ドライバー遺伝子変異陽性非小細胞肺癌における内因性および外因性のPD-L2発現制御メカニズム

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Intrinsic and Extrinsic Regulation of PD-L2 Expression in Oncogene-Driven Non-Small Cell Lung Cancer

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Abstract:	Introduction: The interaction of programmed cell death-ligand 2 (PD-L2) with programmed cell death-1 (PD-1) is implicated in tumor immune escape. The regulation of PD-L2 expression in tumor cells has remained unclear, however. We here examined the intrinsic and extrinsic regulation of PD-L2 expression in non-small cell lung cancer (NSCLC). Methods: PD-L2 expression was examined by reverse transcription and real-time PCR analysis, and by flow cytometry. Results: BEAS-2B cells stably expressing an activated mutant form of the epidermal growth factor receptor (EGFR) or the EML4-ALK fusion oncoprotein manifested increased expression of PD-L2 at both mRNA and protein levels. Furthermore, treatment of NSCLC cell lines that harbor such driver oncogenes with corresponding EGFR or ALK tyrosine kinase inhibitors or depletion of EGFR or ALK by siRNA transfection suppressed expression of PD-L2, demonstrating that activating EGFR mutations or EML4-ALK fusion intrinsically induce PD-L2 expression. We also found that IFN-γ extrinsically induced expression of PD-L2 via STAT1 signaling in NSCLC cells. Oncogene-driven expression of PD-L2 in NSCLC cells was inhibited by

	knockdown of the transcription factors STAT3 or c-FOS. IFN-γ also activated STAT3 and c-FOS, suggesting that these proteins may also contribute to the extrinsic induction of PD-L2 expression. Conclusions: Expression of PD-L2 is induced intrinsically by activating EGFR mutations or EML4-ALK fusion as well as extrinsically by IFN-γ, with STAT3 and c-FOS possibly contributing to both intrinsic and extrinsic pathways. Our results therefore provide insight into the complexity of tumor immune escape in NSCLC.

Intrinsic and Extrinsic Regulation of PD-L2 Expression in Oncogene-Driven Non–Small Cell Lung Cancer

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ABSTRACT

Introduction: The interaction of programmed cell death–ligand 2 (PD-L2) with programmed cell death–1 (PD-1) is implicated in tumor immune escape. The regulation of PD-L2 expression in tumor cells has remained unclear, however. We here examined intrinsic and extrinsic regulation of PD-L2 expression in non–small cell lung cancer (NSCLC).

Methods: PD-L2 expression was evaluated by reverse transcription and real-time polymerase chain reaction analysis and by flow cytometry.

Results: BEAS-2B cells stably expressing an activated mutant form of the epidermal growth factor receptor (EGFR) or the EML4-ALK fusion oncoprotein manifested increased expression of PD-L2 at both mRNA and protein levels. Furthermore, treatment of NSCLC cell lines that harbor such driver oncogenes with corresponding EGFR or ALK tyrosine kinase inhibitors or depletion of EGFR or ALK by siRNA transfection suppressed expression of PD-L2, demonstrating that activating *EGFR* mutations or *EML4-ALK* fusion intrinsically induce PD-L2 expression. We also found that interferon-γ extrinsically induced expression of PD-L2 via STAT1 signaling in NSCLC cells. Oncogene-driven expression of PD-L2 in NSCLC cells was inhibited by knockdown of the transcription factors STAT3 or c-FOS. Interferon-γ also activated STAT3 and c-FOS, suggesting that these proteins may also contribute to the extrinsic induction of PD-L2 expression.

Conclusions: Expression of PD-L2 is induced intrinsically by activating *EGFR* mutations or *EML4-ALK* fusion as well as extrinsically by interferon-γ, with STAT3 and c-FOS possibly contributing to both intrinsic and extrinsic pathways. Our results thus provide insight into the complexity of tumor immune escape in NSCLC.

Keywords: Non–small cell lung cancer; Programmed cell death–ligand 2 (PD-L2); Epidermal growth factor receptor (EGFR); EML4-ALK; Interferon-γ

Introduction

Immune-checkpoint blockade has shown promising clinical activity in the treatment of several types of cancer. Treatment with antibodies that target programmed cell death–1 (PD-1, also known as CD279) or its ligand PD-L1 (also known as B7-H1 or CD274) has thus demonstrated durable efficacy for various malignant tumors.[1-7] PD-L2 (also known as B7-DC or CD273) is another ligand of PD-1, and the interaction of PD-L2 with PD-1 also inhibits T cell activation.[8, 9] PD-L1 and PD-L2 compete for binding to PD-1,[10] with the relative affinity of PD-L2 for PD-1 being two to six times that of PD-L1.[11] PD-L1 is expressed in various immune and nonimmune cell types, including tumor cells, whereas PD-L2 expression was initially thought to be restricted to antigen presenting cells such as dendritic cells and macrophages.[8, 12] However, PD-L2 has recently been shown to be expressed by several malignant tumor cell types and therefore to have a potential role in tumor immune escape.[13-17] Indeed, some patients whose tumors do not express PD-L1 respond to treatment with antibodies to PD-1, whereas some PD-L1–positive patients do not respond to treatment with antibodies to PD-1 or to PD-L1,[1, 3, 4] suggesting that the interaction of PD-L2 with PD-1 might contribute to tumor immune escape in some cases. However, little has been known of the regulation of PD-L2 expression in tumor cells. We and others have previously shown that PD-L1 expression is intrinsically induced by activating mutations of the epidermal growth factor receptor gene (*EGFR*) or by the echinoderm microtubule-associated protein–like 4 (*EML4*)–anaplastic lymphoma kinase (*ALK*) fusion gene in non–small cell lung cancer (NSCLC) cells.[18-21] We have now investigated the role of activating *EGFR* mutations and the *EML4-ALK* fusion gene in the regulation of PD-L2 expression in NSCLC cells. In addition, we examined extrinsic regulation of PD-L2 expression by interferon (IFN)–γ in these cells.

Materials and Methods

Cell culture and reagents

PC-9, 11_18, and H3122 cells were obtained as previously described.[21] HCC827, H1975, H1650, H2228, H1299, A549, H23, H2122, H1437, and BEAS-2B cells were obtained from American Type Culture Collection. All cells were maintained under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium or Dulbecco's modified Eagle's medium, each supplemented with 10% fetal bovine serum. Erlotinib (Selleckchem), alectinib (Selleckchem), and osimertinib (AstraZeneca) were each dissolved in dimethyl sulfoxide (DMSO, Wako). Recombinant human IFN-γ (Peprotech) was dissolved in

phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin. All reagents were stored at –20° or –80°C.

Flow cytometric analysis

Cells were stained either with biotinylated mouse monoclonal antibodies to human PD-L1 (eBioscience) or an immunoglobulin (Ig) G1 κ isotype control (eBioscience), followed by phycoerythrin (PE)–labeled streptavidin (BD Biosciences), or with PE-labeled mouse monoclonal antibodies to human PD-L2 (BioLegend) or a PE-labeled IgG2a κ isotype control (BioLegend), for flow cytometric analysis with a FACS Verse instrument (BD Biosciences). The relative median fluorescence intensity (MFI) ratio was calculated as PD-L1 or PD-L2 MFI/isotype control MFI.

RNA extraction, RT, and real-time PCR analysis

Total RNA was extracted from cells with the use of an RNeasy Mini Kit (Qiagen) and was subjected to reverse transcription (RT) with the use of PrimeScript RT Master Mix (Takara). Real-time polymerase chain reaction (PCR) analysis was performed in triplicate with the use of SYBR Premix Ex Taq (Takara) and a Thermal Cycler Dice Real Time System (Takara). The PCR primers (forward and reverse, respectively) were as follows: PD-L1 (5'-CAATGTGACCAGCACACTGAGAA-3' and 5'-GGCATAATAAGATGGCTCCCAGAA-3'), PD-L2 (5'-AAAGACCTGTCACCACAACAAAG-3' and 5'-AAAGTGCTGGGTCATCCAAAG-3'), STAT3 (5'-GGTCTGGCTGGACAATATCATTG-3' and 5'-ATGATGTACCCTTCGTTCCAAAG-3'), c-FOS (5'-AGAATCCGAAGGGAAAGGAA-3' and 5'-CTTCTCCTTCAGCAGGTTGG-3'), STAT1 (5'-ATCACATTCACATGGGTGG-3' and 5'-CTTCAGGGGATTCTCAGGAATA-3'), and 18S rRNA (5'-ACTCAACACGGGAAACCTCA-3' and 5'-AACCAGACAAATCGCTCCAC-3'). The abundance of each mRNA was normalized by that of 18S rRNA.

