Cancer Research

# The Hippo Pathway Component TAZ Promotes Immune Evasion in Human Cancer through PD-L1

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## Abstract

The Hippo pathway component WW domain-containing transcription regulator 1 (TAZ) is a transcriptional coactivator and an oncogene in breast and lung cancer. Transcriptional targets of TAZ that modulate immune cell function in the tumor microenvironment are poorly understood. Here, we perform a comprehensive screen for immune-related genes regulated by TAZ and its paralog YAP using NanoString gene expression profiling. We identify the immune checkpoint molecule *PD-L1* as a target of Hippo signaling. The upstream kinases of the Hippo pathway, mammalian STE20-like kinase 1 and 2 (MST1/2), and large tumor suppressor 1 and 2 (LATS1/2), suppress PD-L1 expression while TAZ and YAP enhance PD-L1 levels in breast and lung cancer cell lines. PD-L1 expression in cancer cell lines is determined by TAZ activity and TAZ/YAP/TEAD increase *PD-L1* promoter activity. Critically, TAZ-induced PD-L1 upregulation in human cancer cells is sufficient to inhibit T-cell function. The relationship between TAZ and PD-L1 is not conserved in multiple mouse cell lines, likely due to differences between the human and mouse *PD-L1* promoters. To explore the extent of divergence in TAZ immune-related targets between human and mouse cells, we performed a second NanoString screen using mouse cell lines. We show that many targets of TAZ may be differentially regulated between these species. These findings highlight the role of Hippo signaling in modifying human/murine physiologic/pathologic immune responses and provide evidence implicating TAZ in human cancer immune evasion.

**Significance:** Human-specific activation of PD-L1 by a novel Hippo signaling pathway in cancer immune evasion may have a significant impact on research in immunotherapy. *Cancer Res; 78(6); 1457–70. ©2018 AACR.*



## Introduction

Almost all neoplasms show some degree of immune cell presence (1). In the tumor microenvironment, cancer cells interact with immune cells often restraining their antineoplastic roles and enhancing tumor-promoting functions. Cancer cell–intrinsic processes (i.e., aberrant expression of oncogenes and tumor suppressors) are critical for this polarization of the immune response. Indeed, dysregulated signaling pathways within malignant cells can upregulate genes that lead to immune evasion such as *PD-L1* (*CD274*).

PD-L1 is a key protein that governs the interaction between tumor-infiltrating T lymphocytes and cancer cells. As an immune checkpoint molecule, PD-L1 on cancer cells binds to its receptor, PD-1, on T cells to suppress their function. Specifically, PD-1/PD-L1 binding inhibits T-cell activation/proliferation/IL2 production, promotes anergy/exhaustion, and initiates T-cell apoptosis (2). Blockade of PD-1 or PD-L1 with mAbs reverses many of these phenomena and can restore T-cell function. Thus, the PD-1/PD-L1 axis has gained recognition as an exciting therapeutic target for treating multiple cancer types. While PD-1 and PD-L1 blocking agents have shown tremendous success in treating melanoma, bladder cancer and non–small cell lung cancer, response rates to these therapies are low in breast cancer clinical trials (3–6). Therefore, there is an urgent need to develop a better understanding of the immunobiology of breast cancer to establish methods for stratifying patients who will respond well to immunotherapy. Furthermore, given that there is great interest in exploiting PD-L1 as a biomarker, it is vital that we fully comprehend the cellular networks affecting its expression across varying cancer types.

Over the past decade, the Hippo signaling pathway has emerged as a central player in regulating many aspects of tumor biology (7–9). When the Hippo pathway is activated by upstream signals, MST1/2 kinases (homologs of *Drosophila* "Hippo") phosphorylate and activate LATS1/2 kinases (10–12). LATS1/2 subsequently phosphorylate transcriptional coactivators TAZ and YAP at key serine residues (S89 and S127, respectively) to inhibit their translocation into the nucleus, interaction with the TEAD family of transcription factors and activation of downstream genes (e.g., *CTGF, CYR61*; refs. 13–15). TAZ is an oncogene in breast and lung cancer where alterations in its activity have been associated with chemotherapy resistance and metastasis (16–18). There is early evidence that the Hippo pathway can influence immune cell recruitment and activation as well as the anticancer immune response (19–22). However, there has been no systematic search for TAZ and YAP transcriptional targets that act on immune cells

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**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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**doi:** 10.1158/0008-5472.CAN-17-3139

during development and disease. Indeed, whether dysregulated Hippo signaling affects the immune system in the context of breast cancer is unknown. Finally, it remains unclear whether the relationship between Hippo signaling and the immune system is conserved between human cancers and the model organisms that are commonly used to study these diseases.

In our study, we have performed the first comprehensive screen for immune-related transcriptional targets of TAZ and YAP using NanoString gene expression profiling. We identified 71 genes that are transcriptionally regulated by TAZ or YAP overexpression that are relevant to immunology. We have further validated *PD-L1* as a *bona fide* transcriptional target of the Hippo signaling pathway in human cells. Surprisingly, we observed that the relationship between TAZ and PD-L1 is not conserved in multiple mouse cell lines due to differences between the human and mouse *PD-L1* promoter sequences. Therefore, we provide evidence that the Hippo pathway plays critical roles in modulating the immune system and directing human cancer immune evasion.

## Materials and Methods

### Cell lines

To induce TAZ, YAP, or LATS overexpression in our inducible cell lines, cells were treated with 1 μg/mL doxycycline (Dox) for 48 hours. For some experiments, cells were treated as follows: 5 nmol/L 12-O-tetradecanoylphorbol-13-acetate (TPA) 1 hour, 200 ng/mL EGF 30 minutes, 4 μg/mL insulin 30 minutes, 1 μmol/L sphingosine-1-phosphate (S1P) 30 minutes, 10 μmol/L forskolin + 100 μmol/L 3-isobutyl-1-methylxanthine (IBMX) 1 hour, 2 μmol/L glucagon 1 hour, 100 nmol/L wortmannin 1 hour, 10 μmol/L GSK2334470 4 hours, serum starvation 4 hours, 10 μmol/L LY3009120 4 hours, 100 nmol/L dasatanib 24 hours, 1 μmol/L fluvastatin 24 hours, 10 μmol/L pazopanib 24 hours, 10 μmol/L rottlerin 18 hours. Culture media for cell lines are listed in the Supplementary Data. Cell lines were purchased from ATCC. The identity of our cell lines has not been recently authenticated or *Mycoplasma* tested. All experiments were conducted using cells with passage number less than 40.

### Site-directed mutagenesis, plasmid construction, and establishment of stable cell lines

Site-directed mutagenesis was performed using overlapping PCR. Mouse *TAZ* and *TEAD4* cDNAs were synthesized by reverse transcription from E10 cells. For transient gene expression, cDNAs were cloned into pCDNA3.1. For inducible overexpression, cDNAs were cloned into a puromycin-resistant modified pTRIPZ. Stable overexpression constructs were created in a HA-tagged, hygromycin-resistant modified WPI. Methods for lentivirus production/infection are as described previously (15). To create the human *PD-L1* promoter reporter, nucleotides −221 to +21 were PCR-amplified from HeLa cell gDNA and cloned into pGL3-basic. The mouse *Pd-l1* promoter (nucleotides −1723 to +220) was constructed using gDNA extracted from C57BL/6 mice. Deletions in the promoter reporters were made by overlapping PCR. See Supplementary Data for primers used in cloning.

### NanoString analysis

RNA was collected from each condition in biological replicates using RNAzol RT reagent (Sigma) and was cleared of residual gDNA using the RNeasy Mini Kit (Qiagen). Three-hundred nanograms of RNA was used per NanoString reaction using the nCounter Human Immunology v2 Gene Expression Panel or the nCounter Mouse Immunology v1 Gene Expression Panel. nSolver 3.0 software was used for background subtraction, normalization, and data analysis.

### Quantitative real-time PCR

Total RNA collection and quantitative (q)RT-PCR protocols using SYBR Green reagents are as previously described (15). Primers are in Supplementary Data.

### Western blot, flow cytometry, and antibodies

Methods for Western blot analysis are as described in ref. 15. Antibodies for Western blot analysis were as follows: PD-L1 (E1L3N), PD-L2 (D7U8C), YAP/TAZ (D24E4), MST1 (D8B9Q), MST2, LATS1 (C66B5), and phospho-YAP (S127) from Cell Signaling Technology; YAP (H-125) from Santa Cruz Biotechnology; TAZ (M2-616) from BD Biosciences; TEAD1-4 (EPR15629) from Abcam; LATS2 (BL2213) from Bethyl Laboratories; β-actin (AC-15) from Sigma. Flow cytometry was performed according to a standard protocol from Abcam using a PE-conjugated antibody for human PD-L1 (MIH1) from eBioscience (isotype control mouse IgG1 (B11/6) from Abcam) or a PE-conjugated antibody for mouse PD-L1 (MIH5) from eBioscience (isotype control rat IgG2a from eBioscience).

