PD-L1 degradation is regulated by electrostatic membrane association of its cytoplasmic domain

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The cytoplasmic domain of PD-L1 (PD-L1-CD) regulates PD-L1 degradation and stability through various mechanisms, making it an attractive target for blocking PD-L1-related cancer signaling. Here, by using NMR and biochemical techniques we find that the membrane association of PD-L1-CD is mediated by electrostatic interactions between acidic phospholipids and basic residues in the N-terminal region. The absence of the acidic phospholipids and replacement of the basic residues with acidic residues abolish the membrane association. Moreover, the basic-to-acidic mutations also decrease the cellular abundance of PD-L1, implicating that the electrostatic interaction with the plasma membrane mediates the cellular levels of PD-L1. Interestingly, distinct from its reported function as an activator of AMPK in tumor cells, the type 2 diabetes drug metformin enhances the membrane dissociation of PD-L1-CD by disrupting the electrostatic interaction, thereby decreasing the cellular abundance of PD-L1. Collectively, our study reveals an unusual regulatory mechanism that controls the PD-L1 level in tumor cells, suggesting an alternative strategy to improve the efficacy of PD-L1-related immunotherapies.
The important discovery that programmed death ligand 1 (PD-L1) expression on tumor cells inhibits programmed cell death 1 (PD-1) on T cells to escape immune surveillance has opened an era of tumor immunotherapy. PD-L1 is a type 1 transmembrane protein containing an extracellular domain (ECD), a transmembrane domain (TMD), and a cytoplasmic domain (CD). It is highly expressed on the surface of many cancer cells. Early functional and structural studies have revealed that the canonical immunoglobulin (Ig)-like ECD of PD-L1 binds to PD-1 on T cells to inhibit their tumor-killing activity. The PD-L1-ECD-targeting drugs that were developed to recover the tumor-killing activity of T cells have achieved great clinical success in treating a wide range of cancers. However, the therapeutic resistance to these anti-PD-L1-ECD drugs has been increasingly observed in patients, highlighting the need for alternative strategies to overcome the acquired resistance.

The small cytoplasmic domain of PD-L1 (PD-L1-CD) is involved in multiple regulation pathways controlling PD-L1 protein stability and degradation, and has been a potential new target inside the cell for the immune surveillance. PD-L1-CD was reported to bind to the cullin ligase (SPOP) that decreases the cellular abundance of PD-L1, hence a potential new target inside the cell for the immune pathways controlling PD-L1 protein stability and degradation, lysosome. Xu and Hung lab separately demonstrated that palmitoylation of C272 stabilizes the membrane insertion of PD-L1 and that was fused to the C-terminus of a His6-SUMO sequence with a PreScission protease (3 C) cleavage site in between. The fusion protein was purified by nickel affinity chromatography followed by 3 C cleavage and reverse-phase HPLC to remove the His6-SUMO tag.

The purified CD260–290 protein was incubated with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) liposomes supplemented with 25% 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), or 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS), or 5 or 15% cardiolipin (CL), respectively. After an ultracentrifugation, CD260–290 was found in the pellet associated with the liposomes containing the acidic phospholipid DMPG, DMPS or CL but not the zwitterionic phospholipid DMPE and/or DMPC (Fig. 1a). These results indicate that the acidic phospholipids are required for the membrane binding of CD260–290.

We further used solution NMR spectroscopy to study the PD-L1-CD-membrane interaction as previously reported. Isotope-labeled CD260–290 was reconstituted into the zwitterionic DMPC/1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPE) bicelles (molar ratio q = 0.8) containing DMPG at different molar ratios (Supplementary Fig. 2a). Two-dimensional (2D) 1H–15N transverse relaxation optimized spectroscopy (TROSY) experiments were used to monitor the chemical shift and intensity perturbations. A continuous fashion during the titration was observed on the N-terminal residues of CD260–290 (Supplementary Fig. 2a), i.e., a single resonance peak for each perturbed resonance of CD260–290 at any titration point, indicating that CD260–290 is in fast exchange on the chemical shift time scale between membrane-bound and unbound forms. We noticed that CD260–290 in DMPC/DHPE bicelles (molar ratio q = 0.8) did not have detectable chemical shift differences from the protein in solution (Fig. 1b and Supplementary Fig. 2d), while CD260–290 in acidic DMPG/DHPE bicelles (q = 0.8) displayed significant chemical shift and peak intensity changes in most N-terminal resonance peaks (Fig. 1b and Supplementary Fig. 2d), suggesting that CD260–290 specifically binds to acidic phospholipids in the bicelles. Moreover, addition of CD260–290 to 1-palmitoyl-2-oleoyl-sn-glycero-3-(phospho-rac-(1-glycerol)) (POPG)/DHPE bicelles (q = 0.8) induced chemical shift changes similar to those induced by DMPG/DHPE bicelles (Supplementary Fig. 2e).

Results
Acidic lipids regulate membrane interaction of PD-L1-CD. To explore whether acidic lipids regulate PD-L1-CD protein–membrane interactions, we expressed the residues 260–290 of human PD-L1 (CD260–290) (Supplementary Fig. 1a) in E. coli that was fused to the C-terminus of a His6-SUMO sequence with a PreScission protease (3 C) cleavage site in between. The fusion protein was purified by nickel affinity chromatography followed by 3 C cleavage and reverse-phase HPLC to remove the His6-SUMO tag (Supplementary Fig. 1b–d).