Immunoblot analysis

Cells were rinsed with ice-cold PBS before lysis with SDS sample buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl [pH 6.8], and protease and phosphatase inhibitor cocktails), and then the lysates were incubated at 95°C for 5 min. Or, nuclear and cytoplasmic extracts of cells were prepared with the use of NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Protein was quantitated with the use of a DC Protein Assay Kit (Bio-Rad), portions (30 to 50 μg) of the lysates or extracts were fractionated by SDS-polyacrylamide

gel electrophoresis on a 10% gel, the separated proteins were transferred to a polyvinylidene difluoride membrane, and the membrane was then incubated overnight at 4°C with rabbit primary antibodies. Primary antibodies included those to phosphorylated EGFR (Y1068), EGFR, phosphorylated ALK (Y1064), ALK, phosphorylated STAT1 (Y701), STAT1, phosphorylated STAT3 (Y705), phosphorylated STAT3 (S727), STAT3, c-FOS, Lamin B1, and β-actin (all from Cell Signaling Technology). The membrane was subsequently incubated for 1 h at room temperature with horseradish peroxidase–conjugated goat antibodies to rabbit IgG (Abcam), after which immune complexes were detected with the use of Pierce Western Blotting Substrate Plus (Thermo Scientific) and a ChemiDoc XRS$^+$ system (Bio-Rad).

RNA interference

Cells were plated at 60% to 70% confluence in six-well plates and then incubated for 24 h before transient transfection for 48 h with small interfering RNAs (siRNAs) mixed with the RNAiMAX reagent (Invitrogen). The siRNAs specific for EGFR mRNA (EGFR-1, 5′-GCAAAGUGUGUAACGGAAUAGGUAU-3′; EGFR-2, 5′-GCAGUCUUAUCUAACUAUGAUGCAA-3′), ALK mRNA (ALK-1, 5′-ACACCCAAAUUAAUACCAA-3′; ALK-2, 5′-UCAG CAAAUUCAACCACCA-3′), STAT3 mRNA (STAT3-1, 5′-UCAUUGACCUUGUGAAAAA-3′; STAT3-2, 5′-GCAAAAAGUUUCCUACAAA-3′), c-FOS mRNA (c-FOS-1, 5′-CUGUCAACGCGCAGGACUU-3′; c-FOS-2, sc-29221), or STAT1 mRNA (STAT1-1, 5′-CCUACGAACAUGACCCUAU-3′; STAT1-2, 5′-GCGUAAUCUUCAGGAUAAU-3′), as well as a control nontarget siRNA (5′-UUCUCCGAACGUGUCACG-3′), were obtained from JBioS and Nippon EGT, with the exception of c-FOS-2 (sc-29221 from Santa Cruz Biotechnology). Data are presented for one of the two siRNAs corresponding to each target (siRNA-1 in each case) in the main figures, with those for each second siRNA (siRNA-2) being shown in the supplementary figures as indicated.

Plasmid constructs for EGFR or EML4-ALK expression

The plasmid pBabe-EGFR-Del1, which encodes human EGFR with an exon-19 deletion (Ex19del, E746–A750), was kindly provided by M. Meyerson (Addgene plasmid #32062). An expression vector for EML4-ALK (variant 3) was established as previously described.[21] The coding sequences for both the EGFR Ex19del and EML4-ALK proteins were amplified by PCR with PrimeSTAR GXL DNA Polymerase (Takara), and the PCR products were verified by sequencing and then ligated into the pQCXIP retroviral vector

(Clontech) between the NotI and EcoRI sites with the use of an In-Fusion HD cloning kit (Clontech).

Stable cell lines

The pQCXIP vectors encoding the EGFR Ex19del mutant or EML4-ALK were introduced into Amphopack-293 cells (Clontech) by transfection for 48 h with the Xfect reagent (Clontech). The culture supernatants were then passed through a 0.45-μm filter, incubated overnight at 4°C with a Retro-X concentrator (Clontech), and centrifuged at 1500 × *g* for 45 min at 4°C for isolation of retrovirus pellets. BEAS-2B cells were infected with the retroviruses in the presence of polybrene (8 μg/ml, Nacalai Tesque) for 24 h and were then cultured in complete growth medium for an additional 24 h. The infected cells were then selected by culture in the presence of puromycin (1 μg/ml, Invivogen).

Promoter constructs

Human genomic DNA was isolated from BEAS-2B cells with the use of a DNeasy Tissue Kit (Qiagen). The candidate promoter regions of the PD-L1 (–1019 to +110 bp relative to the transcription start site) and PD-L2 (–982 to +99 bp) genes were amplified from the genomic DNA by PCR, and the PCR products were ligated into the pGL4.1 luciferase vector (Promega) between the KpnI and XhoI sites with the use of an In-Fusion HD cloning kit (Clontech). Mutations were introduced into putative STAT3 or c-FOS binding sites within the PD-L1 and PD-L2 gene promoter regions by site-directed mutagenesis with the use of a KOD Plus Mutagenesis Kit (Toyobo) and the following primers (forward and reverse, respectively, with the mutated sequence underlined): PD-L1 STAT3 (5′-GGGGAAGAAAACTGGACTGACATGTTTCA-3′ and 5′-ATGAGATTTTCACCGGGAAGAGTTTC-3′), PD-L1 c-FOS (5′-ATAACAAGGGAAGGAAAGGCAAACAACGAAGAGTCC-3′ and 5′-TCAACTGCAGTTCAAAATACTGCAT-3′), PD-L2 STAT3 (5′-GGGGTGGCACAGCACTAAGACATGCTGGT-3′ and 5′-ATTGACTCATTTCCTAGGGCTTCTGT-3′), and PD-L2 c-FOS (5′-TAACGAGGATTTCCTGGCACAGCACTAAGACATG-3′ and 5′-TTCCTAGGGCTTCTGTAACACATGA-3′). Each promoter region was verified by direct sequencing.

Luciferase reporter assay

Cells cultured in 24-well plates were transfected for 24 h with 2.5 ng of the pGL4.73 *Renilla* luciferase vector (Promega) and 200 ng of PD-L1 or PD-L2 gene promoter vectors with the use of the Lipofectamine 3000 reagent (Invitrogen). Cell extracts were then

assayed for firefly and *Renilla* luciferase activities with the use of a Dual-Glo Luciferase Assay System (Promega). Firefly luciferase activity was normalized by that of *Renilla* luciferase.

Statistical analysis

Data are presented as means ± SD and were analyzed with the unpaired Student's *t* test as performed with GraphPad Prism 7 software (GraphPad Software). A *P* value of <0.05 was considered statistically significant.

Results

PD-L2 expression is induced by activating *EGFR* mutation or *EML4-ALK* in BEAS-2B cells

To investigate the effects of driver oncogenes on the expression of PD-L2 and PD-L1, we established BEAS-2B human bronchial epithelial cells that stably express either human EGFR with an activating (Ex19del) mutation or the EML4-ALK fusion protein. Immunoblot analysis confirmed the expression of total and phosphorylated EGFR (Fig. 1A) or ALK (Fig. 1B) in the respective stably transfected cells. Consistent with previous results,[18, 20, 21] the expression of PD-L1 at both mRNA and protein levels was increased by expression of the EGFR Ex19del mutant or EML4-ALK in a manner sensitive to inhibition by treatment with EGFR (erlotinib) or ALK (alectinib) tyrosine kinase inhibitors (TKIs). Moreover, we found that the abundance of both PD-L2 mRNA and protein in BEAS-2B cells was also increased by expression of the EGFR Ex19del mutant or EML4-ALK, and that these effects were inhibited by treatment of the cells with erlotinib (Fig. 1A) or alectinib (Fig. 1B), respectively. These results thus indicated that the expression of PD-L2 is up-regulated by activated mutant forms of EGFR and by the EML4-ALK fusion protein.

PD-L2 expression is intrinsically regulated by activating *EGFR* mutations or *EML4-ALK* in NSCLC cell lines

To investigate the regulation of PD-L2 expression in NSCLC cell lines, we measured PD-L2 mRNA and protein levels in five cell lines (HCC827, H1975, PC-9, 11_18, H1650) that harbor activating *EGFR* mutations, two cell lines (H3122, H2228) that harbor the *EML4-ALK* fusion gene, and five cell lines (A549, H2122, H1299, H23, H1437) that are wild type (WT) for both *EGFR* and *ALK*. HCC827, PC-9, and H1650 harbor an activating in-frame deletion [del(E746–A750)] in exon 19 of *EGFR*; 11-18 harbors an activating point mutation (L858R) in exon 21; and H1975 harbors the L858R point mutation in exon

21 as well as a secondary mutation (T790M) in exon 20. RT and real-time PCR analysis detected PD-L2 mRNA at only a low level in cell lines WT for *EGFR* and *ALK* (Supplementary Fig. 1A). In contrast, most cell lines positive for the driver oncogenes, including HCC827, H1975, PC-9, H1650, and H2228, manifested a high level of PD-L2 mRNA (Supplementary Fig. 1A). Flow cytometric analysis revealed that the five cell lines WT for *EGFR* and *ALK* did not express PD-L2 at the cell surface, whereas PC-9, H1975, and H2228 cells manifested substantial surface expression of PD-L2 (Supplementary Fig. 1B). We also examined the Cancer Cell Line Encyclopedia (CCLE) database (http://www.broadinstitute.org/ccle) to investigate PD-L2 gene expression in additional NSCLC cell lines. Expression of the PD-L2 gene in cell lines positive for activating *EGFR* mutations or *EML4-ALK*, including two additional *EGFR*-mutated lines, was consistent with our data (Supplementary Fig. 1C). Analysis of the CCLE database also revealed that some cell lines positive for *KRAS* mutations or WT for known driver oncogenes manifest a high level of PD-L2 gene expression.

