# Eya3 promotes breast tumor–associated immune suppression via threonine phosphatase–mediated PD-L1 upregulation

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**Eya proteins are critical developmental regulators that are highly expressed in embryogenesis but downregulated after development. Amplification and/or re-expression of Eyas occurs in many tumor types. In breast cancer, Eyas regulate tumor progression by acting as transcriptional cofactors and tyrosine phosphatases. Intriguingly, Eyas harbor a separate threonine (Thr) phosphatase activity, which was previously implicated in innate immunity. Here we describe what we believe to be a novel role for Eya3 in mediating triple-negative breast cancer–associated immune suppression. Eya3 loss decreases tumor growth in immune-competent mice and is associated with increased numbers of infiltrated CD8$^+$ T cells, which, when depleted, reverse the effects of Eya3 knockdown. Mechanistically, Eya3 utilizes its Thr phosphatase activity to dephosphorylate Myc at pT58, resulting in a stabilized form. We show that Myc is required for Eya3-mediated increases in PD-L1, and that rescue of PD-L1 in Eya3-knockdown cells restores tumor progression. Finally, we demonstrate that Eya3 significantly correlates with PD-L1 in human breast tumors, and that tumors expressing high levels of Eya3 have a decreased CD8$^+$ T cell signature. Our data uncover a role for Eya3 in mediating tumor-associated immune suppression, and suggest that its inhibition may enhance checkpoint therapies.**

## Introduction

The eyes absent family of proteins (Eyas 1–4) are crucial regulators of embryogenesis, contributing to the development of diverse tissues such as the ear (1, 2), eye (3), craniofacial complex (4, 5), colon (6, 7), esophagus (8), heart (9, 10), kidney (1, 11), lung (11, 12), and muscle (11, 13). The ability of Eya proteins to contribute to the proper development of numerous organs is likely due in part to their diverse functions, acting both as transcriptional regulators and as phosphatases (14, 15).

The best-described function of the Eya proteins is as transcriptional cofactors to the Six family of homeoproteins (16, 17). All members of the Eya family act as transactivating proteins when bound to Six family members, and the Six/Eya complex acts as a bipartite transcription factor in the development of many organs (18, 19). Described Six/Eya transcriptional targets include c-Myc, cyclin A/D, VEGF-C, and TGF-βRI (20–25). In addition to acting as transcriptional cofactors, the Eyas belong to the haloacid dehydrogenase (HAD) superfamily, as they contain intrinsic tyrosine (Tyr) phosphatase activity in their C-termini (14, 15, 26). The Tyr phosphatase activity of Eya proteins has been shown to regulate cell proliferation and apoptosis (1, 26–29), migration (28, 30, 31), invasion (28), survival (32), and angiogenesis (30). Whether these functions are solely due to the Tyr phosphatase activity of Eya, or whether the interaction with Six family members also plays a role, is not clear. Since the Six1/Eya interaction has been shown to mediate TGF-β signaling and epithelial-mesenchymal transition (EMT) (33, 34), this interaction likely contributes to migratory and invasive properties mediated by Eya. Because Eya proteins mediate key developmental properties that are utilized in tumors, it is not surprising that their misexpression is observed in numerous cancers.

Eya family members are expressed and/or amplified in Ewing sarcoma (35), lung (36), ovarian (37), and breast cancers (28, 33, 38), as well as Wilms tumors (39). In breast cancer, Eyas play a role in tumor progression and metastasis, in large part via their action with Six1 (33, 34). Currently, novel compounds are being created to inhibit the Six/Eya interaction (40) and the Tyr phosphatase activity of Eyas (31, 40, 41), which may have utility as tools to dissect the different functions of Eya, and as potential antimetastatic agents. However, these compounds do not target the third described activity of Eyas: a novel threonine (Thr) phosphatase activity found in their N-termini (42–44). Intriguingly, the Thr phosphatase activity of Eya regulates the innate immune response to denatured DNA and double-stranded RNA in mouse embryonic fibroblasts and in *Drosophila* by promoting the expression of CXCL10 and IFN-β (43, 44). This novel link of Eya proteins with the innate immune system suggests that Eya expression in tumor cells may have additional functions through regulation of antitumor immune responses.

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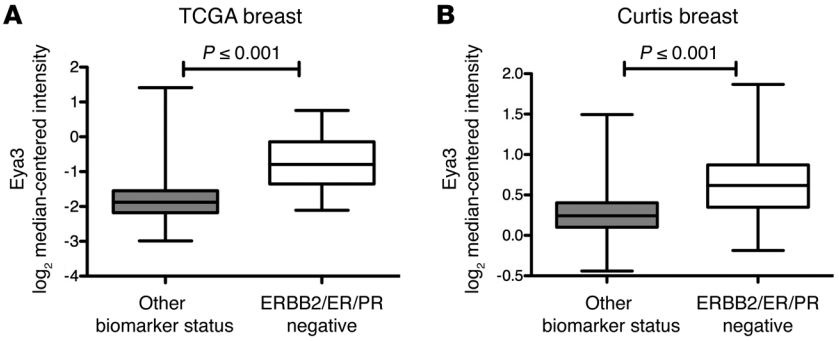


**Figure 1. Eya3 is expressed in TNBCs.** Eya3 gene expression ($log_2$ median-centered intensity) graphed as mean with SD in data sets separated by TNBC and other biomarker status. Data sets were categorized as $ER^-PR^-ERBB2^-$ if negative for ER, PR, and ERBB2 protein and "other biomarker status" if tumors were positive for either ER, PR, or ERBB2 protein. Data were obtained from the Oncomine portal (https://www.oncomine.com). Data represent mean ± SEM, and significance was measured by a 2-tailed Student's *t* test. (**A**) Eya3 expression from TCGA data set. Other biomarker status, $n = 250$; $ER^-PR^-ERBB2^-$, $n = 46$. (**B**) Eya3 expression from Curtis data set (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3440846/). Other biomarker status, $n = 1,340$; $ER^-PR^-ERBB2^-$, $n = 211$. Tumors with no biomarker status were excluded.

Evading immune destruction is now recognized as a hallmark of cancer (45), and numerous mechanisms by which cancer cells evade immune detection have been discovered. These mechanisms include cancer cell secretion of factors that suppress $CD8^+$ T cell function and differentiation/proliferation (46–48), secretion of factors to attract or modify additional immune cells that suppress $CD8^+$ T cell function (46, 49, 50), the ability of cancer cells to become unrecognizable to $CD8^+$ T cells through loss of antigen processing/presentation (51), and cancer cell presentation of ligands that render $CD8^+$ T cells unable to function and/or lead to $CD8^+$ T cell apoptosis (46, 48, 52).

PD-L1 is an immunosuppressive ligand that is normally expressed by myeloid-lineage cells and can bind to its receptor, PD-1, on activated, functional $CD8^+$ cytotoxic T cells. Binding of PD-L1 to PD-1 causes inhibition of $CD8^+$ T cell proliferation and function while promoting $CD8^+$ T cell apoptosis and anergy/exhaustion (53–57). PD-L1 is an important part of the adaptive immune response as it serves to prevent autoimmunity (58) and fetal-maternal rejection during pregnancy (59). PD-L1 is normally expressed in myeloid cells in addition to a handful of epithelial cell types, including cells of the lung, heart, and placenta (60). It is often overexpressed in cancers, including breast cancer, where it dampens the $CD8^+$ T cell tumor response and is associated with tumor aggressiveness and poor prognosis (53, 61–67). One of the most highly aggressive and metastatic types of breast cancer is the triple-negative breast cancer (TNBC) subtype. TNBCs are highly immunogenic, because of their high genomic instability and mutational load, and yet are frequently seen to possess low levels of infiltrated $CD8^+$ T cells (68–75). The loss of $CD8^+$ T cells in TNBC correlates with high levels of PD-L1 in these tumors, and clinical trials using immunotherapies against PD-L1/PD-1 in TNBC are showing favorable outcomes (76, 77).

Here we show, for the first time to our knowledge, that Eya3 expression in TNBC promotes tumor growth by regulating the adaptive immune response. We demonstrate that high Eya3 results in decreased numbers of $CD8^+$ T cells in tumors, and leads to $CD8^+$ T cell exhaustion. Mechanistically, we demonstrate that Eya3, through its Thr phosphatase activity, and through regulating c-Myc, upregulates PD-L1, and that this upregulation is required for Eya3-mediated alterations in adaptive immune response and tumor growth. Further, we demonstrate that Eya3 and PD-L1 are significantly associated in breast cancer, and that breast cancer patients with high Eya3 have a significantly reduced $CD8^+$ T cell signature. Our findings suggest a potential role for Eya3 as a biomarker or target to enhance immune therapies in TNBC.

## Results

*Eya3 decreases the number of $CD8^+$ cytotoxic T cells in mammary tumors*. By examining public gene expression data sets, we found that Eya3 is most highly expressed in the TNBC subtype compared with other subtypes of breast cancer (Figure 1). Thus, to determine the role of Eya3 in TNBC, and to specifically examine whether Eya3 has any function in tumor immunity, we knocked down Eya3 in 2 immune-competent murine TNBC cell lines: the BALB/c-derived 66cl4 cell line (78) and the FVB/N-Tg(MMTV-PyVmT)–derived Met1 cell line (79). Knockdown (KD) was confirmed using quantitative reverse transcription PCR (RT-qPCR) and Western blot analysis (Figure 2, A and B). To determine whether KD of Eya3 altered parameters previously associated with Eya3 in human breast cancer cell lines (28), we performed cell growth assays using IncuCyte Live-Cell Analysis from Essen BioScience and in vitro migration assays. Knockdown of Eya3 decreased the proliferative and migratory abilities of 66cl4 cells, similar to what has been previously reported in human TNBC cell lines (28). However, in Met1 cells, KD of Eya3 had no effect on proliferation, although it did still significantly affect migration, suggesting that Eya3's ability to promote proliferation may be context-dependent (Figure 2, C and D).

To determine the role of Eya3 in TNBC growth in vivo, in the context of an intact immune system, we orthotopically injected 66cl4 and Met1 scramble (SCR) and Eya3-KD cell lines into the mammary fat pads of BALB/c and FVB/N mice, respectively. Although KD of Eya3 inhibited proliferation in vitro in 66cl4 cells (Figure 2C), KD of Eya3 significantly delayed tumor growth in vivo in both mouse models without any clear alteration in proliferation, when examined at the time of sacrifice (Figure 3, A–D, and Figure 4, A–D). Because the 66cl4-SCR and Eya3-KD cell lines were clonal (whereas the Met1-KD lines were pooled populations), we repeated this in vivo experiment using two 66cl4-SCR and two 66cl4-Eya3-KD clones, to ensure that our tumor growth effects were not due to clonal variability. Importantly, we observed a similar, and consistent, significant delay in tumor growth with Eya3-KD when compared with both SCR clones (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI96784DS1).

