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Cytidine Deaminase APOBEC3A Regulates PD-L1 Expression in Cancer Cells in a JNK/c-JUN-dependent Manner

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Abstract

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DATA AND MATERIALS AVAILABILITY

All transgenic constructs and transgenic cell lines are available upon request. Please contact the corresponding authors with requests.

Programmed death-ligand 1 (PD-L1) promotes tumor immune evasion by engaging the PD-1 receptor and inhibiting T-cell activity. While the regulation of PD-L1 expression is not fully understood, its expression is associated with tumor mutational burden and response to immune checkpoint therapy. Here, we report that Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A (APOBEC3A) is an important regulator of *PD-L1* expression. Using an APOBEC3A inducible expression system as well as siRNA against endogenous APOBECA, we found that APOBEC3A regulates PD-L1 mRNA and protein levels as well as PD-L1 cell surface expression in cancer. Mechanistically, APOBEC3A-induced PD-L1 expression was dependent on APOBEC3A catalytic activity as catalytically dead APOBEC3A mutants (E72A) failed to induce PD-L1 expression. Furthermore, APOBEC3A-induced PD-L1 expression was dependent on replication-associated DNA damage and JNK/c-JUN signaling but not interferon signaling. In addition, we confirmed the relevance of these finding in patient tumors as APOBEC3A expression and mutational signature correlated with *PD-L1* expression in multiple patient cancer types. These data provide a novel link between APOBECA, its DNA mutagenic activity and PD-L1-mediated anti-tumoral immunity. This work nominates APOBEC3A as a mechanism of immune evasion and a potential biomarker for the therapeutic efficacy of immune checkpoint blockade.

Keywords

Cancer; APOBEC3A; PD-L1; c-JUN; Immune Checkpoint Blockade; Interferon

INTRODUCTION

Immune checkpoints including programmed cell-death 1 ligand (PD-L1, CD274 or B7-H1) inhibit T-cell function and contribute to cancer evasion of immune surveillance (1,2). Pharmacologic targeting of immune checkpoints with either PD-L1 or PD-1 blocking antibodies are now FDA approved for the treatment of melanoma, non-small cell lung cancer (NSCLC), bladder cancer, breast cancer, and many other cancer types (3). Expression of PD-L1 on either tumor cells or tumor-infiltrating immune cells has been described as a relevant, albeit imperfect, predictive biomarker for the response to anti-PD-1 and/or anti-PD-L1 therapies (4,5). Therefore, a better understanding of the processes that regulate PD-L1 expression is critical for identification of the tumors that are most likely to respond to immunotherapy (6).

Recent studies have established a link between the genomic alterations in cancer cells and the response to checkpoint blockade in various tumor types (7–10). Tumor mutational burden correlates with response to immune checkpoint blockade (11). Furthermore, increased frequency and quality of tumor neoantigens also correlates with response to immune checkpoint blockade (12). Disruptions in DNA damage repair pathways including mismatch repair deficiency are thought to increase the tumor neoantigen burden and thus increase susceptibility to immune checkpoint blockade by promoting adaptive tumor immune responses which in turn are counterbalanced by the activation of immune inhibitory mechanisms such as expression of PD-L1. In addition to mutational burden, cytoplasmic DNA (single- or double-stranded) and/or micronuclei occurring as a result of DNA damage or inhibition of the DNA damage response enhance tumoral PD-L1 expression

and sensitivity to immune checkpoint blockade by stimulating tumor cell type I interferon (IFN) innate immune responses (13–15). Taken together, these data indicate that diverse types of DNA damage acting through both adaptive and innate mechanisms to induce PD-L1 expression.

DNA editing enzymes, including the APOBEC family, can contribute to DNA damage. The human genome contains 11 *APOBEC* genes, which forms a superfamily of cytidine deaminases which convert the cytidines of DNA and RNA to uracil and play crucial role in innate immunity and viral restriction (16). The *APOBEC3* gene cluster at 22q13 encodes all 7 APOBEC3 deaminases (APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H). Physiologically, APOBEC family members are IFN-stimulated genes upregulated during coordinated immune responses to viral infection. APOBECs cause degradation and mutation of retroviruses by converting cytosine-to-uracil in single-stranded DNA ultimately resulting in cytosine-to-thymine mutations (17–19). Pathophysiologically, APOBEC3 enzymes, especially APOBEC3A and APOBEC3B, are implicated as a major source of mutations in cancers (17,20–22). Although the precise mechanisms of APOBEC overexpression in cancer are unclear, NFκB and P53 pathways have been implicated as well as aberrant, sustained APOBEC expression following viral infection (23). Excessive activation of APOBEC3 family members in cancer leads to somatic hypermutation also known as kataegis which contributes to tumor mutational burden and genomic instability (17,24). Correlative studies have identified that APOBEC3A expression is associated with PD-L1 expression (25,26). The mechanisms connecting APOBEC3A and PD-L1, however, are unclear although coordinated IFN-mediated expression and tumor mutational burden have been suggested (26). A causative role of cytosine deamination by APOBEC3A leading to increased enhanced PD-L1 expression has not been established. Furthermore, the influence APOBEC3A cytosine deamination on tumor intrinsic signaling pathways leading to PD-L1 expression is not established.

In this study, we investigated the signaling pathways regulating PD-L1 mRNA, protein, and cell surface expression in response to APOBEC3A. We demonstrate for the first time that APOBEC3A regulates PD-L1 expression through its deaminase activity. Furthermore, our data show that APOBEC3A-induced PD-L1 expression is independent of IFN signaling, but dependent on replication-associated DNA damage and JNK/c-JUN signaling. This novel molecular mechanism of regulating PD-L1 expression in cancer cells may be useful in the identification of new therapeutic targets or biomarkers that lead to enhanced efficacy of PD1/PD-L1-targeted therapies.

MATERIALS AND METHODS

Cell lines

U2OS (ATCC Cat# HTB-96, RRID:CVCL_0042), SKBR3 (ATCC Cat# HTB-30, RRID:CVCL_0033), and NCI-H2347 (ATCC Cat# CRL-5942, RRID:CVCL_1550) cells were purchased from the American Tissue Culture Collection (ATCC) in 2018. U2OS cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 100 U/mL of penicillin and 100 mg/mL of streptomycin, and supplemented with 10% fetal bovine serum (FBS). SKBR3 cells were maintained in McCoy's 5A supplemented

with 10% FBS and 1% penicillin/streptomycin. NCI-H2347 cells were maintained in RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin. Inducible U2OS-HA-APOBEC3A cells constructed with a dual vector system (pLKO-EGFP-TetR and pLKO-TetO-A3A) were generously provided by Matthew Weitzman (University of Pennsylvania) and Abby Green (Washington University School of Medicine) (27). U2OS cell lines expressing FLAG-APOBEC3A (WT and E72A) under a doxycycline-inducible promoter (pInducer20) were developed in Lee Zou's laboratory (Harvard Medical School) as previously described (28). For APOBEC3A expression, cells were incubated with doxycycline (1 μg/mL) every other day to achieve continuous induction. Cells were tested for mycoplasma through a luciferase linked assay (Lonza). Extended cell culture was minimized; experiments were completed in under six months.

Reagents

Doxycycline was purchased from Sigma-Aldrich. Recombinant active human IFNγ and IFNβ were purchased from InvivoGen. Small interference RNAs targeting human c-JUN were purchased from Dharmacon. Human IFN receptor neutralization antibody anti-IFNAR2 was purchased from PDI Assay Science. The JAK kinase inhibitor Ruxolitinib was purchased from STEMCELL Technologies. The MEK and JNK inhibitors trametinib and SP600125, respectively, were purchased from Cell Signaling Technology. AZD6738 (AstraZeneca) and roscovitine (Cell Signaling) were each dissolved in DMSO and stored in aliquots at −20°C. Human APOBEC3A and scramble siRNAs were purchased from Ambion and transfected into cells by using Oligofectamine according to the manufacturer's protocol. APOBEC3A knockdown was maximal at 3 days following transfection, at which point cells were used for additional experiments.