We next examined whether PD-L2 expression is dependent on activated EGFR signaling in PC-9 cells, which harbor an activating *EGFR* mutation and also express PD-L1 and PD-L2 at high levels. Treatment of these cells with the EGFR-TKI erlotinib inhibited EGFR phosphorylation and resulted in down-regulation of PD-L2 expression as well as that of PD-L1 expression at both mRNA and protein levels (Fig. 2A). We also examined the participation of activated EGFR signaling in regulation of PD-L2 expression in H1975 cells, which harbor both an activating *EGFR* mutation as well as a secondary mutation (T790M) in exon 20 of *EGFR* that contributes to EGFR-TKI resistance. Treatment with osimertinib, a third-generation EGFR-TKI that inhibits the activation of EGFR harboring T790M, attenuated the phosphorylation of EGFR and reduced the amounts of PD-L2 mRNA and protein as well as those of PD-L1 mRNA and protein in these cells, whereas erlotinib had no such effects (Fig. 2B). To exclude the possibility of off-target effects of erlotinib in PC-9 cells, we instead silenced EGFR expression by transfection with an siRNA specific for EGFR mRNA. Both the abundance of PD-L2 and PD-L1 mRNAs and surface expression of both proteins were down-regulated by transfection with the EGFR siRNA compared with those in cells transfected with a control siRNA (Fig. 2C). We obtained similar results with a second siRNA targeting a different sequence within EGFR mRNA (Supplementary Fig. 2A). These findings thus indicated that PD-L2 expression is up-regulated at both mRNA and protein levels in cells with activating *EGFR* mutations.

We next tested whether PD-L2 expression is dependent on EML4-ALK signaling in H2228 cells, which harbor the *EML4-ALK* fusion gene and also highly express PD-L1 and PD-L2. Treatment of these cells with the ALK-TKI alectinib inhibited EML4-ALK phosphorylation and induced down-regulation of PD-L2 expression as well as that of PD-L1 expression at both mRNA and protein levels (Fig. 2D). To exclude potential nonspecific effects of alectinib, we also silenced EML4-ALK expression in H2228 cells by transfection with an siRNA specific for ALK mRNA. Depletion of EML4-ALK was associated with down-regulation of both PD-L2 and PD-L1 mRNA and surface protein levels in H2228 cells (Fig. 2E). Similar results were obtained with a second siRNA targeting a different sequence within ALK mRNA (Supplementary Fig. 2B). These results thus indicated that PD-L2 expression is also up-regulated as a result of constitutive activation of ALK signaling.

PD-L2 expression is extrinsically regulated by IFN-γ in NSCLC cell lines

Our data revealed intrinsic induction of both PD-L2 and PD-L1 expression by activating *EGFR* mutations or *EML4-ALK* fusion in NSCLC cell lines. Although extrinsic induction by IFN-γ, a cytokine that plays a key role in inflammation,[9, 22, 23] is also an important mechanism for regulation of PD-L1 expression in tumor cells, little has been known of such extrinsic regulation of PD-L2 expression. We therefore examined the effect of IFN-γ on PD-L2 expression in NSCLC cell lines. Consistent with previous results,[9] the surface expression of PD-L1 was up-regulated by IFN-γ stimulation in PC-9, H1975, and H2228 cells, all of which harbor *EGFR* or *ALK* driver oncogenes, as well as in A549 cells, which are WT for these genes (Fig. 3). The surface expression of PD-L2 was also up-regulated by IFN-γ treatment in these four cell lines. These results thus showed that expression of PD-L2, like that of PD-L1, is extrinsically induced by IFN-γ stimulation in NSCLC cells.

Transcriptional regulation of PD-L2 and PD-L1 expression by intrinsic and extrinsic pathways

To identify transcription factors that regulate transcription of the PD-L2 and PD-L1 genes in oncogene-driven NSCLC cell lines, we first searched for potential binding sites in the promoter regions of these genes, including 1000 bp upstream from the transcription start site, with the use of the JASPAR database (http://jaspar.genereg.net). The promoter regions of both human PD-L1 and PD-L2 genes were found to harbor putative binding sites for STAT3 (signal transducer and activator of transcription 3) and c-FOS, both of which are downstream transcription factors of EGFR and ALK signaling pathways (Supplementary Fig. 3A). To investigate whether STAT3 or c-FOS regulates PD-L2 or

PD-L1 gene transcription via these binding sites in oncogene-driven NSCLC cell lines, we performed luciferase reporter assays with WT and mutant promoter constructs. Mutation of the putative binding sites for STAT3 or c-FOS resulted in significant attenuation of PD-L2 and PD-L1 gene promoter activity in H1975 cells (Supplementary Fig. 3B). To investigate further whether driver oncogenes induce PD-L2 expression via STAT3 or c-FOS, we examined the effects of EGFR-TKI or ALK-TKI treatment on the activation of STAT3 or c-FOS in PC-9 and H2228 cells. Treatment of these cells with the corresponding TKI inhibited phosphorylation of STAT3 and the nuclear abundance of c-FOS in both cells (Supplementary Fig. 4A). In BEAS-2B cells stably expressing the EML4-ALK fusion protein, forced expression of EML4-ALK increased the level of STAT3 phosphorylation and the nuclear abundance of c-FOS (Supplementary Fig. 4B). We further silenced STAT3 or c-FOS expression in these cells by transfection with specific siRNAs, finding that the up-regulation of PD-L2 expression by activated EML4-ALK signaling was attenuated by knockdown of STAT3 or c-FOS (Supplementary Fig. 4C). To confirm the contribution of STAT3 and c-FOS to regulation of PD-L2 and PD-L1 expression in oncogene-driven NSCLC cells, we investigated the effects of STAT3 or c-FOS knockdown on PD-L2 and PD-L1 expression in PC-9 and H1975 cells. In PC-9 cells, knockdown of STAT3 resulted in down-regulation of the surface expression of PD-L2 and PD-L1 (Fig. 4A and E), whereas depletion of c-FOS reduced PD-L2 surface expression without affecting that of PD-L1 (Fig. 4B and E). On the other hand, depletion of STAT3 or c-FOS attenuated the expression of both PD-L2 and PD-L1 at the surface of H1975 cells (Fig. 4C–E). We obtained similar results with additional siRNAs targeting different sequences within STAT3 or c-FOS mRNAs (Supplementary Fig. 5). Together, these findings thus showed that expression of both PD-L2 and PD-L1 is regulated via STAT3 and c-FOS in oncogene-driven NSCLC cells, although the pattern of regulation appears to differ depending on the cell line.

IFN-γ has been shown to induce PD-L1 expression via STAT1, a key transcription factor in IFN-γ signaling.[24-27] To investigate the regulation of PD-L2 expression by downstream signaling of IFN-γ, we silenced STAT1 expression by transfection with an siRNA specific for STAT1 mRNA in PC-9, H1975, and H2228 cells. The induction of PD-L1 expression by IFN-γ was inhibited by depletion of STAT1 in all three cell lines, whereas that of PD-L2 expression was also attenuated by knockdown of STAT1 but to a lesser extent in PC-9 and H1975 cells than was that of PD-L1 (Fig. 5A). Similar results were obtained with a second siRNA targeting a different sequence within

STAT1 mRNA (Supplementary Fig. 6). These observations thus indicated that IFN-γ–STAT1 signaling induces PD-L2 as well as PD-L1 expression in NSCLC cells.

Finally, to investigate whether IFN-γ activates additional transcription factors in oncogene-driven NSCLC cells, we examined the effects of IFN-γ on the phosphorylation of STAT3 and the nuclear translocation of c-FOS in PC-9, H1975, and H2228 cells (Fig. 5B). Consistent with previous results,[26, 28] IFN-γ markedly increased the amount of phosphorylated STAT1 in all three cell lines. It also increased the phosphorylation of STAT3 and the nuclear abundance of c-FOS, but not the phosphorylation of EGFR or ALK, implicating both STAT3 and c-FOS, but not EGFR or ALK, as participants in IFN-γ signaling in oncogene-driven NSCLC cells.

