Cyclin D–CDK4 kinase destabilizes PD–L1 via cullin 3–SPOP to control cancer immune surveillance

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Cyclin-dependent kinases play crucial roles in regulating the stability of cell cycle–related proteins during cell cycle progression14,15. Therefore, we adopted a genetic method to ablate each major cyclin and found that ablating all three D-type cyclins (D1, D2 and D3), but not cyclin A (A1 and A2) or cyclin E (E1 and E2), strongly increased PD-L1 protein abundance in mouse embryonic fibroblasts (MEFs) (Fig. 2a and Extended Data Fig. 2a–e). Using MEFs lacking individual D-type cyclins, we observed that depletion of cyclin D1, and to a lesser extent cyclin D2 or D3, upregulated PD-L1 protein levels (Fig. 2b, c). Conversely, reintroduction of cyclin D1, to and to a lesser extent cyclin D2 or D3, suppressed PD-L1 protein abundance in triple knockout Ccnd1−/−Ccnd2−/−Ccnd3−/− MEFs (Extended Data Fig. 2f). In further support of a physiological role for cyclin D1 in negatively regulating PD-L1 protein level in vivo, mammary carcinomas in Ccnd1−/− MMTV-Wnt1 or MMTV-Myc mice displayed increased PD-L1 protein levels, as compared to tumours arising in Ccnd1+/+ animals (Fig. 2d and Extended Data Fig. 2g).

Depletion of the cyclin D catalytic partner cyclin–dependent kinase 4 (CDK4), but not CDK616 nor the cyclin A and cyclin E binding-partner, CDK217, also increased PD-L1 protein abundance in cells (Fig. 2e, f and Extended Data Fig. 2h–j). Conversely, ectopic expression of wild-type CDK4, but not the kinase-dead N158F mutant, decreased PD-L1 levels (Extended Data Fig. 2k, l). Furthermore, treatment of multiple cancer cell lines with two different selective inhibitors of CDK4/6 kinase, palbociclib or ribociclib18, upregulated PD-L1 protein abundance and stability even in RB1-deficient cells (Fig. 2g, h and Extended Data Fig. 2m–q).

RB1 is frequently inactivated in human cancers18,19. In agreement with previous reports20,21, we found that RB1-deficient cancer cells often displayed high levels of the cyclin D–CDK4/6 inhibitor, p16 (also known as CDKN2A). Consistent with the notion that cyclin D1–CDK4 kinase suppresses PD-L1 levels, we observed that upregulation of p16 correlated with increased PD-L1 abundance (Extended Data Fig. 2r). Moreover, in RB1-proficient/p16-low cancer cell lines, higher PD-L1 levels correlated with relatively low CDK4 expression (Extended Data Fig. 2s–u). In addition, ectopic expression of p16 in RB1-proficient/p16-low cell lines (MC7F and T47D) or an RB1-deficient/p16-low cell line (HLF) increased PD-L1 protein abundance (Extended Data Fig. 2v–x), whereas depletion of p16 in RB1-deficient/p16-high cell lines (MDA-MB-436, BT549, and HCC1937) had the opposite effect (Extended Data Fig. 2v–x), further documenting an inverse correlation between CDK4 activity and PD-L1 expression.

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LETTER

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To extend these observations to an in vivo setting, we treated transgenic MMTV-Erbb2 mice bearing autochthonous breast cancers, or mice carrying allografts of murine MC38 or B16-F10 cancer cell lines with palbociclib, and monitored PD-L1 levels. Inhibition of CDK4/6 led to a significant upregulation of PD-L1 in all these cancer models, which was accompanied by a reduction in the number of infiltrating CD3+ T lymphocytes (TILs) (Fig. 2i–k and Extended Data Fig. 3a–c). We also observed that palbociclib treatment significantly increased PD-L1 protein levels in various organs of normal mice (Extended Data Fig. 3d–h). Collectively, these results demonstrate that cyclin D–CDK4 kinase plays a rate-limiting role in regulating PD-L1 levels in vivo.

To understand how cyclin D–CDK4 regulates PD-L1 levels, we first determined that treatment of cells with proteasome inhibitor MG132, or with cullin-based ubiquitin E3 ligase inhibitor MLN492422 increased PD-L1 protein levels (Fig. 3a). To identify which cullin family regulates PD-L1, we screened the potential interaction of increased PD-L1 protein levels (Fig. 3a). To identify which cullin family E3 ligase(s) regulates PD-L1, we screened the potential interaction of PD-L1 with each cullin family protein and found that cullin 3, and the other cullin family E3 ligase(s) recognize their downstream subunit (Extended Data Fig. 4a, b). These results indicate that in addition to cullin 1/β-TRCP (β-transducin repeat containing E3 ubiquitin protein ligase)11, the cullin 3-based E3 ligase(s) might play a role in regulating PD-L1 stability. Consistent with this notion, depletion of cullin 3 increased the protein abundance of endogenous PD-L1 (Extended Data Fig. 4c).

Cullin 3-based E3 ubiquitin ligases recognize their downstream substrates through substrate-recruiting adaptor proteins12. We found that SPOP, but not the other adaptor proteins we examined, interacted with PD-L1 in cells (Fig. 3c, d). We further determined that deletion of the C-tail, or the last eight amino acids of PD-L1 (283–290), disrupted binding of PD-L1 to SPOP, and rendered PD-L1 resistant to SPOP-mediated poly-ubiquitination (Extended Data Fig. 4d–h), indicating that the 283–290 region of PD-L1 might represent the potential binding motif for SPOP. Notably, a cancer-derived PD-L1 mutant containing a T290M mutation located within the SPOP-binding motif (cBioPortal) also lost its ability to interact with SPOP and became more stable in vivo. To extend these observations to an in vivo setting, we treated transgenic MMTV-Erbb2 mice bearing autochthonous breast cancers, or mice carrying allografts of murine MC38 or B16-F10 cancer cell lines with palbociclib, and monitored PD-L1 levels. Inhibition of CDK4/6 led to a significant upregulation of PD-L1 in all these cancer models, which was accompanied by a reduction in the number of infiltrating CD3+ T lymphocytes (TILs) (Fig. 2i–k and Extended Data Fig. 3a–c). We also observed that palbociclib treatment significantly increased PD-L1 protein levels in various organs of normal mice (Extended Data Fig. 3d–h). Collectively, these results demonstrate that cyclin D–CDK4 kinase plays a rate-limiting role in regulating PD-L1 levels in vivo.

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deleting its substrate-interacting MATH domain increased and stabilized PD-L1 in cells (Fig. 3e, f and Extended Data Fig. 5a–m). However, deleting known SPOP substrates including AR, ERG, TRIM24 or DEK in wild-type SPOP or SPOP−/− human tumour cell lines did not lead to obvious changes in PD-L1 levels (Extended Data Fig. 5n–u), arguing against the possibility of secondary effects for the observed elevation of PD-L1 after SPOP depletion.

SPOP mutations occur in 10–15% of human prostate cancers, and are largely clustered within the MATH domain24,25 (Extended Data Fig. 6a). Notably, these cancer-derived SPOP mutants failed to promote PD-L1 degradation owing to their deficiency in binding to PD-L1, which leads to a failure in the promotion of PD-L1 poly-ubiquitination (Fig. 3g–i and Extended Data Fig. 6b, c). This behaviour resembles the elongin C-encoding ELOC hotspot mutants in clear cell renal carcinoma that result in deficiencies in the ability of cullin 2–elongin B/C–VHL E3 ligase complex to promote HIF-1α degradation26. We also observed that in human tumour mutations in the PD-L1 C-tail (degron) are mutually exclusive with mutations in the substrate-interacting MATH domain of SPOP (Extended Data Fig. 6d, e).