The purified CD260–290 protein was incubated with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) liposomes supplemented with 25% 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS), or 5 or 15% cardiolipin (CL), respectively. After an ultracentrifugation, CD260–290 was found in the pellet associated with the liposomes containing the acidic phospholipid DMPG, DMPS or CL but not the zwitterionic phospholipid DMPE and/or DMPC (Fig. 1a). These results indicate that the acidic phospholipids are required for the membrane binding of CD260–290.

Despite of the high biological relevance of PD-L1-CD in regulating PD-L1 levels in tumor cells, the molecular recognition and interaction of PD-L1–CD that control PD-L1 levels remain a mystery. Previous studies showed that PD-L1 is highly expressed on the plasma membrane in tumor cells, of which the inner leaflet is enriched in acidic phospholipids, including phosphatidylserine (PS) and phosphatidylinositol (PI). The asymmetric distribution of lipids results in negative charges on the cytoplasmic face of the plasma membrane. Moreover, recent studies revealed that PD-L1 is not only located on the cell membrane, but also in extracellular vesicles, such as exosomes, which facilitates tumor cells to evade immune surveillance. Exosomes are also enriched in acidic lipids such as PS, indicating that PD-L1 has a preference to locate in an acidic lipid-rich environment. The palmitoylation of C272 stabilizes the membrane insertion of PD-L1-CD and thus suppresses PD-L1 degradation, suggesting that the membrane association of PD-L1-CD has a significant effect on stabilizing PD-L1. More broadly, the asymmetric lipid distribution with excess negative charges on the plasma membrane appears to regulate the signaling via several type 1 transmembrane proteins, such as CD36/C and CD28 in T cells.

Therefore, in this study we performed structural and functional investigations of the lipid regulation of PD-L1-CD. We found that the membrane insertion of PD-L1-CD is regulated by negatively charged acidic phospholipids. The electrostatic interactions between acidic phospholipids and basic residues in PD-L1-CD proximal to the TM domain are critical for the membrane association. Using nuclear magnetic resonance (NMR) technology, we determined how PD-L1-CD binds to a lipid bicelle, in which three arginine residues are inserted into the lipid bilayer. Sequestration of these arginines into the plasma membrane enhanced the PD-L1 degradation in cells. Interestingly, we observed that metformin can disrupt the interaction between PD-L1-CD and membranes. Further, mutagenesis and cell-based functional assay showed that disruption of the membrane association by metformin reduces PD-L1 levels. And, the PD-L1-CD membrane interaction controls the abundance of PD-L1 in tumor cells and thereby regulates the immunosurveillance. Thus, our study uncovers a molecular mechanism that regulates PD-L1 levels, providing insights into PD-L1-mediated immune evasion of tumor cells that can be potentially targeted by drugs.
To test the effect of acidic lipids in live cells, we used fluorescence resonance energy transfer (FRET) to monitor the membrane binding of PD-L1-CD following an established protocol, in which the monomeric teal fluorescent protein (mTFP) was the donor and the octadecyl rhodamine B (R18) was the acceptor (Fig. 1c). The mTFP was fused to the C-terminus of full length PD-L1 (PD-L1-mTFP). The PD-L1-mTFP fusion protein was stably expressed in RKO cells whose plasma membranes were labeled with the R18 dye. The FRET efficiency between PD-L1-mTFP and the R18 dyes in the membranes was first measured using the donor dequenching method. The result showed that after a treatment of the cells with PS, which increases the content of acidic lipids in the plasma membrane, a higher FRET efficiency was detected compared to the solvent-treated cells, indicating stronger interactions between PD-L1-CD and the membrane with more acidic phospholipids (Fig. 1d). This result was further confirmed by the FRET efficiency determined using the donor quenching approach (Supplementary Fig. 3). In addition, a lower FRET efficiency was obtained when the RKO cells were treated with sphingosine (Sph, a membrane-permeant base), as expected for the reduced negativity of the plasma membrane by Sph (Fig. 1d).

The effects of PS and Sph on the endogenous PD-L1 level on the RKO cell surface were further examined by confocal imaging.
Acidic phospholipids are required for recruitment of PD-L1-CD into model and native membranes. a CD$_{260-290}$ was incubated without or with DMPC liposomes supplemented with DMPE, DMPG, DMPS, or CL as indicated on the bottom. The fractions from the pellet (P) and the supernatant (S) were separated by ultracentrifugation and analyzed by SDS-PAGE. Two independent experiments were performed with similar results. b Superoimposed 1H-15N TROSY-HSQC spectra of CD$_{260-290}$ in a solution buffer (25 mM MES pH 6.5) (blue) and in the same solution plus DMPC/DH$_4$PC (green) or DMPC/DH$_4$PC (red) bilayers. The results are representative of three independent experiments. c Schematic illustration of the FRET assay for measuring the interaction between mTFP (blue) fused to the C-terminal of PD-L1 and R18 dye (red) incorporated into the plasma membrane. The membrane contains acidic (pink) and other (green) lipids. The basic patch in PD-L1-CD is indicated by + symbols. d FRET detected lipid effects on PD-L1-CD-membrane interaction. Fluorescent images of the cells treated with DMSO, 5 μM PS or 2 μM Sph were taken to detect the mTFP or R18 dye emission before or after photobleaching (BP or AP) the R18 dye, respectively. Scale bars = 10 μm. The FRET statistical results from cells treated with DMSO (n = 24), PS (n = 18) or Sph (n = 17) are shown as the mean ± standard deviation (SD). Statistical differences were determined by unpaired two-sided Student’s t-test. e Immunofluorescence detection of endogenous PD-L1 in RKO cells treated with DMSO, 5 μM PS or 2 μM Sph. Scale bars = 5 μm in all panels. The fluorescence intensities from the cells were quantified and shown as mean ± SD from n = 20 cells under each treatment. P values were determined by two-sided Student’s t-test. f Cell surface levels of PD-L1 in RKO cells analyzed by flow cytometry after treatment with DMSO, 5 μM PS or 2 μM Sph for 6 h. g PD-L1 level (left) and ubiquitination (right) in RKO cells analyzed by western blotting. RKO cells were cultured for 12 h with or without 5 μM PS or 2 μM Sph. Two independent experiments were performed with similar results. Source data are provided as a Source Data file.