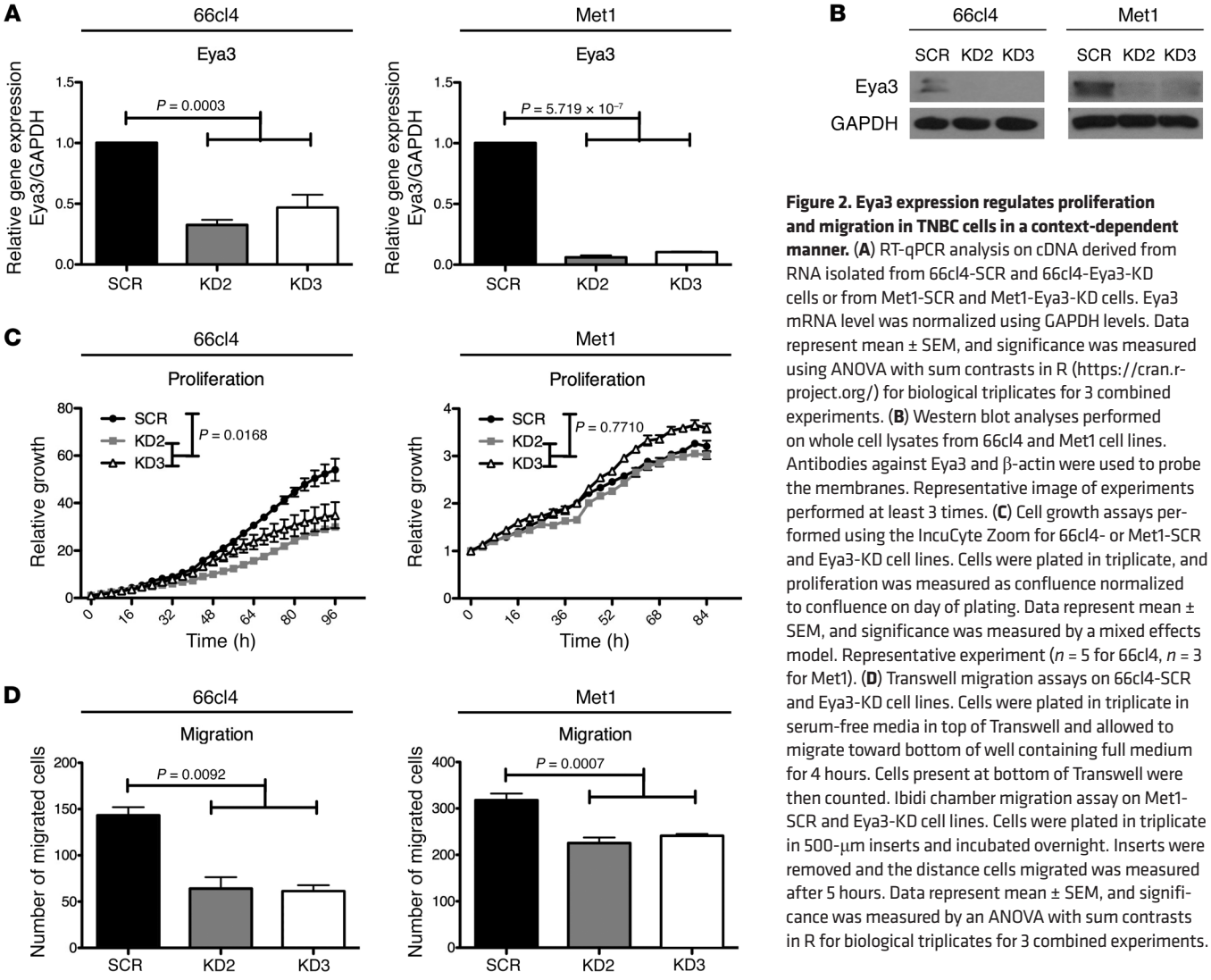


**Figure 2. Eya3 expression regulates proliferation and migration in TNBC cells in a context-dependent manner.** (**A**) RT-qPCR analysis on cDNA derived from RNA isolated from 66cl4-SCR and 66cl4-Eya3-KD cells or from Met1-SCR and Met1-Eya3-KD cells. Eya3 mRNA level was normalized using GAPDH levels. Data represent mean ± SEM, and significance was measured using ANOVA with sum contrasts in R (https://cran.r-project.org/) for biological triplicates for 3 combined experiments. (**B**) Western blot analyses performed on whole cell lysates from 66cl4 and Met1 cell lines. Antibodies against Eya3 and β-actin were used to probe the membranes. Representative image of experiments performed at least 3 times. (**C**) Cell growth assays performed using the IncuCyte Zoom for 66cl4- or Met1-SCR and Eya3-KD cell lines. Cells were plated in triplicate, and proliferation was measured as confluence normalized to confluence on day of plating. Data represent mean ± SEM, and significance was measured by a mixed effects model. Representative experiment ($n$ = 5 for 66cl4, $n$ = 3 for Met1). (**D**) Transwell migration assays on 66cl4-SCR and Eya3-KD cell lines. Cells were plated in triplicate in serum-free media in top of Transwell and allowed to migrate toward bottom of well containing full medium for 4 hours. Cells present at bottom of Transwell were then counted. Ibidi chamber migration assay on Met1-SCR and Eya3-KD cell lines. Cells were plated in triplicate in 500-μm inserts and incubated overnight. Inserts were removed and the distance cells migrated was measured after 5 hours. Data represent mean ± SEM, and significance was measured by an ANOVA with sum contrasts in R for biological triplicates for 3 combined experiments.

As we did not observe proliferative differences in vivo, yet the tumor growth was dramatically inhibited with Eya3 KD, we asked whether the immune system may be playing a role in inhibiting the tumor when Eya3 levels were decreased. In both the 66cl4 and Met1 models, tumors that expressed Eya3 contained fewer $CD8^+$ T cells as determined by immunohistochemistry (IHC) and flow cytometry when compared with their Eya3-KD counterparts (Figure 3, E–G, and Figure 4, E–G). Interestingly, Eya3 expression did not significantly affect the number of $CD4^+$ T cells seen within the tumors, as assessed using flow cytometry in both models (Figure 3H and Figure 4H). Additionally, we observed significant increases in the populations of $NK1.1^+$ natural killer cells, $CD11c^+$ dendritic cells, and $F4/80^+$, $CD80^+$, and $CD206^+$ macrophages in response to Eya3 KD. No significant differences in $CD4^+CD25^+$ T cells or $CD11b^+$ myeloid cells were observed upon Eya3 KD (Supplemental Figure 2). Because the presence of infiltrated $CD8^+$ T cells in breast cancer is known to inversely correlate with tumor growth and aggressiveness, particularly in TNBC, and because their presence is a good predictor of overall survival, prognoses, and response to chemotherapies (72, 80–83), we focused on the role of Eya3 in regulating $CD8^+$ T cells.

*Eya3 increases tumor growth by suppressing $CD8^+$ T cells.* To determine whether decreased numbers of $CD8^+$ T cells within the tumor are critical for Eya3-mediated enhancement of tumor growth, we injected a depleting anti-CD8α antibody into BALB/c and FVB mice. Three days after injection of anti-CD8α or control IgG antibody, we performed flow cytometry on blood from the treated mice, which confirmed that the anti-CD8α–injected mice had significantly reduced $CD8^+$ T cells in comparison with IgG-injected control mice (Figure 5A and Figure 6A). We then injected the 66cl4-SCR and Eya3-KD or Met1-SCR and Eya3-KD cell lines into the mammary fat pads of the BALB/C and FVB mice, respectively. The mice continued to receive control or CD8-depleting antibodies weekly for the continuation of the study. As previously observed, both 66cl4 and Met1 tumors expressing Eya3 grew more rapidly than Eya3-KD tumors in IgG-injected control mice (Figure 5B and Figure 6B). Depletion of $CD8^+$ T cells increased the growth of Eya3-KD tumors almost to the levels of SCR tumors in both the 66cl4 and Met1 tumor models, suggesting that the ability of Eya3 to enhance tumor growth is due in large part to its ability to regulate cytotoxic T cells. While depletion of $CD8^+$ T cells in the SCR controls also enhanced tumor growth, as expected, it did not