Discussion

We and others have previously shown that PD-L1 expression is induced by activating *EGFR* mutations or *EML4-ALK* fusion in NSCLC cell lines.[18-21] However, little has been known of the regulation of PD-L2 expression by driver oncogenes. We have now shown that BEAS-2B cells stably expressing an activated mutant form of EGFR or the EML4-ALK fusion protein manifested increased amounts of PD-L2 mRNA and protein. Furthermore, either inhibition of activated EGFR or ALK signaling by corresponding TKIs or transfection with EGFR or ALK siRNAs suppressed the expression of PD-L2 in oncogene-driven NSCLC cells. These data thus indicate that activating *EGFR* mutations or *EML4-ALK* fusion induce intrinsic up-regulation of PD-L2 expression as well as that of PD-L1 expression in NSCLC cells. We further showed that knockdown of STAT3 or c-FOS inhibited PD-L2 expression in oncogene-driven NSCLC cells, thus implicating these transcription factors in the intrinsic regulation of PD-L2 expression. STAT3 is a downstream transcription factor of EGFR and EML4-ALK signaling[29, 30] and has previously been shown to induce the expression of PD-L1 by binding to the PD-L1 gene promoter.[31-33] We similarly found that STAT3 knockdown resulted in down-regulation of PD-L1 expression in oncogene-driven NSCLC cells. The human PD-L1 and PD-L2 genes share 37% sequence identity as well as a similar overall structural organization of their promoter regions.[8, 9] The PD-L2 gene promoter also contains putative binding sites for STAT3,[28] consistent with our findings implicating STAT3 in the regulation of both PD-L2 and PD-L1 expression. c-FOS is activated in response to EGFR signaling and regulates the expression of various genes by forming heterodimers with other transcription factors.[34-36] Although, as far as we are aware, the regulation of PD-1 ligand expression by c-FOS has

not previously been described, we identified potential binding sites for c-FOS in the promoter regions of both PD-L2 and PD-L1 genes. We further found that depletion of c-FOS suppressed both PD-L2 and PD-L1 expression in NSCLC cells, thus implicating c-FOS in regulation of the expression of both PD-1 ligands.

We showed that driver oncogenes intrinsically induce PD-L2 as well as PD-L1 expression via STAT3, c-FOS, or both transcription factors in NSCLC cells. IFN-γ produced by tumor-infiltrating lymphocytes (TILs) has previously been shown to mediate the extrinsic up-regulation of PD-L1 expression in tumor cells.[9, 22, 23, 27] We have now shown that IFN-γ–STAT1 signaling also up-regulates the expression of PD-L2 in NSCLC cell lines. The expression of PD-L1 on the surface of tumor cells in the tumor microenvironment is thought to be induced largely in response to stimulation by IFN-γ released from TILs.[23, 37] Our findings that IFN-γ up-regulated the expression of both PD-L2 and PD-L1 in NSCLC cells suggest that expression of both PD-1 ligands is induced in NSCLC by the presence of a high number of TILs in tumor tissue. Our results are consistent with those of a recent study showing that PD-L2 expression generally correlates with PD-L1 expression in human tumor samples.[38]

The IFN-γ signaling pathway is complex and mediated by various transcription factors and other signaling proteins.[24, 39] STAT3 has been implicated as a downstream transcription factor of IFN-γ signaling.[28, 39, 40] We have now shown that IFN-γ increased the phosphorylation of STAT3 as well as that of STAT1 in NSCLC cells. Furthermore, we found that c-FOS was also activated in response to IFN-γ stimulation in these cells. These data thus suggest that STAT3 and c-FOS might contribute to the extrinsic induction of PD-L1 and PD-L2 expression by IFN-γ as well as to the intrinsic induction of these PD-1 ligands in oncogene-driven NSCLC cells. Indeed, STAT3 was recently shown to mediate induction of PD-L2 expression by IFN-β or IFN-γ through binding to the PD-L2 gene promoter in melanoma cells.[28] We also found that knockdown of STAT3 or c-FOS with specific siRNAs inhibited the IFN-γ–induced expression of both PD-L2 and PD-L1 in PC-9 and H1975 cells (data not shown), suggesting that these transcription factors indeed mediate the extrinsic induction of PD-1 ligands. Together, our data thus indicate that STAT3 and c-FOS contribute to both intrinsic and extrinsic regulation of PD-L2 and PD-L1 expression in NSCLC cells (Fig. 6).

In conclusion, we have shown that PD-L2 expression is induced intrinsically by activating *EGFR* mutations or *EML4-ALK* fusion as well as extrinsically by IFN-γ–STAT1 signaling in NSCLC cells. Furthermore, STAT3 and c-FOS appear to be common

transcription factors in both the intrinsic and extrinsic induction of both PD-L1 and PD-L2 expression (Fig. 6). Our findings thus provide a better understanding of the regulation of PD-L2 expression in tumor cells and of the complex nature of tumor immunity in oncogene-driven NSCLC.

Acknowledgments

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Figure Legends

Figure 1. Up-regulation of PD-L2 and PD-L1 expression by activating *EGFR* mutation or *EML4-ALK* in transfected BEAS-2B cells. BEAS-2B cells stably expressing an Ex19del mutant form of EGFR (**A**) or the EML4-ALK fusion protein (**B**), or those stably infected with the corresponding empty virus, were incubated for 48 h in the presence of erlotinib (100 nM) (**A**), alectinib (100 nM) (**B**), or DMSO vehicle. The cells were then lysed and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR or ALK or with those to β-actin (loading control), as indicated (left panels). The bands detected by the antibodies to phosphorylated or total ALK correspond to the EML4-ALK fusion protein. The cells were also subjected to RT and real-time PCR analysis of relative PD-L1 or PD-L2 mRNA abundance (middle panels); data are means ± SD of triplicates from one experiment and are representative of three independent experiments. In addition, the cells were subjected to flow cytometric analysis of PD-L1 and PD-L2 expression at the cell surface (right panels). Immunoblot and flow cytometric data are representative of three independent experiments. $*P < 0.05$, $***P < 0.001$ (Student's *t* test).

Figure 2. Up-regulation of PD-L2 and PD-L1 expression by activating *EGFR* mutations or *EML4-ALK* in NSCLC cell lines. (**A**, **B**, and **D**) PC-9, H1975, and H2228 cells, respectively, were incubated in the presence of DMSO vehicle or either 100 nM erlotinib for 24 h (**A**), 100 nM erlotinib or 100 nM osimertinib for 48 h (**B**), or 100 nM alectinib for 24 h (**D**). The cells were then lysed and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR or ALK or with those to β-actin (loading control), as indicated (left panels). The bands detected by the antibodies to phosphorylated or total ALK correspond to the EML4-ALK fusion protein. The cells were also subjected to RT and real-time PCR analysis of relative PD-L1 or PD-L2 mRNA abundance (middle panels); data are means ± SD of triplicates from one experiment and are representative of three independent experiments. In addition, the cells were subjected to flow cytometric analysis of PD-L1 and PD-L2 expression at the cell surface (right panels). (**C** and **E**) PC-9 and H2228 cells, respectively, were transfected with nontargeting (NT) or EGFR (**C**) or ALK (**E**) siRNAs for 48 h and were then subjected to immunoblot, RT and real-time PCR, and flow cytometric analyses as above. All immunoblot and flow cytometric data are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t* test); NS, not significant.

Figure 3. Up-regulation of PD-L2 and PD-L1 expression by IFN-γ in NSCLC cell lines. A549, PC-9, H1975, or H2228 cells were incubated in the absence or presence of IFN-γ (100 ng/ml) for 24 h, after which surface expression of PD-L1 and PD-L2 was determined by flow cytometry. Representative profiles as well as the relative median fluorescence intensity (MFI) ratios (PD-L1/isotype or PD-L2/isotype) as means ± SD from three independent experiments are shown. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t* test).

Figure 4. Transcriptional control of PD-L2 and PD-L1 expression by STAT3 and c-FOS in NSCLC cells. (**A**–**D**) PC-9 cells (**A** and **B**) and H1975 cells (**C** and **D**) were transfected with nontargeting (NT), STAT3 (**A** and **C**), or c-FOS (**B** and **D**) siRNAs for 48 h, after which cell surface expression of PD-L1 (left panels) and PD-L2 (right panels) was measured by flow cytometry. Representative profiles are shown. (**E**) The relative PD-L1/isotype and PD-L2/isotype MFI ratios were determined as means ± SD from three independent experiments. **P* < 0.05 (Student's *t* test).

Figure 5. Regulation of PD-L2 and PD-L1 expression by IFN-γ signaling molecules. (**A**) PC-9, H1975, and H2228 cells were transfected with nontargeting (NT) or STAT1 siRNAs for 48 h, for the final 24 h of which the cells were also exposed to IFN-γ (100 ng/ml) or PBS vehicle. The cells were then assayed for surface PD-L1 (left panels) and PD-L2 (right panels) expression by flow cytometry. (**B**) PC-9, H1975, and H2228 cells were incubated with IFN-γ (100 ng/ml) or PBS vehicle for 24 h, after which cytoplasmic and nuclear fractions were prepared from cell lysates and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR, ALK, STAT1, STAT3, or c-FOS as well as with those to β-actin (cytoplasmic loading control) or to Lamin B1 (nuclear loading control). All results are representative of three independent experiments.

Figure 6. Model for intrinsic and extrinsic regulation of PD-L2 and PD-L1 expression in oncogene-driven NSCLC cells. Activating *EGFR* mutations or *EML4-ALK* fusion intrinsically induce PD-L2 as well as PD-L1 expression via the transcription factors STAT3 and c-FOS. IFN-γ signaling extrinsically induces the expression of both PD-1 ligands via STAT3 and c-FOS in addition to STAT1. The regulation of PD-L2 and PD-L1 expression by intrinsic and extrinsic pathways thus shares common transcription factors in

NSCLC cells.