To further explore the effect of SPOP mutations on tumorigenesis, we generated tumour cell lines expressing wild-type SPOP or cancer-derived SPOP mutants. We found that cells expressing mutant SPOP displayed increased levels of endogenous PD-L1 protein, as compared to cells expressing wild-type SPOP (Fig. 3j and Extended Data Fig. 6f–i). Upon inoculation into immunoprophilic mice, the growth of implanted tumours expressing the cancer-derived SPOP(F102C)
Figure 4 | Cyclin D1–CDK4-mediated phosphorylation of SPOP stabilizes SPOP by recruiting 14–3–3γ to disrupt its binding with FZR1.  

**a, c,** Immunoblot of WCL derived from HeLa cells, synchronized in M phase by nocodazole treatment before release for the indicated times, with or without FZR1 knockdown using sgRNAs targeting SPOP (sgSPOP) (a) or knockdown of FZR1 with shRNA (e).  
**b, d,** Immunoblot of immunoprecipitates and WCL derived from MDA-MB-231 (b) or HEK293T (c) cells, or Ni–NTA pull-down products derived from HeLa cells (d) transfected with the indicated constructs and treated with MG132 (30 μM) for 6 h.  
**f, i,** In vitro kinase assays showing that cyclin D1–CDK4 phosphorylates recombinant SPOP at Ser6, not Ser222.  
**g,** Immunoblot analysis of immunoprecipitates and WCL derived from HEK293T cells transfected with indicated constructs and treated with MG132 (10 μM) (g, h) or MG132 with palbociclib (1 μM) for 12 h (i), or HeLa cells (with and without SPOP knockdown) treated with palbociclib (0.5, 1 μM) for 48 h (j).  
**k, l,** HeLa released from nocodazole (0.001, Gehan–Breslow–Wilcoxon test. Experiment repeated twice.  

We found that SPOP protein abundance fluctuated during the cell cycle and displayed an inverse correlation with PD-L1 protein levels (Figs 1a, 4a). Depleting SPOP resulted in stabilization of PD-L1 across the cell cycle (Fig. 4a and Extended Data Fig. 8a). We noted that the APC/C (anaphase-promoting complex, also known as cyclosome) E3 ligase adaptor protein FZR1 (also known as CDC20 homologue 1, CDH1) displayed an inverse correlation with SPOP protein levels during the cell cycle (Figs 1a, c, 4a). Furthermore, depletion of FZR1, but not CDC20, increased SPOP protein abundance, which was accompanied by a simultaneous reduction in PD-L1 protein levels (Extended Data Fig. 8b, c). Consistent with these results, we detected a physical interaction between the endogenous SPOP and FZR1 proteins (Fig. 4b and Extended Data Fig. 8d, e), and identified an evolutionarily conserved destruction-box (D-box) motif (RXXLXXXXN)29 in SPOP (Extended Data Fig. 8f). Deleting the D-box motif in SPOP disrupted its binding to FZR1 and rendered SPOP resistant to FZR1-mediated poly-ubiquitination and degradation (Fig. 4c, d and Extended Data Fig. 8g–i). Moreover, depletion of FZR1 led to SPOP stabilization, which subsequently resulted in a reduction in PD-L1 protein levels during cell cycle progression (Fig. 4e). Taken together, these results indicate that FZR1 is a physiologically important upstream E3 ligase responsible for negatively regulating SPOP protein stability.

To determine how the cyclin D–CDK4 kinase affects this mechanism, we established that cyclin D1–CDK4 directly phosphorylates SPOP at Ser6 (p-Ser6), but not Ser222, the only two conserved
serine–proline motifs in SPOP (Fig. 4f and Extended Data Fig. 9a–d). Treatment of cells with palbociclib reduced the phosphorylation of SPOP in cells (Extended Data Fig. 9e). We observed that 14-3-3- protein physically interacted with SPOP in a p-Ser6-dependent manner and disrupted the interaction of SPOP with FZR1 in cells (Fig. 4g, h and Extended Data Fig. 9f–h). Inhibition of SPOP-p-Ser6 decreased the interaction of SPOP with 14-3-3- and increased its binding to FZR1, leading to increased SPOP poly-ubiquitination (Fig. 4i and Extended Data Fig. 9i–p). Consequently, palbociclib treatment decreased SPOP protein abundance and increased PD-L1 levels in wild-type SPOP, but not in SPOP-deficient cells (Fig. 4j). Moreover, depletion of 14-3-3- markedly upregulated PD-L1 levels and stabilized PD-L1 during cell cycle progression (Extended Data Fig. 9q–t).

Recent clinical studies revealed that the success of PD1–PD1 blockade correlates with PD-L1 expression levels in tumour cells. Observing that inhibition of CDK4/6 increased PD-L1 levels, we hypothesized that inhibitors of CDK4/6 kinases might synergize with anti-PD-1–PD-L1 therapy to elicit an enhanced therapeutic efficacy. Indeed, we observed that treatment of immunoproficient mouse bearing CT26 tumours with palbociclib plus an anti-PD-1 antibody markedly retarded tumour progression and resulted in complete tumour regression. Using mice bearing tumours derived from mouse tumour MC38 cells, treatment of tumour-bearing mice with palbociclib decreased the absolute number of TILs in 8 complete responses out of 12 treated mice (Fig. 4k and Extended Data Fig. 10a). Moreover, combining CDK4/6 inhibitor with anti-PD-1 therapy resulted in a significant improvement in overall survival compared with either treatment alone (Fig. 4l). Similar results were obtained using mice bearing tumours derived from mouse tumour MC38 cells. (Extended Data Fig. 10b, c). As expected from our earlier observations, treatment of tumour-bearing mice with palbociclib decreased the absolute number of TILs, including CD3+, CD4+, CD8+, granulocyte B+ and IFNγ+ cells. Notably, the addition of anti-PD-1 antibody to palbociclib treatment restored essentially normal numbers of TILs (Extended Data Fig. 10d–i).

A recent study revealed that another inhibitor of CDK4/6, abemaciclib, increased the immunogenicity of cancer cells via an Rbl-dependent mechanism, first by activating tumour cell expression of endogenous retroviral elements, and then by stimulating the production of type III interferons and antigen presentation by tumour cells. Together with our demonstration that cyclin D–CDK4 regulates PD-L1 stability through cullin 3–SPOP (Extended Data Fig. 10k), these studies provide a complementary molecular rationale for combining CDK4/6 inhibitor treatment with immunotherapy targeting the PD-1–PD-L1 interaction to enhance tumour regression.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper. Correspondence and requests for materials should be addressed to G.E. (gordon_freeman@dfci.harvard.edu) or W.W. (wwei2@bidmc.harvard.edu). Details are available in the online version of the paper.

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Author Contributions J.Z., X.B. and H.W. performed most of the experiments with assistance from Y.Z., Y.G., N.T.N., Y.T., Y.C., F.W., X.D., J.G., Y.H., C.F., S.R. and Y.S.Y., S.R., and Y.S. performed immunohistochemistry for human prostate cancer samples. Y.G., Y.T. and Y.C. helped with mouse work. J.Z., X.B., H.W., G.J.F., P.S., and W.W. supervised the study. J.Z. and H.W. wrote the manuscript with help from X.B., H.W., P.S., and G.J.F. All authors commented on the manuscript.

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METHODS

Cell culture, transmissions and virion isolation. HEK293T, HEK293, HeLa, MDA-MB-231, MCF7, Hs578T cells and MEFs (wild-type, Cnd1Δ−/−, Cnd2Δ−/−, Cnd3Δ−/−, Cnd1Δ−/− Cnd2Δ−/− Cnd3Δ−/−, Cnd1Δ90/Cnd2Δ90/Cnd3Δ90, Cdkt4Δ−/−, Ccn1Δ−/− Ccn2Δ−/−, Ccn1Δ−/− Ccn2Δ−/− and Spa9Δ−/−) MEFs (a gift from N. Mitsiades) were cultured in MEM medium supplemented with 10% FBS (Gibco), 100 μg of penicillin and 100 μg/ml streptomycin (Gibco). HLF, HepG2, Huh7 and HaCaT were cultured in RPMI medium supplemented with 10% FBS. MDA-MB-231 wild-type PD-L1 and PD-L1 knockout cells were a gift from M.-C. Hung, BT549, T47D, ZR75-1, HCC1954, HCC1937, MDA-MB436, MDA-MB468 and SKBR3 cells were from the A. Toker laboratory, and were cultured in RPMI medium or McCoy’s 5A (Corning) medium supplemented with 10% FBS. PC3, DU145, 22Rv1, LNCaP and C4-2 were gifts from the P. P. Pandolfi group, and cultured in RPMI medium (Corning) with 10% FBS. The mouse tumour-derived MC38 cell line was a gift from A. Sharpe. Mouse tumour-derived CT26, 4T1 and B16-F10 cell lines were routinely cultured in the laboratory of G.J.F. in MEM medium supplemented with 10% FBS (Gibco), 100 μg of penicillin and 100 μg/ml streptomycin (Gibco). All cell lines were routinely tested for mycoplasma contamination and found to be negative.