### Transient gene knockdown with siRNA

siRNAs were transfected into cells using Lipofectamine RNAiMAX reagent (Invitrogen) at a final concentration of 50 nmol/L and according to the manufacturer's instructions. MST1/2 were knocked down using siRNA from the TriFECTa RNAi kit from IDT. See Supplementary Data for other siRNA sequences. Knockdown efficiency was determined 48 hours after transfection by Western blot analysis.

### Stable gene knockout using CRISPR–Cas9

CRISPR–Cas9 constructs for TAZ knockout were generated using the lentiCRISPRv1 vector (23, 24). Clonal cell lines were established by limiting dilution plating. CRISPR-Cas9–resistant addback TAZ constructs were created by mutating the PAM sequence in the *TAZ* cDNA by overlapping PCR. sgRNA sequences and primers for the addback mutations are listed in the Supplementary Data. Addback constructs were cloned into WPI.

### Dual luciferase assays

One-hundred nanograms of promoter reporter was cotransfected into SK-BR-3 using PolyJet transfection reagent (SignaGen) alongside 200 ng of *TAZ/YAP* construct, 200 ng of *TEAD* construct, and 10 ng of *Renilla* luciferase pRL-TK plasmid as an internal control. Total DNA was adjusted to 510 ng/well using pCDNA3.1. After 48 hours, dual luciferase assay was performed using a kit from Promega. Relative promoter activity was calculated as the ratio of Firefly luciferase signal to *Renilla* luciferase signal. All measurements were normalized to promoter reporter alone.

### Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) from Cell Signaling Technology. MCF10A-TAZ-S89A cells were induced with doxycycline for 72 hours. Ten micrograms of chromatin was incubated overnight with 2 μg antibody for TAZ (M2-616 from BD Biosciences) or with 2 μg normal mouse IgG (Santa

Cruz Biotechnology). The *PD-L1*, *CTGF*, and *CYR61* promoters were PCR-amplified using primers in the Supplementary Data. Total chromatin extract ("input") was used as a positive control for PCR.

### T-cell apoptosis assays

Cancer cell TAZ-S89A overexpression was induced with doxycycline for 48 hours. Jurkat T cells were activated with 1 μg/mL phytohaemagglutinin (PHA) and 50 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 hours to induce PD-1 expression. Following doxycycline treatment, $5 \times 10^5$ cancer cells were plated in Jurkat cell media into each well of a 12-well plate. The following day, $5 \times 10^4$ activated T cells were added to each well of the 12-well plate. After 24 hours (MCF10A coculture) or 5 hours of coculture (A549, H1299 coculture), T cells were collected. T-cell apoptosis was measured using the Caspase-Glo 3/7 assay from Promega. For PD-L1 inhibition, H1299 cells were plated in Jurkat cell media containing 20 μg/mL purified anti-PD-L1 antibody (29E.2A3 from BioLegend). T-cell coculture was performed the following day in the presence of 20 μg/mL anti-PD-L1 antibody.

### T-cell IL2 production assay

A549 TAZ-S89A overexpression was induced with doxycycline for 48 hours while Jurkat T cells were stimulated with 1 μg/mL PHA and 50 ng/mL PMA for 24 hours to initiate IL2 and PD-1 expression. A total of $1 \times 10^5$ A549 cells were plated with $1 \times 10^4$ activated T cells in 100 μL T-cell media with PMA/PHA in a 96-well plate. The following day, coculture supernatants were collected and IL2 levels were measured by ELISA using the IL-2 Human ELISA kit from Invitrogen. For PD-L1 inhibition, cells were cocultured in the presence of 20 μg/mL purified anti-PD-L1 antibody (29E.2A3 from BioLegend).

### Statistical analysis

Student *t* test (two-tailed) and ANOVA (with *post hoc* analysis) were used for all statistical analysis.

## Results

### NanoString gene expression profiling reveals immune-related genes regulated by TAZ and YAP

To identify immune-related transcriptional targets of TAZ and YAP, we subjected RNA from MCF10A breast epithelial cell lines overexpressing doxycycline-inducible, constitutively active TAZ (TAZ-S89A), or YAP (YAP-S127A) to NanoString profiling using the nCounter Human Immunology v2 panel of 579 immunology-related genes (Fig. 1A). Genes regulated by TAZ or YAP were defined as having at least a 2-fold, statistically significant change in expression after TAZ-S89A or YAP-S127A induction ($P < 0.1$). By this criterion, 25 genes were upregulated by TAZ-S89A, while 34 genes were downregulated. A further 17 genes were upregulated by YAP-S127A, whereas 19 genes were downregulated. Top candidate TAZ-S89A- and YAP-S127A–regulated genes are heatmapped in Fig. 1B. Many genes were commonly regulated by TAZ-S89A and YAP-S127A (e.g., *S1PR1*, *NLRP3*, *PD-L1/CD274*; Fig. 1C). The fold-change regulations are depicted in Fig. 1D and E. The top TAZ-S89A- and YAP-S127A–upregulated genes were validated by qRT-PCR and were further confirmed to be upregulated by TAZ-S89A/YAP-S127A overexpression in a mesenchymal-like breast cancer cell line, MDA-MB-231 (Fig. 1F; Supplementary Fig. S1A and S1B). In addition, as a control, we verified that none of the top candidates identified by our screen were regulated by doxycycline treatment in wild-type MCF10A (Supplementary Fig. S1C). Therefore, TAZ and YAP modulate the expression of many genes that are relevant to immunology and the tumor immune microenvironment.

### The Hippo pathway regulates PD-L1 expression

Given the well-established role of *PD-L1* in modulating the interaction between cancer and immune cells, we chose this gene for further study. Consistent with our NanoString data, TAZ-S89A and YAP-S127A induced PD-L1 expression at the protein level in MCF10A (Fig. 2A). *PD-L2* (*PDCD1LG2*), a paralog of *PD-L1* and a candidate YAP-regulated gene identified by our screen, was also induced at the protein level by YAP-S127A overexpression. TAZ-S89A–induced PD-L1 expression was apparent by flow cytometry in nonpermeabilized cells, indicating that TAZ-S89A enhances PD-L1 expression at the plasma membrane (Fig. 2B). The relationship between TAZ-S89A overexpression and PD-L1 upregulation was also observable in human immortalized lung/bronchus epithelial cells (HBE-135; Fig. 2C).

We next evaluated whether upstream Hippo pathway components regulate PD-L1 through TAZ and YAP. Transient knockdown of MST1/2 or LATS1/2 in wild-type MCF10A suppressed inhibitory YAP-S127 phosphorylation, enhanced *CYR61* mRNA expression, and increased PD-L1 expression at the protein and mRNA level (Fig. 2D–G; Supplementary Fig. S2A and S2B). Furthermore, LATS2 overexpression in MDA-MB-231 (high endogenous PD-L1) increased YAP-S127 phosphorylation and reduced PD-L1 expression (Fig. 2H). Therefore, the Hippo pathway regulates PD-L1.

Many upstream signaling pathways converge on Hippo signaling (Fig. 2I). We next explored whether our finding that the Hippo pathway affects PD-L1 expression might provide insights into other stimuli that can regulate PD-L1. We exposed MCF10A (low TAZ) and MDA-MB-231 (high TAZ) to culture conditions that inhibit and activate Hippo signaling, respectively (25–31). We identified four treatments [PKC activator (TPA), EGF, insulin and GPCR inhibitor (S1P); Fig. 2J; Supplementary Fig. S2C] that induced both *PD-L1* and *CYR61* mRNA expression in MCF10A and six treatments [PKA activator (Forskolin/IBMX), glucagon, PI3K inhibitor (Wortmannin), PDK inhibitor (GSK2334470), serum starvation, RAF inhibitor (LY3009120); Fig. 2K; Supplementary Fig. S2D] that repressed *PD-L1* mRNA expression in a similar pattern to *CYR61* in MDA-MB-231. Thus, the Hippo pathway may link PD-L1 expression to various other signaling networks and stimuli.

### TAZ determines PD-L1 expression in cancer cell lines

We next set out to determine whether TAZ and PD-L1 are coexpressed in cancer cell lines. Indeed, the expression levels of TAZ are correlated with those of PD-L1 in multiple breast cell lines and a similar (albeit weaker) relationship was observed in lung cell lines (Fig. 3A and B). This was consistent with data from The Cancer Genome Atlas (TCGA) database indicating that *TAZ* and *PD-L1* mRNA expression cooccurs in multiple cancer types including breast invasive carcinomas and lung adenocarcinomas (Supplementary Fig. S3A). Interestingly, relatively fewer datasets from TCGA demonstrated an association between *YAP* and *PD-L1* mRNA expression (Supplementary Fig. S3B).