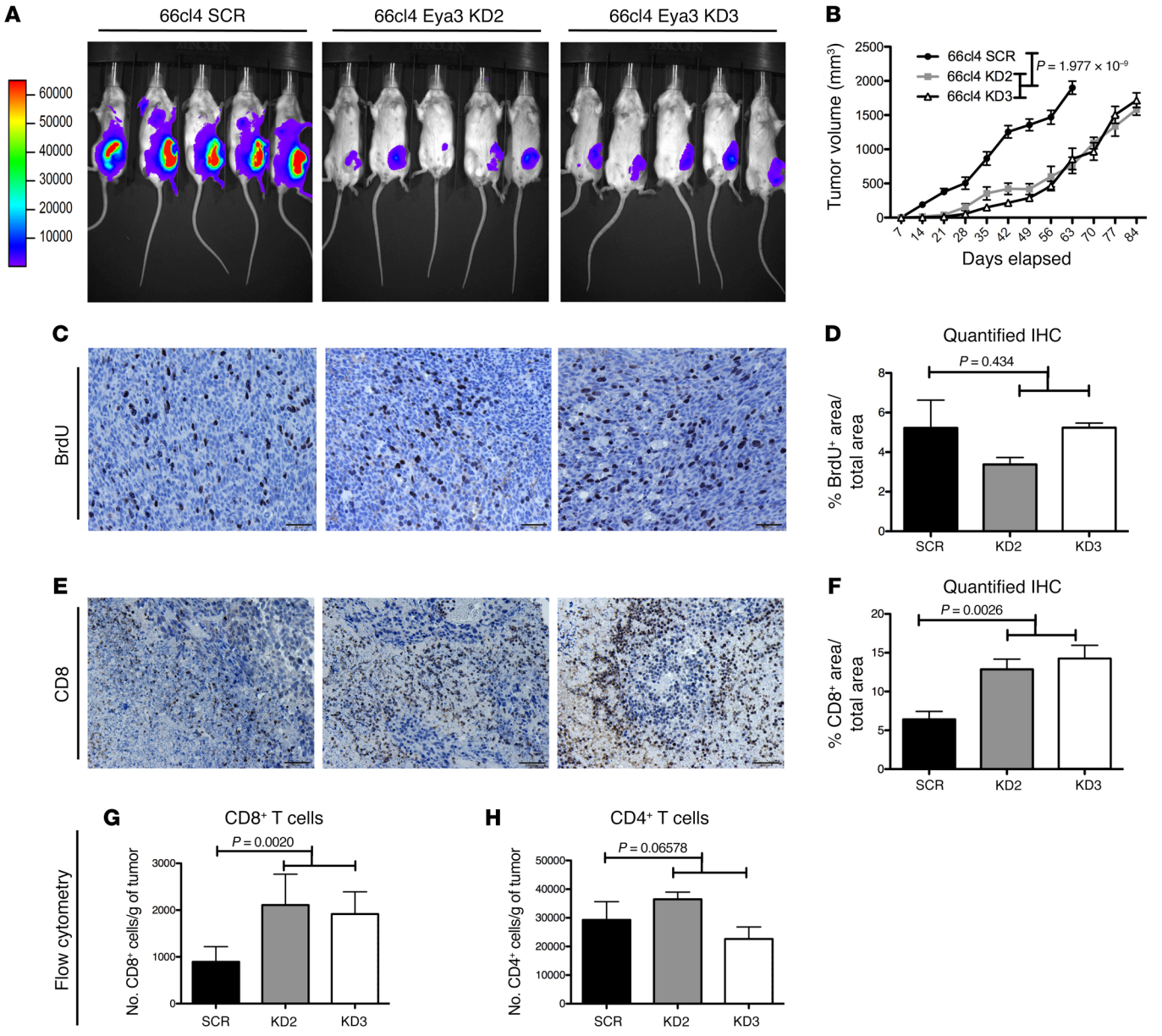


**Figure 3. Eya3 regulates CD8⁺ T cells in 66cl4 mammary carcinoma tumors.** (**A**) Representative bioluminescence images of BALB/c mice bearing 66cl4-SCR or Eya3-KD tumors at week 6 after injection. (**B**) Tumor volume of 66cl4-SCR and Eya3-KD tumors in BALB/c mice as measured using calipers. Each point represents the mean tumor size ± SEM at that time point after injection, and a mixed effects model was used to measure significance. Representative experiment ($n$ = 3); $n$ = 10 mice per cell line. (**C**) Representative pictures, original magnification ×20, of IHC for anti-BrdU staining performed on 66cl4-SCR or Eya3-KD tumors. Five sections per tumor were stained and 5 fields of view photographed for each tumor. (**D**) Quantification of BrdU IHC performed on 66cl4-SCR or Eya3-KD tumors. Data represent mean ± SEM. Significance was measured using ANOVA with sum contrasts in R for 5 tumor sections with 5 fields of view scored for each section. (**E**) Representative pictures, original magnification ×20, of IHC performed for CD8⁺ on 66cl4-SCR or Eya3-KD tumors. Five sections per tumor were stained and 5 fields of view photographed for each tumor. (**F**) Quantification of CD8⁺ IHC shown in **E**. Data represent mean ± SEM. Significance was measured using ANOVA with sum contrasts in R for 5 tumor sections with 5 fields of view scored for each section. (**G** and **H**) Calculated number of CD8⁺ and CD4⁺ T cells present per gram of 66cl4-SCR and Eya3-KD tumor. Tumors were isolated (SCR, $n$ = 5; KD2, $n$ = 5; KD3, $n$ = 7) and analyzed by flow cytometry. CD8⁺ T cells defined as CD45⁺CD3⁺CD8⁺CD4⁻. CD4⁺ T cells defined as CD45⁺CD3⁺CD8⁻CD4⁺. Data represent mean ± SEM. Significance was measured using ANOVA with sum contrasts in R. Representative experiment ($n$ = 2).

enhance the growth to the same extent as observed in the Eya3-KD cells in either tumor model (Figure 5C and Figure 6C).

*Eya3 regulates CD8⁺ T cell function in mammary carcinomas.* The data above demonstrate that regulation of CD8⁺ T cells is critical for Eya3 to enhance tumor growth. While differences in the number of cytotoxic T cells within the tumor were observed with Eya3 KD, their functionality remained unknown. Thus, we repeated injection of 66cl4 cells into the mammary fat pads of a small cohort of BALB/c mice, and isolated tumors 3 weeks after injection to examine the activation state of tumor-associated CD8⁺ T cells. Intriguingly, a higher percentage of CD8⁺ T cells expressed Tim3, Blimp1, T-bet, Eomes, and PD-1, markers asso-

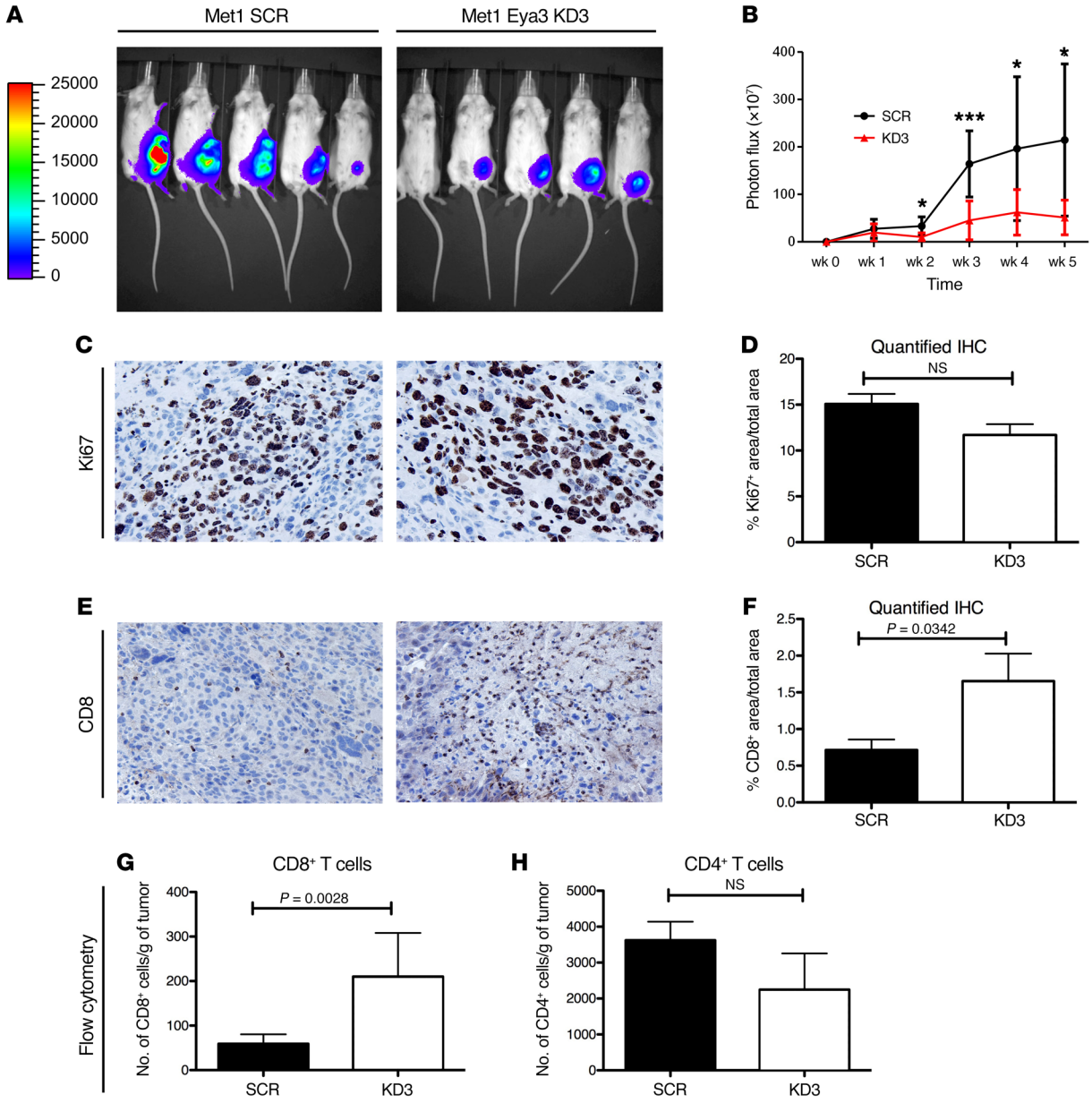


**Figure 4. Eya3 regulates CD8⁺ T cells in Met1 mammary carcinoma tumors.** (**A**) Representative bioluminescence images of FVB mice bearing Met1-SCR or Eya3-KD tumors at week 5 after injection. One Eya3 KD was used, as Eya3 KD2 lost Eya3 suppression. (**B**) Tumor volume of Met1-SCR and Eya3-KD tumors in FVB mice as measured by luciferase signal of primary tumor over time. Points represent the tumor size at a given time after injection ± SEM. A mixed effects model was used to measure significance. $n = 10$ mice per cell line. (**C**) Representative pictures, original magnification ×40, of IHC for Ki67 staining performed on 66cl4-SCR or Eya3-KD tumors. Five sections per tumor were stained and 5 fields of view photographed for each tumor. (**D**) Quantification of Ki67 IHC performed on Met1-SCR or Eya3-KD tumors. Data represent mean ± SEM. Significance was determined using a 2-tailed Student's *t* test on quantification from 5 tumor sections. Five fields of view were scored for each section. (**E**) Representative pictures, original magnification ×20, of IHC for CD8⁺ performed on Met1-SCR or Eya3-KD tumors. Five sections per tumor were stained and 5 fields of view photographed for each tumor. (**F**) Quantification of CD8⁺ IHC performed on Met1-SCR or Eya3-KD tumors. Data represent mean ± SEM. Significance was measured using a 2-tailed Student's *t* test for 5 tumor sections with 5 fields of view scored for each section. (**G** and **H**) Calculated number of CD8⁺ and CD4⁺ T cells present per gram of Met1-SCR and Eya3-KD tumors. Tumors were isolated (SCR, $n = 7$; KD3, $n = 8$) and analyzed using flow cytometry. CD8⁺ T cells defined as $CD45^+CD3^+CD8^+CD4^-$. $CD4^+$ T cells defined as $CD45^+CD3^+CD8^-CD4^+$. Data represent mean ± SEM. Significance was measured using a 2-tailed Student's *t* test. $^*P < 0.05$, $^{***}P < 0.001$. **C**–**H** are from a repeated experiment and are not from the mice pictured in **A** and **B**.

ciated with T cell exhaustion or loss of function (84), in 66cl4-SCR tumors than in 66cl4-Eya3-KD tumors (Figure 7, A–E). Conversely, 66cl4-Eya3-KD tumors, when compared with the 66cl4-SCR counterparts, contained a significantly higher percentage of CD8⁺ T cells expressing IL-2 and IFN-γ, and the CD8⁺ T cells also showed trends of increased CD44 and TNF-α expression, all markers associated with cytotoxic T cell activation (Figure 7, F–I). Thus, not only does Eya3 expression lead to decreases in the numbers of CD8⁺ T cells, it is associated with indicators of CD8⁺ T cell loss of function.