Cells with 80% confluence were transfected with Lipofectamine Plus reagents in Opti-MEM medium (Invitrogen). 293FT cells were used for packaging of lentiviral and retroviral DNA-expression viruses; subsequent infection of various cell lines were performed in brief. medium, secreted with viruses was collected twice at 48 h and 72 h after transfection. After filtering through 0.45 μm filters, viruses were used to infect the cells in the presence of 4 μg/ml of polybrene (Sigma-Aldrich). Cells were split 48 h after infection and selected using hygromycin B (200 μg/ml) or puromycin (1 μg/ml) for three days. Cells were collected and lysed in EBC buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40) supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Calbiochem) for immunoblot analysis.

Reagents. Nocodazole (M1404) and taxol (T7402) were purchased from Sigma, thymidine (50-89-5) and cycloheximide (66-81-9) from Acros Organics, polaricidin (PD032991, S1116) and ribociclib (LEE011, S7440) from Selleckchem and Abmole, and MG132 (BML-PI102-0005) from Enzo Life Science. MLN4924 was a gift from W. Kaelin. Plasmids. MYC-tagged cullin 1, cullin 2, cullin 3, cullin 4A, cullin 4B and cullin 5; Flag-tagged wild-type and mutant PD-L1 (Y87C, F102C and W131G); delta MATH; C-cul3; HA-tagged wild-type PD-L1 (HA tag in the N terminus of PD-L1) was provided by M.-C. Hung. HA–FZR1, Flag-tagged wild-type SPOP and mutants (Y87C, F102C and W131G); delta MATH; pS10-H3 (3377), anti-p-S780-Rb (8180), anti-pS807/S811-Rb (8516), anti-Rb (sc-7355), anti-CDK2, CDK4 and CDK6 were generated in this study. pET-28a-His–SPOP (wild-type, S6A, S222A and S6A/S222A mutants), Flag-SPOP ΔD-box (TRCN0000057587) were purchased from Open Biosystems. pcDNA3-PD-L1, pcDNA3-β-gal (PC3-2759), anti-GST (2625), rabbit polyclonal anti-Myc-Tag antibody (2278) and pS6A, HA-tagged CDK2, CDK4 and CDK6 were generated in this study.

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For immunoblotting analyses. Cells were lysed in EBC buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP-40) supplemented with protease inhibitors (Complete Mini, Roche) and phosphatase inhibitors (phosphatase inhibitor cocktail set I and II, Calbiochem). Protein concentrations were measured using a Beckman Coulter DU-800 spectrophotometer using the Bio-Rad protein assay reagent. Equal amounts of protein were resolved by SDS–PAGE and immunoblotted with indicated antibodies. For immunoprecipitations analysis, 1,000 μg total cell lysates were incubated with the primary antibody-conjugated beads for 4 h at 4 °C. The recovered immunocomplexes were washed four times with NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40) before being resolved by SDS–PAGE and immunoblotted with indicated antibodies.

Immunohistochemistry for cell pellets, xenografted tumours or human prostate tumour specimens. The cultured cells (MDA-MB-231 wild-type PD-L1 and PD-L1 knockout cells; HPB-ALL shScramble and shCD8 cells; KE73 shScramble and shCD8 cells) were washed and fixed in 4% paraformaldehyde for 20 min. Cell pellets or xenografted (MDA-MB-231 wild-type PD-L1 and PD-L1 knockout) tumours were embedded into tissue freezing medium (TFM) and frozen. Cryostat sections (10 μm) were placed on Superfrost Plus Slides (Thermo Scientific) and samples were then permeabilized in 0.1% Triton X-100/PBS for 10 min. For immunohistochemistry analysis, we used UltraSensitive SP (mouse) IHC Kit (K7T901, Fuzhou Maixin Biotech) or VECTASTAIN Elite ABC reagent (DAB Peroxidase Substrate kit, Vector Laboratories, SK-4100) following the manufacturer’s instructions with minor modification. The sections were incubated with 3% H2O2 for 15 min at room temperature to block endogenous peroxidase activity. After incubating in normal goat serum for 1 h to block non-specific binding of IgG, sections were treated with primary antibody (PD-L1, 298B.3G6, 18 μg/ml; CD8α, sc-55212, clone 2C8/144B, dilution 1:40) at 4 °C overnight. Sections were then incubated for 30 min with biotinylated goat-anti-mouse IgG secondary antibodies (Fuzhou Maixin Biotech), followed by incubation with streptavidin-conjugated HRP (Fuzhou Maixin Biotech), and were developed with 3,3′-diaminobenzidine (DAB-2031, Fuzhou Maixin Biotech). Images were taken using an Olympus microscopic camera and matched software.

The prostate tumour specimens were obtained from Shanghai Changhang Hospital. Usage of these specimens was approved by the Institute Review Board of Shanghai Changhang Hospital. For immunohistochemistry, the paraffin-embedded prostate tissue samples were deparaffinized in xylene (three times for 10 min), dehydrated through a series of graded alcohols (100%, 95%, 85%, and 75%) to water. Samples were then subjected to heat-mediated antigen retrieval at 95 °C for 20 min. The subsequent staining steps were the same as those described above.

The expression level of PD-L1 in prostate cancer tumour samples was determined according to the intensity of the staining as 0, negative; 1, weak expression; 2, intermediate expression and 3, strong expression. The number of intraprostatic CD8+ TILs were counted as previously described. In brief, three independent areas with the most abundant infiltration were selected under a microscopic field at 200× magnification (0.0625 mm2). The number of intraprostatic CD8+ TILs was counted as previously described. Mann–Whitney test was used to compare the difference in PD-L1 expression between wild-type and mutated SPOP cases. A Student’s t-test was used to determine P values of the difference in CD8+ TILs between wild-type and mutant SPOP cases. P < 0.05 was considered significant.

In vitro cyclin D−/− CDK4 kinase assays. Kinase assays were performed in a final volume of 30 μl of a kinase buffer as described previously: 50 mM HEPES (pH 7.5), 10 mM MgCl2, 1 mM dithiothreitol (DTT), 1 mM EGTA, 0.1 mM Na3VO4, containing 10 μM ATP and 0.4 μM I-IAIP (Perkin Elmer). 0.2 μg of cyclin D1−/− CDK4 (0142-0143-1, Pro-Qinase), cyclin D2−/− CDK4 (0142-0375-1, Pro-Qinase), cyclin D3−/− CDK4 (0142-0373-1, Pro-Qinase) were used as kinase controls. 2 μg of His+SPop, His+SPop, His+SPop-S222A, or His+SPop-S6A222A mutant proteins immobilized on Ni–NTA beads were used as kinase substrates. 0.1 μg of Rb C-terminal recombinant protein (SC-4112, Santa Cruz) was used as a positive control for kinase assays. 2 μg of BSA was used as a negative control. After
60 min incubation at 30 °C, proteins were denatured, resolved on SDS–PAGE, transferred to nitrocellulose membranes and exposed to X-ray films.