Supplementary Figure 1. Expression of PD-L2 at mRNA and surface protein levels in NSCLC cell lines. (**A**) RT and real-time PCR analysis of PD-L2 mRNA abundance (normalized by that of 18S rRNA) in NSCLC cell lines positive or negative for activating *EGFR* mutations or the *EML4-ALK* fusion gene. Data are means of triplicates from one experiment and are representative of three independent experiments. (**B**) Flow cytometric analysis of PD-L2 expression at the cell surface for representative NSCLC cell lines. Data are representative of three independent experiments. (**C**) PD-L2 gene expression in NSCLC cell lines presented as reads per kilobase of exon per million mapped reads (RPKM). Data are from the Cancer Cell Line Encyclopedia database.

Supplementary Figure 2. Efficiency of EGFR and ALK knockdown in NSCLC cells. PC-9 (**A**) and H2228 (**B**) cells were transfected with nontargeting (NT) or EGFR-1 or EGFR-2 (**A**) or ALK-1 or ALK-2 (**B**) siRNAs for 48 h, lysed, and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR or ALK or with those to β-actin (loading control), as indicated (left panels). In addition, the cells were subjected to flow cytometric analysis of PD-L2 expression at the cell surface (right panels). Data are representative of three independent experiments.

Supplementary Figure 3. Regulation of PD-L1 and PD-L2 gene promoter activity by STAT3 and c-FOS. (**A**) Putative STAT3 and c-FOS binding sites (red letters) identified in the promoter regions of the PD-L1 (–1019 to +110 bp relative to the transcription start site [TSS]) and PD-L2 (–982 to +99 bp) genes by analysis of the JASPAR database. The nucleotides of these sites that were mutated for promoter activity assays are underlined. (**B**) Luciferase reporter assays performed in H1975 cells for the activity of WT forms of the PD-L1 and PD-L2 gene promoters as well as of mutant (mut) forms of the promoters in which the putative STAT3 or c-FOS binding sites were altered. Data are means ± SD from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test).

Supplementary Figure 4. Regulation of PD-L2 expression by driver oncogenes through STAT3 and c-FOS in NSCLC cell lines or BEAS-2B cells stably expressing the EML4-ALK fusion protein. (**A**) PC-9 and H2228 cells, respectively, were incubated in the presence of DMSO vehicle or either 100 nM erlotinib for 24 h, or 100 nM alectinib for 24

h. Cytoplasmic and nuclear fractions prepared from PC-9 and H2228 cells (A) or BEAS-2B cells stably expressing the EML4-ALK fusion protein (B) were subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR, ALK, STAT3, with those to c-FOS, or with those to β-actin (cytoplasmic loading control) or to Lamin B1 (nuclear loading control). (**C**) The cells were also transfected with nontargeting (NT), STAT3 (STAT3-1), or c-FOS (c-FOS-1) siRNAs for 48 h, after which cell surface expression of PD-L2 was measured by flow cytometry. Data are representative of three independent experiments.

Supplementary Figure 5. Efficiency of STAT3 and c-FOS knockdown in NSCLC cells. (**A**) PC-9 cells were transfected with nontargeting (NT), STAT3-1, STAT3-2, c-FOS-1, or c-FOS-2 siRNAs for 48 h, after which the corresponding relative abundance of STAT3 or c-FOS mRNAs was determined by RT and real-time PCR analysis (data are means ± SD of triplicates from one experiment). The cells were also lysed and subjected to immunoblot analysis with antibodies to phosphorylated (p) STAT3 (Y705), total STAT3 or with those to β-actin (loading control). Alternatively, a nuclear fraction prepared from the cells was subjected to immunoblot analysis with antibodies to c-FOS or to Lamin B1 (loading control). (**B**) PC-9 cells transfected with NT, STAT3-2, or c-FOS-2 siRNAs for 48 h were subjected to flow cytometric analysis of PD-L2 expression at the cell surface. Data are representative of three independent experiments.

Supplementary Figure 6. Efficiency of STAT1 knockdown in NSCLC cells. (**A**) PC-9 cells were transfected with nontargeting (NT), STAT1-1, or STAT1-2 siRNAs for 48 h, for the final 24 h of which the cells were also exposed to IFN-γ (100 ng/ml). The relative abundance of STAT1 mRNA was then determined by RT and real-time PCR analysis (data are means ± SD of triplicates from one experiment). The cells were also lysed and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of STAT1 or with those to β-actin (loading control). (**B**) PC-9 cells treated as in (**A**) were subjected to flow cytometric analysis of PD-L2 expression at the cell surface. Data are representative of three independent experiments.

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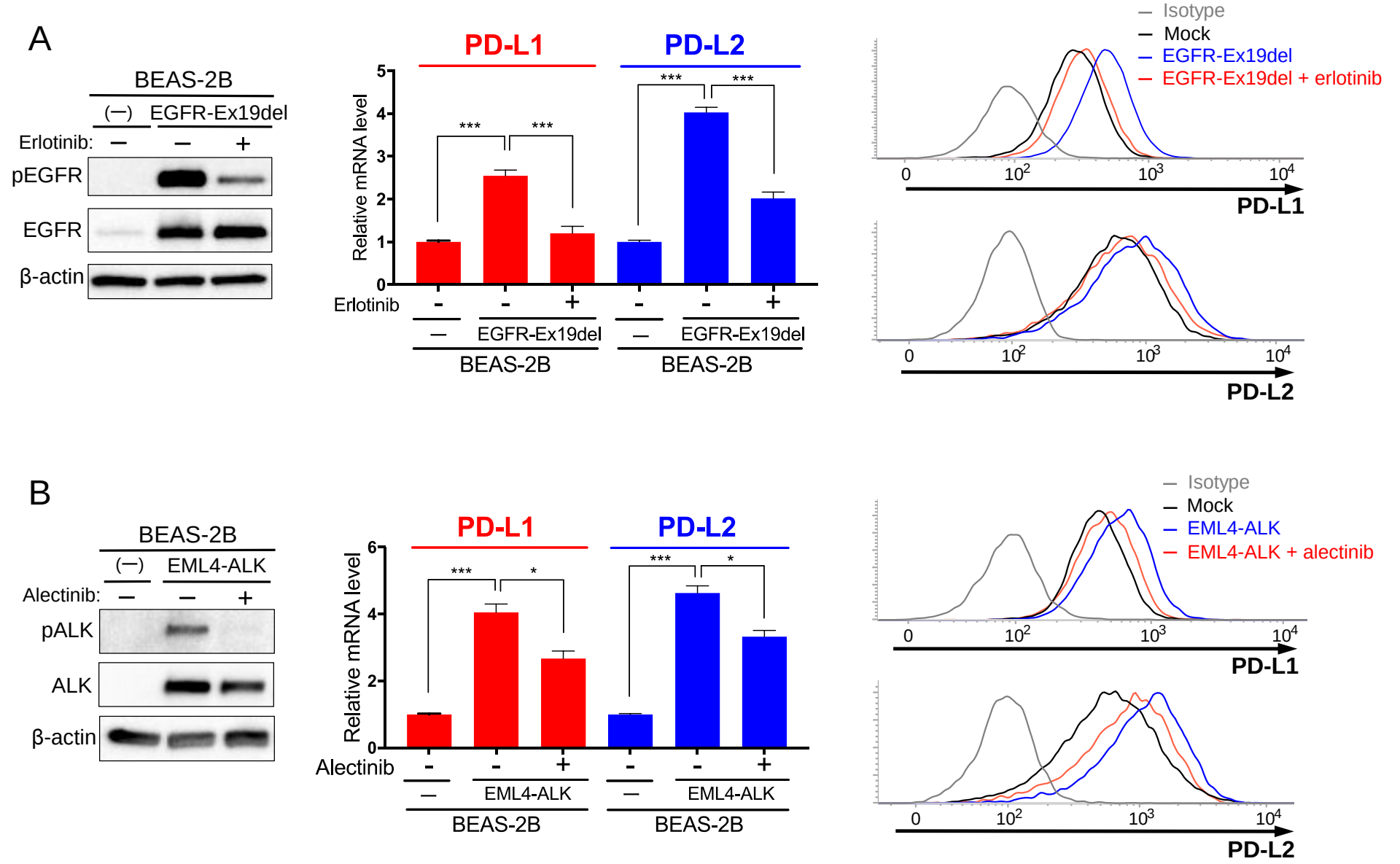
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Figure 2.