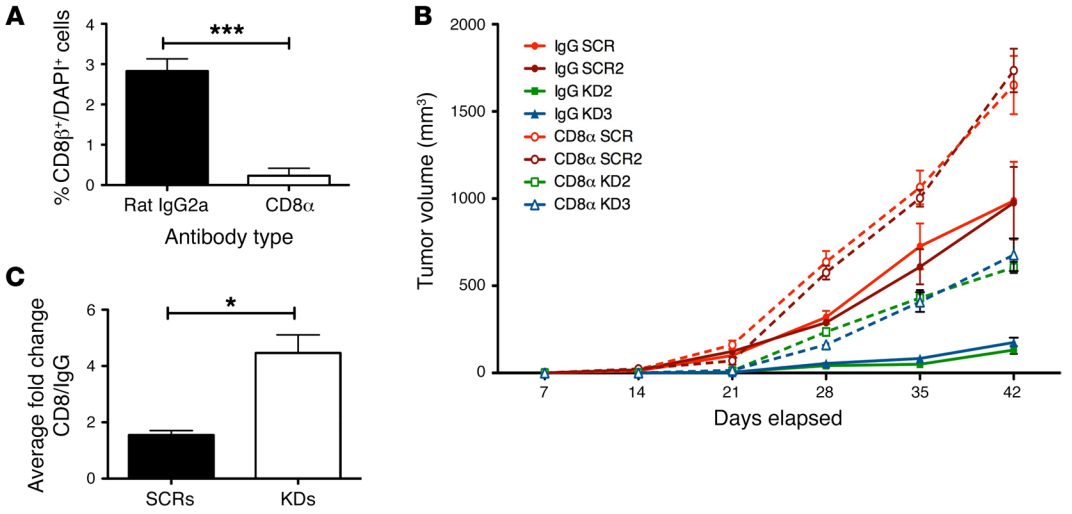


**Figure 5. Eya3 regulation of CD8⁺ T cells is required for increased 66cl4 mammary carcinoma growth.** (**A**) Percentage of CD8⁺ cells in 50 μl blood from animals treated with rat IgG or CD8α-depleting antibody as measured by flow cytometry. Blood was isolated from 10 mice per group. Data represent mean ± SEM. Significance was measured using a 2-tailed Student's *t* test. (**B**) Tumor volume of 66cl4-SCR and Eya3-KD tumors treated with rat IgG or CD8⁺-depleting antibody, as measured using calipers. Each point represents mean of tumors from mice in that condition ± SEM, and a mixed effects model was used to measure significance. *n* = 5 mice per group. Solid lines and filled symbols represent mice treated with IgG antibody. Dotted lines and open symbols represent mice treated with anti-CD8⁺ antibody. (**C**) Fold change of tumor growth between IgG-treated 66cl4-SCR and SCR2 tumors and CD8⁺-treated 66cl4-SCR and SCR2 tumors and fold change of tumor growth between IgG-treated 66cl4-Eya3 KD2 and KD3 tumors and CD8⁺-treated 66cl4-Eya3 KD2 and KD3 tumors. Data represent mean ± SEM. Significance was measured using a 2-tailed Student's *t* test of average fold change of CD8⁺/IgG tumor size averaged for every time point over the experimental time course. *n* = 5 mice per group. $^*P < 0.05$, $^{***}P < 0.001$.

While the expression of Eya3 did not affect the proliferation of mammary carcinoma cells in vivo when examined at the endpoint of the study (Figures 3 and 4), the fact that its presence leads to T cell exhaustion suggests that the growth differences could be attributed to different apoptotic rates in the tumor in response to the immune system. Thus, we examined apoptosis of tumor cells 5 weeks after injection using propidium iodide (PI) and annexin V in the same study as outlined above in which we examined T cell markers. Intriguingly, we observed that high expression of Eya3 protected tumor cells from both early-stage (as shown by the presence of annexin V) and late-stage (as shown as by the presence of annexin V and PI) apoptosis (Figure 7, J–K), suggesting that death of the tumor cells significantly contributes to tumor size when Eya3 is knocked down.

*Eya3 increases PD-L1 expression through Thr phosphatase–mediated regulation of c-Myc.* Cancers promote exhaustion of tumor-infiltrating CD8⁺ T cells via numerous mechanisms. As PD-L1 expression on tumor cells, including TNBC, is known to lead to tumor-associated immunosuppression (53, 61, 63, 66, 67, 76), we asked whether Eya3 regulates PD-L1. We observed that PD-L1 mRNA expression is reduced in both 66cl4 and Met1-Eya3-KD cells compared with their control counterparts (Figure 8A and Supplemental Figure 3A). Although PD-L1 mRNA levels were increased in the presence of Eya3 expression, we did not observe increased PD-L1 protein on the surface of these cells in culture. The inability to observe surface PD-L1 protein in a culture setting has previously been described (67, 85); thus we orthotopically injected our 66cl4-Eya3-KD and SCR control cell lines into the mammary fat pads of BALB/c mice, isolated the tumors when they had reached 1 $cm^3$ in size, and analyzed the surface of the cancer cells for PD-L1 protein by flow cytometry. We observed increased PD-L1 protein on the surface of tumor cells expressing Eya3 compared with those with Eya3 KD (Figure 8B).

Eya proteins are known to contain several domains with differing activities, including a transactivation domain, a Tyr phosphatase domain, and a completely separable Thr phosphatase domain (42, 86). As the Thr phosphatase domain has previously been implicated in innate immunity (43), we wanted to determine whether this domain is responsible for the altered PD-L1 expression. To this end, we created the previously described H79A mutation to render Eya3 Thr phosphatase dead (42). We confirmed the mutation efficacy by pulling down Eya3 from HEK293FT cells and examining its ability to dephosphorylate a Thr-phosphorylated peptide (Figure 8C). We then re-expressed WT Eya3 or Thr phosphatase–dead (H79A) Eya3 containing wobble mutations to avoid knockdown by Eya3 shRNAs in our 66cl4 and Met1-Eya3-KD cell lines, as well as previously described Eya3 mutants that render the protein Tyr phosphatase dead (D262N) (28) or unable to bind Six family members (A520R) (the equivalent of the A532R mutant of Eya2) (34). These latter constructs also contained wobble mutations to avoid knockdown by Eya3 shRNA within the 66cl4- and Met1-Eya3-KD cells (Figure 8D and Supplemental Figure 3B). Our data demonstrate that cells containing WT Eya3 upregulate PD-L1, whereas those containing the Thr phosphatase–dead Eya3 (H79A), even when expressed very efficiently, did not upregulate PD-L1 (Figure 8E and Supplemental Figure 3C). In contrast, the Tyr phosphatase–dead Eya3 (D262N), even when not highly expressed, was able to restore PD-L1 levels to a degree similar to that observed in the control cells, as well as observed with WT Eya3 add-back, suggesting that this activity of Eya3 is not required for its ability to modulate PD-L1 (Figure 8E and Supplemental Figure 3C). Additionally, the Six1-binding mutant of Eya3 (A520R) was only able to partially restore PD-L1 levels, particularly in the Met1 model

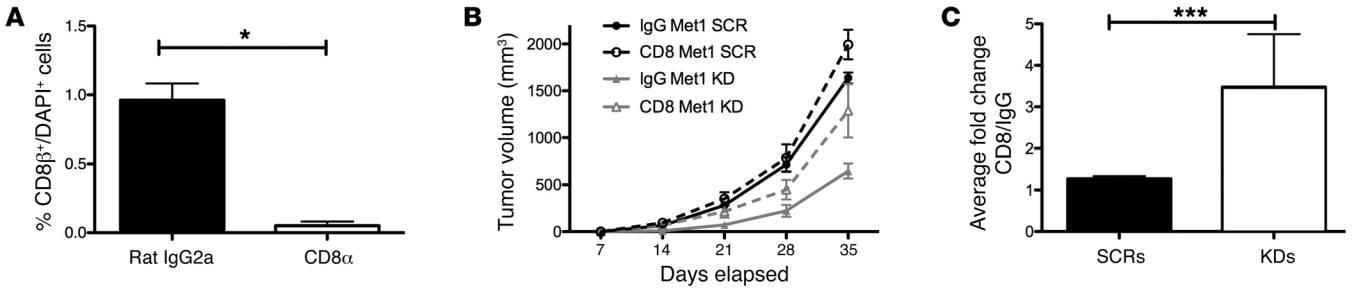


**Figure 6. Eya3 regulation of CD8⁺ T cells is required for increased Met1 mammary carcinoma growth.** (**A**) Percentage of CD8⁺ cells in 50 μl blood from animals treated with rat IgG or CD8α-depleting antibody as measured by flow cytometry. Blood was isolated from 5 mice per group. Data represent mean ± SEM. Significance was measured using a 2-tailed Student's *t* test. (**B**) Tumor volume of Met1-SCR and Eya3-KD tumors treated with rat IgG or CD8⁺-depleting antibody, as measured using calipers. Each dot represents mean of tumors from mice in that condition ± SEM, and a mixed effects model was used to measure significance. *n* = 5 mice per group. Solid lines and filled symbols represent mice treated with IgG antibody. Dotted lines and open symbols represent mice treated with anti-CD8⁺ antibody. (**C**) Fold change of tumor growth between IgG-treated Met1-SCR tumors and CD8⁺-treated Met1-SCR tumors and fold change of tumor growth between IgG-treated Met1-Eya3 KD tumors and CD8⁺-treated Met1-Eya3-KD tumors. Data represent mean ± SEM. Significance was measured using a 2-tailed Student's *t* test of average fold change of CD8⁺/IgG tumor size averaged for every time point over the experimental time course. *n* = 5 mice per group. $*P < 0.05$, $***P < 0.001$.

(Figure 8E and Supplemental Figure 3C), suggesting that the interaction with Six family members may be in part required to mediate upregulation of PD-L1 mRNA, but that it is not the main activity of Eya3 contributing to this effect. These data demonstrate that Eya3 mediates upregulation of PD-L1 mainly through its Thr phosphatase activity.

One known target of the Eya3 Thr phosphatase is c-Myc. The Eya3 Thr phosphatase stabilizes c-Myc by dephosphorylating it at pT58, preventing its degradation (87, 88, 89). As c-Myc is a transcriptional regulator of PD-L1 (90), we explored the possibility that regulation of c-Myc levels may be one mechanism by which the Eya3 Thr phosphatase regulates PD-L1 expression. We observed that the degradable pT58 form of c-Myc is increased when Eya3 expression is lost in both the 66cl4 and Met1 systems (Figure 9, A and E). This pT58 form of c-Myc is decreased when WT Eya3 is re-expressed, but is not decreased when Eya3 is Thr phosphatase–dead (Figure 9, A–E). These data suggest that Eya3 is stabilizing c-Myc levels in both systems via changing its phosphorylation state. Thus, to determine whether Eya3 is regulating PD-L1 mRNA levels via c-Myc, we knocked down c-Myc in the presence of either WT Eya3 or the Eya3 H79A mutant (Figure 9, B, C, F, and G). Our data demonstrate that c-Myc is required downstream of WT Eya3 to upregulate PD-L1 mRNA in both systems, but that it is not required downstream of the Eya3 H79A mutant (where PD-L1 is not increased compared with in the presence of WT Eya3) (Figure 9, D and H).

*PD-L1 mediates increased growth with Eya3 overexpression*. As we observed decreased PD-L1 on the surface of the mammary cancer cells in response to Eya3 KD, we asked whether PD-L1 expression was the means by which Eya3 regulates CD8⁺ T cells and tumor growth. To this end, we stably re-expressed PD-L1 in the 66cl4-Eya3-KD cells, which was confirmed by RT-qPCR (Figure 10A), and injected the cell lines into the mammary fat pads of BALB/c mice. Restoration of PD-L1 in Eya3-KD cells led to rescued levels of PD-L1 on the surface of the tumor cells compared with 66cl4-SCR control cells (Figure 10B). As previously observed, tumors expressing Eya3 (66cl4-SCR) had an increased rate of growth when compared with Eya3-KD tumors (Figure 10, C and D). Importantly, restoration of PD-L1 significantly restored tumor growth (Figure 10C). Tumors isolated at 42 days after injection exhibited similar proliferation rates, as measured by BrdU incorporation, in all of the groups (Figure 10E), again demonstrating that alterations in proliferation were not mediating the effects of Eya3 in this context. Further, live CD8⁺ T cells were increased in 66cl4-Eya3-KD tumors compared with 66cl4-SCR tumors, and re-expression of PD-L1 in 66cl4-Eya3-KD tumors decreased the numbers of live CD8⁺ T cells back to that observed in 66cl4-SCR control tumors (Figure 10F). Conversely, 66cl4-SCR tumors and KD tumors containing PD-L1 contained large numbers of dead CD8⁺ T cells when compared with 66cl4-Eya3-KD tumors (Figure 10G). Together, these data demonstrate that tumor growth mediated by Eya3 is due in large part to its ability to increase PD-L1 expression, altering T cell function and tumor immunity.