Quantitative RT-PCR

Total RNA from the U2OS cells was obtained by using the RNeasy Mini Kit (Qiagen). Approximately 0.1 μg of total RNA was used for reverse transcription. Reverse transcription was performed by a two-step protocol based on the manufacture's instruction (Invitrogen). *PD-L1* and *GAPDH* mRNA copy numbers were determined by real-time quantitative PCR using a DNA Master SYBR Green I kit. The quantitative RT-PCR primers are: PD-L1 forward: 5'-GGAGATTAGATCCTGAGGAAAACCA-3'; PD-L1 reverse: 5'-AACGGAAGATGAATGTCAGTGCTA-3', GAPDH forward: 5'-CTCCTCTGACTTCAACAGCGA-3'; GAPDH reverse: 5'-CCAAATTCGTTGTCATACCAGGA-3'.

Human phospho-kinase antibody array

The Human Phospho-Kinase Array Kit (#ARY003B; R&D Systems) was used according to the manufacturer's protocol. Briefly, U2OS cells were lysed and incubated with the array membranes followed by washing and incubation with a biotinylated antibody cocktail. The levels of individual phospho-kinase proteins were assessed by streptavidin-horseradish peroxidase, followed by chemiluminescence detection. Image J (RRID:SCR_003070) software was used to quantify the density of each phospho-kinase protein which was then normalized to the average of an internal control.

Chromatin immunoprecipitation

U2OS cells (HA-A3A) were crosslinked with 1% formaldehyde and then sonicated on ice to shear the DNA to lengths between 200 and 1000 base pairs. Soluble chromatin fragments were incubated at 4°C overnight with 5 μg of Acetyl-Histone H3 (Lys9/Lys14) antibody (Cell Signaling, #9677). Normal rabbit IgG was used as a negative control for validating the ChIP assay. Isolated DNA fragments were purified, and qPCR was performed using 2 μl of DNA in technical triplicate. The primers covering the upstream of the human *PD-L1* gene were from − 455 bp to − 356 bp: Forward 5'-ATG GGT CTG CTG CTG ACT TT-3' and Reverse 5'-GGC GTC CCC CTT TCT GAT AA-3'.

Western blotting

Western blotting was performed as previously described (15). Cells were harvested and lysed in SDS sample buffer containing protease and phosphatase inhibitors. After sonicating and boiling at 95°C for 10 minutes, samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). The antibodies used in this study were: HA-Tag (Cell Signaling Technology; #3724, RRID:AB_1549585), FLAG M2 monoclonal antibody (Sigma-Aldrich Cat# P2983, RRID:AB_439685), PD-L1 (Cell Signaling Technology; E1L3N, #13684, RRID:AB_2687655), Phospho-STAT1 (Tyr701; Cell Signaling Technology; 58D6, #9167), STAT1 (Cell Signaling Technology; #9172, RRID:AB_2198300), Phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204; Cell Signaling Technology; #4370, RRID:AB_2315112), p44/42 MAPK (ERK1/2; Cell Signaling Technology; #4695, RRID:AB_390779), c-JUN (Cell Signaling Technology; #9165, RRID:AB_2130165), Phospho-c-JUN (Ser73; Cell Signaling Technology; #9164, RRID:AB_330892), β-Actin (C4; Santa Cruz, sc-47778, RRID:AB_626632), GAPDH (Cell Signaling Technology; #5174, RRID:AB_10622025).

Flow cytometry

U2OS cells were treated with doxycycline for 1–5 days and then harvested by trypsinization followed by washing with ice cold PBS and staining R-phycoerythrin conjugated anti-Human PD-L1 antibody (BioLegend Cat# 329706, RRID:AB_940368) for 30 minutes on ice. Flow cytometry analysis was performed on a BD LSR or Fortessa flow cytometer. Data are reported as the change in the MFI (mean fluorescence intensity) of PD-L1–isotype compared to isotype control. Alternatively, fixed cells were stained overnight with an anti-γH2AX antibody (JBW301, EMD Millipore, Cat# 05–636, RRID:AB_309864) followed by incubation with a FITC-conjugated anti-mouse secondary antibody (Sigma) as previously described (29). Samples were then stained with propidium iodide to assess total DNA content and analyzed on a FACScan flow cytometer (BD Biosciences) with FlowJo software (Tree Star, RRID:SCR_008520).

TCGA analysis

Completed data sets, which included expressions of genes (RNAseqv2, Level_3, RSEM_genes_normalized) and APOBEC mutational signatures (Mutation_APOBEC, Level_4) were downloaded from the TCGA using the Broad Institute Genome Data Analysis Center (GDAC) Firehose portal through Firebrowse (http://gdac.broadinstitute.org). The

mutational signature analysis was done by using latest R packages “SignatureAnalyzer” as previously described (20,30). c-Jun gene signatures were used as previously described (31).

Statistical and patient survival analyses

GraphPad Prism6 software (GraphPad Prism, RRID:SCR_002798) and R were used for statistical analysis. The two-tailed paired Student t test or one-way ANOVA were used to verify whether the difference between groups was statistically significant. P values < 0.05 were considered to be statistically significant.

RESULTS

APOBEC3A regulates PD-L1 expression

Genomically unstable cancers such as those with microsatellite instability (MSI) have increased mutational and neoantigen burden that is associated with increased expression of PD-L1 and sensitivity to anti-PD-1/PD-L1 therapies (32). Given that the most potent human APOBEC3 deaminase, APOBEC3A, is able to induce DNA damage and genomic instability, we sought to determine whether APOBEC3A would cause PD-L1 expression. We therefore examined PD-L1 levels in U2OS osteosarcoma cells stably expressing APOBEC3A under the control of an inducible promoter. We found that induction of APOBEC3A protein in response to doxycycline treatment was followed by increased expression of PD-L1 protein (days 3 and 5) (Figure 1A and 1B). The possibility that PD-L1 upregulation was induced by doxycycline itself rather than APOBEC3A expression, was excluded by the finding that PD-L1 expression was not induced by doxycycline in U2OS parental cells (Figure S1A). PD-L1 is a transmembrane protein in tumor which binds to PD-1 on $CD8^+$ T cells to promote immune evasion. Therefore, we next investigated the effect of APOBEC3A on PD-L1 expression on the cell surface by flow cytometry. Consistent with the increase in total cellular PD-L1 protein levels, we found a significant increase in cell surface PD-L1 expression in response to APOBEC3A induction as assessed by flow cytometry mean fluorescence intensity (MFI) (Figure 1C and 1D). This increase was comparable to that by IFNγ, an established regulator of PD-L1 expression (Figure S1B) (33). To determine whether the observed increase in PD-L1 protein in response to APOBEC3A induction was attributable to increased *PD-L1* transcript levels, mRNA was isolated at different time points and quantified by real-time PCR. Notably, *PD-L1* (*CD274*) mRNA was upregulated at days 1, 3, and 5 after doxycycline treatment, indicating that APOBEC3A-induced PD-L1 expression is regulated at the transcriptional level (Figure 1E). These findings were confirmed in SKOV3 ovarian cancer cells stably expressing a doxycycline inducible APOBEC3A (Figure S1C). To investigate the importance of endogenous APOBEC3A to PD-L1 expression, we assessed the effects of siRNA targeting endogenous APOBEC3A on PD-L1 expression in additional cell lines (34). SKBR3, a human breast cancer model that selectively expresses APOBEC3A but not APOBEC3B, and NCI-H2347 cells, a human lung adenocarcinoma model with high levels of endogenous APOBEC3A expression were selected for these studies (34,35). We found in both SKBR3 and NCI-H2347 cells that siAPOBEC3A caused a reduction in both PD-L1 protein and mRNA levels (Figure 1F, 1G, S1D and S1E). Taken together, these findings demonstrate that APOBEC3A induces PD-L1 expression in cancer cells.