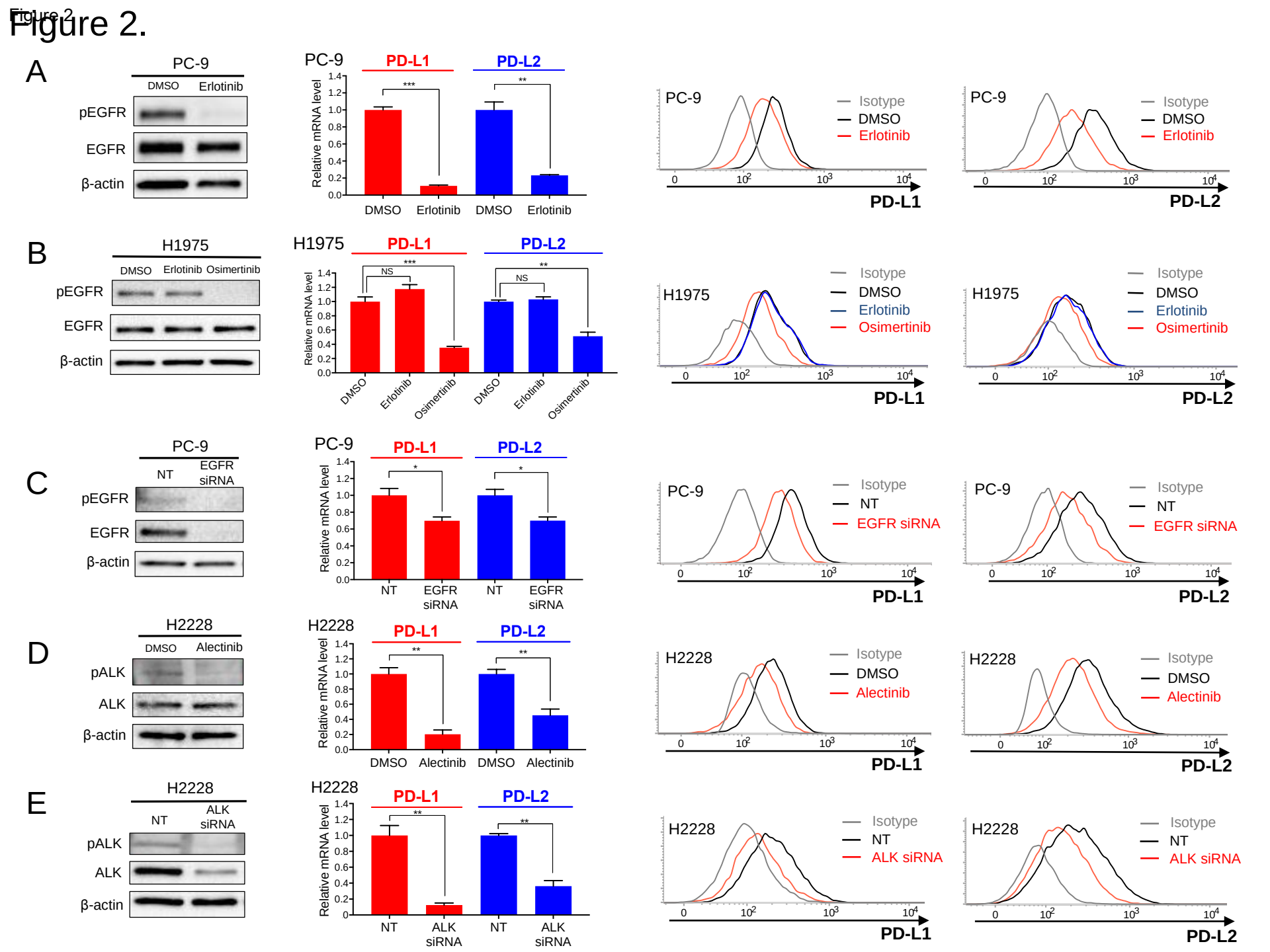
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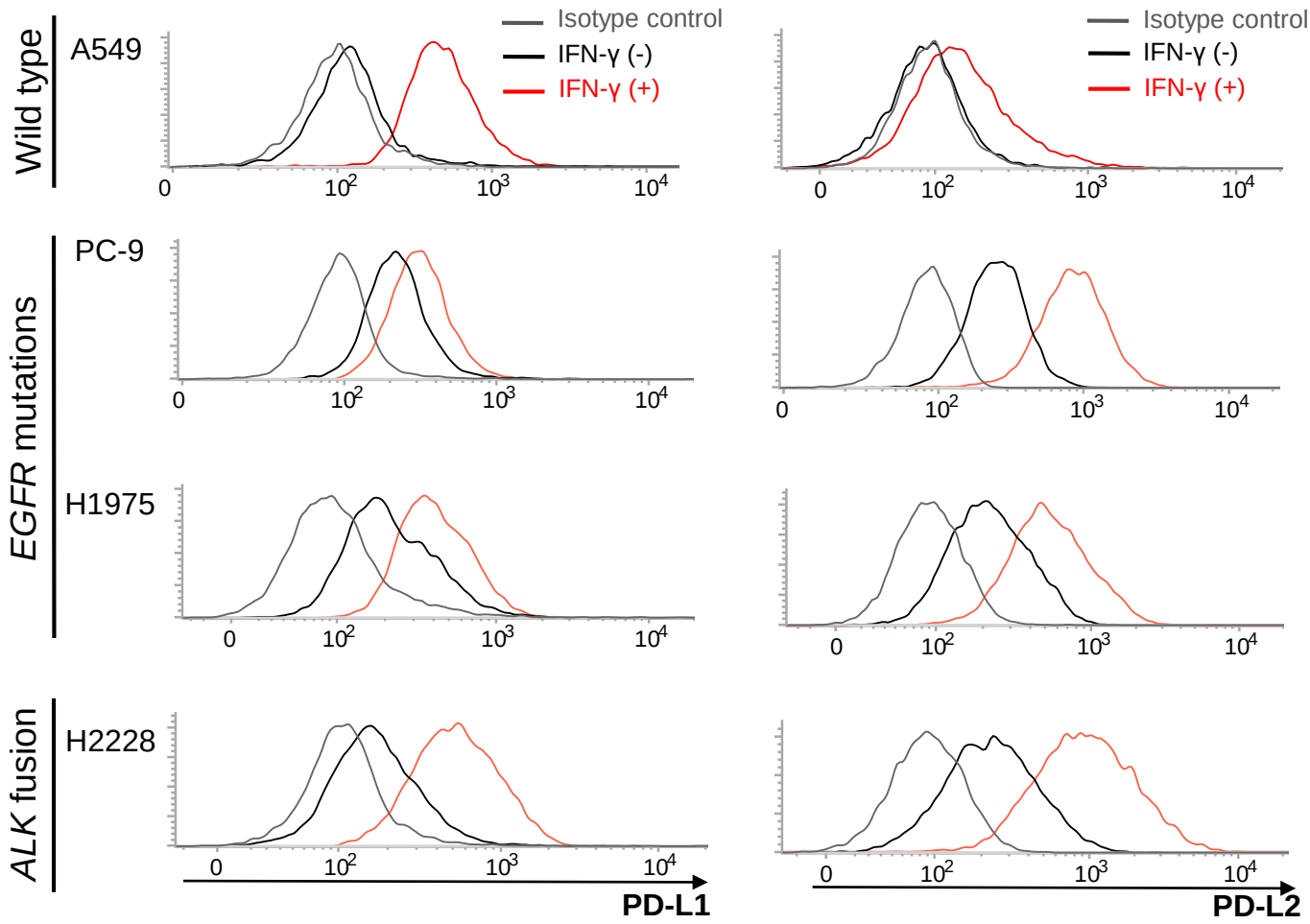
Figure 3.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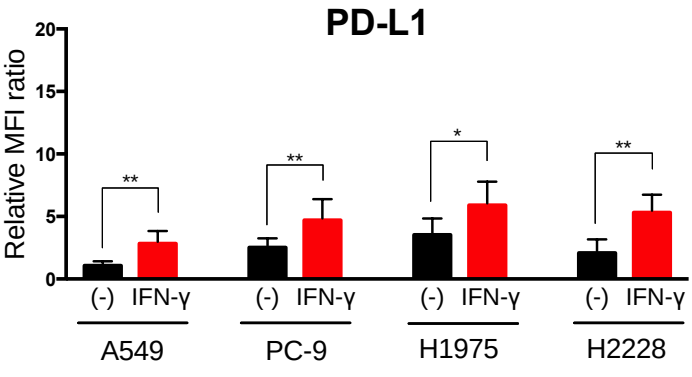
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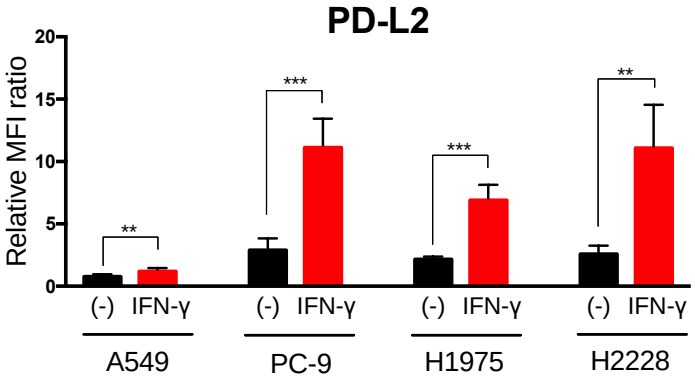
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Figure 4.