*Eya3 expression correlates with PD-L1 expression and a decreased CD8⁺ T cell signature in human breast cancers*. To determine whether Eya3 is associated with T cell suppression in human settings, we first examined whether Eya3 regulates PD-L1 expression in human breast cancer cells. We knocked down Eya3 in the human TNBC cell line MDA-MB-231 (91). KD was determined by RT-qPCR, and, as observed in the murine systems, KD of Eya3 resulted in a reduction of PD-L1 expression (Supplemental Figure 4, A and B). We further examined whether Eya3 expression and PD-L1 expression correlate in human breast cancer data sets. In The Cancer Genome Atlas (TCGA; https://www.oncomine.org) breast cancer data sets we observed a highly significant correlation between Eya3 and PD-L1 expression for all breast cancer subtypes and TNBCs (Figure 11, A and B). Because cells other than tumor cells in the microenvironment likely also express PD-L1, and immunohistochemical analysis of PD-L1 in the many cell types within a tumor can be difficult to interpret (61, 92–94), we used gene expression analysis to examine whether the correlation between Eya3 and PD-L1 appeared specific to the tumor cells. To this end, we identified breast cancers, using gene expression signatures, with a purity of greater than 80% tumor cells (Figure 11, C and D), and found that these tumors showed a strong significant correlation between Eya3 and

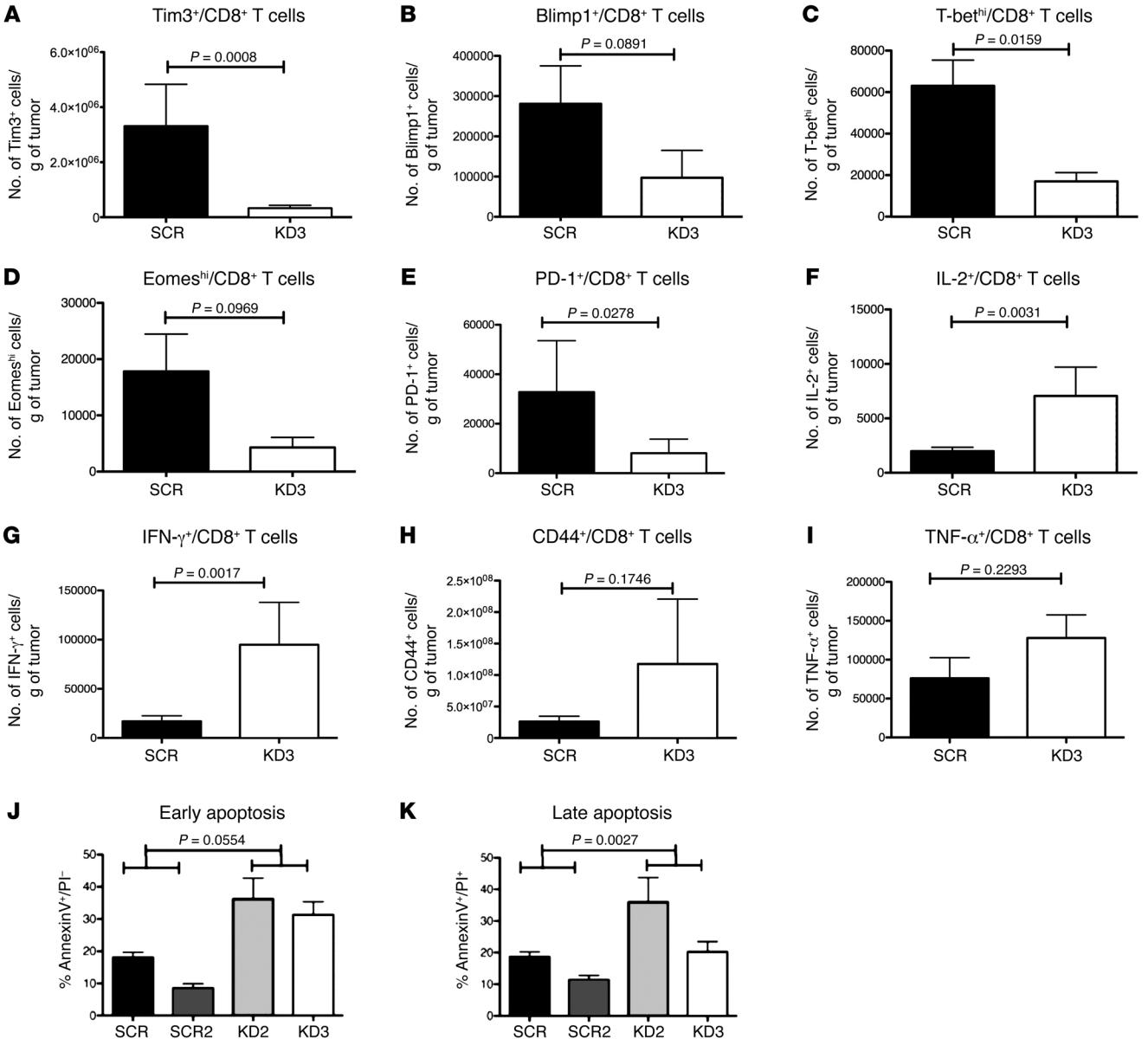


**Figure 7. Eya3 regulates CD8⁺ T cell exhaustion and promotes apoptosis of mammary carcinoma cells.** (**A**–**I**) Number of immune cells per gram of 66cl4-SCR and Eya3 KD3 tumors. Tumors were isolated 3 weeks after cancer cell injection (SCR, $n = 5$; KD3, $n = 5$), digested, and treated with brefeldin A, ionomycin, and PMA for 4 hours, and analyzed by flow cytometry. Representative experiment ($n = 2$). Data represent mean ± SEM. Significance was measured using a 2-tailed Student's $t$ test. (**A**) Tim3⁺CD8⁺ T cells were defined as GhostRed780⁻B220⁻MHCII⁻CD3⁺CD8⁺CD4⁻Tim3⁺ (Ghost Red 780, Tonbo Biosciences). (**B**) Blimp1⁺CD8⁺ T cells were defined as GhostRed780⁻B220⁻MHCII⁻CD3⁺CD8⁺CD4⁻Blimp1⁺. (**C**) T-bet$^{hi}$CD8⁺ T cells were defined as GhostRed780⁻B220⁻MHCII⁻CD3⁺CD8⁺CD4⁻T-bet$^{hi}$. (**D**) Eomes$^{hi}$CD8⁺ T cells were defined as GhostRed780⁻B220⁻MHCII⁻CD3⁺CD8⁺CD4⁻Eomes$^{hi}$. (**E**) PD-1⁺CD8⁺ T cells were defined as GhostRed780⁻B220⁻MHCII⁻CD45⁺CD3⁺CD8⁺CD4⁻PD-1⁺. (**F**) IL-2⁺CD8⁺ T cells were defined as GhostRed780⁻B220⁻MHCII⁻CD3⁺CD8⁺CD4⁻IL-2⁺. (**G**) IFN-γ⁺CD8⁺ T cells were defined as GhostRed780⁻B220⁻MHCII⁻CD45⁺CD3⁺CD8⁺CD4⁻IFN-γ⁺. (**H**) CD44⁺CD8⁺ T cells were defined as GhostRed780⁻B220⁻MHCII⁻CD3⁺CD8⁺CD4⁻CD44⁺. (**I**) TNF-α⁺CD8⁺ T cells were defined as GhostRed780⁻B220⁻MHCII⁻CD3⁺CD8⁺CD4⁻TNF-α⁺. Number of apoptotic cells per gram of 66cl4-SCR and Eya3 KD3 tumors. (**J** and **K**) Tumors were isolated 5 weeks after cancer cell injection (SCR, $n = 5$; SCR2, $n = 5$; KD2, $n = 5$; KD3, $n = 5$) and treated as outlined above. Data represent mean ± SEM. Significance was measured using ANOVA with sum contrasts in R. (**J**) Early-apoptotic carcinoma cells were defined as B220⁻MHCII⁻CD45⁻annexinV⁺PI⁻. (**K**) Late-apoptotic carcinoma cells were defined as B220⁻MHCII⁻CD45⁻annexinV⁺PI⁺.

PD-L1. In contrast, those tumors that were less than 60% pure tumor cells did not show a correlation between Eya3 and PD-L1 (Figure 11, C and D). These data suggest that Eya3 is contributing to PD-L1 expression specifically on the tumor cells. To further determine whether Eya3 expression correlates with a reduced CD8⁺ T cell signature within a tumor, we used the TCGA RNA sequencing data set to perform Cibersort analysis (95), to estimate the abundance of the different subtypes of T cells within the tumor. Importantly, we found that high Eya3 expression significantly correlates with lower estimates of tumor-infiltrating CD8⁺ T cells in breast cancer patients, including those with the TNBC subtype (Figure 11, E and F).