Kinase assays of endogenous CDK4–cyclin D1 from mouse organs. The endogenous CDK4 was immunoprecipitated using 6 μg of anti-CDK4 antibody (Santa Cruz, sc-23896) from 2.5 mg of lysates (buffer: 20 mM Tris–HCl pH 8.0, 0.1 M KC1, 5 mM MgCl2, 10% glycerol, 0.1% Tween-20, 0.1% NP40) of livers or brains isolated from C57BL/6 mice. The association of cyclin D1 with CDK4 was confirmed by immunoblotting with an anti-cyclin D1 antibody (Abcam, ab134175). The immunoprecipitated endogenous cyclin D1-CDK4 was used as kinase, and 0.5 μg of Bc R-Combinant recombiant protein (SC-4112) was used as the kinase substrate.

In vivo ubiquitination assays. PC3 or HeLa cells with 80% confluence were transfected with His–ubiquitin and the indicated constructs. Thirty-six hours after transfection, cells were treated with 30 μg/ml MGI32 for 6 h and lysed in buffer A (6 M guanidine–HCl, 0.1 M Na2HPO4/NaH2PO4, and 10 mM imidazole pH 8.0). After sonication, the lysates were incubated with Ni–NTA beads (QIAGEN) for 3 h at room temperature. Subsequently, the His pull-down products were washed twice with buffer A, twice with buffer A/TI (1 volume buffer A and 3 volumes buffer TI), and once with buffer TI (25 mM Tris–HCl and 20 mM imidazole pH 6.8). The pull-down proteins were resolved for SDS–PAGE for immunoblotting.

Protein half-life assays. Cells were transfected or treated under indicated conditions. For half-life studies, cycloheximide (20 μg/ml −1) was added to the medium. At indicated time points thereafter, cells were collected and protein abundance was measured by immunoblot analysis.

Cell synchronization and FACS analyses. Cells synchronized with nocodazole arrest and double thymidine treatment as described previously were collected at the indicated time points and stained with propidium iodide (Roche) according to the manufacturer’s instructions. Cells were fixed by 70% ethanol at −20 °C overnight and washed three times using cold PBS. The samples were digested with RNase for 30 min at 37 °C and stained with propidium iodide (Roche) according to the manufacturer’s instructions. Stained cells were sorted with BD FACSCanto II Flow Cytometer. The results were analysed by ModFit LT 4.1 and FSC express 5 softwares.

Brdu/PI labelling and FACS analyses. Cells were incubated with or without BrdU (75 μM, Sigma) containing medium for 1 h. Cells were collected and washed once with cold PBS for centrifugation for 5 min at 270g. Cells were re-suspended in 200 μl cold PBS and 5 ml of cold 90% ethanol was added for fixation overnight. After centrifugation for 5 min at 270g, cells were washed once using 5 ml PBS and 0.5 ml 2 N HCl-0.5% Triton X-100 was added for 30 min at room temperature. After adding in 5 ml PBS, samples were centrifuged for 5 min at 270g and re-suspended in 1 ml Na2B4O7 (pH 8.5). Samples were re-suspended in 200 μl anti-BrdU diluted (1:40) in PBS with 0.5% Tween 20 and 1% BSA and were incubated 30 min at room temperature. After adding 5 ml 20% HEPES-PBS (pH 7.4) with 0.5% Tween 20, samples were centrifuged for 5 min at 270g and re-suspended in 0.5 ml PBS with propidium iodide (PI, 5 μg/ml −1, Sigma) and RNase A (200 μg/ml −1, Roche). After incubating 30 min at room temperature, samples were transferred into FACS tubes and analysed by flow cytometry.

Real-time RT–PCR analyses. Total RNAs were extracted using the QIAGEN RNeasy mini kit, and reverse transcription reactions were performed using the ABI Taqman Reverse Transcription Reagents (N808-0234). reactions were performed with the ABI-7500 Fast Real-time PCR system and SYBR green qPCR Mastermix (400288) from Agilent Technologies Stratagene. The following primers were used human GAPDH: forward, 5′-GGAGGAGATGCCTCCAAAAAT-3′; reverse, 5′-GGCTTGTGTTCATCTCTCAGTG-3′; mouse GAPDH: forward, 5′-AGGTGGTGTAAGGAAGTTG-3′; reverse, 5′-GGGTGTTGCTGAACAGCA-3′; human PD-L1 (also known as CD274): forward, 5′-TGCGATTTGCTGTAACGG-3′; reverse, 5′-GAGGTGGAGGCTGCTGACG-3′; mouse PD-L1 (also known as CD274): forward, 5′-GGTCCCAAAGGAGCTTACTG-3′; reverse, 5′-GTACGGTGGACTGGATGTC-3′.

Generation of Cdcd-deficient MEFs. Cdcd−/−Cdcd−/−Cdcd−/− and Cdcd−/+ Cdcd−/+ Cdcd−/+ MEFS were derived from E13.5 mouse embryos as described previously.[39,40]

Generation of mouse tumours. Cdcd−/− mice were mated with MMTV-Myc or MMTV-Wnt1 mice (from the Jackson Laboratory) yielding Cdcd−/− MMTV-Myc or Cdcd−/− MMTV-Wnt1, as well as control Cdcd−/+ MMTV-Myc or Cdcd−/+ MMTV-Wnt1 mice. Mammary tumours were dissected from multiparous females and analysed by flow cytometry.

In vivo experimental therapy in MC38 and CT26 mice tumour models. Animal studies were approved by Dana–Farber Cancer Institute Institutional Animal Care and Use Committee (IACUC; protocol number 04-047), and performed in accordance with the Guide for the care and use of laboratory animals. MC38 or CT26 tumours were established by subcutaneous injection of 107 MC38 or CT26 tumour cells in 100 μl HBSS into the right flank of 6-week-old C57BL/6 (for MC38) or BALB/c (for CT26) female mice (Jackson Laboratory). Tumour sizes were measured every three days by caliper after implantation and tumour volume was calculated by length × width 2 /2. Seven days after tumour cells were injected, animals were pooled and randomly divided into four groups with comparable average tumour size. Laboratory members who measured the mice were blinded to the treatment groups. Mice were grouped into control antibody treatment, anti-PD-1 antibody treatment (clone 29F.1A12), CDK4/6 inhibitor treatment and anti-PD-1 antibody and CDK4/6 inhibitor treatment. As illustrated in Extended Data Fig. 10a, control and anti-PD-1 antibody treatments were conducted by intraperitoneal injection (200 μg per mouse in 200 μl HBSS saline buffer) every three days for a total of three injections. Subsequently, tumours were collected and analysed by FACS.

Cells (1 × 106 B16-F10) stably expressing wild-type SPOP or F102C mutant were injected subcutaneously into 6-week-old C57BL/6 female mice (from the Jackson Laboratory). Three days after tumour cells were injected, control, wild-type and knockout cells were seeded in chambers (754374, Thermo Fisher Scientific). Cells were fixed with 4% paraformaldehyde for 20 min, followed by 0.1% Triton X-100 in PBS for 10 min. Cells were pre-blocked with 2% BSA/PBS for 45 min, then incubated with primary antibodies against PD-L1 (PD-L1-29B8, 1:200, vials 1:250) for 2.5 h at room temperature and followed with secondary anti-mouse antibodies conjugated with Alexa-Fluor-568 (Invitrogen, 1:250). Hoechst (Life Technology, 1:10,000) was used to stain the nuclei. TEM-embedded μm-thick tissue sections were fixed with 2% paraformaldehyde–PBS for 30 min, and permeabilized in 0.1% Triton X-100 in PBS for 10 min. Tissue sections were pre-blocked with 2% BSA/PBS for 45 min, then incubated with primary antibodies against PD-L1 (PD-L1-29B8, 1:200, vials 1:250) and anti-rabbit antibodies conjugated with Alexa-Fluor–488 (Invitrogen, 1:250). Hoechst (Life Technology, 1:10,000) was used to stain the nuclei. Tissue sections were mounted with fluoromount-G (SouthernBiotech) at 4 °C overnight. Tissue sections were examined with a fluorescence microscope using a 20× objective lens. CD3+ cell numbers were counted in an area of 3.67 × 104 μm2.