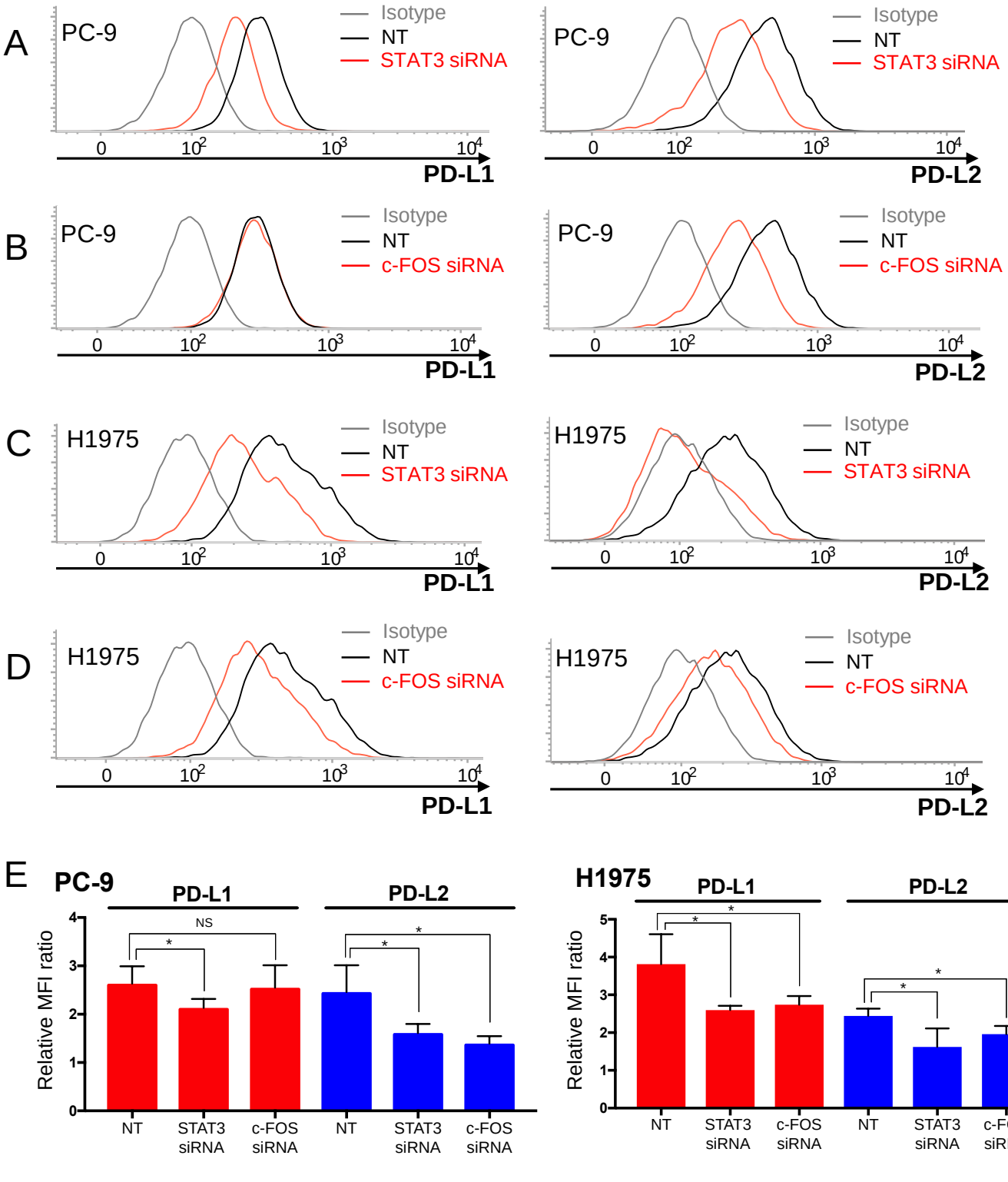
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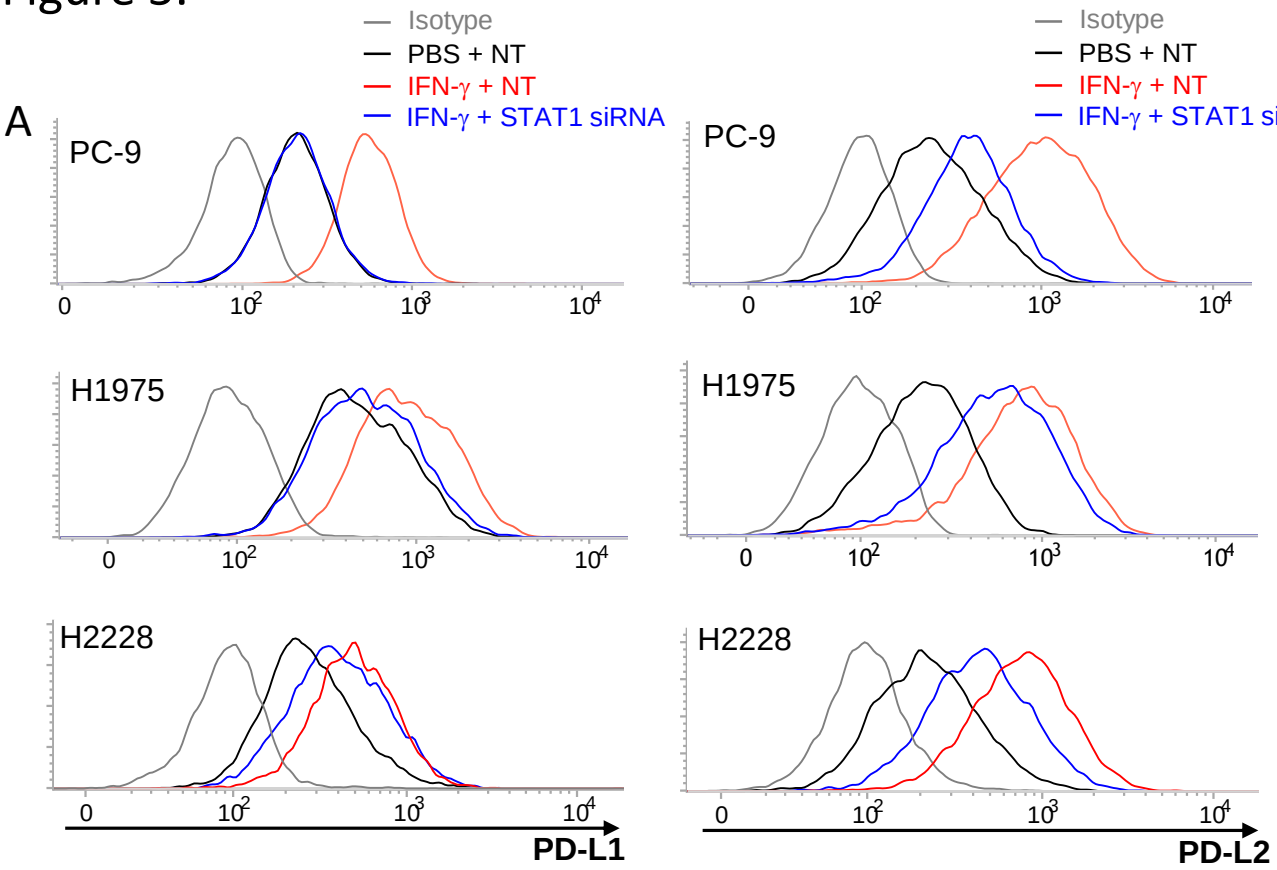
Figure 5.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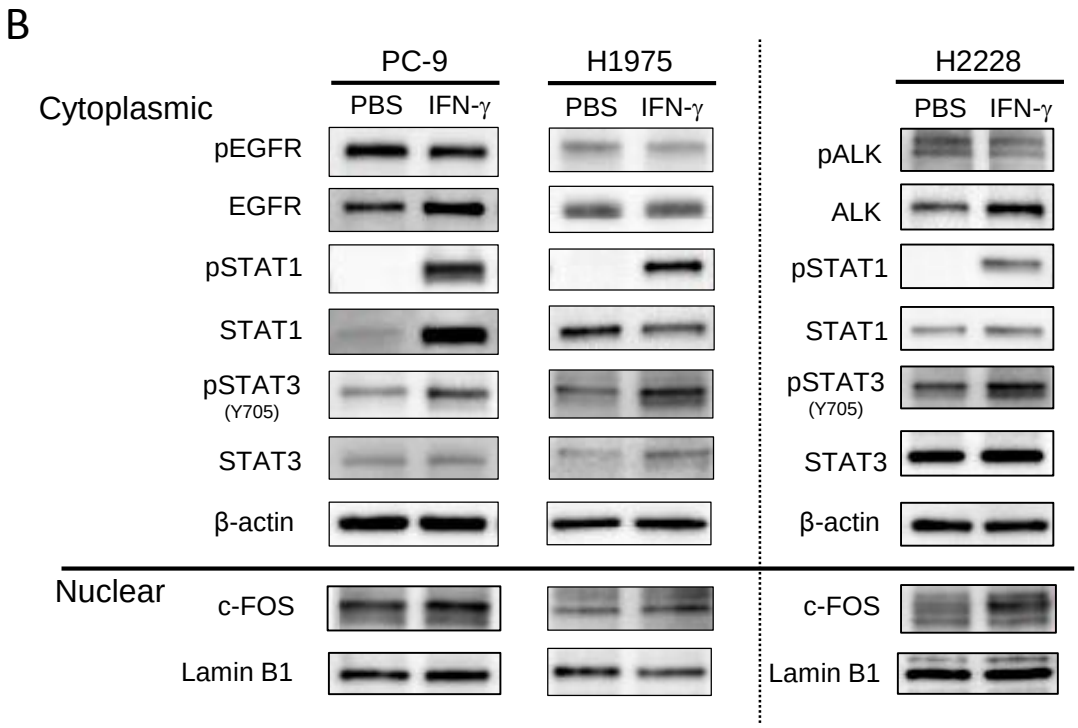
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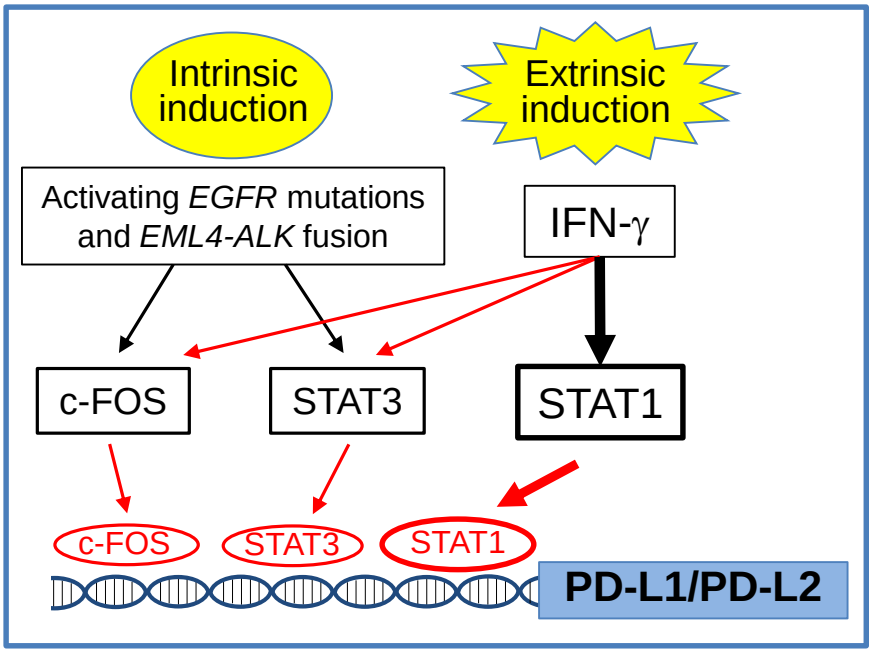
Lamin B1