To test whether TAZ is required for high PD-L1 expression in certain cancer cells, we knocked out TAZ in Hs578T and MDA-MB-

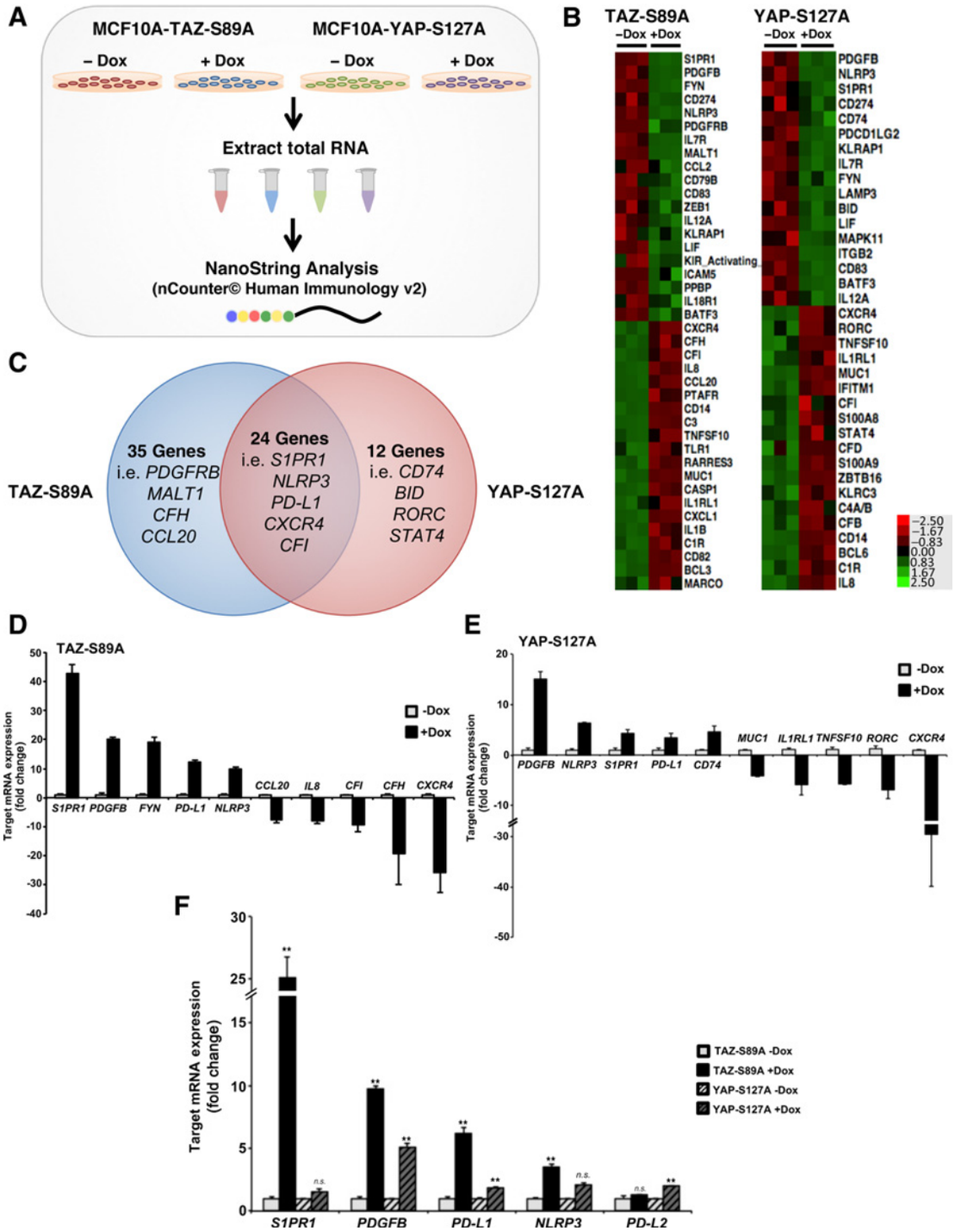



**Figure 1.**

NanoString gene expression profiling reveals novel immune-related transcriptional targets of TAZ and YAP. **A,** Experimental design to determine immune-related transcriptional targets of TAZ and YAP. MCF10A-TAZ-S89A or YAP-S127A cell lines were induced for 48 hours with doxycycline (Dox) before RNA was collected (biological triplicate) and subjected to NanoString analysis using the nCounter Human Immunology v2 gene expression panel. **B,** Heatmap summarizing the top up- (green) and downregulated (red) genes affected by TAZ-S89A or YAP-S127A expression (+Dox; color scale denotes Euclidean distance of mRNA count). **C,** Comparison of candidate immune-related genes regulated by TAZ-S89A and/or YAP-S127A. **D** and **E,** Summary of the top up- and downregulated genes affected by TAZ-S89A (**D**) and YAP-S127A (**E**) overexpression from NanoString analysis (NanoString; mean + SEM, $n = 3$ biological replicates). **F,** Validation of the top immune-related genes upregulated by TAZ-S89A and YAP-S127A (qRT-PCR; mean + SEM, $n = 2$ biological replicates, **, $P < 0.01$; n.s., not significant). See also Supplementary Fig. S1.

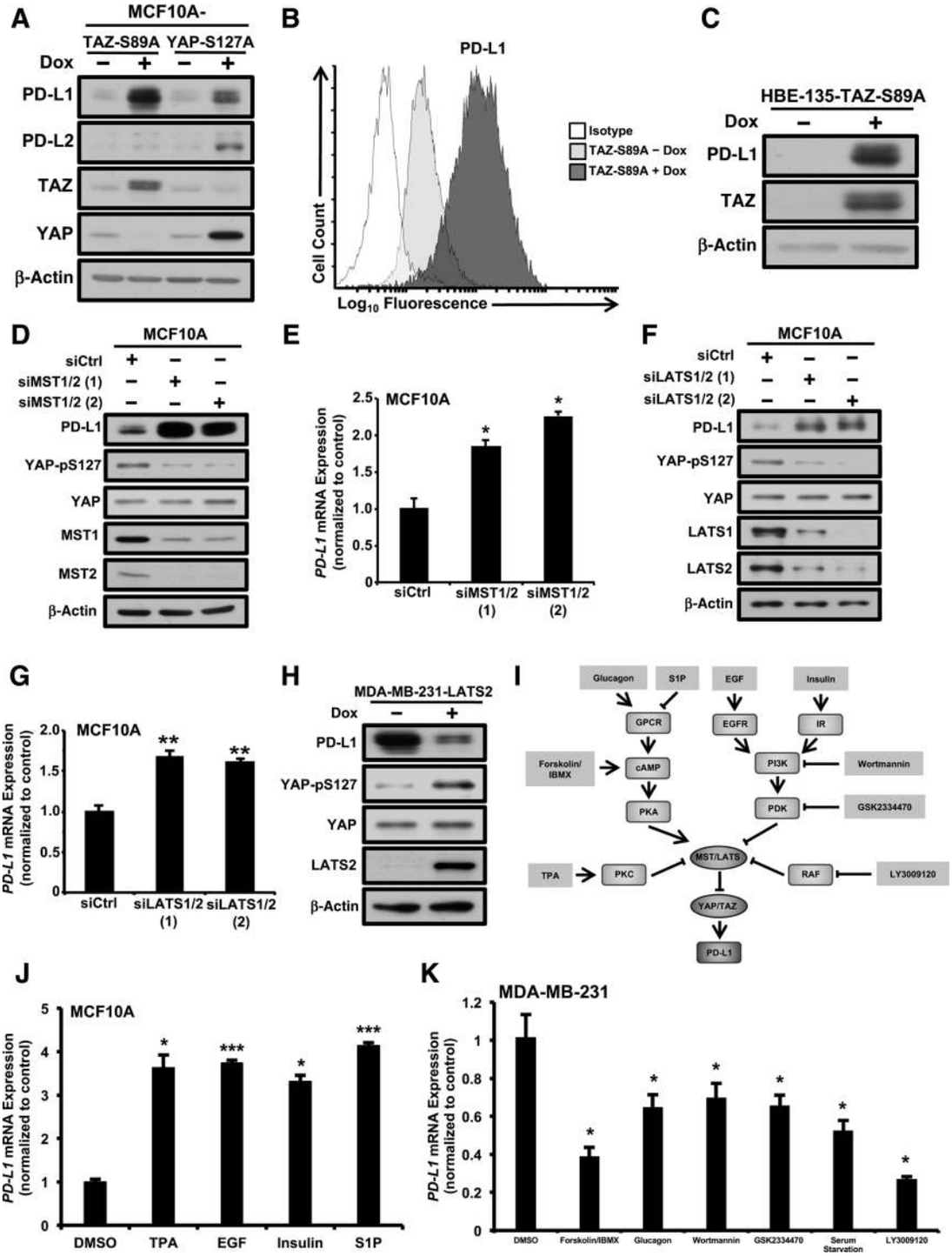



**Figure 2.**

The Hippo pathway regulates PD-L1 expression. **A,** TAZ-S89A and YAP-S127A induce PD-L1 and PD-L2 protein expression in MCF10A. **B,** TAZ-S89A overexpression in MCF10A enhances membrane PD-L1 expression. **C,** TAZ-S89A overexpression enhances PD-L1 expression in HBE-135. **D** and **E,** Transient knockdown of MST1/2 or LATS1/2 (**F** and **G**) in MCF10A reduces YAP-S127 phosphorylation and upregulates PD-L1 protein and mRNA expression (qRT-PCR; mean + SEM, $n = 2$ technical replicates, *, $P < 0.05$; **, $P < 0.01$). **H,** LATS2 overexpression in MDA-MB-231 enhances YAP-S127 phosphorylation and inhibits PD-L1 expression. **I,** Schematic diagram of a selection of cellular pathways that converge on Hippo signaling and drugs/ligands that act on these pathways. **J** and **K,** Various treatments that act upstream of Hippo signaling enhance *PD-L1* mRNA expression in MCF10A (**J**) or diminish *PD-L1* expression in MDA-MB-231 (**K**; qRT-PCR; mean + SEM, $n = 2$ technical replicates, *, $P < 0.05$; ***, $P < 0.001$). See also Supplementary Fig. S2.