Membrane insertion of PD-L1-CD. We next studied how CD$_{260-290}$ interacts with the membrane using solution NMR spectroscopy. As mentioned above, a series of NMR spectra at different ratios of DMPG/DMPC showed that the chemical shifts of the N-terminal resonances moved continuously in straight lines (Supplementary Fig. 2a), indicating that CD$_{260-290}$ exists a two-state shift from membrane unbound to bound. To trap CD$_{260-290}$ in a membrane-bound state, we chose 100% DMPG/DH$_4$PC or 100% POPG/DH$_4$PC for the further investigation on protein–membrane interaction. The CD$_{260-290}$ protein was first reconstituted in DMPG/DH$_4$PC bicelles (q = 0.8). Essentially complete backbone and side-chain resonance assignments were accomplished through a standard set of triple resonance experiments (Fig. 2a). The intramolecular NOE restraints showed that the cytoplasmic domain are largely dynamic with no NOEs characteristic of secondary structures.

The membrane-bound protein was then characterized using the protein–lipid intermolecular NOEs measured by the protein–lipid NOESY experiments ($t_{mix} = 200$ ms) (Fig. 2b), in which CD$_{260-290}$ was completely deuterated to eliminate any signals from the backbone amides to side chains and reconstituted in the bicelles containing the DH$_4$PC with deuterated acyl chains. 100% POPG/DH$_4$PC bicelles were used in this experiment to keep the lipid acyl chains with the same length as the native membranes. Three arginine residues (R260/R262/R265) showed strong NOEs (distance of < 5 Å) to lipid acyl chains and headgroups (Fig. 2b, c), suggesting that these residues adopt an orientation toward the interior of the lipid bilayer. Such an orientation can facilitate the ionic interactions between the basic residue side chains and the phosphate groups in the lipid headgroups. V269 showed strong NOE signal to the methyl but weak signal to the methylene of lipid acyl chains, suggesting it is deeply inserted into the lipid bilayer (Fig. 2b).

In addition to NOE-based determination of CD$_{260-290}$ membrane insertion, we performed paramagnetic relaxation enhancement analysis for qualitative validation of the membrane-embedded CD$_{260-290}$ conformation. Two paramagnetic relaxation enhancement (PRE) probes were used to measure residue-specific depth immersion of the protein in the lipid bilayer region of the bicelles, as previously described (Supplementary Fig. 4a). The residue-specific PRE amplitudes (PRE$_{amp}$) from the water-soluble paramagnetic probe Gd-DOTA indicate that the N-terminal half of CD$_{260-290}$ is mostly inserted into the lipid bilayer with residues 265–270 displaying the lowest PRE$_{amp}$ inferring their membrane partition, while the C-terminal half is exposed to water with high PRE$_{amp}$ (Supplementary Fig. 4b). The results from the lipophilic PRE probe 16-DSA (Supplementary Fig. 4c) showed a clear cutoff of PRE$_{amp}$ between the membrane-embedded residues 260–274 and the water exposed residues 275–290 (Supplementary Fig. 4d). In short, the PRE data from the paramagnetic probe titrations are consistent with the protein–lipid NOE data, strengthen a model for partially membrane-embedded CD$_{260-290}$.

Key arginine residues in PD-L1-CD are critical to the membrane interaction. In the membrane-bound CD$_{260-290}$ several basic residues in the N-terminal half of CD$_{260-290}$ including R260, R262, K263, R265, K270, and K271, may interact with the lipid headgroups. Sequence analysis of PD-L1-CD shows that this basic region in the juxtamembrane segment is evolutionarily conserved between species, though the exact positions of the basic residues are not well conserved (Supplementary Fig. 5a, b).