APOBEC3A cytidine deaminase activity is required for PD-L1 expression

The cytosine deaminase activity of APOBEC3A generates DNA substitutions, most frequently cytosine-to-thymine, by using ssDNA as a substrate during DNA replication. Therefore, we next investigated whether the deaminase activity of APOBEC3A was required for PD-L1 induction. We began by utilizing an independent, isogenic APOBEC3A model system consisting of doxycycline-inducible wild type or catalytically dead mutant (E72A) FLAG-APOBEC3A which had previously been shown to express low and likely more physiological relevant levels of APOBEC3A (Figure 2A) (27,28). Consistent with our initial observation that wild type APOBEC3A overexpression induced PD-L1 expression (Figure 1), APOBEC3A expression at more physiologically relevant levels also resulted in an increase in PD-L1 protein levels at days 1, 3, and 5 after doxycycline treatment (Figure 2B and 2C). Next we compared the effects of wild type and mutant APOBEC3A on PD-L1 protein levels and found in contrast to wild type APOBEC3A, that mutant APOBEC3A did not cause a significant increase in PD-L1 protein expression (Figure 2D and S2A). In addition, no elevation of PD-L1 on the cell surface was found in APOBEC3A (E72A) expressing cells by flow cytometry (Figure 2E and S2B). Correspondingly, at the transcriptional level APOBEC3A (E72A) expression failed to cause the elevation of *PD-L1* mRNA levels seen in wild type cells (Figure 2F). Taken together, these data demonstrate that APOBEC3A cytosine deaminase activity is required for the induction of PD-L1 expression.

APOBEC3A-mediated PD-L1 expression is independent of the IFN/JAK/STAT pathway

PD-L1 expression is mainly regulated by IFN signaling (33). Our finding that PD-L1 expression was elevated in response to APOBEC3A induction prompted us to evaluate the involvement of IFN/JAK/STAT signaling in this process. Using phosphorylated STAT1 (pSTAT1 Y701) as a surrogate for IFN pathway activation, we found no detectable STAT1 phosphorylation in response to APOBEC3A expression at any time point (Figure 3A). In contrast, IFNγ treatment strongly induced pSTAT1 and PD-L1 expression levels (Figure 3A and 3B). Furthermore, ruxolitinib, a potent JAK1/2 inhibitor, failed to block APOBEC3A-induced PD-L1 expression, whereas IFNγ-mediated stimulation of both STAT1 phosphorylation and PD-L1 expression were inhibited by ruxolitinib (Figure 3A and 3B). Further supporting an IFN-independent mechanism, ruxolitinib failed to inhibit cell surface PD-L1 expression in response to APOBEC3A induction (Figure 3C and S3A). IFNα and IFNβ are endogenously expressed type I IFNs in cancer cells that can act in an autocrine fashion thereby activating JAK/STAT signaling and PD-L1 expression (33). Therefore, we assessed the requirement for IFNβ signaling in APOBEC3A -mediated PD-L1 expression. As shown in Figure 3D and 3E, although IFNβ blocking antibody efficiently inhibited STAT1 phosphorylation and PD-L1 expression in U2OS cells treated with IFNβ, it had minimal effect on PD-L1 protein levels in response to APOBEC3A expression. Taken together, our findings demonstrate that enhanced expression of PD-L1 in response to APOBEC3A induction occurs independently of the interferon signaling.

APOBEC3A-mediated PD-L1 expression is associated with replication-mediated DNA damage

Given the lack of involvement of interferon signaling in APOBEC3A-mediated PD-L1 expression as well as the lack of convincing evidence that MEK/ERK1/2 signaling was involved specifically in APOBECA-mediated PD-L1 expression (in contrast to regulation of baseline PD-L1 expression; Figure S3B and S3C), we next investigated the role of DNA damage to PD-L1 expression in response to APOBEC3A. As APOBEC3A causes replication-associated DNA damage (27,28), we hypothesized that replication-associated DNA damage was a mechanism of PD-L1 expression in response to APOBEC3A. Inhibition of DNA replication with the CDK1/2 inhibitor roscovitine did diminish APOBEC3A-induced DNA damage as measured by γH2AX positivity in U2OS (HA-A3A) cells (Figure 4A and S4). This reduction in replication-associated DNA damage coincided with a reduction in both APOBEC3A-induced PD-L1 protein levels and cell surface expression (Figures 4B and 4C). As inhibition of ATR potentiates APOBEC3A-mediated DNA replication stress and damage (28), we next assessed the effects of the ATR inhibitor AZD6738 on APOBEC3A-mediated PD-L1 expression. We found that AZD6738 increased APOBEC3A-induced DNA damage marked by and an increase in γH2AX positive cells (Figure 4D and S4). Importantly, this increase in APOBEC3A-mediated DNA damage in response to AZD6738 was accompanied by an increase in PD-L1 protein levels as well as PD-L1 cell surface expression (Figures 4E and 4F). Taken together, these data support our hypothesis that APOBEC3A-induced PD-L1 expression is mediated by replication-associated DNA damage.

JNK/c-JUN signaling is required for APOBEC3A-mediated PD-L1 expression

To further explore the mechanisms connecting APOBEC3A-induced DNA damage and PD-L1 expression, we performed a phospho-kinase screen to identify proteins that are phosphorylated in response to APOBEC3A expression. We observed Serine (Ser)63-phosphorylation of c-JUN, representing active c-JUN (36), was the most altered (3.6-fold increase) of the kinase substrate phospho-proteins included in this screen following APOBEC3A induction (Figure 5A). Based on the identification of phospho-c-JUN in the screening array, we next investigated whether APOBEC3A-induced PD-L1 expression was dependent on c-JUN. To this end, we depleted c-JUN in doxycycline-inducible HA-APOBEC3A cells. We found under control siRNA conditions that phospho-c-JUN protein levels were increased, an effect that was paralleled by increased PD-L1 expression in response to APOBEC3A induction (Figure 5B). More importantly, depletion of c-JUN significantly inhibited PD-L1 expression in response to APOBEC3A induction (Figure 5B and 5C). c-JUN transcriptional activity is regulated by c-JUN N-terminal kinase (JNK)-mediated phosphorylation of the c-JUN NH2-terminal transactivation domain, including Ser63. Therefore, we next evaluated the effect of JNK inhibition on APOBEC3A-mediated PD-L1 expression. Treatment with the JNK inhibitor SP600125 not only caused a reduction of c-JUN phosphorylation, but also a significant decrease of PD-L1 protein levels in response to APOBEC3A induction (Figure 5D and 5E). Furthermore, consistent with the hypothesis that the JNK/c-JUN modulates PD-L1 expression in response to APOBEC3A, roscovitine diminished and AZD6738 augmented phospho-c-JUN protein levels (Figure S5A). Consistent with this observation, increased c-JUN phosphorylation and PD-L1 protein

expression upon APOBEC3A induction was also blocked by SP600125 in SKOV3 cells stably expressing doxycycline-inducible APOBEC3A (Figure S5B). Furthermore, chromatin immunoprecipitation assays revealed increased *PD-L1* promoter activity in response to APOBEC3A induction that was inhibited by JNK inhibition (Figure S5C). We confirmed that knockdown of endogenous APOBEC3A in NCI-H2347 and SKBR3 diminished c-JUN phosphorylation (Figure S5D). Overall, these results strongly support our conclusion that APOBEC3A-induced PD-L1 expression is dependent upon the JNK/c-JUN signaling pathway.