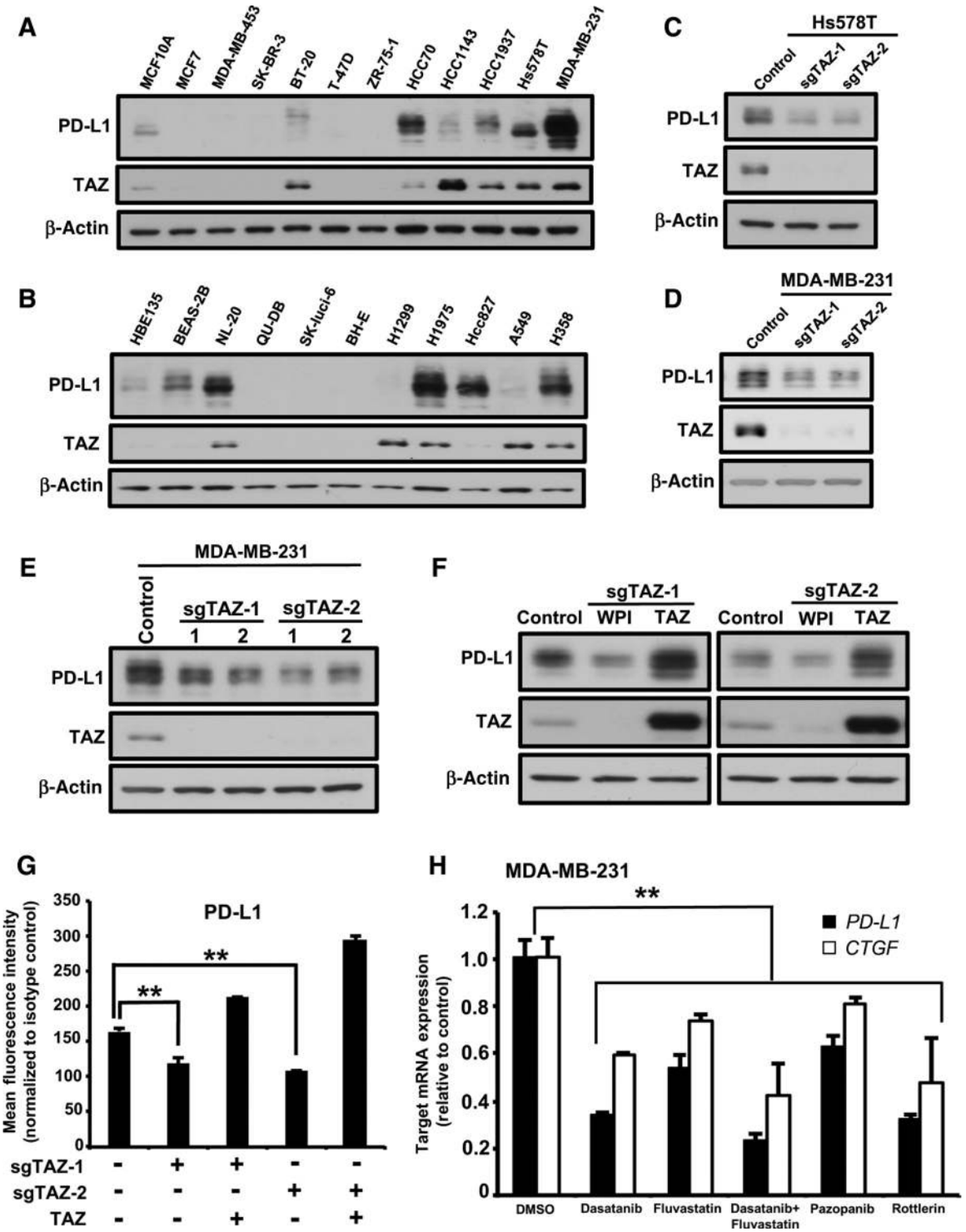



**Figure 3.**

TAZ determines PD-L1 expression in cancer cell lines. TAZ and PD-L1 are coexpressed in breast (**A**) and lung (**B**) cancer cell lines. TAZ knockout in Hs578T (**C**) or MDA-MB-231 (**D**) reduces PD-L1 expression. Stable knockout of TAZ was achieved using CRISPR/Cas9 with two different sgRNA sequences targeting *TAZ* (sgTAZ-1 and sgTAZ-2). **E,** Clonal TAZ-knockout MDA-MB-231 cell lines show reduced PD-L1 expression. **F** and **G,** Addback of PAM- mutated TAZ into clonal TAZ-knockout MDA-MB-231 cell lines restores PD-L1 expression (WPI, empty vector; flow cytometry; mean + SEM, $n = 2$ technical replicates, **, $P < 0.01$). **H,** Pharmacologic inhibition of TAZ and YAP in MDA-MB-231 reduces *PD-L1* mRNA expression (qRT-PCR; mean + SEM, $n = 3$ biological replicates, **, $P < 0.01$). See also Supplementary Fig. S3.

231 using CRISPR-Cas9. TAZ knockout decreased PD-L1 protein in both cell lines (Fig. 3C and D). Clonal cell lines derived from the heterogeneous TAZ knockout MDA-MB-231 cell lines also showed reduced PD-L1 expression compared with control cells (Fig. 3E). PD-L1 expression in TAZ-knockout MDA-MB-231 cell lines was rescued by addback of CRISPR-Cas9–resistant TAZ constructs (Fig. 3F and G). We further confirmed that PD-L1 expression in wild-type MDA-MB-231 depends on TAZ activity by pharmacologically inhibiting TAZ/YAP function (32, 33). Inhibition of TAZ and YAP reduced *PD-L1* mRNA expression to a greater extent than *CTGF* (Fig. 3H). Therefore, PD-L1 expression in cancer cell lines is determined by the activity of TAZ.

### TAZ transcriptionally activates PD-L1 through the TEAD family of transcription factors

TAZ and YAP regulate gene expression by binding to transcription factors such as the TEAD family (i.e., TEAD1–4; refs. 34, 35). To establish whether TEADs play a role in the regulation of PD-L1 by TAZ, we overexpressed a constitutively active, TEAD-binding mutant form of TAZ (TAZ-S89A-F52/53A) in MCF10A and found that this construct had a diminished ability to induce PD-L1 (Fig. 4A and B). Similarly, knockdown of TEAD1/3/4 in MCF10A-TAZ-S89A reduced TAZ-induced PD-L1 expression (Fig. 4C). Finally, addback of TEAD-binding mutant TAZ (TAZ-F52/53A) into TAZ-knockout MDA-MB-231 cells could not restore PD-L1 expression (Fig. 4D). Therefore, TAZ regulates PD-L1 expression primarily through TEAD transcription factors.

To further characterize the transcriptional regulation of PD-L1 by TAZ, we constructed a luciferase-based reporter for the minimal *PD-L1* promoter (nucleotides −221 to +21). We cotransfected this reporter into SK-BR-3 breast cancer cells alongside TAZ-S89A and TEAD1-4. TAZ-S89A and TEAD1-4 coexpression dramatically increased *PD-L1* promoter activity (Fig. 4E). TEAD-binding mutant TAZ (TAZ-F52/53A), TAZ without its C-terminal transcriptional coactivation domain (TAZ-Δ227), and TAZ-binding mutant TEAD4 (TEAD4-Y429H) all could not activate the *PD-L1* promoter (Fig. 4F). Like TAZ, a similar increase in *PD-L1* promoter activity was induced by YAP-S127A and TEAD4 (Fig. 4G).