The 3D NOESY spectra showed that R260, R262 and R265 have the strongest NOE signals to lipid headgroups and acyl chains, whereas K270 and K271 have weak NOE signals to lipids (Fig. 2b). We made R260E/R262E/R265E (CD$_{260-290}$RE) and K270E/K271E (CD$_{260-290}$KE) mutants to determine the contribution of these positively charged residues to the membrane interaction. The 2D TROSY spectra showed that the addition of CD$_{260-290}$RE mutant to the DMPG/DH$_4$PC bicelles did not induce the resonance shifts that was induced by the wild type (WT) protein (CD$_{260-290}$WT) (Figs. 1b and 3a). In contrast, obvious chemical shift perturbations were observed in the spectra of CD$_{260-290}$KE mutant upon the addition of the bicelles (Supplementary Fig. 5c). These spectral data indicate that the CD$_{260-290}$RE mutant loses the capacity to bind the membrane even it contains the acidic lipids, but the CD$_{260-290}$KE mutant retains the membrane binding capacity. This conclusion was further supported by the liposome binding assay, in which only CD$_{260-290}$KE mutant was detected in the DMPG liposome pellet after both mutants were incubated with the liposomes (Fig. 3b and Supplementary Fig. 5d). As a negative control, the R260K/R262K/R265K (CD$_{260-290}$KK) mutant interact...
with the lipid bicelles similar to the WT protein as detected by the NMR spectroscopy (Supplementary Fig. 5e) and the liposome binding assay (Supplementary Fig. 5f), implying that the positive charges at these positions instead of the specific residues dictate the ionic protein–lipid interaction.

To validate the role of the positively charged residues in the membrane binding we defined in vitro, we expressed wildtype CD\textsubscript{260-290} with a C-terminal GFP tag (CD\textsubscript{260-290\ WT}-GFP) in HEK293T cells, and determined the subcellular localization by confocal imaging. CD\textsubscript{260-290\ WT}-GFP is mainly localized on the plasma membrane (Fig. 3c), while CD\textsubscript{260-290\ 3RE}-GFP mutant is dispersed in the cytoplasm (Fig. 3d). These results were further confirmed by the FRET measurement with the mTFP-tagged full-length PD-L1\textsubscript{3RE} mutant (PD-L1\textsubscript{3RE}-mTFP) expressed in the RKO cells showing a lower FRET efficiency to the R18 dyes in the plasma membrane than the full-length wildtype PD-L1 (PD-L1\textsubscript{WT}-mTFP) in the absence of PS (Fig. 3e), while the presence of PS didn’t affect the FRET efficiency on PD-L1\textsubscript{3RE} (Fig. 3e). These mutagenesis data demonstrate that PD-L1-CD binds to the negatively charged membrane via the ionic interaction of the three positively charged juxtamembrane arginine residues, replacement of which with the negatively charged glutamic acid residues can potently disrupt the membrane binding.

**PD-L1-CD-membrane interaction regulates PD-L1 stability.** As we mentioned above, PD-L1-CD regulates PD-L1 stability and degradation\textsuperscript{14,15}, and stabilizing the PD-L1-CD-membrane association by palmitoylation\textsuperscript{16} suppresses the degradation of PD-L1. To study whether the membrane interaction of PD-L1-CD facilitated by acidic lipids plays a role in the stability of PD-L1, we examined the level of full-length PD-L1\textsubscript{WT} and PD-L1\textsubscript{3RE} mutant expressed in HEK293T cells. The cycloheximide (CHX)-chase assay\textsuperscript{39} revealed accelerated reduction of PD-L1\textsubscript{3RE} level than PD-L1\textsubscript{WT} level (Fig. 4a-c). Cell-surface expression of PD-L1 monitored by flow cytometry showed a more substantial reduction of PD-L1\textsubscript{3RE} mutant compared to the wild-type protein (Fig. 4d). MG132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucine), an inhibitor that can effectively block the proteosome activity\textsuperscript{30}, was introduced to the HEK293T cells expressing PD-L1\textsubscript{WT} or PD-L1\textsubscript{3RE} mutant to inhibit their degradation. Western blot analysis indicated that MG132 restored the level of both proteins in HEK293T cells with a more significant increase observed for PD-L1\textsubscript{3RE} mutant (Fig. 4e).

To demonstrate that the proteosome mediated PD-L1 degradation involves ubiquitination, we determined the level of the cellular ubiquitination of PD-L1\textsubscript{3RE} mutant relative to PD-L1\textsubscript{WT}. The HEK293T cells expressing PD-L1\textsubscript{WT} or PD-L1\textsubscript{3RE} were first treated with MG132, then subjected to PD-L1 immunoprecipitation (IP), followed by immunoblotting analysis with anti-V5 antibody to detect the ubiquitination of full length PD-L1\textsubscript{WT} and PD-L1\textsubscript{3RE} mutant. We found more ubiquitination for PD-L1\textsubscript{3RE} mutant in the presence of MG132 compared to the wild-type protein (Fig. 4f), indicating that the mutant undergoes

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**Fig. 2 The NMR characterization of membrane-bound CD\textsubscript{260-290}.** a \textsuperscript{1}H\textsubscript{15N} TROSY-HSQC spectrum of CD\textsubscript{260-290} in POPG/DH\textsubscript{6PC b}icelles with backbone resonances assigned. The spectrum was recorded at \textsuperscript{1}H frequency of 600 MHz using \textsuperscript{15N, 2H}-labeled protein. b Lipid NOEs from CD\textsubscript{260-290} in POPG/ DH\textsubscript{6PC} bicelles. Strips from 3D \textsuperscript{15N}-edited NOESY-TROSY-HSQC (\textsuperscript{τNOE} = 200 ms) spectra recorded using 0.8 mM \textsuperscript{15N, 2H}-labeled CD\textsubscript{260-290} in the bicelles with protonated POPG and deuterated DH\textsubscript{6PC} show the NOEs exclusively between backbone amide protons of CD\textsubscript{260-290} and methyl protons or water protons (*), mostly for the residues in the N-terminal half of the protein. The spectrum was recorded at \textsuperscript{1}H frequency of 900 MHz. c Strips from the same experiment mentioned above showed the NOEs from the side chains of three arginine residues to the lipid acyl chains and headgroups of POPG.
Fig. 3 Polybasic residues mediate the membrane interaction of PD-L1-CD in vitro and in cells. 