Single-cell generation from tumour tissue and flow cytometry analysis. Tumour tissues were minced and digested with 5 ml of 2 mg ml−1 collagenase (Sigma) in DMEM for 1 h at 37 °C. Tissue sections were then collected by centrifugation and washed through a 70-μm strainer in DMEM. Cells were collected and washed twice in cold PBS with 2% BSA/PBS. Cells were then resuspended with 2% BSA/PBS and 1 ml DCCM medium. Cells (1 × 105 MC38 or CT26 tumour cells in 100 μl HBSS saline buffer) were then resuspended in 2% BSA/PBS and 1 ml DCCM medium. Cells (1 × 105 MC38 or CT26 tumour cells in 100 μl HBSS saline buffer) were then resuspended in 2% BSA/PBS and 1 ml DCCM medium. Cells (1 × 105 MC38 or CT26 tumour cells in 100 μl HBSS saline buffer) were then resuspended in 2% BSA/PBS and 1 ml DCCM medium. Cells (1 × 105 MC38 or CT26 tumour cells in 100 μl HBSS saline buffer) were then resuspended in 2% BSA/PBS and 1 ml DCCM medium.
a break every week for one day. For survival studies, animals were monitored for
tumour volumes every three days for 120 days (MC38) or 57 days (CT26) after
initial treatment, until tumour volume exceeded 2,000 mm$^3$ (MC38) or 1,500 mm$^3$
(CT26), or until tumours had an ulcer with a diameter of 1 cm. Statistical anal-
ysis was conducted using the GraphPad Prism software (GraphPad Software).
Kaplan–Meier curves and corresponding Gehan-Breslow-Wilcoxon tests were used
to evaluate the statistical differences between groups in survival studies. $P < 0.05$
was considered significant.

T cell analysis for MC38-implanted tumours. MC38-implanted tumours were
established by subcutaneously injecting $1 \times 10^5$ MC38 cells into the right flanks
of 6-week-old C57BL/6 female mice (Jackson Laboratory). On the day of tumour
cell injection, mice were randomly divided into four groups: control antibody
treatment, anti-PD-1 antibody treatment, CDK4/6 inhibitor treatment, and
anti-PD-1 antibody plus CDK4/6 inhibitor treatment. Control and anti-PD-1
antibody treatments were conducted by intraperitoneal injection (200 μg per
mouse in 200 μl HBSS saline buffer) every three days for a total of 4 injections.
The treatment of palbociclib was given by daily oral gavage with the dosage
of 200 mg kg$^{-1}$ for 9 days, with a break after 7 days. Tumours were then col-
lected and single cells were generated from tumour tissues as described above.
After cells were filtered through 40-μm strainer, cells were fixed in 0.5 ml per
tube Fixation buffer (420801, Biolegend) in the dark for 20 min at room tem-
perature. Cells were then washed with PBS and 2% BSA. The fixed cells were
suspended in Intracellular Staining Perm Wash Buffer (421002, Biolegend) and
centrifuged twice to permeabilize the cells. Cells were then co-stained with anti-
bodies against CD3 (100236, APC conjugated, Biolegend), granzyme B (515403,
FITC-conjugated, Biolegend) or IFNγ (505808, phycoerythrin (PE)-conjugated,
Biolegend), or co-stained with antibodies against CD3 (100236, APC-conjugated,
Biolegend), CD4 (100510, FITC-conjugated, Biolegend) and CD8 (100708, PE
conjugated, Biolegend). The corresponding isotype IgG controls were used for
controls. The cells were incubated with corresponding antibodies for 30 min
at room temperature. Cells were washed by PBS with 2% BSA and analysed by
flow cytometry.

Data availability. Gel source images for Figs 1–4 and Extended Data Figs 1–9 are
available in Supplementary Fig. 1. Source Data for Figs 2j, k, 3j–l, n, o, 4k, l and
Extended Data Figs 3c, e, 6j, l, n–p, r, s, 10b–j are available with the online version
of this paper. All other data supporting the findings of this study are available from
the corresponding authors upon reasonable request.

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Extended Data Figure 1 | PD-L1 fluctuates during cell cycle progression. 

a, b, Immunoblot of WCL derived from MDA-MB-231 (a) or HCC1954 (b) cells synchronized in M phase by nocodazole treatment before release back into the cell cycle for the indicated times. 

c, d, Quantitative reverse transcription PCR (qRT–PCR) analyses of relative mRNA levels of PD-L1 (also known as CD274) and GAPDH from samples derived from HeLa cells synchronized in M phase by nocodazole treatment before release back to the cell cycle for the indicated times. 

e, Immunoblot of WCL derived from untreated HeLa cells or HeLa cells pre-treated with IFNγ (10 ng ml⁻¹) for 12 h and then synchronized in M phase by nocodazole treatment before release back into the cell cycle for the indicated times. 

f, Immunoblot of WCL derived from HeLa cells stably expressing wild-type HA–MYC, or HA–MYC(T58A/S62A). Empty vector was used as a negative control. 

g, Immunoblot of WCL derived from wild-type HeLa cells or HeLa cells stably expressing HA–MYC; cells were synchronized in M phase by nocodazole treatment before release back into the cell cycle for the indicated times. 

h–k, Immunoblot of WCL derived from MC38, CT26, 4T1 or B16-F10 mouse tumour cells treated with the indicated concentration of nocodazole for 20 h before collecting. 

l–m, Immunoblot of WCL derived from 4T1 or CT26 mouse tumour cells treated with the indicated concentration of taxol for 20 h before collecting.
Extended Data Figure 2 | See next page for caption.
Extended Data Figure 2 | Cyclin D–CDK4 negatively regulates PD-L1 protein stability. a, b, Immunoblot analysis of WCL derived from wild-type and Ccna1−/−Ccna2−/− (a) or Ccne1−/−Ccne2−/− (b) MEFs. c, qRT–PCR analysis of relative PD-L1 mRNA levels from wild-type and Ccnd1−/−Ccnd2−/−Ccnd3−/− MEFs. Data are mean ± s.d., n = 5. NS, not significant, two-tailed t-test. d, Cell cycle profiles for wild-type and Ccnd1−/−Ccnd2−/−Ccnd3−/− MEFs, which were labelled with BrdU and propidium iodide and analysed by FACS. e, Immunoblot analysis of WCL derived from Ccnd1fl/fl Ccnd2−/−Ccnd3fl/fl MEFs upon expression of Cre resulting in ablation of Ccnd1 and Ccnd3. Cre was delivered via viral infection with pLenti-Cre (pLenti-EGFP used as a negative control). Cells were selected with puromycin (1 μg ml−1) for 72 h before collecting. f, Immunoblot analysis of WCL derived from Ccnd1−/−Ccnd2−/−Ccnd3−/− MEFs with stable reexpression of Ccnd1, Ccnd2 or Ccnd3. Empty vector was used as a negative control. g, Immunoblot analysis of WCL derived from mouse mammary tumours induced by MMTV-Myc in Ccnd1+/+ or Ccnd1−/− females. n = 5 mice per experimental group. h, Immunoblot analysis of WCL derived from wild-type and Cdk6−/− MEFs. i, j, Immunoblot analysis of WCL derived from MDA-MB-231 cells stably expressing shCDK6 or shCDK2 as well as shScramble as a negative control. k, l, Immunoblot analysis of WCL derived from MDA-MB-231 cells transfected with indicated constructs (k), the intensity of PD-L1 band was quantified by the ImageJ software (l). m, Immunoblot analysis of WCL derived from RB1 depleted MDA-MB-231 cells (with shScramble as a negative control) treated with palbociclib where indicated. n, o, Immunoblot analysis of WCL derived from mouse CT26 or 4T1 tumour cell lines treated with or without palbociclib or ribociclib, respectively. p, q, Immunoblot analysis of WCL derived from MDA-MB-231 cells pre-treated with palbociclib (1 μM) for 36 h before treatment with cycloheximide (CHX) for the indicated time (p) and PD-L1 protein abundance was quantified by ImageJ as indicated (q). r, Immunoblot analysis of WCL derived from 19 different cancer cell lines with indicated antibodies. s–u, Immunoblot analysis of WCL derived from MCF7 (s), T47D (t) or HLF (u) stably expressing p16 as well as empty vector as a negative control. v–x, Immunoblot analysis of WCL derived from MDA-MB-436 (v), BT549 (w) or HCC1937 (x) stably expressing three independent shRNAs targeting p16 (also known as Cdkn2a) as well as shScramble as a negative control. © 2018 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Extended Data Figure 3 | See next page for caption.
Extended Data Figure 3 | Treatment with the CDK4/6 inhibitor palbociclib increases PD-L1 levels in vivo. a, b, Immunoblot analysis of WCL derived from MC38 or B16-F10 implanted tumours, in mice treated with palbociclib (150 mg kg$^{-1}$ body weight, by gastric gavage) or vehicle for 7 days. $n = 5$ mice per experimental group. c, FACS analysis for PD-L1 or CD3$^+$ T cell populations from B16-F10-implanted tumours in mice treated with vehicle or palbociclib for 7 days. $n = 5$ mice per experimental group. d, Immunoblot analysis of WCL derived from the indicated organs in mice treated with palbociclib (150 mg kg$^{-1}$ body weight, by gastric gavage) or vehicle for 7 days. $n = 5$ mice per experimental group. e, Quantification of PD-L1 protein bands intensity in d using ImageJ. $n = 5$ mice per experimental group. f, Immunoblot analysis of WCL derived from 15 different organs dissected from mice with or without palbociclib treatment and MMTV-c-Myc induced breast tumours. g, Quantification of PD-L1 protein bands intensity in f using ImageJ. $n = 3$ biological replicates. h, In vitro kinase assay using immunoprecipitated cyclin D1–CDK4 kinase complex from livers or brains by anti-CDK4 antibody and recombinant Rb as a substrate. Note that cyclin D1–CDK4 complex in non-dividing organs (livers and brains) displayed kinase activity, which might explain why the CDK4/6 inhibitor increased PD-L1 in these organs. Data are mean ± s.d. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, two-tailed t-test.
Extended Data Figure 4 | Cullin 3–SPOP promotes PD-L1 ubiquitination and subsequent degradation largely through interaction with the cytoplasmic tail of PD-L1. 

