids:

Coding sequences of 4E-BP1, GSK3α and GSK3β were cloned into pCDH-CMV-MCS-EF1-Puro vector (CD510B-1, System Biosciences). Coding sequences of eIF4E, PD-L1, CDK7, CDK8, CDK9, CDK11, CDK12 and CDK13 were cloned into pBiFC-VN155(I152L) vector (27097, Addgene). Thr37, Thr46, Ser65 and Thr70 of 4E-BP1 were replaced by Ala to express 4E-BP1-4A. Ser209 of eIF4E was replaced by Asp or Ala. The original sequence between PD-L1 coding sequence and the poly(A) tail of pBiFC-PD-L1 was replaced by PD-L1 v1 or v4 3' UTR to study their effects on mRNA stability. Intron 4 of PD-L1 gene was inserted between exon 4 and exon 5 of pBiFC-PD-L1, and 4 canonical PAS (AATAAA) motifs were deleted to study intronic polyadenylation. All plasmids mentioned here were transiently transfected into Hela cells. Other plasmids used in this paper would be described in detail in the corresponding Methods sections.

Precipitation of secreted proteins:

Secreted PD-L1-v4 in the conditioned medium was precipitated by chloroform-methanol extraction method. Cells were grown in serum-free DMEM medium for 24 h. The conditioned medium was centrifuged at 2,000 g for 10 min to remove cell debris. 2 mL supernatant was mixed with 2 mL methanol and 500 µL chloroform, incubated on ice for 30 min, then centrifuged at 12,000 for 15 min. The protein layer was collected, washed with 1 mL methanol and dissolved in 1×loading buffer.

RiboTag RNA coimmunoprecipitation:

Stable A375 and Hela cells expressing RPL22-3×HA were used for RiboTag assays.

12 h after IFN-γ and DMSO/AR-A014418 (40 μM) treatment, the cells were harvested and lysed in homogenization buffer (50 mM Tris pH 7.4, 100 mM KCl, 12 mM $MgCl_2$, 1% NP-40, 1 mM DTT, proteinase inhibitors (PR20016, Proteintech), 400 U/mL RNasin (B600478, Sangon) and 200 μg/mL cycloheximide) on ice for 30 min. Cycloheximide was used to stabilize mRNAs onto ribosomes. All the buffers used in the RiboTag assays were prepared in DEPC treated water. The lysates were then centrifuged at 12,000g for 15 min. Mouse monoclonal anti-HA antibody was added to the supernatants and incubated overnight with rotation. After adding protein A/G magnetic beads, the mixture was further incubated for 8 h. The beads were collected and washed with high salt wash buffer (50 mM Tris pH 7.4, 300 mM KCl, 12 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 400 U/mL RNase Inhibitor, 200 μg/mL cycloheximide) for three times. Ribosome-bound (beads-bound) mRNAs were extracted using RNA Isolation Kit (B610583, Sangon) and subjected to RT-qPCR analysis. A portion of supernatants before antibody incubation and beads after washing were analyzed by western blot to confirm successful immunoprecipitation of ribosomes.

Thermal shift assays:

To analyze thermal stability of CDK12 and CDK13, which are located in the nucleus, A375 cells were lysed in lysis buffer (20 mM HEPES pH 7.4, 300 mM KCl, 0.2% NP-40, 1 mM DTT, proteinase inhibitors and 10 U/mL DNase I (2270A, TaKaRa)) on ice for 30 min. The lysates were then centrifuged at 20,000g for 15 min. The supernatant was mixed with dilution buffer (20 mM HEPES pH 7.4, 1 mM DTT, proteinase inhibitors) at 1:1 and incubated with DMSO, AR-A014418 (final concentration 160 μM) or THZ531 (final concentration 1.6 μM) on ice for 1 h. Samples were aliquoted into PCR tubes and exposed to a thermal gradient ranging from 42 °C to 52 °C for 3 min. Tubes were spun down at 20,000 g, 20 min to separate protein aggregates from soluble proteins. Supernatants were collected and analyzed by western blot.