a) Superimposed 2D $^{1}$$H$-$^{15}$N TROSY-HSQC spectra of CD$_{260-290}^{3RE}$ mutant in solution (red) and with DMPG/DHPC bicelles (blue). The spectra were recorded at $^{1}$$H$ frequency of 600 MHz using [$^{15}$N, $^{1}$$H$]-labeled protein. 

b) Wildtype and mutant CD$_{260-290}$ association with liposomes (n = 3 independent experiments). The liposome binding assay was performed with (labeled as "liposome") or without liposomes (labeled as "No liposome") as in Fig.1a but for CD$_{260-290}^{WT}$ and CD$_{260-290}^{3RE}$ mutant. The fractions after centrifugation were analyzed by SDS-PAGE, in which "S" represents the supernatant and "P" represents the pellet. 

c, d Localization of wildtype and mutant CD$_{260-290}$ in HEK293T cells. Confocal microscopy images of HEK293T cells expressing CD$_{260-290}^{WT}$-GFP (c) or CD$_{260-290}^{3RE}$ (d) protein are shown. The experiment was repeated twice with similar results. Scale bars, 5 μm. 

e) Plasma membrane interaction of wildtype and mutant PD-L1 detected by FRET. The FRET efficiency was determined for RKO cells expressing PD-L1$_{WT}$-mTFP (left top), PD-L1$_{3RE}$-mTFP (right top) and PD-L1$_{3RE}$-mTFP treated with 5 μM PS (left bottom) for 2h. A similar whisker-dot plot shows the statistical results from n = 24, 22, or 25 WT or 3RE mutant-expressing cells, respectively (right bottom). Data are represented as mean ± SD. P values were determined by unpaired two-sided Student’s t-test. Source data are provided as a Source Data file.
Fig. 4 The 3RE mutation reduces cellular PD-L1 protein levels. a–c Cellular levels of PD-L1WT or PD-L13RE mutant. HEK293T cells expressing exogenous PD-L1WT or PD-L13RE mutant were treated with 20 μM cycloheximide (CHX) for 2, 4, 6, or 8 h. The PD-L1 level was analyzed by western blot (a, b). The intensities of the PD-L1 protein bands on the blots were quantified by ImageJ analysis, and the statistical data shown in (c) are the mean ± SD (n = 3 independent experiments). P values by two-sided Student’s t-test were indicated. d Surface level of PD-L1WT or PD-L13RE mutant in HeLa cells determined by flow-cytometric analysis. e Cellular level of PD-L1WT or PD-L13RE mutant in HEK293T cells treated with 10 μM of the proteasome inhibitor MG132 for 4 h (+ MG132) or not (- MG132) determined by western blot. f Ubiquitination of PD-L1WT or PD-L13RE mutant in HEK293T cells examined by V5 immunoblotting after IP with PD-L1 antibody, along with the control cells that did not express these proteins (vector). All data shown above are representative of three independent experiments. Source data are provided as a Source Data file.

more degradation by the proteasome dependent on the ubiquitination. We further performed the CHX-chase assay and the ubiquitination examination on PD-L1WT and PD-L13RE in RKO cells, showing consistent results that PD-L13RE degraded faster than PD-L1WT with higher ubiquitination levels (Supplementary Fig. 6). Collectively, these observations suggest that the impaired membrane-binding capability of PD-L13RE mutant promotes its ubiquitination-dependent degradation. Thus, the membrane association of PD-L1-CD enhances PD-L1 protein stability.

Metformin promotes PD-L1 degradation by PD-L1-CD-membrane dissociation. The critical role of PD-L1-CD in PD-L1 stability and degradation prompted us to search for therapeutics that can alter the membrane association of PD-L1-CD and hence control the PD-L1 level. Metformin has been reported to possess antitumor effects against various cancer types in recent years31–33, though it has been a popular drug for the treatment of type 2 diabetes for long time34. However, how metformin acts on cancer cells remains controversial due to the use of suprapharmacological concentrations in almost all previous studies35. Considering both arginine and metformin contain the same positively charged guanidinium group (Supplementary Fig. 7a), it is possible that metformin may compete with the arginine residues in PD-L1-CD for the lipid headgroups and thereby disrupt the membrane binding of PD-L1-CD, which allows the access to the enzymes that can ubiquitinate and degrade PD-L1.