APOBEC3 expression and activity positively correlates with *PD-L1* expression levels in patient tumors

The finding that APOBEC3A increases *PD-L1* mRNA and protein levels led us to investigate a potential correlation between *APOBEC3A* and *PD-L1* expression in human cancer. Analysis of whole genome alterations have identified that APOBEC mutational signatures occur predominantly in non-small cell lung, breast, bladder, cervical, and head and neck cancers (37,38). Furthermore, APOBEC3A is the predominant isoform contributing to APOBEC-associated mutations in these cancer types (34,35,39). Analysis of the cancer genome atlas (TCGA) lung adenocarcinoma (LUAD), bladder (BLCA), breast (BRCA), cervical (CERC), and head and neck (HNSC) cohorts revealed a positive correlation between *APOBEC3A* expression and *PD-L1* (*CD274*) expression, consistent with previous results (Figure 6A)(25,26). We observed that APOBEC3B expression was also correlated with PD-L1 expression to a lesser extent (Figure S6A). To understand if APOBEC3A catalytic activity correlated with *PD-L1* expression, we correlated the presence of a previously validated APOBEC mutational signature with *PD-L1* expression (37). Interestingly, again, in TCGA lung, bladder, breast, cervical, and head and neck cohorts, we observed higher expression of *PD-L1* in samples with an APOBEC mutational signature (Figure 6B). These data support the link between PD-L1 and APOBEC expression and catalytic activity. Our data identified that JNK/c-JUN is required for APOBEC3A-mediated PD-L1 expression (Figure 5). We confirmed that c-JUN activity correlated with PD-L1 expression in in TCGA lung, bladder, breast, cervical, and head and neck cohorts (Figure S6B). Taken together the results of this study support a model in which APOBEC3A positively regulates PD-L1 expression in a JNK/c-JUN-dependent manner, a finding which is likely applicable to multiple solid tumor types (Figure 7).

DISCUSSION

In this study, we have identified that APOBEC3A is a potent inducer of PD-L1 expression in cancer cells as well as cancer patients. Further, we identified that APOBEC3A-mediated PD-L1 expression is dependent on its cytidine deaminase enzymatic activity. While type I IFNs are generally considered the most prominent soluble inflammatory signaling to promote PD-L1 expression, we found that APOBEC3A-mediated induction of PD-L1 did not occur through interferon signaling but rather through DNA damage and replication stress which activates the JNK/c-JUN pathway. Given that the PD-L1/PD-1 axis is a critical mechanism used by tumor cells to evade immune surveillance, the findings of our study are important in that they reveal a novel tumor-cell intrinsic mechanism which

connects APOBEC3A cytidine deaminase activity and its enzyme-signature mutations with PD-L1 expression. This coordination between APOBEC3A-mediated mutagenesis and PD-L1 expression is likely to simultaneously promote both tumorigenesis as well as an immunosuppressive tumor microenvironment. Importantly, APOBEC3A-mediated PD-L1 expression is both a mechanism of immune evasion as well as targetable vulnerability in tumor cells that can be therapeutically leveraged with antibodies targeting the PD-L1/PD-1 axis.

Consistent with this finding, it has been shown that APOBEC3A correlates with PD-L1 in multiple cancer types (25,26). Furthermore, others have identified that tumoral PD-L1 overexpression is highly correlated with APOBEC driven localized hyper-mutation (i.e., kataegis), as well as with overexpression of APOBEC3 members (26). Base excision repair (BER) is the primary response to G:U mismatches, and depletion of BER enzymes, such as DNA glycosylase, also promotes PD-L1 expression (40). Consistent with this, oxidative stress-induced reactive oxygen species (ROS), which also lead to base damage and SSBs, increase PD-L1 mRNA expression and protein levels (40).

We have identified that APOBEC3A regulates PD-L1 by causing DNA replication stress and damage. Multiple signaling pathways have been implicated in the regulation of PD-L1 levels, including IFN, β-catenin, MEK/ERK and PI3K/AKT(41–43). We did not observe that IFN or MEK/ERK signaling was responsible for APOBEC3A-mediated induction of PD-L1. Rather, we observed that APOBEC3A-mediated PD-L1 expression required JNK/c-JUN. The JNK/c-JUN pathway has been implicated in responses to DNA damage (44). c-JUN activation has also been linked to constitutive PD-L1 expression in BRAF mutant melanoma cells, and targeting of the JNK/c-JUN pathway and or STAT3 inhibits PD-L1 expression (45,46). In addition, the EGFR and PDK1 oncogenic signaling cascade mediates expression of PD-L1 via c-JUN in ovarian cancer and NSCLC (47,48). While it is suggested that PD-L1 and APOBEC3 correlate secondary to coordinated gene transcription following interferon signaling or increased tumor mutational burden (26), our work suggests a novel intracellular link between APOBEC3A and PD-L1 expression in cancer cells.

As APOBEC3A enzyme-signature mutations in tumors may regulate PD-L1 expression through multiple mechanisms in patients, the biological significance of the APOBEC3A/JNK/c-JUN axis on PD-L1 expression needs to be evaluated *in vivo*. The lack of a murine APOBEC3A homolog, however, limits these investigations as only a single APOBEC3 family member, mAPOBEC3, exists in mice which shares ~30% sequence identity with human APOBEC3G [45]. Future studies which establish a murine model with transgenic expression of human APOBEC3A might circumvent this problem.

Although knowledge of the molecular mechanisms that control PD-L1 expression in tumors has greatly advanced in recent years, identification of novel PD-L1 regulators will further improve our ability to predict responses PD-1/PD-L1-targeting therapeutic agents. Our work identifies that APOBEC3A intrinsically promotes PD-L1 expression in cancer cells, which highlights the connection between DNA damage and tumoral innate immunity. While microsatellite instability is now recognized as an indication for anti-PD-L1 therapy regardless of tumor type, it is unknown if other mutational signatures are also predictive

biomarkers of immunotherapy response. Our work suggests that patients with APOBEC3 driven mutational signatures might derive significant benefit from immune checkpoint blockade. Future studies are needed to confirm the relevance of these findings in cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Implications:

APOBEC3A catalytic activity induces replication-associated DNA damage to promote PD-L1 expression implying that APOBEC3A-driven mutagenesis represents both a mechanism of tumor immune evasion and a therapeutically targetable vulnerability in cancer cells.

