CEACAM1 is a direct SOX10 target and inhibits melanoma immune infiltration and stemness

Highlights

- CEACAM1 is a direct genetic target of SOX10
- Loss of SOX10 and CEACAM1 inhibits tumor growth in immune-competent mice
- CEACAM1 expression inhibits CD8+ T-cell infiltration
- Loss of CEACAM1 and SOX10 enhances cancer stem cell properties in melanoma
CEACAM1 is a direct SOX10 target and inhibits melanoma immune infiltration and stemness

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SUMMARY

SOX10 is a key regulator of melanoma progression and promotes a melanocytic/differentiated state. Here we identified melanoma cell lines lacking SOX10 expression which retain their in vivo growth capabilities. More importantly, we find that SOX10 can regulate T-cell infiltration in melanoma while also decreasing common cancer stem cell (CSC) properties. We show that SOX10 regulates CEACAM1, a surface protein with immunomodulatory properties. SOX10 directly binds to a distal CEACAM1 promoter region approximately 3-4kbps from the CEACAM1 transcriptional start site. Furthermore, we show that a SOX10-CEACAM1 axis can suppress CD8+ T-cell infiltration as well as reduce CSC pool within tumors, leading to reduced tumor growth. Overall, these results identify SOX10 as a direct regulator of CEACAM1, and uncover both a pro- and anti-tumorigenic roles for SOX10 in melanoma.

INTRODUCTION

The Sox family (SRY-HMG box) of transcription factors plays critical roles in tissue homeostasis, organogenesis, and cell fate decisions in many developmental processes.1–3 The SoxE group, including Sox8, 9, and 10, has been extensively studied in the context of reproductive system development, neural crest cell-derived tissues and cell types such as melanocytes. Although the role of the SOX proteins in cancer progression remains elusive and controversial,1 SOX10 has been shown to play an important role in melanoma maintenance and progression in the context of haploinsufficiency.4–6 Similarly, studies on SOX9- or SOX10-floxed animals have been performed in the context of the NRASQ61 allele, inducing senescence and nevi formation.7

BRAF(V600E) and NRAS(Q12,Q13,Q61) accounts for about 80% of melanoma mutations.8–12 The design of highly specific BRAFV600E (Vemurafinib), MEK (Trametinib) and immune checkpoint inhibitors (PD-1 and CTLA-4) has produced good response rates in melanoma.8,10–17 However, most responses are transient with most patients becoming resistant and progressing within a year due to the reactivation of the MAPK pathway in 70% of cases.7,10 Similarly, immunotherapies to PD-1 and CTLA-4 have improved overall survival in patients with melanoma but with only a subset showing long-term benefits.8,11–17 However, most responses are transient due to the activation of resistance mechanisms. Strikingly, drug resistance is often associated with resistance to immune checkpoint inhibitors, leading to a cross-resistance phenomenon.9,10,15

A number of studies have identified distinct subpopulations of melanoma cells with variable sensitivity to therapy.18–23 Genomic studies have revealed the existence of major subpopulations characterized by differential expression of AXL, EGFR, NGFR, SOX9, and SOX10 in drug-resistant cells. Upon acquired resistance, the sensitive pool (AXL-, EGFR-, NGFR-, SOX9-, SOX10+) undergoes a switch to an AXL+, EGFR+, NGFR+, SOX9+, SOX10- phenotype.21–25 This phenotype switch involves transcriptional reprogramming rather than mutational evolution or expansion of resistant subpopulations.21,22,26,27 However, the mechanisms of drug resistance are far more complex than initially thought as intermediate populations may exist as well as a neural crest stem cell-like (NCSC) state that can be induced through phenotype switching, regardless of their initial driver mutations.28