a, A schematic illustration of PD-L1 with N-terminal signal peptide, extracellular domain, transmembrane domain, cytoplasmic tail and the potential SPOP-binding motif in PD-L1.

b, d, Immunoblot analysis of WCL and glutathione S-transferase (GST) pull-down precipitates derived from 293T cells transfected with indicated constructs and treated with MG132 (10 μM) for 12 h before collecting.

c, e, g, Immunoblot analysis of WCL and immunoprecipitate derived from HEK293T cells transfected with indicated constructs and treated with MG132 (10 μM) for 12 h before collecting.

f, h, Immunoblot analysis of WCL and Ni–NTA pull-down products derived from the lysates of PC3 cells transfected with the indicated constructs. Cells were treated with MG132 (30 μM) for 6 h before collecting and lysis in denaturing buffer.

i, k, Immunoblot of WCL and Ni–NTA pull-down products derived from the lysates of PC3 cells transfected with the indicated constructs. Cells were treated with MG132 (30 μM) for 6 h before collecting and lysis in denaturing buffer.

j, l, Immunoblot analysis of WCL derived from 293T cells transfected with indicated constructs.

h, Immunoblot analysis of WCL and immunoprecipitate derived from 293T cells transfected with indicated constructs and treated with MG132 (10 μM) for 12 h before collecting.

i, Immunoblot of WCL derived from HEK293T cells transfected with wild-type HA–PD-L1 or the T290M mutant. Cells which were treated with CHX for indicated times before collecting.

k, Immunoblot of WCL and Ni–NTA pull-down products derived from the lysates of PC3 cells transfected with the indicated constructs. Cells were treated with MG132 (30 μM) for 6 h before collecting and lysis in denaturing buffer.

l, Immunoblot analysis of WCL derived from 293T cells transfected with indicated constructs.
Extended Data Figure 5 | See next page for caption.
Extended Data Figure 5 | SPOP negatively regulates PD-L1 protein stability in a poly-ubiquitination-dependent manner. a–c, Immunoblot analysis of WCL derived from 293T cells transfected with indicated constructs. d, e, Immunoblot analysis of WCL derived from 293T cells transfected with indicated constructs. Thirty-six hours after transfection, cells were treated with 20 μg ml$^{-1}$ CHX for indicated length of time (d). The PD-L1 protein abundances were quantified using ImageJ (e). f, Immunoblot of WCL and Ni–NTA pull-down products derived from the lysates of PC3 cells transfected with the indicated constructs. Cells were treated with MG132 (30 μM) for 6 h before collecting and lysis in the denaturing buffer. g, A schematic illustration of SPOP with MATH and BTB domains interacting with substrate and cullin 3, respectively. h, Immunoblot analysis of WCL and immunoprecipitate derived from HEK293T cells transfected with indicated constructs and treated with MG132 (10μM) for 12 h before collecting. i, Immunoblot analysis of WCL derived from HEK293T cells transfected with indicated constructs. j, qRT–PCR analysis of relative mRNA levels of PD-L1 (also known as Cd274) from Spop$^{+/+}$ and Spop$^{-/-}$ MEFs. Data are mean ± s.d., n = 5. NS, not significant, two-tailed t-test. k, Immunoblot analysis of WCL derived from PC3 cells infected with indicated lentiviral shRNAs against SPOP and selected with puromycin (1 μg ml$^{-1}$) for 72 h before collecting. l, m, Immunoblot analysis of WCL derived from C4-2 cells with depletion of SPOP using sgRNA, treated with CHX for indicated time points before collecting (l). The PD-L1 protein abundance was quantified using ImageJ (m). n, o, Immunoblot analysis of WCL derived from LNCaP cells stably expressing shAR or shERG as well as shScr as a negative control. p, q, Immunoblot analysis of WCL derived from DU145 cells stably expressing shTRIM24 or shDEK as well as shScr as a negative control. r–u, Immunoblot analysis of WCL derived from C4-2 wild-type SPOP and SPOP$^{-/-}$ cells that stably expressed shAR, shERG, shTRIM24, or shDEK as well as shScramble.
Extended Data Figure 6 | See next page for caption.
Extended Data Figure 6 | Cancer-derived SPOP mutations fail to promote PD-L1 degradation. a, The mutation frequency (mutated cases/total cases) of SPOP across 24 cancer types from The Cancer Genome Atlas (TCGA) database. Mutations are categorized as occurring in the MATH domain, in the BTB domain or at any other position of the gene, including untranslated regions (UTRs). Because some patient cases contain mutations of two or three categories, the proportions of the colours are allocated mutation-wise, instead of case-wise. b, The distribution of mutation positions of SPOP in 24 cancer types from the TCGA database. Mutations without altering amino acid sequence have been discarded. c, Immunoblot analysis of WCL derived from HEK293T cells transfected with indicated constructs. d, The mutation frequency (mutated cases/total cases) of PD-L1 across 19 cancer types from the TCGA database. e, Oncoplot of PD-L1 and SPOP across all 39 cancer types in the TCGA database. Only PD-L1 or SPOP mutant tumours are shown; only mutations or truncations in the C-terminal tail of PD-L1 or in the MATH domain of SPOP are counted. f, Immunoblot of WCL derived from B16-F10 mouse tumour cell line stably expressing the indicated SPOP constructs. g, h, Growth curve and cell cycle profile of B16-F10 cells stably expressing wild-type SPOP and the F102C mutant, empty vector was used as a negative control. i, Cell cycle profile of 22Rv1 cells stably expressing wild-type SPOP and the F102C mutant, empty vector was used as a negative control. j, Relative cell-surface PD-L1 expression in 4T1-implemented tumours expressing wild-type SPOP or the F102C mutant measured using FACS analysis. n = 5 mice per experimental group. k, B16-F10 tumours derived from cells stably expressing wild-type SPOP or the F102C mutant and implanted in C57BL/6 mice were dissected and photographed after euthanization of mice. l, FACS analysis of the absolute number of CD3+ TILs from 4T1 tumours stably expressing wild-type SPOP or the F102C mutant. n = 5 mice per experimental group. m, B16-F10 tumour cells stably expressing wild-type SPOP or the F102C mutant were implanted into C57BL/6 mice. Tumour-bearing mice were treated with anti-PD-L1 antibody; tumours were then dissected and photographed. n = 7 mice per experimental group. n, The weight of B16-F10 cell derived tumours implanted in C57BL/6 mice that were subsequently treated with anti-PD-L1 antibody. 12 mice per experimental group. o, FACS analysis of relative cell surface expression of PD-L1 in implanted tumours derived from B16-F10 cells expressing wild-type SPOP or the F102C mutant. Animals were treated with anti-PD-L1 antibody. n = 5 mice per experimental group. p, FACS analysis of the absolute number of CD3+ TILs from B16-F10 tumours stably expressing wild-type SPOP or the F102C mutant in mice treated with anti-PD-L1 antibody. n = 7 mice per experimental group. q, B16-F10 cells stably expressing wild-type SPOP or the F102C mutant were inoculated into Tcra−−/− mice. After two weeks, tumours were dissected and photographed. n = 7 mice per experimental group. r, Tumour weights of B16-F10 cells stably expressing wild-type SPOP or the F102C mutant and implanted into Tcra−−/− mice were dissected and recorded. n = 7 mice per experimental group. s, FACS analysis of relative cell surface expression of PD-L1 from B16-F10 tumours stably expressing wild-type SPOP or the F102C mutant arising in Tcra−−/− mice. n = 7 mice per experimental group. Data are mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001, NS, not significant, two-tailed t-test.
Extended Data Figure 7 | Validation of anti-PD-L1 and anti-CD8 antibodies through using PD-L1 knockout or shCD8 cells.