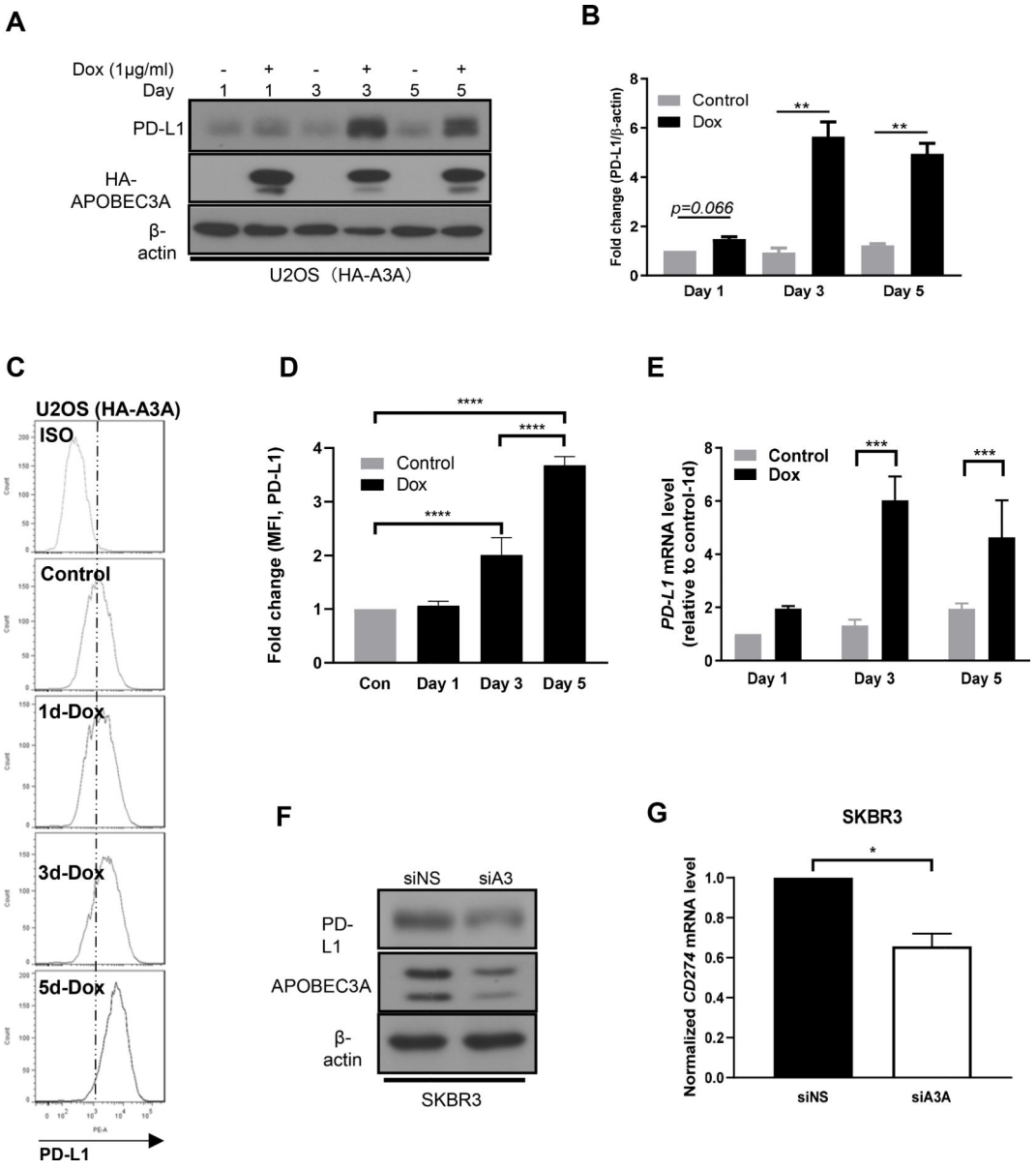

Figure 1. APOBEC3A regulates PD-L1 expression.

A, PD-L1 and HA-tagged APOBEC3A protein levels in doxycycline-inducible HA-APOBEC3A U2OS osteosarcoma cells (U2OS HA-A3A) were analyzed by Western blot at the indicated times following doxycycline (Dox; 1μg/ml) treatment (1, 3, 5 days). **B,** Quantitation of PD-L1 protein levels normalized to β-actin in U2OS (HA-A3A) cells treated with doxycycline as described in A. Data are the mean of 3 independent experiments ± SEM. **C,** Cell surface expression of PD-L1 was measured by flow cytometry in U2OS (HA-A3A) cells at 1, 3 and 5 days after doxycycline treatment. **D,** Quantitation of PD-L1 cell surface expression. Data are the mean ± SEM fold- change in MFI (mean fluorescence intensity) relative to control from 3 independent experiments. **E,** *PD-L1* mRNA expression in U2OS cells was assessed at the indicated time points following treatment with doxycycline (1μg/ml) by real-time qPCR. Data are the mean of 3 independent experiments,

performed in triplicate, mean ± SEM. **F,** PD-L1 protein levels in SKBR3 cells at 3 days post-transfection with siAPOBEC3A. **G,** PD-L1 mRNA levels in SKBR3 cells at 3 days post-transfection with siAPOBEC3A. Statistical significance was determined using Student's two-tailed *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

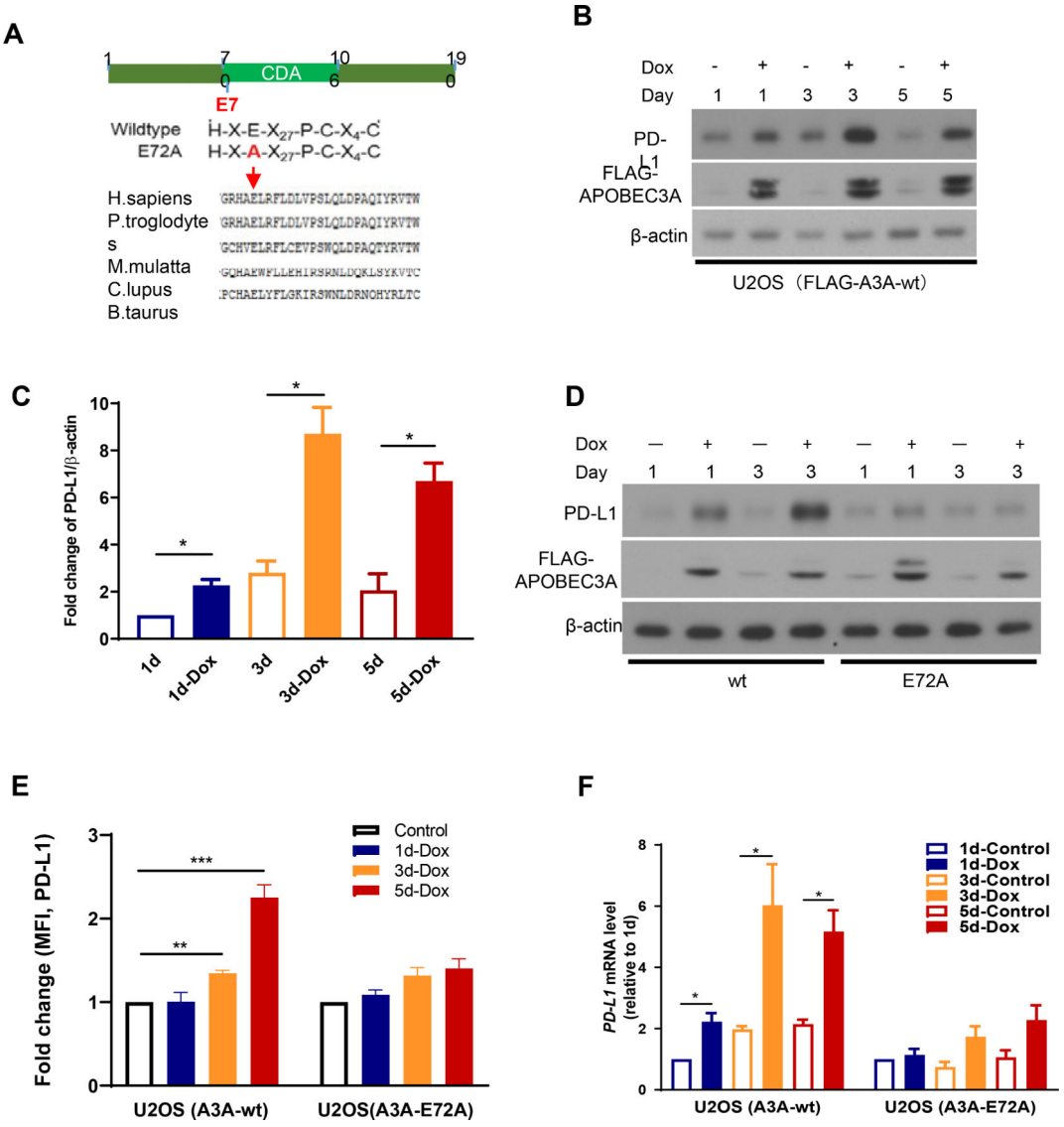

Figure 2. APOBEC3A-induced PD-L1 expression depends on its cytidine deaminase activity.

A, Human APOBEC3A protein domain structure including the conserved E72 site and the E72A mutant in the cytidine deaminase domain (CDA) of APOBEC3A across species. **B,** PD-L1 and FLAG-tagged APOBEC3A protein levels in U2OS (FLAG-A3A) cells were analyzed by Western blot at the indicated times after doxycycline (1μg/ml) treatment. **C,** Quantitation of PD-L1 protein levels normalized to β-actin in U2OS (FLAG-A3A) cells treated as described in A. Data are the mean of 4 independent experiments ± SEM. **D,** PD-L1 and FLAG-APOBEC3A proteins were detected by Western blot in U2OS (A3A-wt) and U2OS (A3A-E72A) cells at 1 and 3 days following doxycycline (1μg/ml) treatment. **E,** Cell-surface expression of PD-L1 in U2OS A3A-wt and A3A-E72A cells were measured at 1, 3 and 5 days after doxycycline (1μg/ml) treatment by flow cytometry. Data are the mean ± SEM of 3 independent experiments. **F,** *PD-L1* mRNA expression in U2OS A3A-wt and A3A-E72A cells was assessed at the indicated time points following treatment with doxycycline (1μg/ml) by real-time qPCR. Data are the mean ± SEM of 3 independent

experiments, performed in triplicate. Statistical significance was determined using Student's two-tailed t-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