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A number of transcription factors have been shown to coordinate phenotype switching. Strikingly, SOX10 expression is associated with the highly proliferative and Vemurafenib (Vemu) sensitive cell populations. Supporting this, suppression of Sox10 induces EGFR, PDGFRβ, and confers resistance to BRAF inhibitors. SOX10 typically act in concert with its downstream target MITF, a bHLH transcription factor. Interestingly, Sox9 was found to be expressed exclusively in the SOX10+ invasive samples together with TEAD and AP-1. A reduction in Sox10 expression was also observed to be associated with increased Sox9 and reduced proliferation, suggesting a negative feedback loop between Sox9 and Sox10. Although numerous other transcription factors have been shown to be important for different aspects of drug resistance and progression, SOX9 and SOX10 appear to be critical to maintain the stem-like and invasive properties of tumors. Recently the cellular inhibitor of apoptosis protein-1/2 (cIAP1/2) were found to be upregulated in SOX10-deficient cells, contributing to drug resistance in the SOX10+ cells. Similarly, the expression of CEACAM1 in SOX10+ cells provides a growth advantage in CD8+ T-cell depleted animals. These data suggest that SOX10 might regulate aspects of both drug sensitivity and immune surveillance in melanoma cells.

Although a positive correlation between SOX10 and CEACAM1 expression has been previously observed in melanoma, we show here that CEACAM1 is a direct target of SOX10. We found that SOX10 regulates CD4+ and CD8+ T-cell infiltration in melanoma while also depleting the CSC properties, primarily the self-renewal capabilities. When SOX10 and CEACAM1 co-exist this perpetuates these effects. In all, we have identified two major contradictory roles of SOX10 not previously known in melanoma.

RESULTS

SOX10+ cells display a growth advantage in immune-competent mice but reduced self-renewal properties

The role of Sox10 in human melanoma is well established. Interrogation of the Firehose human melanoma dataset shows that high SOX10 expression is correlated with a decrease in survival (Figure 1A). To gain further insight into Sox10-driven phenotypes, we set out to identify cell lines with differential SOX10 expression. To achieve this, the YUMM panel of murine congenic melanoma cell lines was assessed for Sox10 expression. Using Western blotting we identified the YUMM1.1 as a SOX10 high cell line, YUMM2.1 showed intermediate levels and YUMM1.7 & YUMM1.9 cells showed little to no expression (Figure 1B). This was further validated at the mRNA level, suggesting that Sox10 expression in these cells is likely to be modulated at the transcriptional level (Figure 1C). We next tested the in vivo growth potential of the SOX10+ YUMM1.1 and the SOX10+ YUMM1.7 cell lines in C57 mice. As expected, the SOX10+ cells reached endpoint significantly faster than the SOX10- cells (Figures 1D and 1F). Although the SOX10+ cells did grow slower, they still formed palpable tumors (Figure 1D). Western blot analysis showed that SOX10 was not re-activated in the YUMM1.7 tumors in an immune-competent background, confirming that cells lacking SOX10 did not activate it and retained some in vivo growth potential (Figures 1D and 1F). To test the potential effects of the immune system in this assay, cells were injected in NOD-SCID mice which lack functional T/B cells and have impaired NK cell and macrophage functions. In marked contrast to previously reported results, the SOX10+ cells displayed reduced growth compared to the SOX10- cells (Figures 1E and 1G) in immuno-compromised hosts. One possibility is that SOX10+ tumors have the ability to negatively modulate the immune system in an immune-competent background. Alternatively, SOX10+ cells may have increased cancer stem cell (CSC) potential. Previous findings in our lab and others have identified SOX10 as a CSC marker enhancing tumor sphere-forming potential. Using melanosphere assays we tested the CSC potential of both cell lines. Interestingly, the SOX10+ YUMM1.1 melanoma cells displayed low sphere-forming potential (Figure 1H), suggesting reduced levels of CSCs. This further translated into a decrease in secondary sphere formation, supporting a reduction in self-renewal capabilities. Surprisingly, no secondary spheres could be recovered from the high SOX10 expressing YUMM1.1 cells, whereas the YUMM2.1 cells, expressing intermediate SOX10 levels, generated a low level of secondary spheres (Figures 1H and 1I). Together, these data suggest that high SOX10 populations have reduced stem cell-like content and that SOX10 may play a different role in epithelial-like cancers.