Expression and purification of recombinant proteins:

Expression and purification of functional CDK12/cyclin K and CDK13/cyclin K complexes were performed as previously described [1] with some modifications. The MYC tag sequence in pBiFC-VN155(I152L) vector (27097, Addgene) was replaced with 6×His tag to generate pBiFC-His in our lab. The entire coding sequences of CDK12, CDK13 or cyclin K were cloned into pBiFC-His vector. One 10 cm dish of 293T cells were transfected with 16 μg pBiFC-His/-CDK12/-CDK13 together with 2 μg pBiFC-His-cyclin K using Lipo6000. 48 h after transfection, cells were harvested and lysed in lysis buffer 1 (20 mM HEPES pH 7.9, 15% glycerol, 0.2% NP-40, 300 mM KCl, 1 mM DTT, protease inhibitors, phosphatase inhibitors (PR20015, Proteintech) and 10 U/mL DNase I). Cell lysates were centrifugated at 20,000g for 15 min to separate soluble proteins. Ni Sepharose™ High Performance beads (17-5268-02, GE Healthcare) were washed two times with lysis buffer 1 and added to the supernatants. After incubation at 4 °C for 5 h, the beads were spun down and washed three times with wash buffer (20 mM HEPES pH 7.9, 15% glycerol, 0.2% NP-40, 500 mM KCl, 1 mM DTT) followed by two times with detergent-free buffer (20 mM HEPES pH 7.9, 150 mM KCl and 1 mM DTT) containing 10 mM imidazole. The

His-tagged proteins were eluted from beads with elution buffer (20 mM HEPES pH 7.9, 150 mM KCl and 1 mM DTT) containing 250 mM imidazole and concentrated using Amicon Ultra 30,000 MWCO centrifugal filters (UFC803096, Merck Millipore). Imidazole was removed during protein concentration.

To obtain unphosphorylated RPB1 CTD, the coding sequence of RPB1 (from 1587G to 1970N) was cloned into pGEX-6P-1 GST Expression vector (28-9546-48, GE Healthcare). Recombinant plasmids were then transformed into BL21 competent cells and 0.2 mM IPTG (I6758, Sigma) was used to induce GST-CTD expression for 10 h at 25 °C. Cells were collected and lysed in lysis buffer 2 (20 mM HEPES pH 7.9, 15% glycerol, 0.2% NP-40, 300 mM KCl, 1 mM DTT, protease inhibitors, 10 U/mL DNase I and 1 mg/mL lysozyme (A610308, Sangon)). After centrifugation at 20,000g for 15 min, the supernatants were transferred to clean tubes. Glutathione Sepharose^{TM} 4B beads (17–0756–05, GE Healthcare) were washed two times with lysis buffer 2 and added to the supernatants for 2 h incubation. The beads were washed three times with wash buffer (20 mM HEPES pH 7.9, 15% glycerol, 0.2% NP-40, 500 mM KCl, 1 mM DTT), three times with elution buffer (20 mM HEPES pH 7.9, 150 mM KCl and 1 mM DTT), and finally resuspended in elution buffer for further analysis.

***In vitro* kinase assays:**

For *in vitro* kinase assays, 5 µL His/cyclin K, CDK12/cyclin K or CDK13/cyclin K complexes and 5 µL GST-CTD beads were added to 30 µL kinase buffer (20 mM HEPES pH 7.9, 5 mM $MgCl_2$ and 1 mM DTT). The mixtures were supplemented with DMSO/AR-A014418/THZ531 at indicated concentrations followed by 2 mM ATP, then incubated at 30 °C for 1 h. The reactions were terminated by 5×protein loading buffer.

References:

1. Greifenberg AK, Honig D, Pilarova K, et al. Structural and Functional Analysis of the Cdk13/Cyclin K Complex. *Cell reports* 2016;14(2):320-31. doi: 10.1016/j.celrep.2015.12.025