We performed a deletion scan to determine the *PD-L1* promoter region regulated by TAZ-S89A and TEAD4. Deletion of nucleotides −100 to −40 abolished activation by TAZ-S89A and TEAD4 (Fig. 4H). Within this region, we identified a putative TEAD-response element spanning positions −74 to −62 (CAGGAAAGTCCAA) (Fig. 4I). Deletion of this region dramatically reduced the activation of the *PD-L1* promoter by TAZ-S89A and TEAD4 (Fig. 4J). We confirmed that TAZ binds at the *PD-L1* promoter by ChIP. Specifically, we found that fragments from the proximal *PD-L1* promoter (i.e., positions −221 to +21 and −183 to +58), the *CTGF* promoter and the *CYR61* promoter precipitated with TAZ whereas a more distal fragment from the *PD-L1* promoter (positions −1039 to −808) did not (Fig. 4K). Therefore, TAZ regulates PD-L1 expression by binding to the *PD-L1* promoter through the TEAD family of transcription factors thereby enhancing promoter activity.

### TAZ overexpression in cancer cell lines suppresses T-cell function

To explore the functional significance of TAZ in modulating the anticancer immune response, we cocultured TAZ-overexpressing cancer cell lines with Jurkat T cells. Jurkat T cells were activated with PMA and PHA prior to coculture to induce PD-1 expression (Fig. 5A). TAZ-S89A overexpression in MCF10A was sufficient to increase apoptosis in Jurkat T cells cocultured with these cells (Fig. 5B and C). A similar increase in apoptosis was observed in T cells cocultured with TAZ-S89A–overexpressing A549 or H1299 lung cancer cells (Fig. 5D–G). To evaluate whether TAZ enhances T-cell apoptosis through PD-L1, we cocultured TAZ-S89A–overexpressing H1299 with Jurkat T cells in the presence of a PD-L1 blocking antibody. Indeed, inhibition of PD-L1/PD-1 binding completely suppressed TAZ-S89A–induced T-cell apoptosis (Fig. 5H). Thus, cancer cell TAZ expression regulates T-cell viability through PD-L1.

As a second measure of T-cell function, we measured IL2 production by PMA/PHA–activated Jurkat T cells in coculture with A549. In this system, TAZ-S89A overexpression in A549 was sufficient to suppress IL2 production by T cells and this was reversed when cells were cocultured in the presence of a PD-L1 blocking antibody (Fig. 5I). Collectively, these data are consistent with a model in which TAZ/YAP/TEAD and the upstream Hippo pathway direct cancer immune evasion through the transcriptional regulation of PD-L1 (Fig. 5J).

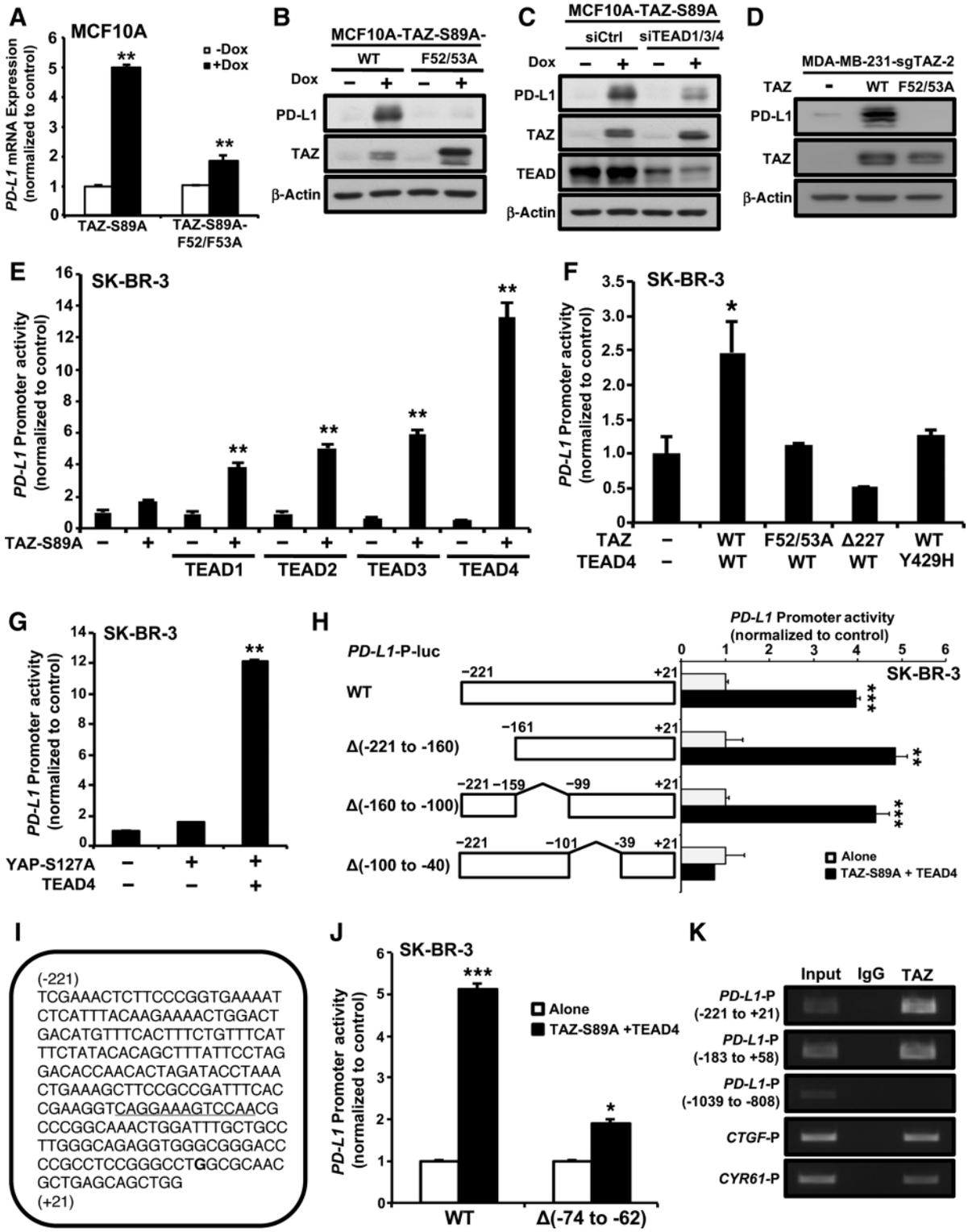
### The relationship between TAZ and PD-L1 is not conserved in mouse cells

Many targets of TAZ and YAP are similarly regulated in human and mouse cell lines (e.g., *CTGF*). However, it has been suggested that there is substantial divergence in the transcriptional programs that act on the human and mouse immune systems (36, 37). To determine whether TAZ upregulates PD-L1 in murine cell lines, we established TAZ-S89A–overexpressing mouse cell lines [three mammary cell lines (HC11, NMuMG, E0771), one lung cell line (E10) and one melanoma cell line (B16-OVA); Fig. 6A)]. While *Ctgf* was upregulated by TAZ in each of these cell lines, TAZ-S89A overexpression caused no change in *Pd-l1* mRNA expression in any of the cell lines examined (Fig. 6B). As a control, *Pd-l1* mRNA was easily detectable in 293T cells overexpressing mouse *Pd-l1* cDNA using the same primers for qRT-PCR (Fig. 6C). Likewise, human TAZ-S89A, mouse TAZ-S89A, or human YAP-S127A overexpression all had no effect on PD-L1 protein levels (Fig. 6D–F; Supplementary Fig. S4A and S4B). Thus, PD-L1 appears to be differentially regulated by TAZ in human and mouse cells.

To investigate the mechanisms underlying this distinction we constructed a luciferase reporter for the mouse *Pd-l1* promoter (nucleotides −1723 to +220). While the human *PD-L1* promoter was activated equally by both human and mouse TAZ-S89A/TEAD4, the mouse *Pd-l1* promoter had a dramatically diminished response to both human and mouse constructs (Fig. 6G). Therefore, the relationship between TAZ and PD-L1 is not conserved in mouse cells and this is likely due to regulatory differences between the human and mouse PD-L1 promoters. It is notable that other top candidate TAZ-regulated genes from our profiling in human cells were also not upregulated by human or mouse TAZ-S89A in HC11 or NMuMG (e.g., *S1pr1*, *Nlrp3*; Fig. 6H and I; Supplementary Fig. S4C and S4D).

### Determination of TAZ immune-related transcriptional targets in mouse cells

Our observation that multiple candidate TAZ targets identified by our human screen were not upregulated in mouse cell lines suggests that there may be broader species-specific differences in the TAZ transcriptional program that have been unrealized in previous work. Given the importance of murine models for



studies of immunology, we set out to explore the extent of divergence between human and mouse TAZ immune-related gene targets. We subjected RNA from HC11-TAZ-S89A and NMuMG-TAZ-S89A to NanoString gene expression profiling using the nCounter Mouse Immunology panel (Fig. 7A). Twenty-eight genes were upregulated by human TAZ-S89A in these cell lines while 56 genes were downregulated by TAZ-S89A (Fig. 7B–D). We observed substantial differences in the gene targets regulated by TAZ in human and mouse cells. Only a minority (14/83) of the candidate TAZ targets identified by our screen in mouse cells were also candidates from our human screen (e.g., *Pdgfb*, *Il12a*, *Cd14*; Fig. 7E). We used qRT-PCR to validate the top upregulated candidate TAZ targets from our screen and confirmed that none of these genes were significantly affected by doxycycline treatment in wild-type cells (Fig. 7F; Supplementary Fig. S5A and S5B). We also explored whether these genes were affected by mouse TAZ-S89A overexpression (in HC11 and NMuMG) or human TAZ-S89A overexpression (in MCF10A). Indeed, both human and mouse TAZ-S89A upregulated the expression of multiple genes identified by our screen (Fig. 7F and G). Furthermore, several of the top candidates from our screen in mouse cells including *Tigit*, *Ptpn22*, *Masp1*, and *Il7* were uniquely upregulated by TAZ in mouse cell lines but not in human cells (Fig. 7F–H). Therefore, we have uncovered multiple genes that may be differentially regulated by TAZ between these two species.