SOX10 modulates the cancer stem cell properties of BRAF + melanomas

To test whether SOX10 expression was sufficient to modulate the CSC potential of melanoma cells, the SOX10+ YUMM1.7 cells were transduced with a retrovirus expressing Sox10. Expression was confirmed by Western Blot analysis (Figure 2A) and the self-renewal capabilities of the SOX10+ and SOX10- YUMM1.7 cells were tested using sphere forming assays in vitro. Interestingly, the expression of SOX10 in the YUMM1.7 cells had no effect on primary sphere formation but slightly decreased the CSC
self-renewal capabilities (2° spheres; Figures 2B and 2C). To further test the role of SOX10 in self-renewal, we knocked out Sox10 in YUMM1.1 cells using CRISPR-Cas9 and guide RNAs targeting the HMG domain in exon two. Western blotting showed a significant SOX10 knockout using two independent guides (Figure 2D).

In vitro sphere forming assays with both Sox10 knockout cell lines showed that Sox10 deletion enhanced the number of primary and secondary spheres (Figures 2E and 2F), suggesting that Sox10 expression negatively modulates CSC content. Similarly, Sox10 ablation in YUMM2.1 expressing intermediate levels of SOX10 led to an increase in the sphere-forming potential, further suggesting that SOX10 decreases the CSC properties of melanoma (Figure S1A and S1B). Together our data show that SOX10 expression reduces the CSC potential of BRAF + melanomas.
SOX10 affects CD8+ T-cell infiltration and is co-expressed with the immune modulator CEACAM1

To further assess the role of SOX10 on tumor growth in vivo, the YUMM1.7 cells expressing SOX10 were injected subcutaneously into immune-competent mice. As expected, SOX10-expressing YUMM1.7 cells showed an increase in tumor growth compared to controls (Figure 3A). One of the hallmarks of melanoma phenotype switching is the ability to evade the immune system. Therefore, one possibility is that the observed in vivo enhanced growth by the SOX10+ cells is due to immune suppression. Following tumor growth, we performed flow cytometry on endpoint tumors. Our analyses show equal levels of CD3+ immune cell infiltration in the tumor microenvironment (Figure S2A). However, flow analysis showed that the SOX10+ tumors had increased CD4+ T-cell infiltration with a decrease in CD8+ T-cell (Figure 3B), suggesting that SOX10-driven melanoma tumors can regulate T-cell infiltration or immune escape. To test this, we repeated the injections using RAG1 mice which lack T/B cells. Interestingly, we found that the SOX10-expressing YUMM1.7 cells grew at the same rate as controls, supporting the notion that SOX10 + melanoma can negatively regulate the immune response (Figures 3C and S2B). This finding was further corroborated by injecting the SOX10-deficient YUMM1.1 cells (Figure 2D) in NCG mice which lack T and B cells while also having an extreme impairment in innate immune function (Figure S2C). As SOX10 expression regulated T cell infiltration in tumors, we analyzed TCGA data for evidence of correlation between immune infiltration and overall survival in melanomas using TIMER2.0. Interestingly, the extent of CD4+ T cell
infiltration within melanomas did not affect overall survival (Figure S2D). However, supporting our data, patients with increased levels of CD8+ T cell infiltrates displayed better survival outcomes (Figure S2E).

Our previous work in breast cancer identified a number of potential SOX10 targets. CEACAM1, a glycoprotein found on the surface of various cell types, was highly expressed in SOX10 + breast cancer cells. Strikingly, CEACAM1 is believed to play multiple roles in inhibiting cytotoxic lymphocyte function. To assess whether SOX10 could be driving the expression of ligands inhibiting CD8+ T cell function, we tested a panel of common T cell modulators including CEACAM1, PDL1, PDL2, CD80, and CD86. Conventional
ligands such as PDL1, PDL2, CD80, and CD86 had no correlation with SOX10 expression in our various YUMM lines as determined by flow cytometry (Figure S3A). However, CEACAM1 expression was only observed in SOX10 expressing YUMM lines with YUMM1.1 displaying the highest CEACAM1 and SOX10 levels (Figure 3D). Supporting this, using TCGA data on CBioPortal we found that CEACAM1 and SOX10 mRNA levels are positively correlated in melanoma (Figure S3B). This significant correlation between SOX10 and CEACAM1 in melanoma prompted us to test whether CEACAM1 expression was SOX10-dependent. Using flow cytometry we identified multiple human melanoma cell lines with both SOX10high/CEACAM1high and SOX10high/CEACAM1med levels (Figures 3E and S3C). This was also observed in the YUMM lines, suggesting that this correlation is conserved in murine cell lines. We found that the YUMM1.7 cells expressed little to no SOX10 or CEACAM1 protein (Q2; Figure 3F). In contrast, in both the YUMM1.1 and YUMM2.1, a large proportion of CEACAM1 positive cells are also SOX10 positive.