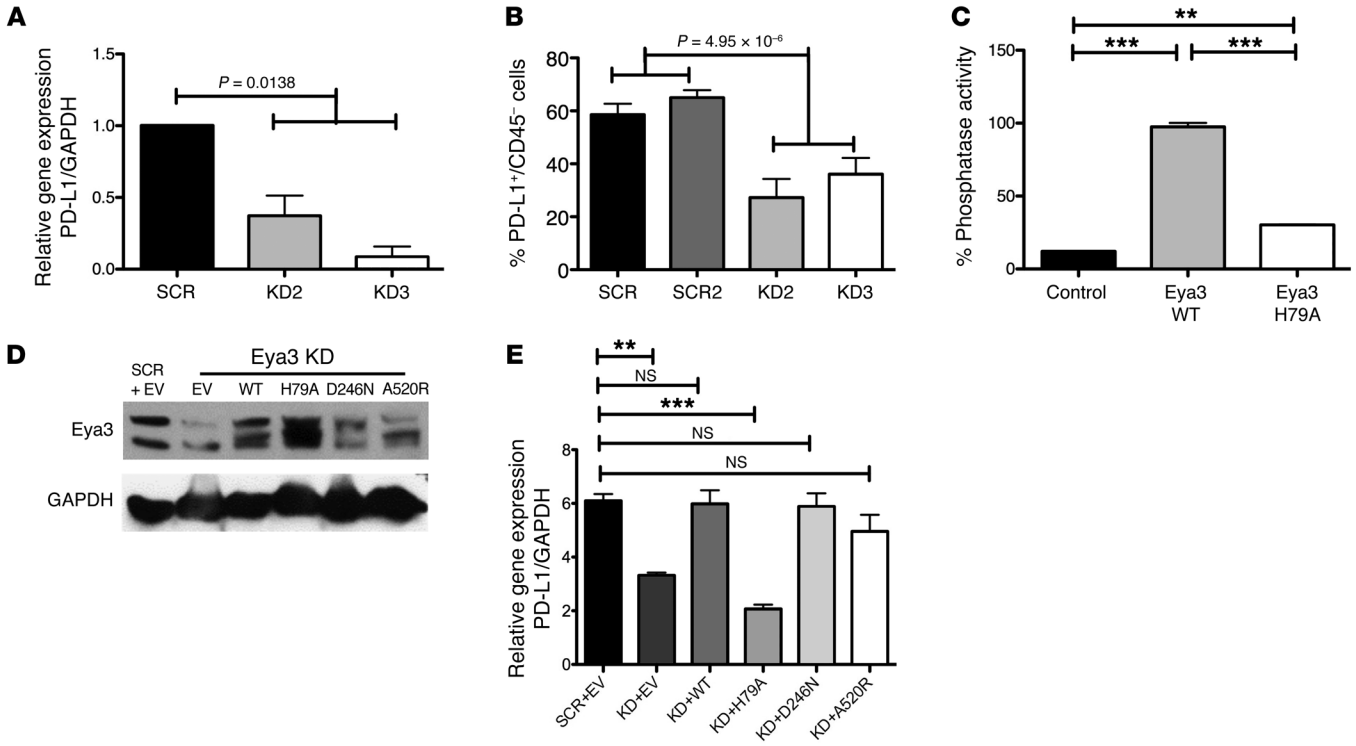


**Figure 8. Eya3 Thr phosphatase activity regulates PD-L1 expression in 66cl4 mammary carcinoma cells.** (**A**) RT-qPCR analysis on cDNA derived from RNA isolated from 66cl4-SCR and Eya3-KD cells. PD-L1 was normalized to GAPDH levels. Data represent mean ± SEM, and significance was measured using ANOVA with sum contrasts in R for biological triplicates for 3 combined experiments. (**B**) Calculated percentage of PD-L1$^+$ cancer cells present per gram of 66cl4-SCR and Eya3-KD tumors. Tumors were isolated (SCR, $n$ = 5; SCR2, $n$ = 5; KD2, $n$ = 4; KD3, $n$ = 5) and analyzed by flow cytometry. Data represent mean ± SEM, and significance was measured using ANOVA with sum contrasts in R. PD-L1 cancer cells were defined as GhostRed780$^-$luciferase$^+$CD45$^-$PD-L1$^+$. (**C**) FLAG-Eya3 WT and H79A Thr phosphatase–dead protein was isolated from HEK293FT cells using FLAG tag pull-down. Thr phosphatase activity was measured using an equal amount of Eya3 WT and H79A mutant protein in a malachite green phosphorylation assay in duplicate. Control indicates reaction with buffer and phospho-substrate without Eya3 protein added. Data represent mean ± SD. Significance was measured using ANOVA. Representative experiment ($n$ = 4). (**D**) 66cl4-Eya3-KD cells were stably rescued with empty vector (EV), Eya3 WT, Eya3 H79A, Eya3 D262N, or Eya3 A520R. Western blot analysis performed on membranes containing whole cell lysates from these cells, as well as the 66cl4-SCR +EV control cells, and antibodies against Eya3 and GAPDH were used to probe the membranes. Representative image of experiments performed at least 3 times. (**E**) RT-qPCR analysis for PD-L1 using cDNA derived from cell lines shown in **D**. PD-L1 normalized to GAPDH. Data represent mean ± SEM. Significance was measured using ANOVA. Representative experiment ($n$ = 3). $^{**}P < 0.01$, $^{***}P < 0.001$.

## Discussion

In this study, we identify an entirely new function for Eya3 in the promotion of breast cancer growth through altering the adaptive immune response to tumors. Previous studies have shown that members of the Eya family can promote breast cancer through 2 of its known biochemical functions, as Six family transcriptional cofactors and as Tyr phosphatases (28, 33). However, that Eya further regulates tumor growth and progression via its Thr phosphatase activity had remained, to our knowledge, unknown until our studies. Eyas are known to promote Six family–mediated transcription, resulting in enhanced breast cancer cell proliferation (20), lymphangiogenesis (22), EMT (33, 34), and stem cell phenotypes (23, 24, 33, 96), all of which likely contribute to their ability to mediate metastasis (34). Interestingly, the Tyr phosphatase activity of the Eya proteins has also been implicated in promoting breast cancer growth and metastasis through regulation of proliferation (38), migration/invasion (28), angiogenesis (30), and cell survival (29, 32). While it has been argued that the Eya Tyr phosphatase is mediating these functions through a cytoplasmic role independent of Six family members (28), it is more likely that both functions of Eya cooperatively mediate its tumor-promoting roles. Indeed, the Tyr phosphatase activity of Eya has been implicated in Six-mediated transcription (26), and thus this activity may both influence transcription and regulate cytoplasmic targets that are unrelated to transcription.

The Thr phosphatase activity of Eya has not, until recently, been explored in tumor progression. Eyas act as dual phosphatases, but via 2 different domains, as opposed to through 1 domain as is typically observed in dual phosphatases (97). Previous studies demonstrated that the Thr phosphatase activity of Eya mediates the innate immune response to denatured DNA/double-stranded RNA (43, 44). These studies have significant implications for the role of Eyas in tumor progression, as the Eyas are amplified and/or overexpressed in numerous tumor types (28, 33, 35–39, 98), but may alternatively be silenced via methylation in other tumor types (99, 100). Surprisingly, the role of Eyas had never before been examined in immune-competent tumor models. Thus, we believe our work is the first to demonstrate that Eya proteins regulate adaptive immunity (in any context), and that Eya-mediated regulation of this immune pathway has consequences for breast cancer growth and progression.

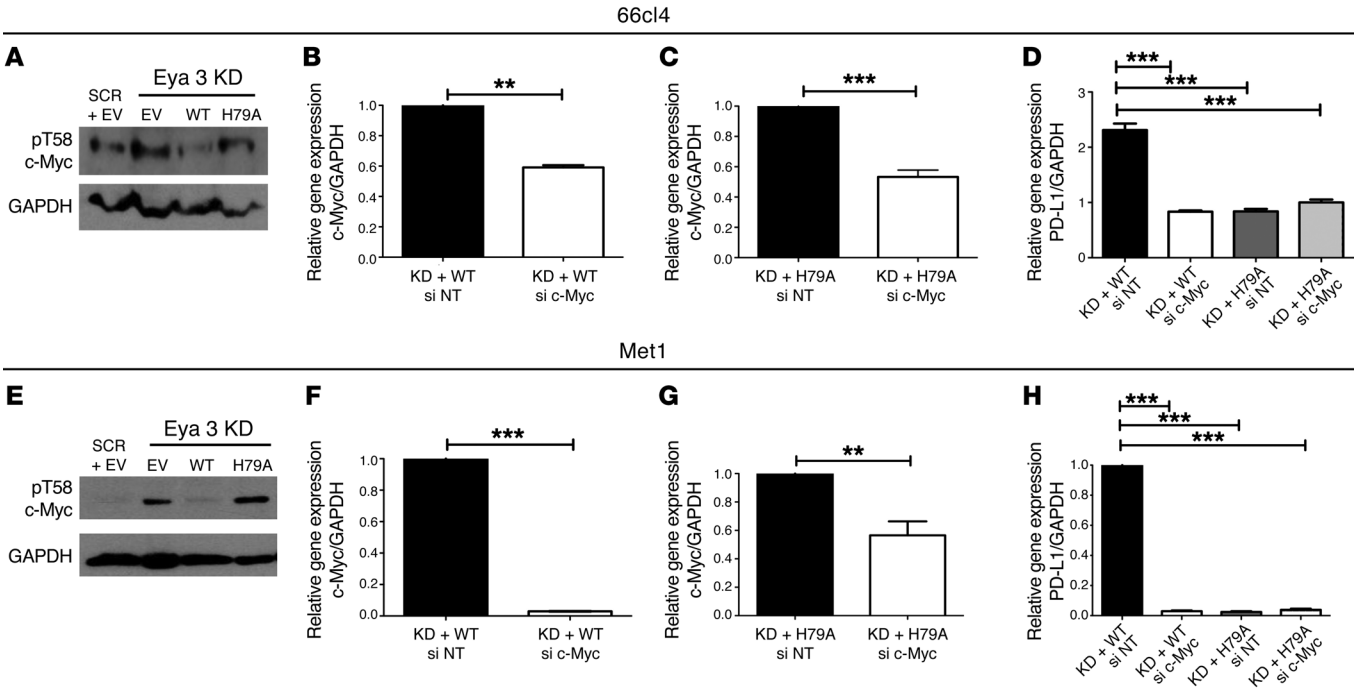


**Figure 9. The Eya3 Thr phosphatase regulates PD-L1 expression through Myc.** (**A**) Western blot analysis performed on membranes containing whole cell lysates from 66cl4-SCR+EV, Eya3 KD+EV, Eya3 KD+WT Eya3, and Eya3 KD+H79A Eya3 rescue cells. Antibodies against pT58 c-Myc and GAPDH were used to probe the membranes. Representative image of experiments performed at least 3 times. (**B** and **C**) RT-qPCR analysis on cell lines shown in **A** 48 hours after transfection with si NT or si c-Myc siRNA. c-Myc normalized to GAPDH. Data represent mean ± SEM. Significance was measured using a 2-tailed Student's *t* test for biological triplicates. Representative experiment ($n = 4$). (**D**) RT-qPCR analysis on cell lines shown in **A**. PD-L1 normalized to GAPDH. Data represent mean ± SEM. Significance was measured using ANOVA for biological triplicates. Representative experiment ($n = 4$). (**E**) Western blot analysis performed on membranes containing whole cell lysates from Met1-SCR+EV, Eya3 KD+EV, Eya3 KD+WT Eya3, and Eya3 KD+H79A Eya3 rescue cells. Antibodies against pT58 c-Myc and GAPDH were used to probe the membranes. Representative image of experiments performed at least 3 times. (**F** and **G**) RT-qPCR analysis on cell lines shown in **D** 48 hours after transfection with si NT or si c-Myc siRNA. c-Myc normalized to GAPDH. Data represent mean ± SEM. Significance was measured using a 2-tailed Student's *t* test for biological triplicates. Representative experiment ($n = 4$). (**H**) RT-qPCR analysis on cell lines shown in **D**. PD-L1 normalized to GAPDH. Data represent mean ± SEM. Significance was measured using ANOVA for biological triplicates. Representative experiment ($n = 4$). $^{**}P < 0.01$, $^{***}P < 0.001$.