## Discussion

The Hippo signaling pathway plays critical roles in cancer development and progression. In cancer cells, dysregulation of the Hippo transducers TAZ and YAP leads to aberrant expression of their downstream gene targets and endows cells with numerous "hallmarks of cancer" (9). In our study, we performed the first comprehensive screen for immune-related transcriptional targets of TAZ and YAP using NanoString profiling. In doing so, we uncovered a novel function for TAZ in promoting cancer immune evasion through the transcriptional regulation of PD-L1.

PD-L1 is a critical mediator in the interaction between effector T lymphocytes in the tumor microenvironment and tumor cells. Indeed, the PD-1/PD-L1 axis is a key target for immunotherapies. In our study, we showed that both TAZ and YAP transcriptionally regulate PD-L1 in human cancer cells. We characterized the molecular mechanisms by which TAZ enhances PD-L1 expression by binding to the *PD-L1* promoter through the TEAD family of transcription factors. Most significantly, we demonstrated that these observations have functional importance in coculture experiments where TAZ overexpression in cancer cells was sufficient to disrupt T-cell function through PD-L1.

These findings provide new insights into how PD-L1 is regulated in breast cancer. PD-L1 is expressed by a significant portion of triple-negative breast cancers (38). However, whether high tumor cell PD-L1 levels predict a favorable or unfavorable prognosis for breast cancer patients is unclear and likely depends on the circumstances surrounding PD-L1 upregulation (39, 40). PD-L1 may be expressed due to tumor cell–intrinsic processes (e.g., oncogenic signaling through PI3K, STAT3, HIF-1α, or TAZ) but may also occur secondary to a robust anticancer immune response (e.g., through IFNγ signaling and NF-κB; ref. 41). In each of these scenarios, PD-L1 expression likely reflects different disease pathology. Thus, knowledge of tumor PD-L1 status on its own may be insufficient as a biomarker for predicting patient prognosis and response to immunotherapy. A better understanding of the mechanisms regulating PD-L1 expression may allow us to more accurately interpret what PD-L1 positivity means and may reveal superior biomarkers and therapeutic targets for cancer treatment. Furthermore, given that ongoing clinical trials applying anti-PD-1 and anti-PD-L1 therapies to breast cancer patients have shown only limited success, there is an urgent need to develop tests that can stratify patients for immunotherapy (3–5). Our findings suggest that future work exploring tumor TAZ status as a prognostic and predictive factor for cancer immunotherapy may be warranted. In addition, as we have shown that pharmacologic inactivation of TAZ/YAP significantly inhibited PD-L1 expression, our data also highlight the therapeutic potential of targeting the Hippo pathway for cancer treatment either as a monotherapy or in combination with PD-L1–targeted immunotherapies.

It should also be noted that PD-L1 plays multiple roles in immune evasion and cancer biology outside of its effects on T-cell function. For example, PD-1/PD-L1 binding regulates tumor cell phagocytosis by tumor-associated macrophages and several groups have proposed that PD-L1 has tumor cell–intrinsic functions (42–44). Therefore, it is possible that TAZ regulates the immune system more broadly through PD-L1 or that signaling downstream of PD-L1 contributes to the oncogenic potential of TAZ. These areas represent interesting directions for future work.

While characterizing PD-L1 as a transcriptional target of TAZ, we were surprised to find that the relationship between PD-L1 and TAZ was not apparent in mouse cell lines. This was unexpected, as

**Figure 4.**

TAZ transcriptionally activates PD-L1 expression at its promoter through the TEAD family of transcription factors. **A** and **B,** Overexpression of TEAD-binding mutant TAZ (TAZ-S89A-F52/53A) leads to less *PD-L1* mRNA (**A**) and protein (**B**) upregulation in MCF10A compared with TAZ-S89A (qRT-PCR; mean + SEM, $n = 2$ technical replicates, **, $P < 0.01$; WT, wild type). **C,** Transient knockout of TEAD1/3/4 transcription factors diminishes PD-L1 induction by TAZ-S89A in MCF10A. **D,** Addback of TEAD-binding mutant TAZ-F52/53A into clonal TAZ-knockout MDA-MB-231 cell lines cannot rescue PD-L1 expression. WT, wild type. **E,** TAZ-S89A and TEAD1–4 enhance *PD-L1* promoter (−221 to +21) activity in SK-BR-3 (luciferase assay; mean + SEM, $n = 3$ biological replicates, **, $P < 0.01$). **F,** Wild-type (WT) TAZ and TEAD4 increase *PD-L1* promoter activity while TEAD-binding mutant TAZ (TAZ-F52/53A), TAZ without its transcriptional coactivation domain (TAZ-Δ227), and TAZ-binding mutant TEAD4 (TEAD4-Y429H) cannot activate the *PD-L1* promoter (luciferase assay; mean + SEM, $n = 3$ biological replicates, *, $P < 0.05$). **G,** YAP-S127A and TEAD4 enhance *PD-L1* promoter activity in SK-BR-3 (luciferase assay; mean + SEM, $n = 3$ biological replicates, **, $P < 0.01$). **H,** Deletion scan to identify the region of the *PD-L1* promoter (*PD-L1*-P) activated by TAZ-S89A and TEAD4 identifies positions (−100 to −40) as essential for promoter activation (luciferase assay; mean + SEM, $n = 3$ biological replicates, **, $P < 0.01$; ***, $P < 0.001$). **I,** The core *PD-L1* promoter (−221 to +21) contains a putative TEAD-response element (positions −74 to −62; underlined). **J,** Deletion of a putative TEAD-response element in the *PD-L1* promoter (nucleotides −74 to −62) dramatically reduces activation by TAZ-S89A and TEAD4 (luciferase assay; mean + SEM, $n = 3$ biological replicates, *, $P < 0.05$; ***, $P < 0.001$; WT, wild-type *PD-L1* promoter construct). **K,** TAZ binds to the *PD-L1* promoter in MCF10A-TAZ-S89A. Chromatin and associated proteins were crosslinked and a mouse mAb was used to pull down chromatin associated with TAZ (ChIP). Normal mouse IgG was used as a control. Regions of the *PD-L1* promoter (*PD-L1*-P −221 to +21, −183 to +58, or −1039 to −808), the *CTGF* promoter (*CTGF*-P), or the *CYR61* promoter (*CYR61*-P) were amplified by PCR and fragments were visualized by agarose gel electrophoresis. Total chromatin extract ("input") was used as a positive control for PCR.