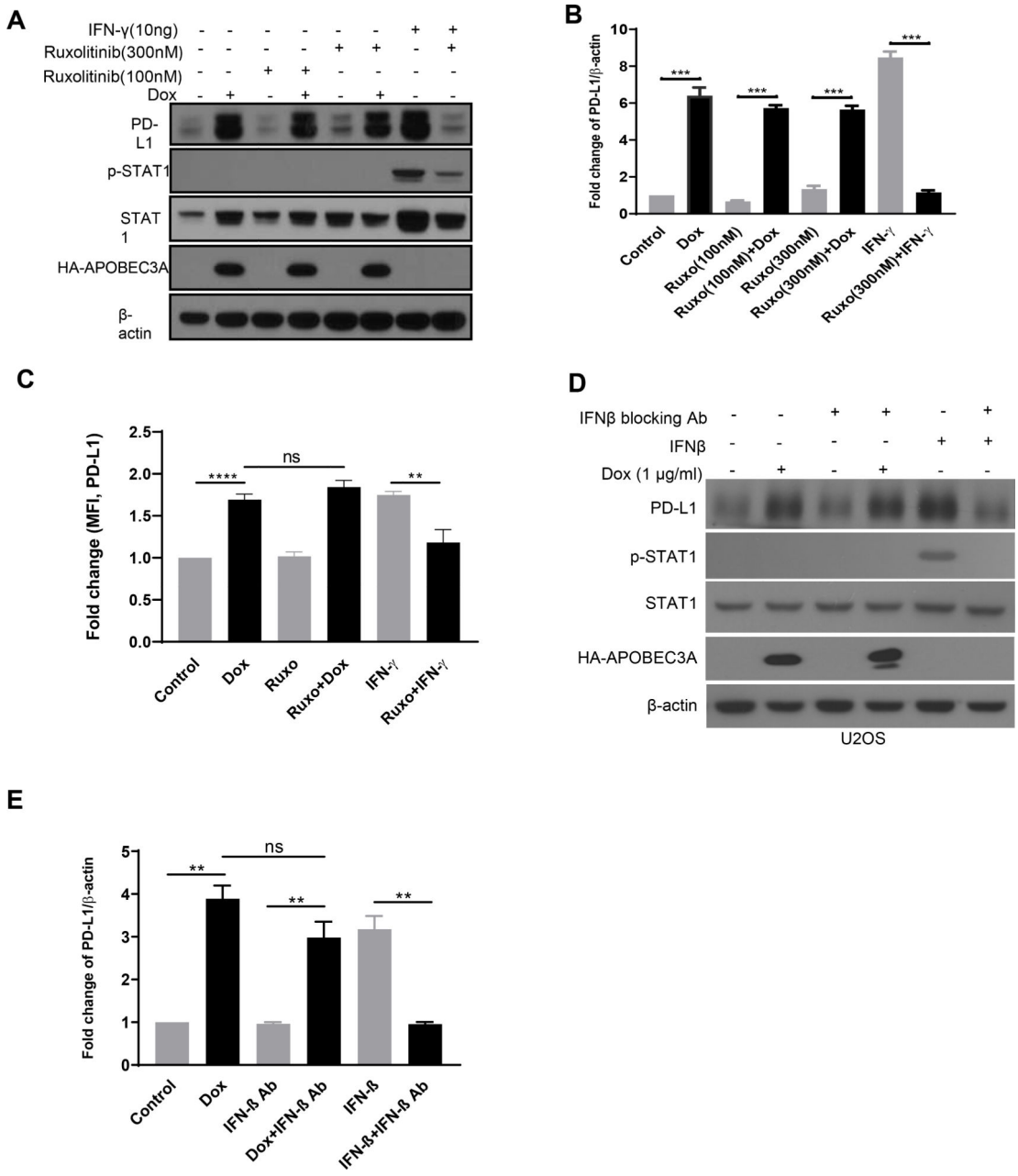

Figure 3: The Type I IFN/JAK/STAT pathway is not required for APOBEC3A-mediated PD-L1 expression.

A, Western blot analysis of PD-L1, pSTAT1, STAT1 in U2OS (HA-A3A) cells at 3 days after treatment with doxycycline (1μg/ml) and the JAK1/2 inhibitor (Ruxo; ruxolitinib; 100nM, 300nM) or IFNγ (10ng/ml; 1 day) with or without ruxolitinib (300nM). **B,** Quantitation of PD-L1 protein levels in U2OS (HA-A3A) treated as described in A. Data are the mean ± SEM of 3 independent experiments. **C,** Cell surface expression of PD-L1 in U2OS (HA-A3A) cells was analyzed by flow cytometry at 3 days after treatment of doxycycline (1μg/ml) or IFNβ (10ng/ml; 1 day) with or without ruxolitinib. Data are the mean ± SEM of 3 independent experiments. **D,** The indicated proteins were detected by

Western blot in U2OS (HA-A3A) cells treated with doxycycline (3 days) or IFNβ (10ng/ml; 1 day) and IFNβ blocking antibody (10ug/ml). **E,** Quantitation of PD-L1 protein levels in U2OS (HA-A3A) treated as described in D. Data are the mean ± SEM of 4 independent experiments. Statistical significance was determined using Student's two-tailed t-test. *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$, ****P<0.0001.

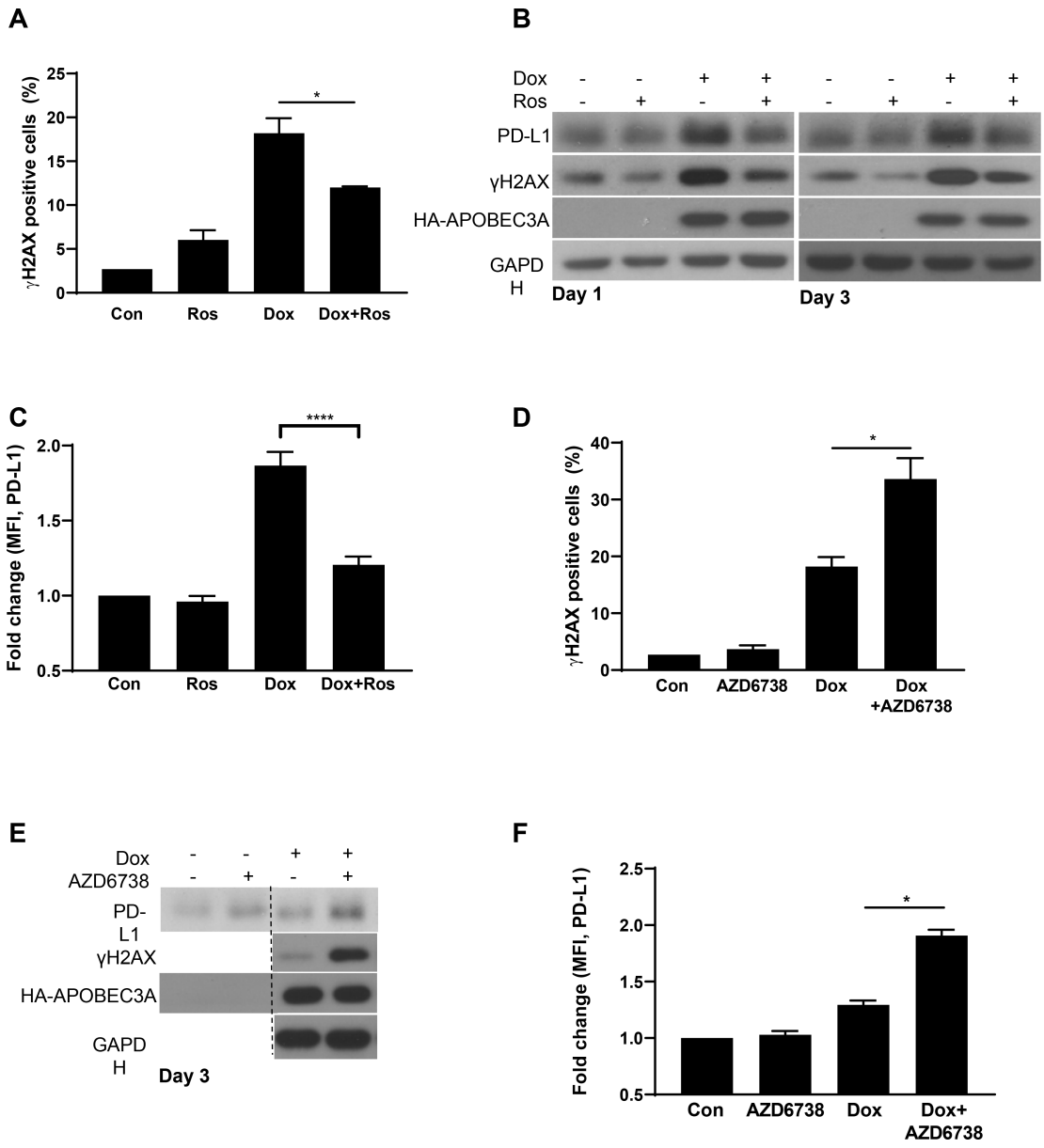

Figure 4. APOBEC3A-mediated PD-L1 expression is dependent on replication-associated DNA damage.