To test this hypothesis, we first titrated metformin to CD260-290 in the presence of lipid bicelles and monitored the changes of protein–membrane interaction using NMR spectroscopy. As the concentration of metformin increases, the resonances of CD260-290 move toward the bicelle-free form in both 15N and 1H dimensions, indicating that the conformation of CD260-290 shifts toward a non-membrane-bound state (Fig. 5a, b). We further performed PRE experiments to verify the role of metformin on the membrane association of CD260-290. Titration of metformin to CD260-290 in the presence of the DMPG/DHPC bicelles containing the lipophilic paramagnetic probe 16-DSA increased the recovery of resonance intensities of the membrane-bound residues (Fig. 5c), suggesting that metformin moves these residues away from the membrane and thus shielded this region from the paramagnetic probe. The liposome binding assay confirmed the negative effect of metformin on the membrane association of PD-L1-CD since increasing the concentration of metformin reduced the binding of PD-L1-CD to the liposomes (Supplementary Fig. 7b). Moreover, the [1H]-15N HetNOE values of the N-terminal 260-275 residues of CD260-290 shifts toward a non-membrane-bound state (Fig. 5a, b). We further performed PRE experiments to verify the role of metformin on the membrane association of CD260-290. Titration of metformin to CD260-290 in the presence of the DMPG/DHPC bicelles containing the lipophilic paramagnetic probe 16-DSA increased the recovery of resonance intensities of the membrane-bound residues (Fig. 5c), suggesting that metformin moves these residues away from the membrane and thus shielded this region from the paramagnetic probe. The liposome binding assay confirmed the negative effect of metformin on the membrane association of PD-L1-CD since increasing the concentration of metformin reduced the binding of PD-L1-CD to the liposomes (Supplementary Fig. 7b). Moreover, the [1H]-15N HetNOE values of the N-terminal 260-275 residues of CD260-290 from the samples with bicelles are more positive and less dynamic than those from the samples without bicelles, while in the presence of metformin, the N-terminal half of CD260-290 in bicelles gave low steady-state [1H]-15N HetNOE values as the samples in solution, indicating CD260-290 is released from the membrane by metformin (Supplementary Fig. 7c).

Next, we tested the effects of metformin on PD-L1 in cells. In the absence and presence of metformin, the messenger RNA level for endogenous PD-L1 in RKO cells was similar (Supplementary Fig. 7d) but the protein level was reduced in the presence of metformin (Supplementary Fig. 7e). Moreover, the ubiquitinated protein level was higher when metformin was present (Supplementary Fig. 7f), which are consistent with previous studies31,36.
Importantly, the FRET imaging showed a significantly lower FRET efficiency after the metformin treatment of the RKO tumor cells expressing PD-L1WT-mTFP (Fig. 5d). This direct observation of the membrane-binding status of PD-L1 via the C-terminal fluorescent tag indicates that metformin can induce the dissociation of PD-L1-CD from the plasma membrane.

Finally, we treated HEK293T cells expressing PD-L1WT or PD-L13RE mutant with metformin and examined the PD-L1 protein level by western blot (Fig. 5e) and flow cytometry (Fig. 5f). The PD-L13RE mutant showed similar protein levels with or without the metformin treatment, indicating that PD-L13RE mutant that is less associated with the plasma membrane is less affected by metformin than PD-L1WT that is more associated with the membrane. The effects of a structural analog of metformin, moroxydine (Supplementary Fig. 8a), on the membrane association of the cytoplasmic domain in vitro and the PD-L1 protein level in cells were also examined by NMR spectroscopy and western blot, respectively. The same results were observed with this metformin analog as the moroxydine titration shifted the resonances of PD-L1-CD in lipid bicelles toward that of the protein in solution (Supplementary Fig. 8b). And, the level of PD-L1 in RKO cells was reduced by the addition of moroxydine, though a higher amount of moroxydine than metformin was required to achieve the same effect (Supplementary Fig. 8a compared to Fig. 5e).

**Discussion**

Recent studies suggest that intracellular expression of PD-L1 and the redistribution to the cell membrane may cause resistance to
anticancer drugs and protect cancer cells from T cell-mediated immune surveillance. Thus, alternative anticancer strategies are needed, and a recent focus is on the factors that control the cellular abundance of PD-L1. The involvement of the cytoplasmic domain in multiple pathways that regulate cellular level of PD-L1 makes it a potentially target for exploring alternative cancer therapeutics in combination with the current immunotherapy. A step forward is to understand how the cytoplasmic domain of PD-L1 interacts with the plasma membrane and how this interaction regulates the PD-L1 level.