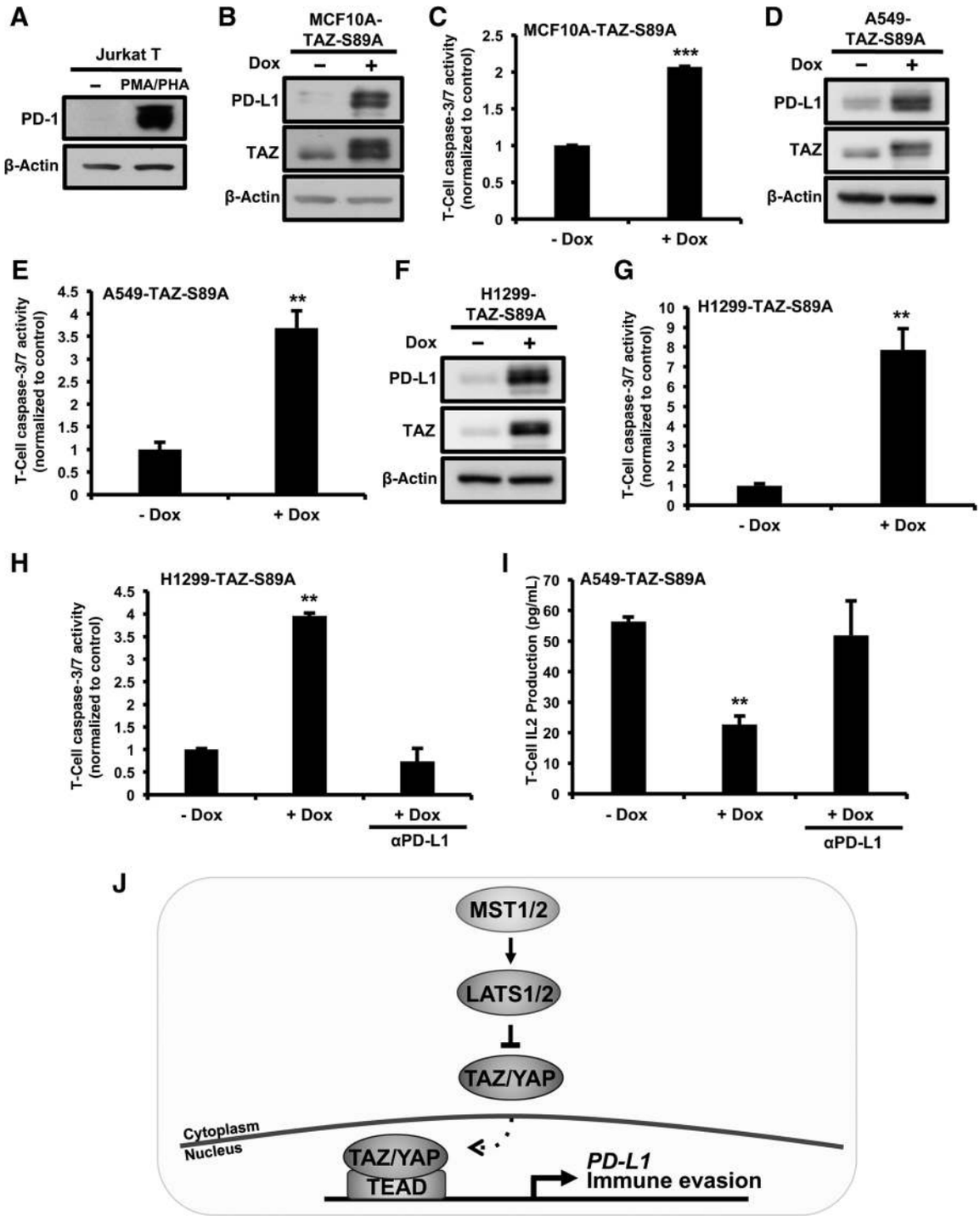



**Figure 5.**

TAZ enhances breast and lung cancer immune evasion. **A,** PD-1 expression in Jurkat T cells can be stimulated by treatment with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL phytohemagglutinin (PHA) for 48 hours. **B–G,** TAZ-S89A overexpression induces PD-L1 expression (**B**) and T-cell apoptosis (**C**) in coculture experiments with MCF10A as well as in A549 (**D** and **E**) and H1299 (**F** and **G**). T-cell caspase-3/7 activities in cells cultured with TAZ-S89A–overexpressing MCF10A/A549/H1299 were normalized to that of T cells cultured with MCF10A/A549/H1299 without TAZ-S89A [caspase-3/7 assay; mean + SEM, $n = 3$ technical replicates (MCF10A) or 4 biological replicates (A549, H1299), **, $P < 0.01$; ***, $P < 0.001$]. **H,** PD-L1 blockade reverses TAZ-S89A–induced T-cell apoptosis (caspase-3/7 assay; mean + SEM, $n = 2$ biological replicates, **, $P < 0.01$). **I,** TAZ-S89A overexpression in A549 suppresses IL2 production by T cells in coculture through PD-L1 (ELISA; mean + SEM, $n = 3$ biological replicates, **, $P < 0.01$). **J,** Model for how the Hippo pathway regulates PD-L1 expression and cancer immune evasion.

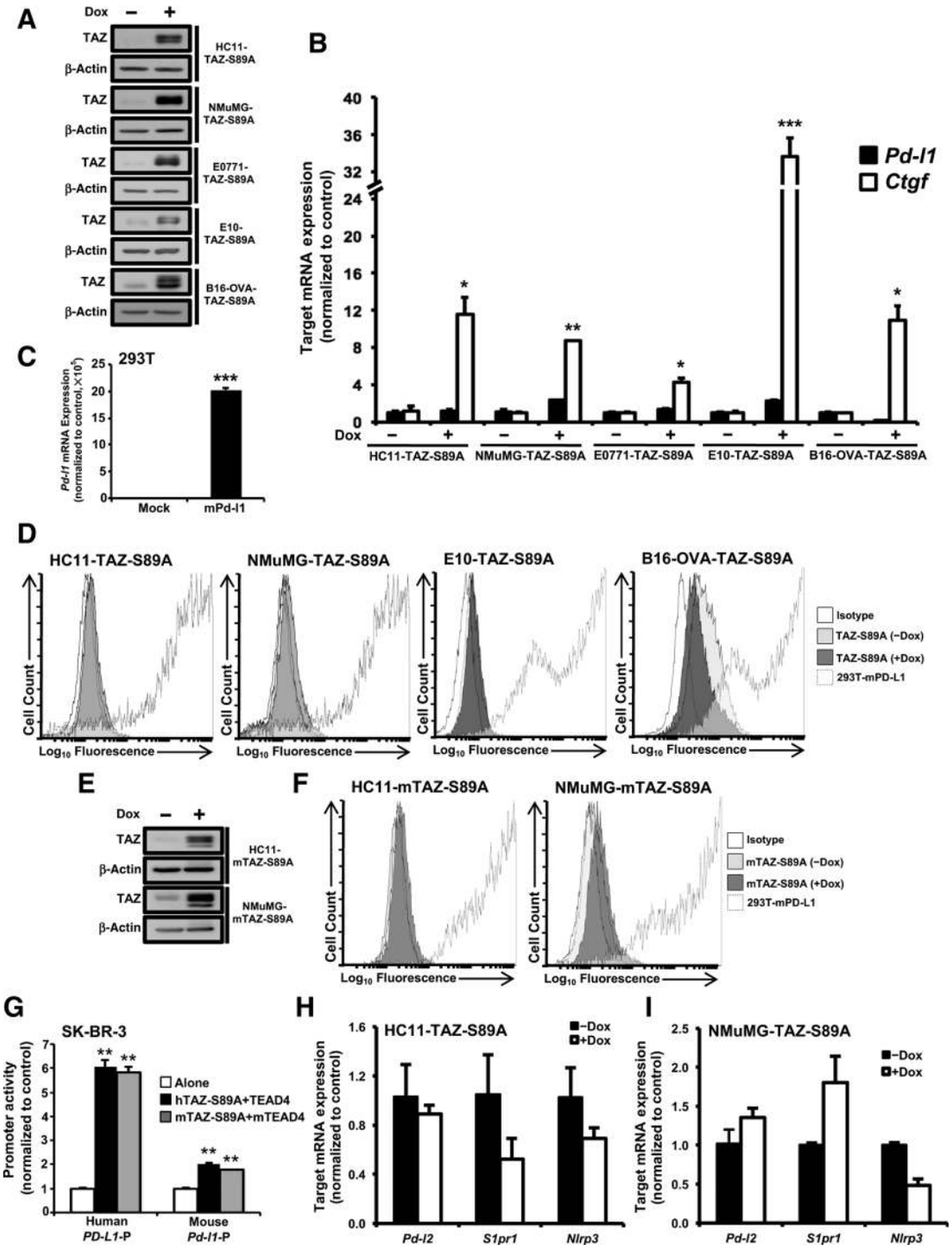



**Figure 6.**

The relationship between TAZ and PD-L1 is not conserved in multiple mouse cell lines. **A,** Establishment of human TAZ-S89A–overexpressing mouse cell lines. **B,** TAZ-S89A overexpression has no effect on *Pd-l1* mRNA expression in HC11, NMuMG, E0771, E10, or B16-OVA mouse cell lines, while *Ctgf* is upregulated by TAZ-S89A in each of these cell lines. (qRT-PCR; mean + SEM, $n = 2$ biological replicates, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **C,** Positive control for the detection of *Pd-l1* mRNA by qRT-PCR. 293T cells were transfected with *Pd-l1* cDNA (qRT-PCR; mean + SEM, $n = 2$ technical replicates, ***, $P < 0.001$). **D,** TAZ-S89A overexpression in HC11, NMuMG, E10, and B16-OVA does not affect membrane PD-L1 protein levels. **E,** Establishment of mouse TAZ-S89A–overexpressing mouse cell lines. **F,** Mouse TAZ-S89A overexpression in HC11 and NMuMG does not upregulate membrane PD-L1 protein levels. **G,** The human *PD-L1* promoter can be activated by either human or mouse TAZ-S89A/TEAD4, while the mouse *Pd-l1* promoter is much less responsive to TAZ-S89A/TEAD4 (luciferase assay; mean + SEM, $n = 3$ biological replicates, **, $P < 0.01$). **H** and **I,** Other candidate transcriptional targets of TAZ identified in our NanoString screen in MCF10A are not similarly regulated by human TAZ-S89A overexpression in HC11 (**H**) or NMuMG (**I**; qRT-PCR; mean + SEM, $n = 2$ biological replicates). See also Supplementary Fig. S4.