A, U2OS (HA-A3A) cells were treated with doxycycline and roscovitine (Ros, 20μM) for 1 day and then analyzed for γH2AX positivity by flow. Data are the mean ± SD of 2 independent experiments. **B,** Western blot analysis of PD-L1 and γH2AX protein levels in U2OS (HA-A3A) cells treated with doxycycline (1μg/ml) and roscovitine for 1 or 3 days. **C,** Cell surface expression of PD-L1 was assessed by flow cytometry in U2OS (HA-A3A) cells after 3 days of treatment with doxycycline (1μg/ml) and roscovitine. Data are the mean ± SEM of 2 independent experiments performed in technical duplicate. **D,** U2OS (HA-A3A) cells were treated with doxycycline and AZD6738 (300nM) for 1 day and then analyzed for γH2AX positivity by flow. Data are the mean ± SD of 2 independent experiments. **E,** Western blot analysis of PD-L1 and γH2AX protein levels in U2OS (HA-A3A) cells treated

with doxycycline (1μg/ml) and AZD6738 (300nM) for 3 days. Data are representative of 2 independent experiments. **F,** Cell surface expression of PD-L1 was assessed by flow cytometry in U2OS (HA-A3A) cells after 1 day of treatment with doxycycline (1μg/ml) and AZD6738 (300nM). Data are the mean ± SD of 2 independent experiments. Statistical significance was determined using Student's two-tailed *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

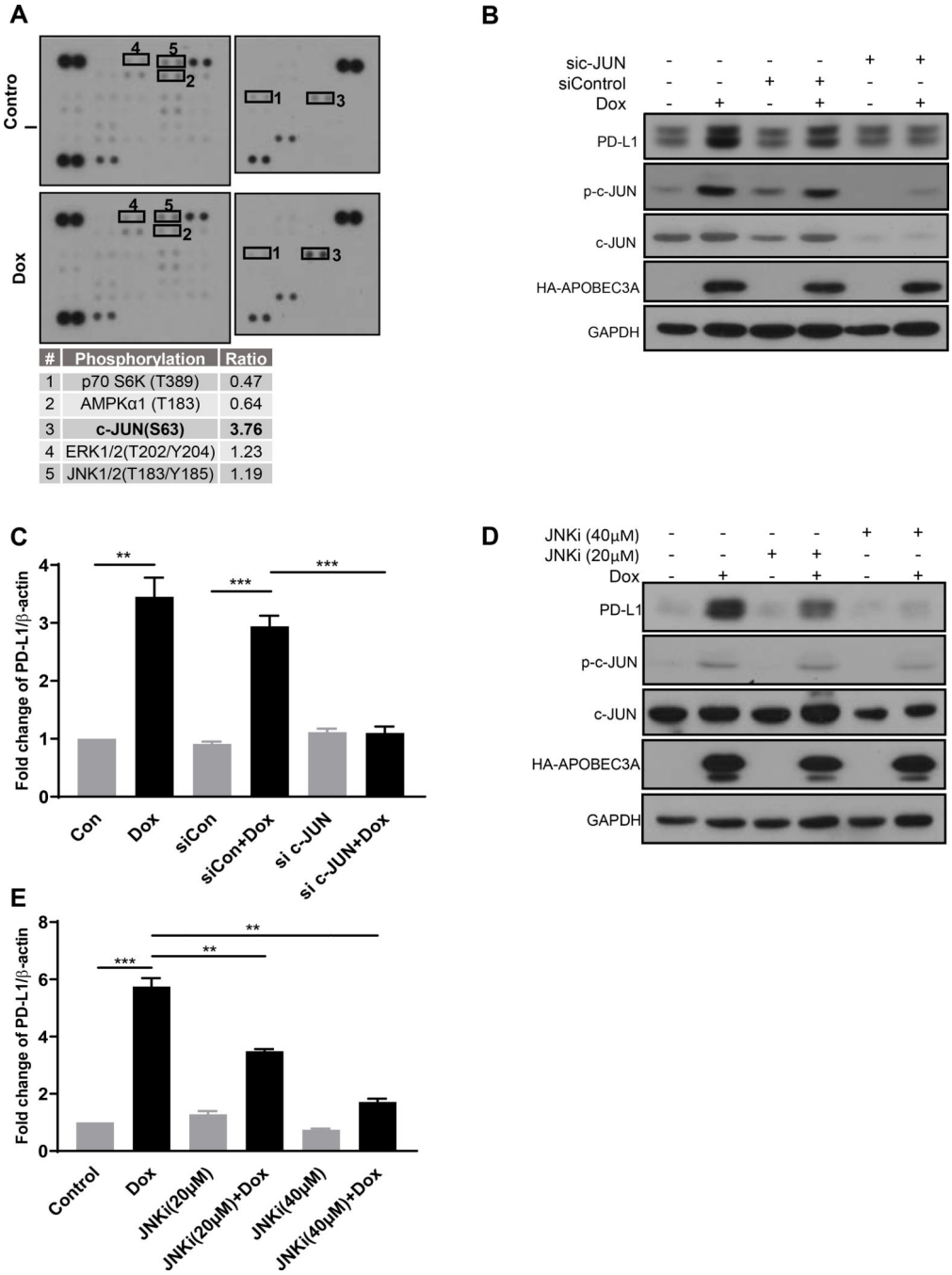

Figure 5. The JNK/c-JUN signaling pathway mediates PD-L1 expression by APOBEC3A.

A, Phospho-kinase arrays were conducted in U2OS (HA-A3A) cells at 3 days after treatment with doxycycline (1μg/ml). The 5 phospho-proteins demonstrating the greatest change in response to APOBEC3A expression are illustrated. **B,** Western blot analysis of PD-L1, p-c-JUN, and c-JUN in U2OS (HA-A3A) cells treated with control or c-JUN siRNAs in the absence or presence of doxycycline. **C,** Quantitation of PD-L1 protein levels in U2OS (HA-A3A) cells treated as described in A. Data are the mean ± SEM of 3 independent experiments. **D,** Western blot analysis of PD-L1, p-c-JUN, and c-JUN in U2OS

(HA-A3A) cells at 3 days following treatment with doxycycline (1μg/ml) and JNK inhibitor (SP600125; 20 and 40μM). **E,** Quantitation of PD-L1 protein levels in U2OS (HA-A3A) cells treated as described in D. Data are the mean ± SEM of 3 independent experiments. Statistical significance was determined using Student's two-tailed *t*-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