KD of Eya3 in 2 different syngeneic triple-negative murine mammary carcinoma cell lines, 66cl4 and Met1, resulted in different in vitro growth phenotypes, with Eya3 KD affecting growth in vitro in 66cl4 cells but not in Met1 cells. Despite these differences in vitro, in vivo transplantation of the Eya3-KD cells into the mammary fat pads of immune-competent mice resulted in a delay of tumor growth for both TNBC lines. Surprisingly, in vivo proliferation of the tumor cells, when examined at the endpoint of the study, was not significantly affected by Eya3 KD in either setting, suggesting that proliferation of the cancer cells is not what drives tumor growth in an immune-competent setting. These data contrast with previous reports suggesting that the proliferative effects of Eyas are important for their tumor-promoting effects (28, 33, 38). It remains possible that proliferative differences between control and Eya3-KD cells exist in vivo at early stages of tumor growth, as our study only examined this parameter when the tumors had reached their maximum size (2 $cm^3$). Nonetheless, our data indicate that Eya3 largely regulates the growth of mammary tumors via effects that do not involve proliferation, and instead involve regulation of the immune system.

Immune cells surrounding and infiltrated into tumors have gained recognition as major regulators of tumor growth (101, 102). Our data demonstrate that Eya3 is responsible for altering the presence of a variety of innate and adaptive immune cell types within a tumor, including natural killer cells, dendritic cells, macrophages, and T cells. All of these immune cell types are known to play roles in inhibiting and/or promoting breast tumor growth (103–105), and the dissection of the subtypes and functions of these cells within Eya3-WT and -KD tumors is an avenue that warrants further exploration. Importantly, we observed striking differences between $CD8^+$ T cells in our mouse models with Eya3 KD, and further demonstrated that Eya3 expression is inversely correlated with the number of $CD8^+$ T cells within human breast tumors. These data, together with the $CD8^+$ T cell depletion experiments, suggest that while Eya3 expression in mammary carcinoma cells leads to alterations in numerous components of the immune system, the effects of Eya3 on $CD8^+$ T cells are critical to its ability to enhance tumor growth.

Our findings are significant as Eyas were previously thought to modulate tumor growth/metastasis via functions unrelated to the immune microenvironment (28). As previously mentioned, Eya proteins influence proliferation, migration, invasion, and EMT (28, 33). Additionally, Eya regulates apoptosis (1, 26–29). We show that Eya3 expression in mammary carcinoma cells, when in an immune-competent setting, protects against apoptosis. Eya3-mediated survival may be due to the ability of Eya3 to pro-

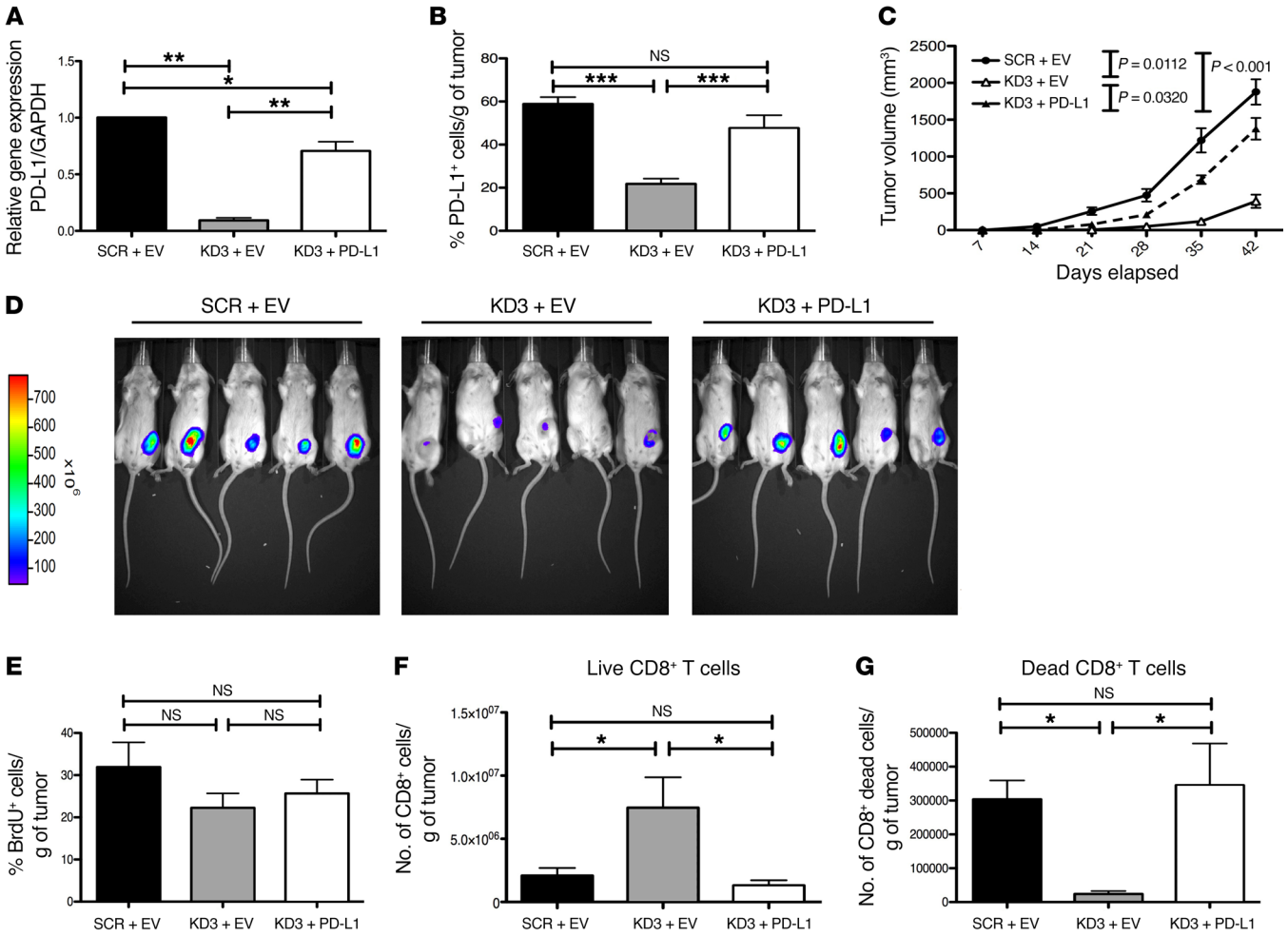


**Figure 10. PD-L1 upregulation is required for Eya3-enhanced mammary carcinoma growth.** 66cl4-SCR and Eya3 KD3 cell lines were stably rescued with empty vector (EV) or PD-L1. (**A**) RT-qPCR analysis on cDNA derived from RNA isolated from 66cl4-SCR+EV, Eya3 KD3+EV, and Eya3 KD3+PD-L1 rescue cells. PD-L1 normalized to GAPDH. Data represent mean ± SEM. Significance was measured using ANOVA for biological triplicates from 3 combined experiments. (**B**) Percentage of PD-L1$^+$ cancer cells present per gram of 66cl4-SCR+EV, Eya3 KD3+EV, and Eya3 KD3+PD-L1 rescue tumors. Tumors were isolated ($n$ = 10 mice per group) and analyzed using flow cytometry. Data represent mean ± SEM. Significance was measured using ANOVA. PD-L1$^+$ cancer cells were defined as GhostRed780$^-$luciferase$^+$CD45$^-$PD-L1$^+$. (**C**) Tumor volume of 66cl4-SCR+EV, Eya3 KD3+EV, and Eya3 KD3+PD-L1 rescue tumors in BALB/c mice as measured using calipers. Each point represents the mean tumor size of mice at that time point after injection ± SEM, and a mixed effects model was used to measure significance. $n$ = 10 mice per group. (**D**) Representative bioluminescence images of BALB/c mice bearing 66cl4-SCR, Eya3 KD3+EV, and Eya3 KD3+PD-L1 tumors at week 4 after injection. (**E**–**G**) Tumors were isolated ($n$ = 10 mice per group) and analyzed by flow cytometry. Data represent mean ± SEM. Significance was measured using ANOVA. (**E**) Calculated percentage of BrdU$^+$ cancer cells present per gram of 66cl4-SCR and Eya3 KD3+PD-L1 rescue tumors. BrdU$^+$ cancer cells were defined as GhostRed780$^-$luciferase$^+$CD45$^-$BrdU$^+$. (**F**) Percentage of live CD8$^+$ T cells per gram of 66cl4-SCR, Eya3 KD3+EV, and Eya3 KD3+PD-L1 rescue tumors. Live CD8$^+$ T cells were defined as GhostRed780$^-$luciferase$^-$CD45$^+$CD3$^+$CD8$^+$. (**G**) Percentage of dead CD8$^+$ T cells present per gram of 66cl4-SCR, Eya3 KD3+EV, and Eya3 KD3+PD-L1 rescue tumors. Dead CD8$^+$ T cells were defined as GhostRed780$^+$luciferase$^-$CD45$^+$CD3$^+$CD8$^+$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

mote apoptotic evasion (1, 26–29), as well as to the ability of Eya3 to inhibit CD8$^+$ T activity within the tumor microenvironment. Therefore, the significant delay in tumor growth attributed to loss of Eya3 expression may be due not to any effect of Eya3 on proliferation, but instead to the ability of Eya3 to promote the survival of cancer cells. However, tumors formed from the 66cl4-Eya3-KD cells eventually catch up to the tumors formed from the 66cl4-SCR control cells. These data suggest that growth is delayed, but not completely halted (in contrast, in the FVB mice, the tumors formed from the Met1-Eya3-KD cells do not ultimately become as large as the tumors formed from the Met1-SCR control cells; Figure 4B). It is possible that in the BALB/c mice, Eya3-KD tumors are ultimately able to overcome the growth delay due to the eventual loss of active CD8$^+$ T cells in the tumor microenvironment through mechanisms unrelated to Eya3. Over time, a buildup of Eya3-independent factors either on the tumor cells or in the microenvironment may lead to the eventual loss of active CD8$^+$ T cells (or other active immune cells in the tumor microenvironment) (106, 107).