a, Immunoblot analysis of WCL derived from PD-L1 knockout MDA-MB-231 cells. b, Immunofluorescence of wild-type and PD-L1 knockout MDA-MB-231 cells using the anti-PD-L1 antibody.

c, d, Immunochemistry using the anti-PD-L1 antibody of wild-type and PD-L1 knockout MDA-MB-231 cells from cultures on glass slides (c) or from implanted tumours (d).

e, f, Immunoblot analysis using the anti-CD8 antibody of WCL derived from HBP-ALL (e) or KE37 (f) cells stably expressing shCD8 as well as shScr as a negative control.

g, h, Immunohistochemistry using the anti-CD8 antibody for HBP-ALL (g) or KE37 (h) cell pellets stably expressing shCD8 or shScr as a negative control. Scale bars, 50 μm.
Extended Data Figure 8 | Depletion of FZR1, but not CDC20, prolongs SPOP proteins stability, which is simultaneously coupled with a decrease in PD-L1 protein levels. a–c, Immunoblot analysis of WCL derived from SPOP-knockout (a), CDC20-knockdown (b) or FZR1-knockdown (c) HeLa cells. d, Immunoblot analysis of WCL and immunoprecipitate derived from 293T cells transfected with indicated constructs and treated with MG132 (10 μM) for 12 h before collecting. e, Immunoblot analysis of WCL and immunoprecipitate derived from HeLa cells transfected with indicated constructs. Thirty-six hours after transfection, cells were treated with CHX at indicated time points before collecting (h). The protein abundances of wild-type SPOP and ΔRXXL mutant were quantified using ImageJ (i).
Extended Data Figure 9 | See next page for caption.
Extended Data Figure 9 | Cyclin D–CDK4-mediated phosphorylation of SPOP at the Ser6 residue promotes its binding with 14-3-3γ to reduce its poly-ubiquitination and subsequent degradation by APC–FZR1.

a, A sequence comparison of conserved SP sites and putative 14-3-3γ binding motif in SPOP. b, Immunoblot analysis of WCL and immunoprecipitate derived from 293T cells transfected with indicated constructs and treated with MG132 (10μM) for 12 h before collecting. c, d, In vitro kinase assays with recombinant Rb and SPOP as substrates and cyclin D1–CDK4, cyclin D2–CDK4 and cyclin D3–CDK4 as kinase complexes were performed. BSA was used as a negative control where indicated. e, Immunoblot analysis of WCL and immunoprecipitate derived from MDA-MB-231 cells transfected with indicated constructs, with and without palbociclib treatment (1μM) for 12 h. f, Streptavidin bead pull-down assay for biotin-labelled SPOP peptide with and without phosphorylation at the Ser6 residue to examine its in vitro association with 14-3-3γ. g, Immunoblot analysis of WCL and GST pull-down precipitates derived from 293T cells transfected with indicated constructs and treated with MG132 (10μM) for 12 h before collecting. h, i, Immunoblot analysis of WCL and immunoprecipitates derived from 293T cells transfected with indicated constructs and treated with MG132 (10μM) for 12 h before collecting. j, k, Immunoblot analysis of WCL derived from 293T cells transfected with indicated constructs. 36 h after transfection, cells were treated with 20μg ml−1 CHX at indicated time points (j). The protein abundance of wild-type SPOP and the S6A mutant were quantified using ImageJ (k). l, p, Immunoblot of WCL and Ni–NTA pull-down products derived from the lysates of PC3 cells transfected with the indicated constructs. Cells were treated with MG132 (30μM) for 6 h before collecting and lysis in denaturing buffer for subsequent assays. m–o, Immunoblot analysis of WCL and immunoprecipitates derived from 293T cells transfected with indicated constructs and treated with MG132 (10μM) with or without palbociclib (1μM) for 12 h before collecting. q–s, Immunoblot of WCLs derived from PC3 (q), BT549 (r) and HeLa (s) cells stably expressing sh14-3-3γ as well as shScr as a negative control. t, Immunoblot of WCL derived from HeLa cells stably expressing shScr or sh14-3-3γ synchronized in M phase by nocodazole treatment before release back into the cell cycle for the indicated times.
Extended Data Figure 10 | See next page for caption.
Extended Data Figure 10 | Combination therapy of anti-PD-1 antibody and CDK4/6 inhibitor in MC38 or CT26 colon cancer mouse model.  

**a**, A schematic of the treatment plan for mice bearing subcutaneous MC38 and CT26 tumours. Female C57BL/6 mice (for MC38) or BALB/c mice (for CT26) were implanted with $1 \times 10^5$ MC38 or CT26 cells subcutaneously and received one of four treatments: control antibody treatment, anti-PD-1 antibody treatment, CDK4/6 inhibitor (palbociclib) treatment, or anti-PD-1 antibody plus CDK4/6 inhibitor combination treatment.  

**b**, MC38 implanted tumour-bearing mice were enrolled in different treatment groups as indicated. Tumour volumes of mice treated with control antibody ($n = 15$), anti-PD-1 antibody ($n = 15$), palbociclib ($n = 14$) or anti-PD-1 antibody plus palbociclib combined therapy ($n = 12$) were measured every three days and plotted individually. Experiment repeated twice.  

**c**, Kaplan–Meier survival curves for each treatment group demonstrate the improved efficacy of combining PD-1 antibody with palbociclib. $*P < 0.05$, Gehan–Breslow–Wilcoxon test. Experiment repeated twice.  

**d, e, g, i**, The absolute numbers of CD3$^+$, CD4$^+$, CD8$^+$, granzyme B$^+$ or IFN$\gamma^+$ TILs in implanted MC38 tumours from mice treated with indicated agents was analysed by FACS. Control: $n = 8$, palbociclib: $n = 10$, anti-PD-1 antibody: $n = 9$, palbociclib and anti-PD-1 antibody: $n = 8$.  