Figure 6.


Intrinsic induction
Extrinsic induction
Activating *EGFR* mutations and *EML4-ALK* fusion
IFN-γ
c-FOS
STAT3
STAT1
c-FOS
STAT3
STAT1
PD-L1/PD-L2

Supplementary Fig. 1

Supplementary Fig. 2

Supplementary Fig. 3

Supplementary Fig. 4

Supplementary Fig. 5

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Supplementary Fig. 6

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Kubo

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Dr. Luis M. Montuenga
Associate Editor
Journal of Thoracic Oncology

Re: JTO-D-17-01565

Dear Dr. Montuenga:

Thank you for your email of 21 February informing us that our manuscript has been found acceptable for publication pending minor revision. We thank the reviewers for insightful comments, which we have now addressed both in the revised paper and in the attached point-by-point responses. We hereby submit the revised version of our manuscript, which we hope is now acceptable for publication in *Journal of Thoracic Oncology*.

We thank you again for your consideration.

Sincerely,

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Response to Reviewer #1

We thank the reviewer for helpful comments, which we feel have helped us to improve our manuscript. Our responses to the points raised are as follows:

1. As suggested by the reviewer, we examined the Cancer Cell Line Encyclopedia (CCLE) database to investigate PD-L2 gene expression in more cell lines. Expression of the PD-L2 gene in cell lines positive for *EGFR* mutations or *EML4-ALK*, including two *EGFR*-mutant cell lines not analyzed in our study, was consistent with our data (new Supplementary Fig. 1C). Analysis of the CCLE database also revealed that some cell lines positive for *KRAS* mutations or wild type for known driver oncogenes showed a high level of PD-L2 gene expression. We have now addressed these data in the Results section of the revised manuscript (page 8, lines 8–14).

2. As requested, we have now included traces for the IgG isotype controls in the flow cytometry experiments shown in Figures 1, 2, 4, and 5 as well as in the supplementary figures of the revised manuscript.

3. The flow cytometry experiments were performed at least three times, as is now mentioned in the legends of all figures with such data.

4. As requested by the reviewer, we have now performed statistical analysis (*t* test) for the flow cytometry data shown in Figures 3 and 4.

5. To investigate whether STAT3 or c-FOS regulates PD-L2 or PD-L1 expression through interaction with their putative binding sites in the corresponding promoter regions, we performed luciferase reporter assays of promoter activity with wild-type and mutated promoter constructs. Mutation of the putative binding sites for STAT3 or c-FOS significantly attenuated the activity of both PD-L2 and PD-L1 gene promoters, indicating that the expression of PD-L2 and PD-L1 is regulated via STAT3 and c-FOS at the transcriptional level (new Supplementary Fig. 3). We have now addressed these new data in the Results section of the revised manuscript (page 9, lines 34–page 10, lines 4) and modified the Materials and Methods section accordingly (page 6, lines 12–page 7, lines 3).

6. To investigate whether driver oncogenes control PD-L2 expression through the activation of STAT3 or c-FOS, we examined the effects of EGFR-TKI or ALK-TKI treatment on the activation of STAT3 or c-FOS in PC-9 and H2228 cells. Treatment of these cells with the corresponding TKI inhibited phosphorylation of STAT3 and the nuclear abundance of c-FOS in both cells (new Supplementary Fig. 4A). In BEAS-2B cells stably expressing the EML4-ALK fusion protein, forced expression of EML4-ALK increased the level of STAT3 phosphorylation and the nuclear abundance of c-FOS (new Supplementary Fig. 4B). We further silenced STAT3 or c-FOS expression in these cells by transfection with specific siRNAs, finding that the up-regulation of PD-L2 expression by activated EML4-ALK signaling was attenuated by knockdown of STAT3 or c-FOS (new Supplementary Fig. 4C). We have now addressed these new data in the Results section of the revised manuscript (page 10, line 4–16).

Response to Reviewer #2

We thank the reviewer for the helpful comments on our manuscript. Our specific responses to the points raised are as follows:

1. We examined five *EGFR*-mutated cell lines for PD-L2 expression in our study. HCC827, PC-9, and H1650 cell lines harbor an activating in-frame deletion [del(E746–A750)] in exon 19 of *EGFR*; 11-18 harbors an activating point mutation (L858R) in exon 21; and H1975 harbors the L858R point mutation in exon 21 as well as a secondary mutation (T790M) in exon 20. We have now included this information in the Results section of the revised manuscript (page 7, lines 32–page 8, lines 1). We also examined the Cancer Cell Line Encyclopedia (CCLE) database to investigate PD-L2 gene expression in more cell lines. Expression of the PD-L2 gene in cell lines positive for *EGFR* mutations or *EML4-ALK*, including two *EGFR*-mutant cell lines not analyzed in our study, was consistent with our data (new Supplementary Fig. 1C). Analysis of the CCLE database also revealed that some cell lines positive for *KRAS* mutations or wild type for known driver oncogenes showed a high level of PD-L2 gene expression. We have now addressed these data in the Results section of the revised manuscript (page 8, line 8–14).

2. According to the reviewer's suggestion, we have now included the data obtained with each second siRNA in the revised manuscript (new Supplementary Figs. 2, 5, and 6).

3. The flow cytometry experiments were performed at least three times, with this information now being included in the corresponding figure legends of the revised manuscript. We have now also performed statistical analysis for the flow cytometry data shown in Figures 3 and 4.

4. In response to the reviewer's comment, we performed additional experiments with H2228 cells, which are positive for the *EML4-ALK* fusion gene. The expression of PD-L2 induced by IFN-γ was also attenuated by knockdown of STAT1 in these cells (new Figure 5A). We also now show that IFN-γ induced the phosphorylation of STAT3 and the nuclear translocation of c-FOS, but not the phosphorylation of EGFR or ALK, in H1975 and H2228 cells (new Figure 5B), implicating both STAT3 and c-FOS as participants in IFN-γ signaling in oncogene-driven NSCLC cells. We have now addressed these new data in the Results section of the revised manuscript (page 10, lines 30 and page 11, lines 5–9).

5. To investigate whether STAT3 or c-FOS regulates PD-L2 or PD-L1 expression through interaction with their putative binding sites in the corresponding promoter regions, we performed luciferase reporter assays of promoter activity with wild-type and mutated promoter constructs. Mutation of the putative binding sites for STAT3 or c-FOS significantly attenuated the activity of both PD-L2 and PD-L1 gene promoters, indicating that the expression of PD-L2 and PD-L1 is regulated via STAT3 and c-FOS at the transcriptional level (new Supplementary Fig. 3). We have now addressed these new data in the Results section of the revised manuscript (page 9, lines 34–page 10, lines 4) and modified the Materials and Methods section accordingly (page 6, lines 12–page 7, lines 3).