Previous studies examining the role of Eyas in tumor progression were conducted in immune-compromised mouse models, where the contribution of the immune system to tumor growth was not assessed. Therefore, it is possible that Eyas contribute to tumor progression via altering numerous tumor cell–autonomous and –non-autonomous properties. Regulation of immune

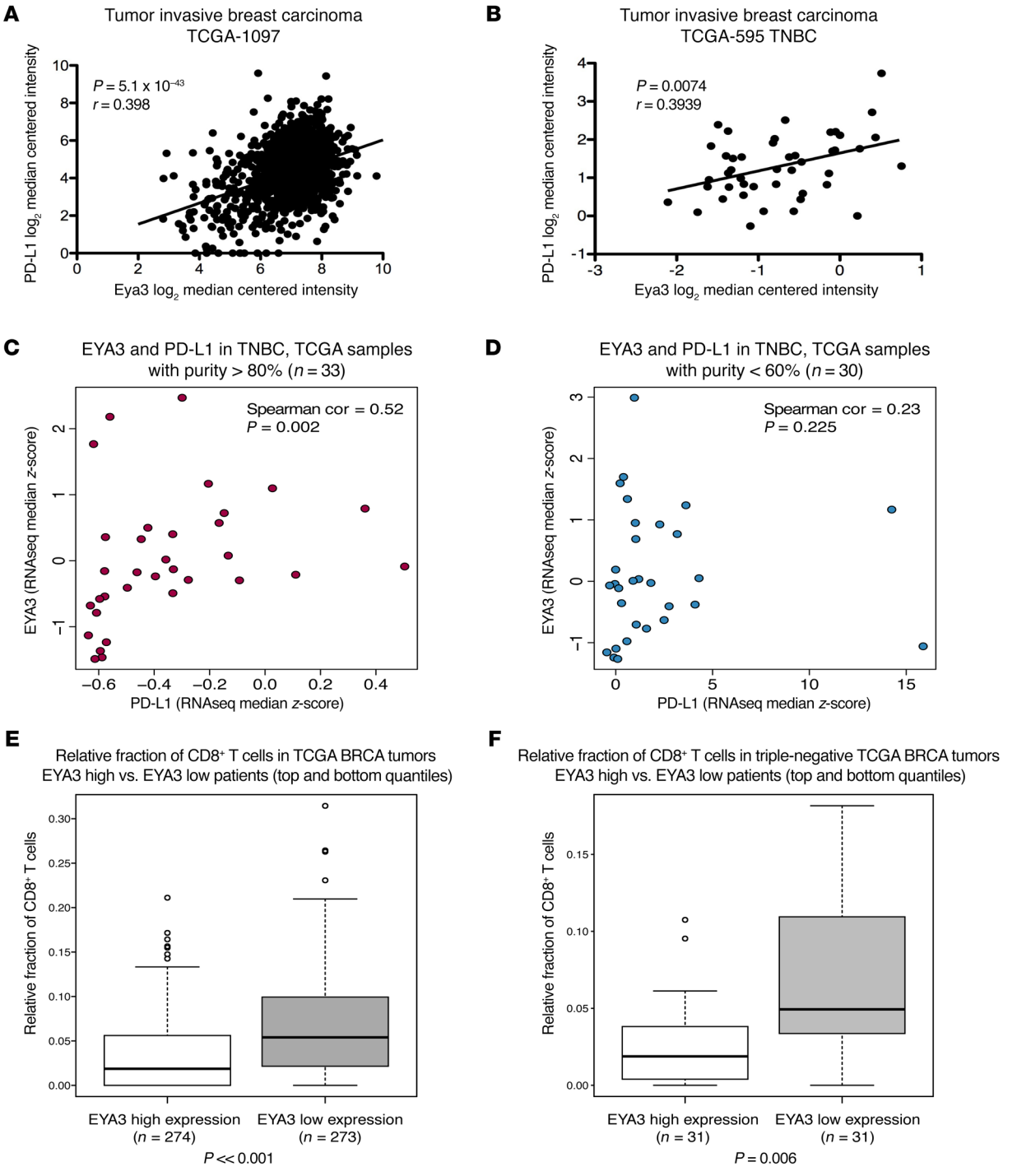


**Figure 11. Eya3 expression, PD-L1 expression, and Myc expression are significantly correlated in human breast tumors.** (**A** and **B**) Correlation of PD-L1 (CD274) and Eya3 expression in human patient tumors from TCGA ($n$ = 1,097) (**A**) and in the TNBC subset from TCGA (data set, $n$ = 595; TNBC subset, $n$ = 46) (**B**). Data sets obtained from the Oncomine portal. Significance was measured using linear regression test and correlation test. (**C** and **D**) Gene expression data for Eya3 and PD-L1/CD274 (RNA-Seq median $Z$ score) were downloaded for TCGA patients directly from cBioPortal for Cancer Genomics (http://www.cbioportal.org/) using the cgdsr R package (https://cran.r-project.org/web/packages/cgdsr/index.html. Tumor purity measures and consensus measurement of purity estimations (CPE) were acquired. Expression values, CPE measures, and receptor status of TCGA patients were compiled for a total of 123 patients who were reported to be triple-negative. Patients were separated based on CPE (>0.80, **C**, or <0.60, **D**). Correlation between Eya3 and PD-L1/CD274 was calculated for each patient group based on Spearman's rank. (**E** and **F**) Expression data from TCGA for all breast cancer subtypes (**E**) and TNBCs (**F**) were stratified for high Eya3 expression (75th percentile and higher) and low Eya3 expression (25th percentile and lower). Data were applied to the Cibersort algorithm, and analysis of relative fraction of $CD8^+$ cytotoxic T cells was graphed. Data represent mean ± SEM. Significance was measured using a Student's $t$ test with multiple-testing correction with the FDR procedure.

detection may be dominant to some of the other functions in vivo, although it should be noted that we did not address metastasis in these models. It is therefore possible that the role of Eya in mediating tumor evasion of the immune system is critical in the primary tumor. However, once tumors have escaped immune detection, the cell-autonomous effects of Eya in mediating tumor migration, invasion, and EMT may play a more critical role. How the different activities of Eya influence tumor immune evasion remains to be thoroughly assessed. In our studies using mutants of Eya3 that abrogate various activities, we observed no difference in PD-L1 mRNA expression when Eya3 was Tyr phosphatase–dead. In contrast, we did observe a partial loss of PD-L1 mRNA upregulation by Eya3 when Eya3 contained a mutation that should abrogate its binding with Six family members (34). The partial requirement of Eya3 for Six family members may be due to the fact that Eya proteins are translocated to the nucleus by Six family members, and that nuclear localization of Eya3 is likely required for dephosphorylation and subsequent stabilization of c-Myc (87). In addition, Six and Eya family members have been shown to mediate transcription of c-Myc (26), and thus the partial requirement for Six interaction could be on multiple levels. Nonetheless, the Thr phosphatase mutation of Eya3 completely abrogated its ability to upregulate PD-L1, suggesting that this activity of Eya3 is most important for its ability to upregulate PD-L1 and mediate immune suppression. In vivo experimentation dissecting each individual function of Eya3 will be required to ultimately understand the relative contribution of the different Eya activities to tumor immune invasion.

Our findings that the Eya3 Thr phosphatase activity regulates PD-L1 expression in TNBC are exciting as the PD-L1/PD-1 pathway is proving to be a remarkably effective cancer target. We show that restoration of PD-L1 expression in Eya3-KD cells is sufficient to induce tumor growth similar to that seen in Eya3-expressing tumors. As we show that Eya3 regulation of $CD8^+$ T cells is necessary to promote tumor growth, it is likely that Eya3 regulates these T cells through PD-L1, rather than another mechanism of $CD8^+$ T cell repression, such as control of immune suppressive cells. In fact, we observed no significant difference in the populations of $CD11b^+Gr1^+$ myeloid-derived suppressor cells in tumors upon Eya3 manipulation, although other immune cell populations may be contributing to altered T cell activity. Additionally, our observation that Eya3 and PD-L1 only correlate in human breast cancer data sets with greater than 80% tumor cell purity strongly suggests that the effects of PD-L1 on tumor growth are due to expression of Eya3 and PD-L1 in the tumor cells themselves, and not due to other $PD\text{-}L1^+$ immunosuppressive cells in the microenvironment.

Our findings have significant clinical implications. No targeted therapies exist for TNBC, and classic chemotherapies do not provide long-term protection for patients, as more than half of TNBC patients become chemoresistant and relapse after treatment (108). However, TNBC tumors are highly immunogenic, and clinical trials are being conducted to treat such tumors with immunotherapies, including anti–PD-L1 immunotherapies (71, 76, 77). Studies have shown that a combination of traditional DNA-damaging and mitotic-halting chemotherapy agents with immunotherapies results in synergistic effects (109, 110). Indeed, several clinical trials combining chemotherapy with anti–PD-L1 immunotherapies in TNBC are under way (74, 111). Eya3 is known to impart cancer cell chemoresistance (32, 35), and as we show that Eya3 regulates PD-L1 expression in tumor cells, treatment with Eya3 inhibitors, chemotherapies, and anti–PD-L1 immunotherapies may represent a potent TNBC therapy. Small-molecule inhibitors against Eya Tyr phosphatase activity have been developed by several groups (30, 31, 41), and we are currently working to develop inhibitors against the Six-Eya interface. In contrast, no inhibitors against the Thr phosphatase activity of Eya exist. Our novel findings that the Eya3 Thr phosphatase leads to upregulation of PD-L1 and tumor immune evasion suggest that its targeting may prove to be beneficial to patients, particularly of the TNBC subtype. However, it should be noted that the correlation between Eya3 and PD-L1 is present in data sets containing all breast cancer subtypes and is present, but not increased, in TNBC data sets. This lack of an increased correlation in TNBC specifically may be due to the fact that there are fewer patient samples in TNBC data sets. Nonetheless, the strong correlation between Eya3 and PD-L1 in data sets containing all breast cancer subtypes suggests that the Eya3/PD-L1 axis may be relevant to additional breast cancer subtypes, although this needs to be experimentally validated.

The Eya Thr phosphatases are unique in that they bear no resemblance to any known Ser/Thr phosphatase, highlighting the need to understand the exact mechanism behind the Eya3 Thr phosphate activity (42, 43). In this study, we show that Eya3 regulates PD-L1 expression in mammary carcinoma cells through regulating c-Myc, as c-Myc knockdown in Eya3 rescue cells inhibits the ability of Eya3 to upregulate PD-L1 (Figure 9), and as c-Myc is known to transcriptionally regulate PD-L1 (90). Additional studies from our laboratory clearly demonstrate that Eya3 increases c-Myc stability via its ability to dephosphorylate c-Myc at pT58, through a mechanism that requires protein phosphatase 2A (112). These findings suggest that targeting the Eya3 Thr phosphatase could lead to downregulation of not only PD-L1, but also other Myc-mediated targets, and thus may influence numerous cancer-associated phenotypes. As the Thr phosphatase function of the Eyas may prove to be an effective cancer therapeutic target for TNBC and potentially other breast cancer subtypes, it is imperative that we work to better understand their function in cancer, and begin to develop means to target this activity.

## Methods

Detailed methods can be found in Supplemental Methods online.

*Statistics*. When conditions tested were over a time period, data were fit using a mixed-effects model and examined for a statistically significant difference between the effect of SCR control versus the effect of Eya3 KD2 and KD3, and reported as the corresponding *P* value.

When exactly 2 conditions were compared, an unpaired 2-tailed Student's *t* test was used and corresponding *P* values were reported. When correlations were tested, a linear regression test and correlation test were performed and corresponding *P* and *r* values were reported. When comparisons were tested with more than 2 conditions, a 1-way ANOVA was used followed by Tukey's multiple-comparisons test. When 2 or more conditions were tested

at a static time point, we tested whether there was a significant difference between SCR versus Eya3 KD2 and KD3. This was done using an ANOVA by comparing the effect of SCR against the average effect of KD2 and KD3 (done using sum contrasts in R) and the corresponding *P* value was reported.

All graphs were created using GraphPad Prism software. If exact *P* values were not stated, reported *P* values are as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

*Study approval*. All animal studies were performed according to protocol B-57716(04)1E reviewed and approved by the Institutional Animal Care and Use Committee at the University of Colorado Anschutz Medical Campus.

## Author contributions

RLV, HZ, JES, RMK, RZ, and HLF conceptualized, designed, and interpreted the experiments in this study. RLV, HZ, LZ, and JK performed experiments and carried out data analyses. MYV, RKP, and JCC designed, performed, and analyzed the Cibersort algorithm data. DG and PR performed statistical analyses. RLV and HLF wrote and edited the manuscript, and all authors provided comments on the manuscript.

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