In this study, we characterized PD-L1-CD in a model membrane system using NMR spectroscopy. Previous studies showed that some disordered proteins display certain structure features when bound to membranes. For example, α-synuclein is predominantly a random coil in aqueous solution but adopts a helical secondary structure when interacts with negatively charged membranes. In our case, no obvious secondary structure forms upon the binding to lipid bicelles, which is similar to the observations on CD28 and CD3ε cytoplasmic tails. We further demonstrated that the electrostatic interactions between a positively charged patch in the cytoplasmic domain and negatively charged phospholipids are critical to the membrane interaction and to the degradation of PD-L1 protein in tumor cells (Fig. 6). In the presence of acidic lipids, the three arginine residues adjacent to the TM domain project their side chains into the headgroups, when substituting the arginines by glutamates the ionic interactions between the positive guanidinium groups of the arginines and the negative phosphate moieties in the lipid headgroups, when substituting the arginines by glutamates completely abolishes the membrane association (Fig. 3a). In contrast, replacement of the arginines with lysines does not reduce the membrane binding (Supplementary Fig. 5e, f), indicating that the electrostatic attraction is the dominant binding force. K263 within this basic patch may also contribute to the membrane association of PD-L1-CD, though we did not investigate its role due to its relatively weak NOE signals to lipids. In addition, the position of the polybasic residues in PD-L1-CD matters since mutations of K270 and K271 that are farther away
from the TM domain do not disrupt the membrane association of PD-L1-CD (Supplementary Fig. 5c, d). Together, only the cluster of basic residues in PD-L1-CD located in the closest proximity to the TM domain is essential for the membrane association. The disruption of PD-L1-CD association with the plasma membrane by the triple arginine mutation leads to a reduction of PD-L1 cellular level. However, it is not clear which of the multiple pathways that regulate PD-L1 level is affected by the PD-L1-CD-membrane dissociation. One possibility is that the membrane partition of the polybasic residues may cause steric hindrance and hence limit the accessibility of themselves or neighboring residues to other downstream proteins, such as SPOP or HIP1R, which promote the ubiquitination-dependent proteosomal or lysosomal PD-L1 degradation in the cells. On the other hand, palmitoylation of PD-L1 has been reported to modulate the protein stability by blocking its degradation. PD-L1 is palmitoylated by DHHC3 acetyltransferase at C272, seven residues away from the basic membrane-binding patch. Previous crystal structures of the DHHC enzymes showed that the substrate palmitoyl-CoA inserts into the cavity formed by the enzymes within the membrane. If C272 were located within the membrane as our lipid NOE signal indicated (Fig. 2b), it would be accessible to the palmitoyl-CoA in the DHHC3 enzyme. When PD-L1-CD is dissociated from the membrane, C272 is less accessible to the enzyme and substrate, which can significantly decrease the palmitoylation and hence the stability of PD-L1. Another possibility is that the effects of PD-L1-CD-membrane dissociation on PD-L1 degradation are a combinational outcome from multiple pathways including promoted PD-L1 degradation by increasing SPOP or HIP1R recruitment to PD-L1-CD and reducing DHHC3 palmitoylation. Further investigations are needed to examine these possibilities.

Calcium is important for cellular signaling that exerts regulatory effects on many enzymes and proteins. Previous studies showed that a calcium influx in T cells can disrupt the membrane interactions of the CD3ζ25,40 and CD2824 tails by shielding the acidic lipid head groups, which releases the motifs in the cytoplasmic domains and mediates the downstream signaling. A high dietary calcium intake has been found to reduce the risk of one or more types of cancer, such as colorectal cancer and rectal cancer, whereas another study suggested that a high calcium intake has been found to reduce the risk of one or more types of cancer, such as colorectal cancer and rectal cancer. Acidic phospholipids act not only as a membrane scaffold, but also involve in the molecular regulation of tumor cell survival. The NMR studies of the membrane-bound PD-L1-CD showed how basic residues in the cytoplasmic tail interact with a membrane to regulate a receptor protein conformation in the membrane, which in turn regulates the receptor stability and hence its function in a signal transduction. We note that some cancer derived mutations found in this cytoplasmic region might enhance the membrane association to escape the immune-surveillance. For instance, D276Y and T290M can increase the residue hydrophobicity with the replacements and stabilize PD-L1 with stronger hydrophobic membrane interactions. R260C might provide a new position for the palmitoylation and therefore improve the stability. It’s not clear whether R262K enhances the membrane interactions of PD-L1, since arginine has a guanidino group but a shorter acyl chain. Further studies of these mutations in biochemical and cell biological systems are required to confirm or refute the expectations. Interestingly, a broad range of transmembrane receptors also have short cytoplasmic domains that are highly basic. For example, the isoelectric point (pI) for B7 family ligands CD80, CD86 and PD-L2 is 11.47, 9.72, and 10.30, respectively. Therefore, we expect that the electrostatic interactions between the receptors and lipids regulating the protein stability and degradation may occur in a general manner, and our study brings valuable insights into the lipid regulation of these transmembrane receptors.

**Methods**

**Reagents and cells.** Lipids and detergents (DMPC, DMPG, DMPE, DMPS, DPPG, DHPC,HPC, Sph and PS (Brain, Porcine)) were from Avanti Polar Lipids. Stable isotopes for NMR spectroscopy experiments were from Cambridge Isotope Laboratories. Anti-human PD-L1 antibodies (ab213524), Alex488-conjugated anti-IgG (ab150077) and anti-IgG-HRP (ab97051) were from Abcam. Anti-V5 antibodies (96025) were from Thermo and FITC anti-PD-L1 antibodies (393606) were from Biologend. MG132 (HY-13299) and metformin (HY-0627) were from MedChem Express (MCE). The HEK293T cell line was a generous gift from Liming Sun (SIBCB). The RKO cell lines for expressing PD-L1 were generous gifts from Jic Xu (Fudan University), other cell lines used in the paper were from Cell Bank of Chinese Academy of Sciences.
Expression and purification of CD260-290 and its mutants. The cytoplasmic domain of human PD-L1 (residues 260-290), named as CD260-290, was fused to an N-terminal 6 His Tag and a small Ubiquitin C-terminal Modifying (SUMO) tag and a PreScission protease (3 C) in the PET28a vector. The construct was transformed into Escherichia coli BL21 (DE3) cells and grown at 37 °C in M9 minimal media until the culture reached an optical density at 600 nm (OD600) of 0.6–0.8. One or more stable isolates were supplemented to the growth media according to the NMR requirements. CD260-290 or variants were purified by a 0.5, 1, 2.5, 3, and 5 mM. The residue-specific PRE amplitude (PREamp) was determined by fitting the peak intensity as a function of [16-DSA].