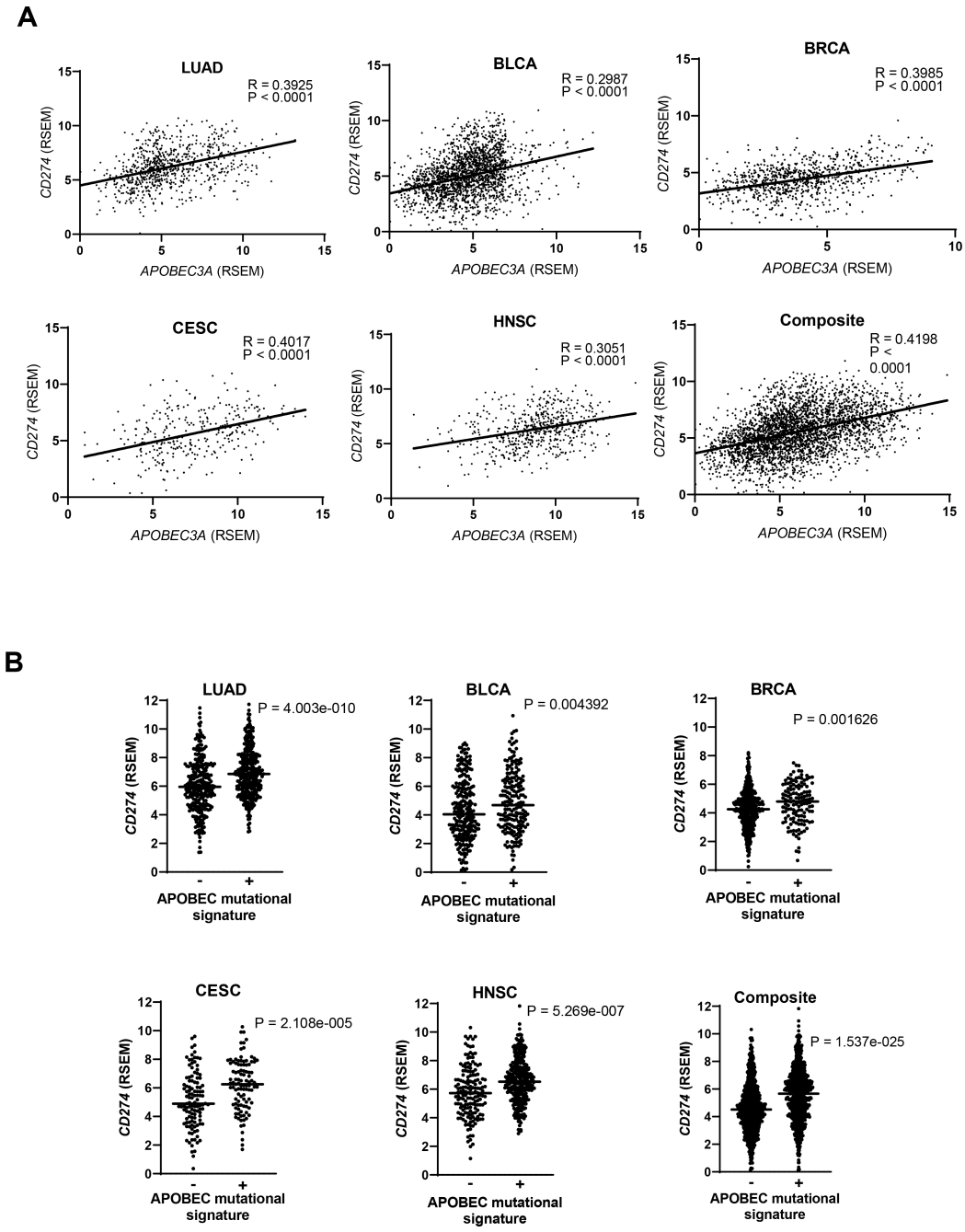

Figure 6. Correlation of APOBEC3A and APOBEC mutational signature with PD-L1 expression in patient tumors.

A. Correlation between *PD-L1* and *APOBEC3A* expression levels in Lung Adenocarcinoma (LUAD), Bladder Urothelial carcinoma (BLCA), Breast invasive cancer (BRCA), Cervical cancer (CESC), Head and neck squamous cell carcinoma (HNSC), and their composite in TCGA (the Cancer Genome Atlas) dataset. Pearson correlation coefficients and *P* values are given. **B.** Correlation between *PD-L1* expression and *APOBEC3A* mutational signature in Lung Adenocarcinoma (LUAD), Bladder Urothelial carcinoma (BLCA), Breast invasive cancer (BRCA), Cervical cancer (CESC), Head and neck squamous cell carcinoma (HNSC),

and their composite in TCGA (the Cancer Genome Atlas) dataset. Pearson correlation coefficients and *P* values are given.

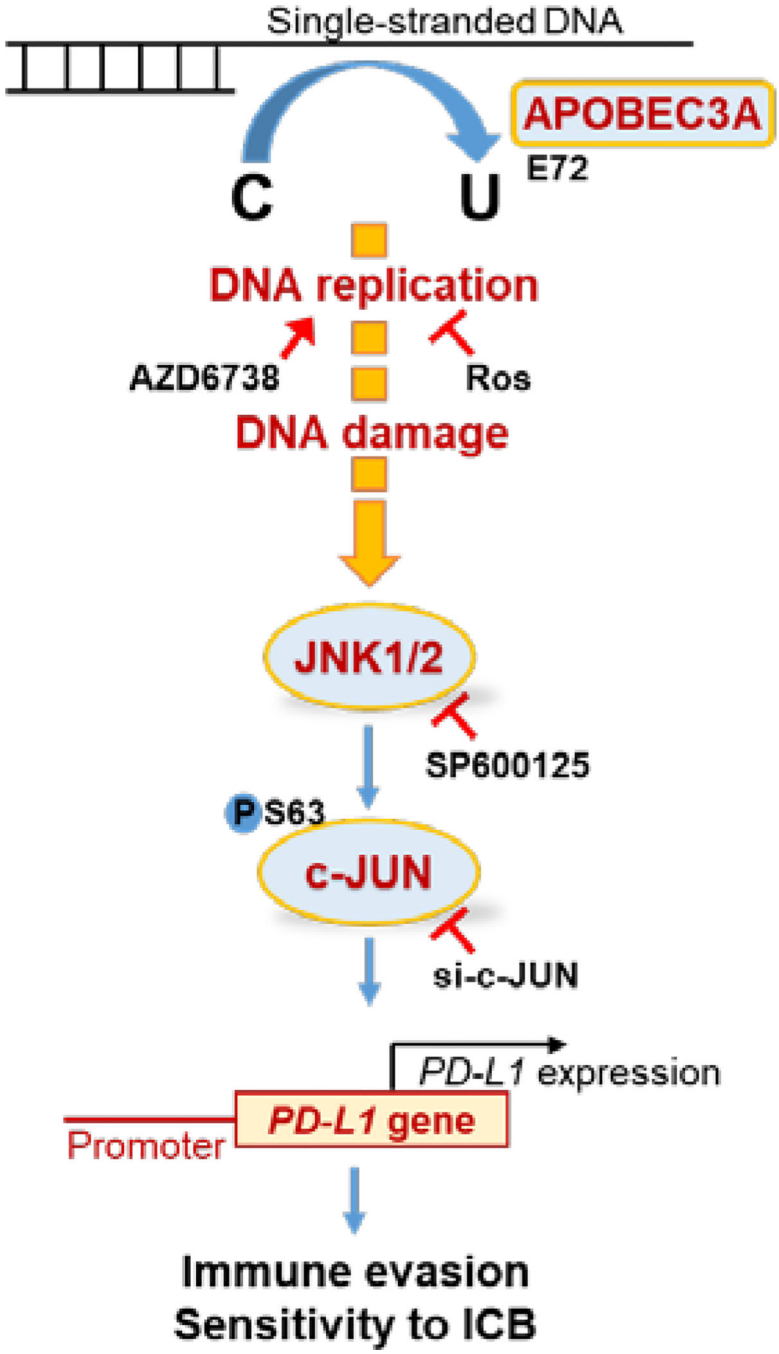

Figure 7. Model for APOBEC3A-induced PD-L1 expression.

The cytidine deaminase APOBEC3A converts C to U in single-stranded DNA. In contrast to catalytically dead APOBEC3A (E72A), catalytically active APOBECA induces expression of PD-L1 via activation of replication-associated DNA damage and induction of the JNK/c-JUN stress response pathway. APOBEC3A-mediated PD-L1 expression represents both a mechanism of immune evasion as well as a targetable vulnerability in cancer cells. ICB, immune checkpoint blockade