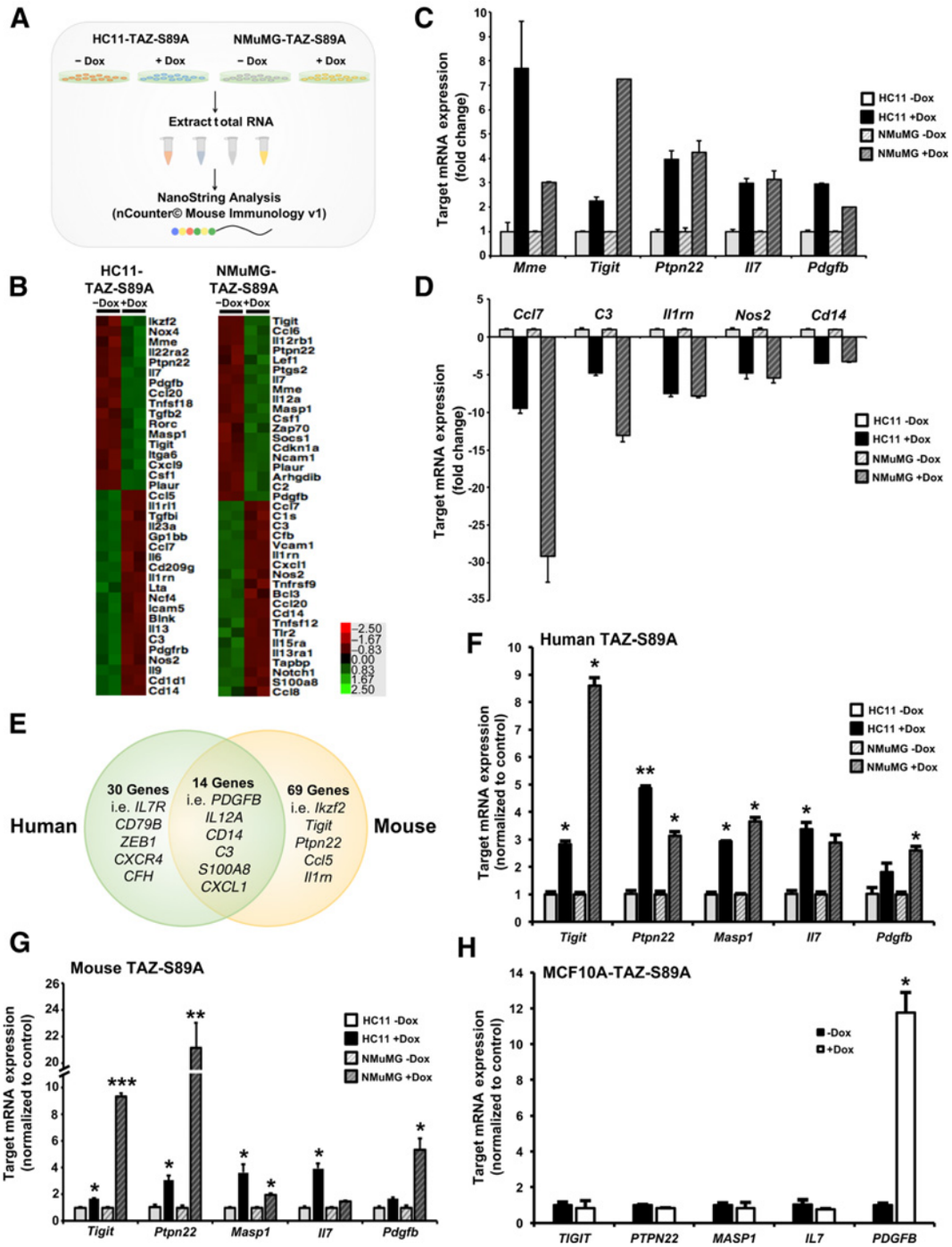



**Figure 7.**

Screen for immune-related transcriptional targets of TAZ in mouse cell lines. **A,** Experimental design to determine immune-related targets of TAZ in mouse cells. HC11-TAZ-S89A or NMuMG-TAZ-S89A cell lines were induced for 48 hours with doxycycline (Dox) before RNA was collected (biological duplicate) and subjected to NanoString analysis using the nCounter Mouse Immunology v1 panel. **B,** Heatmap summarizing the top up- (green) and downregulated (red) genes affected by human TAZ-S89A overexpression (+Dox) in mouse cell lines (color scale denotes Euclidean distance of mRNA count). **C** and **D,** Summary of the top genes up- (**C**) or downregulated (**D**) by TAZ-S89A in HC11 and NMuMG from NanoString gene expression profiling (NanoString; mean + SEM, $n = 2$ biological replicates). **E,** Comparison of candidate immune-related targets of TAZ identified in our human and mouse screens. **F,** Validation of the top genes upregulated by TAZ-S89A overexpression in HC11 and NMuMG (qRT-PCR; mean + SEM, $n = 2$ biological replicates, *, $P < 0.05$; **, $P < 0.01$). **G,** Top candidate TAZ-regulated genes identified by our screen were also upregulated by mouse TAZ-S89A overexpression in HC11 and NMuMG (qRT-PCR; mean + SEM, $n = 2$ biological replicates, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **H,** Multiple candidate targets of TAZ-S89A in HC11 and NMuMG are not regulated by TAZ in human MCF10A cells (qRT-PCR; mean + SEM, $n = 2$ biological replicates, *, $P < 0.05$). See also Supplementary Fig. S5.

many TAZ and YAP transcriptional targets are conserved between these two species including CTGF (35). Moreover, both human and mouse *PD-L1* genes are regulated by other stimuli such as hypoxia (45). However, immune-related transcriptional programs, and regulatory sequences in general, can differ dramatically between human and mice and an alignment of the human and mouse *PD-L1* promoters shows that there are notable differences between these sequences. Thus, our data reveal important species-specific differences in PD-L1 regulation that were previously unrealized in the literature. To the best of our knowledge, this is the first (and only) description of a mechanism of PD-L1 regulation that occurs in human cells but not in mice. This finding could have tremendous significance for future studies and underscores the need to choose appropriate model systems when studying tumor immunology. This conclusion may also help reconcile notable discrepancies between earlier publications that argue that the Hippo pathway both promotes and antagonizes cancer immune evasion. Our study, and another recent study in lung cancer, supports the conclusion that human TAZ suppresses antineoplastic immune responses (46). However, Moroishi and colleagues have observed that loss of LATS1/2 in fact inhibits immune evasion through activation of TAZ and YAP in three different mouse syngeneic tumor models (20). It is possible that species-specific transcriptional regulation by TAZ underlies this distinction.

A number of recent publications have tied the Hippo pathway to other immune-related phenomena including cerebral ischemia reperfusion injury, neurodegeneration, and post-myocardial infarction cardiac remodeling (47–49). The targets of TAZ and YAP revealed by our screen may also contribute to our understanding of how the Hippo pathway influences each of these processes. TAZ and YAP induced dramatic changes in expression for many immune-related genes regulating diverse phenomena ranging from inflammasome formation to complement. Interestingly, many of the top candidate TAZ- and YAP-regulated genes we identified are involved in normal immune cell maturation and differentiation (e.g., *IL7R*; ref. 50). Future work will be necessary to validate these candidates as *bona fide* transcriptional targets of TAZ and YAP, to determine whether these genes are regulated in a species-specific manner and to appreciate the functional significance of these relationships in development and disease.

In summary, in this study we have implicated the Hippo pathway and TAZ as key players in directing cancer immune evasion and have discovered differences in the transcriptional regulation of human and mouse PD-L1. These findings offer compelling evidence that the Hippo pathway and its effectors TAZ and YAP are important regulators of the immune response and present exciting opportunities for future studies.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** H.J. Janse van Rensburg, T. Azad, C.H. Graham, X. Yang
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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** H.J. Janse van Rensburg, T. Azad, B. Snetsinger
**Writing, review, and/or revision of the manuscript:** H.J. Janse van Rensburg, T. Azad, P. Khanal, C.H. Graham, M.J. Rauh, X. Yang
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** H.J. Janse van Rensburg, Y. Hao, B. Snetsinger
**Study supervision:** X. Yang

## Acknowledgments

This work was supported by grants from the Canadian Institute of Health Research (#119325, 148629 to X. Yang), the Canadian Breast Cancer Foundation/Canadian Cancer Society (to X. Yang), and the Canada Foundation for Innovation (to M.J. Rauh). H.J. Janse van Rensburg is supported by a Canada Graduate Scholarship and Queen Elizabeth II Graduate Scholarship in Science and Technology. We would like to thank Canadian Institute of Health Research, the Canadian Breast Cancer Foundation/Canadian Cancer Society, and the Canada Foundation for Innovation for their financial support. We would like to thank Shannyn MacDonald-Goodfellow for her technical expertise and Scott Gerber for providing us with the B16-OVA cell line.

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Received October 12, 2017; revised December 14, 2017; accepted January 10, 2018; published OnlineFirst January 16, 2018.

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