Conformational analysis for immunofluorescence. RKO cells were seeded at approximately 30% confluence in a glass bottom dish (150680, Thermo). After reaching confluence medium, the cells were washed three times with PBS, then fixed by paraformaldehyde for 20 min at room temperature. Cells were washed three times with PBS. Cells were then permeabilized with 0.2% Triton X-100 in PBS and blocked in 5% BSA in PBS for 1 h at room temperature. FITC anti-PD-L1 antibodies were diluted in the blocking buffer (1:100) and incubated cells overnight at 4 °C. After rinsed by PBS three times, cells were treated by SlowFade Diamond Antifade reagent (S36968, Thermo) and sealed by a coverslip. Cells were observed with Zeiss LSM710 confocal microscope (Carl Zeiss) fitted with a 100× oil immersion objective. Micrographs were captured by means of confocal software (ZEN system 2012 Black Edition, Zeiss). All confocal images are representatives of three independent experiments.

Lipophilic PRE and solvent PRE analysis of membrane-bound CD260-290. We cross-validated the NOE-derived membrane insertion using paramagnetic probe titration. All the solvent PRE measurements were performed using a 0.4 mM 13C-labeled CD260-290 in DMPC bilayers with q = 0.8. The solvent PREamp values indicate the protein is more close to the PRE probe. The lipophilic PRE measurements were performed using a 0.4 mM 15N-labeled CD260-290 in DMPC bilayers with q = 0.8. A stock solution of lipophilic paramagnetic agent 16-DSA (Sigma-Aldrich) was prepared at 40 mM concentration in the same NMR sample buffer to prevent changes of q value in the bilayers upon addition of the tritrop. The progress of the titration was monitored by measuring a set of 2D 15N-TROSY-HSQC spectra at each of the following 16-DSA concentrations: 0, 0.1, 0.25, 0.5, 1, 2.5, and 5 mM. The residue-specific PREamp was determined by fitting the peak intensity as a function of 4C α (TH = 0.05 Å).
secondary antibodies (goat-anti-rabbit IgG) were diluted in PBS (1:500) at 4°C for 30 min. After washing twice with PBS, stained cells were collected by BD FACSDiva software in flow cytometry (BD, LSRFortessa). Data were analyzed by FlowJo Software. The gating strategy is shown in Supplementary Fig. 9.

Fluorescence resonance energy transfer measurements and analysis. Human PD-L1 containing a C-terminal mTFP tag (a gift from Chenqi Xu’s lab) with a GSS linker was constructed. The constructs were transfected into RKO cells via lentiviral transduction and stable clones were isolated by single-cell sorting. Cells were injected into a flow chamber set-up with poly-lysin. After staining cells with 1 μM octadecyl rhodamine B (R18, O246, Membrane)-labeling dye for 5 min on ice, FRET efficiencies between the C-terminal mTFP (the donor) and the membrane dye R18 (the receptor) were measured by fluorescence microscopy (Zeiss LSM 710) using the deconvolution method that mTFP was excited at 458-nm and visualized by detection at 470–550 nm; R18 was excited at 561-nm and visualized by detection at 570–650 nm. Fully photobleached R18 signal of whole cells by the 561-nm laser at 100% efficiency for 60 s. The mTFP fluorescence signals were measured before and after R18 photobleaching three times and FRET efficiencies were calculated using the following formula:

\[
\text{FRET efficiency} = \frac{F_{\text{LP}} - F_{\text{LP}}}{F_{\text{LP}}}
\]

where \(F_{\text{LP}}\) and \(F_{\text{LP}}\) represent mTFP fluorescence after and before R18 photo-
bleaching, respectively.

In the FRET detected lipid effects on PD-L1–CD-membrane interaction assay, 1×10⁵ RKO–PD-L1–mTFP cells were seeded in a 6-well plate and cultured in RPMI-1640 medium treated with dimethylsulfoxide (DMSO, negative control), 5 μM phosphatidylserine (PS) or 2 μM sphingosine (Sph) for 2 h, respectively. Cells were collected and fixed into a flow chamber treated with poly-lysin. After staining cells with 1 μM R18, fluorescent images of the cells were taken to detect the mTFP or R18 dye emission before or after photobleaching (BP or AP) the R18 dye. FRET efficiencies were calculated by Eq. 2.

Metformin and moroxidine titration assay. The metformin titration experiments were used to detect metformin effects on CD260–290 in DMPG/DHPC bicelles. 0.4 mM ¹³N-labeled CD260–290 was reconstituted in DMPG/DHPC bicelles. A stock solution of 2 M metformin was added to the NMR sample at the molar ratios of [metformin] : [DMPG] : [Sph] for 2 h, respectively. Cells were collected and 

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

¹H, ¹³C, and ³¹N chemical shifts have been deposited in the Biological Magnetic Resonance Bank under accession number BMRR 36293 (Membrane-bound human PD-L1 cytoplasmic domain), related to the PDB code 6LR. Source data are provided with this paper.

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Author contributions

B.O. and M.W. conceived of the study; Y. C. and Q. W. prepared NMR samples; M.W. and Y.C. performed NMR experiments and data collection with the help of H.X.; M.W. and B.W. analyzed the NMR data; M.W., Y.C., T.X., and R.C. performed the biochemical and imaging experiments with the help of X.L. and Y.Y.; J.L., J.X. and C.X. provided extensive discussions; B.O. and M.W. wrote the paper, and all authors, especially J.L., contributed to editing of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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