**f, h, j**, The percentage of CD4$^+$, CD8$^+$ in CD3$^+$ TILs cells in implanted MC38 tumours from mice treated with indicated agents was analysed by FACS. Control: $n = 8$, palbociclib: $n = 10$, PD-1 antibody: $n = 9$, palbociclib and anti-PD-1 antibody: $n = 8$.  

**k**, A working model of how PD-L1 protein stability is regulated by the cyclin D–CDK4–SPOP–FZR1 signalling pathway. Cyclin D–CDK4 negatively regulates PD-L1 protein stability largely through phosphorylating its upstream physiological E3 ligase SPOP. This phosphorylation promotes SPOP binding to 14-3-3$\gamma$, which subsequently disrupts FZR1-mediated destruction of SPOP. Thus, CDK4/6 inhibitor treatment increases PD-L1 protein levels by inhibiting cyclin D–CDK4-mediated phosphorylation of SPOP to promote its degradation by APC/C. The rise in PD-L1 could present a severe clinical problem for patients receiving CDK4 inhibitor treatment and could be one of the underlying mechanisms accounting for CDK4 inhibitor resistance via evasion of immune surveillance checkpoints. Hence, our work provides a molecular mechanism as well as the rationale for the combination of PD-L1 blockade treatment and CDK4/6 inhibitors as a more efficient anti-cancer clinical option. Data are mean $\pm$ s.d. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-tailed $t$-test.
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

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Experimental design

1. Sample size
   Describe how sample size was determined.
   We determined sample size based on the sufficient statistics. The sample size should be more than 4 across the manuscript. Specifically, for mouse implanted tumor assays, the numbers of mice were included in the figure legends of Fig 2k; Fig. 3i, j, l; Fig. 4k, l; Extended Data Fig. 3c; Extended Data Fig. 6j, k, l, m, n, o, p, q, r, s. For MMTV-oncogene induced breast tumor samples in the presence or absence of Cyclin D1-/-, the numbers of mice were included in the figure legends of Fig. 2d and Extended data Fig. 2g. For Cdk4/6 inhibitor treatment of mice, mice numbers are included in figure legends of Extended data Fig. 3d, e.

2. Data exclusions
   Describe any data exclusions.
   No data was excluded from the experiments.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   Yes, the experimental findings were reliably reproduced.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Yes, for the xenografted mice assay or mice with palbociclib treatment, mice (from the Jackson Laboratory) were randomized into different groups.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Yes. The lab technician help to treat mice using the vehicle or CDK4 inhibitor palbociclib. However, to minimize the likelihood of differential treatment or assessments of outcomes during data collection and analysis, animals were pooled and randomly divided into four groups with comparable average tumor size. Moreover, the lab technician who measured the mice were blinded to the treatment groups.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters
For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a  Confirmed

☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

☐ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ A statement indicating how many times each experiment was replicated

☐ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)

☐ A description of any assumptions or corrections, such as an adjustment for multiple comparisons

☐ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted

☐ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)

☐ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software
Policy information about availability of computer code

7. Software
Describe the software used to analyze the data in this study.

We used the ImageJ software to quantify the protein bands intensity and used the GraphPad and Excel to do the graph figures and statistics.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents
Policy information about availability of materials

8. Materials availability
Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No restriction.

9. Antibodies
Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

All commercial antibodies used in this study were provided with information regarding the dilution, vendor and catalog number (described in the methods/antibody section). We also used the CRISPR KO or shRNAs to specifically deplete endogenous protein to verify anti-PD-L1, anti-SPOP, anti-Cdh1 and anti-CD8 antibodies.

10. Eukaryotic cell lines
a. State the source of each eukaryotic cell line used.

The source of the cell lines was included in the Methods/Cell Culture section.

b. Describe the method of cell line authentication used.

No

c. Report whether the cell lines were tested for mycoplasma contamination.

Yes, cell lines used in this study were routinely tested to be negative for mycoplasma.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

N/A
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

C57BL/6J mice (Jackson lab) were used for the B16-F10 and MC38 implanted tumor assays as well as for the CDK4 inhibitor treatment. Balb/c mice (Jackson lab) were used for the CT26 and 4T1 cells implanted tumor assays. cyclin D1+/+MMTV-Wnt-1 or c-Myc and MMTV-ErbB2 induced mouse mammary tumor models were generated in house.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

No human subject was involved in this study.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
3. All plots are contour plots with outliers or pseudocolor plots.
4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.

The source of cells and processing steps were clearly described below:

Single Cell Generation from Tumor Tissue and Flow Cytometry analysis. Tumor tissues were minced and digested with 5 ml of 2 mg/ml collagenase (Sigma) in DMEM for 1 hour at 37°C. Cells were then collected by centrifuge and filtered through a 70 μm strainer in DMEM. Cell pellets were suspended and lysed in red blood cell lysis buffer for 5 min. The cells were then filtered through a 40 μm strainer in 1 X PBS with 2% BSA. The fixed cells were suspended in Intracellular Staining Perm Wash Buffer (421002, Biolegend) after centrifuge for two times to permeabilize the cells. Cells were then co-stained with antibodies against CD3 (100236, APC conjugated, Biolegend), Granzyme B (515403, FITC conjugated, Biolegend), IFN-γ (505808, PE conjugated, Biolegend) to check the activities of T cells. Or cells were co-stained with antibodies against CD3 (100236, APC conjugated, Biolegend), CD4 (100510, FITC conjugated, Biolegend), CD8 (100708, PE conjugated, Biolegend). The corresponding isotype IgG1 controls were used for controls. The cells were incubated with corresponding antibodies for 30 minutes at room temperature. Cells were washed by 1 × PBS with 2% BSA and analyzed by flow cytometry.

We used the BD LSRFortessa with 4 lasers machine to collect and analyze all the FACS results in the manuscript.

7. Describe the software used to collect and analyze the flow cytometry data.

We used the FASCDiva software version 8.0.2 to collect and analyze the flow cytometry data.

8. Describe the abundance of the relevant cell populations within post-sort fractions.

N/A

9. Describe the gating strategy used.

FSC/SSC gates are exemplified in Supplementary figures. APC- conjugated corresponding isotype IgG stained cells were used as a negative control. The boundaries between “positive” and “negative” staining are also indicated in Supplementary figures.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☑
CORRECTIONS & AMENDMENTS

CORRECTION
https://doi.org/10.1038/s41586-019-1351-8

Author Correction: Cyclin D–CDK4 kinase destabilizes PD–L1 via cullin 3–SPOP to control cancer immune surveillance

Jinfang Zhang, Xia Bu, Haizhen Wang, Yasheng Zhu, Yan Geng, Naoe Taira Nihira, Yuyong Tan, Yanpeng Ci, Fei Wu, Xiangpeng Dai, Jianping Guo, Yu–Han Huang, Caoqi Fan, Shancheng Ren, Yinghao Sun, Gordon J. Freeman, Piotr Sicinski & Wenyi Wei

Correction to: Nature https://doi.org/10.1038/nature25015, published online 16 November 2017.

In the gel source data for Extended Data Fig. 2b (page 7 of Supplementary Fig. 1 of the Letter) of this Letter, the label “IB: Cyclin A” should read “IB: Cyclin E”. Figure 1 of this Amendment shows the original, correct Extended Data Fig. 2b and its corrected Source Data. The original Letter has not been corrected.

**Extended Data Fig. 2b**

| MEFs     | IB: PD-L1 | IB: Cyclin E | IB: Vinculin |
|----------|-----------|--------------|--------------|
| WT       | 55        | 43           |              |
| Ccne1+/− | 55        | 43           |              |
| Ccne2+/− | 55        | 43           |              |

**Corrected gel source data for Extended Data Fig. 2b**

**IB: PD-L1**

55
43

**IB: Cyclin E**

55
43

**IB: Vinculin**

130
95

Fig. 1 | This is the original, correct Extended Data Fig. 2b and its corrected Source Data.