Figure 4. SOX10 directly activates Ceacam1 on a distal enhancer region
(A) YUMM1.1 cells were treated with siRNA at 200nM targeting Sox10 or a non-targeting control for 72 h and Sox10 knockdown was assessed by Immunoblot. (B) 72 h post siRNA transfection cells were isolated and stained for CEACAM1. Representative flow plots were generated with indicated percent CEACAM1+cells. (C) qPCR was used to compare Sox10 knockdown efficiency with Ceacam1 isoform levels of the cells in (A). (D) YUMM1.7 pBABE and YUMM1.7 pBABE-SOX10 cells were collected and analyzed by qPCR for Sox10 and Ceacam1 isoforms. (E) YUMM1.7 pBABE and YUMM1.7 pBABE-SOX10 cells were isolated and stained for CEACAM1. Representative flow plots were generated with indicated percent CEACAM1+cells. (F) Luciferase activity measurements from various Ceacam1 promoter fragments in YUMM1.1 and 2.1 cells. (G) RT-PCR of SOX10 ChIP in YUMM1.1 cells from various regions within the Ceacam1-4135/-2785 fragment showing the fold enrichment over IgG. The red line represents the IgG control set to one. Data is representative of three independent experiments (C, D, F, and G). Graphs show the mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.001, ****p < 0.0001 by one-way ANOVA (C and D) or by two-tailed t test (F).
A large proportion of cells are double-positive, a CEACAM1-/SOX10+ population is also present, suggesting that CEACAM1 expression may also be dependent on additional factors or it is expressed in distinct SOX10+ subpopulations (Figures 3E and 3F). Furthermore, it suggests that SOX10 expression is independent of CEACAM1.

**SOX10 directly activates Ceacam1 on a distal promoter region**

To determine whether SOX10 can modulate the expression of CEACAM1, we transfected two siRNAs targeting SOX10 into the YUMM1.1 and YUMM2.1 melanoma cell lines and confirmed knockdown by western blotting (Figures 4A and S4A). Transfection of the siRNAs resulted in a marked downregulation of CEACAM1 (Figures 4B and S4B). Interestingly, CEACAM1 levels were higher in YUMM1.1 and YUMM2.1 cells with residual SOX10 expression (Figures 4A, 4B, S4A, and S4B). Flow cytometry also revealed that CEACAM1 expression was not reactivated following the long-term CRISPR/Cas9-mediated SOX10 knockout protocol (Figure S4C).

CEACAM1 is expressed as multiple isoforms generated through alternative splicing. Most of these isoforms can be grouped as long (full intracellular domain) or short (lacking the complete ITIM domain) with different functions. Although the specific roles of these isoforms have yet to be fully elucidated, CEACAM1-L isoforms seem to be predominantly expressed and have been found to alter CD8 T-cell activation and regulate metastatic potential of various cancers. To test whether Sox10 preferentially regulates specific CEACAM1 isoforms, we assessed the levels of both long and short isoform groups by qPCR. Sox10 knockdown in the YUMM1.1 decreased both Ceacam1 isoform groups equally, suggesting that Sox10 expression does not affect the splicing mechanisms but overall Ceacam1 expression (Figure 4C). To ultimately test whether SOX10 can activate CEACAM1 expression in melanoma, we assessed CEACAM1 levels in vivo.
Figure 6. SOX10hi/CEACAM1+ cells show decreased CSC pool and CD8+ T-cell infiltration in tumors

(A) Representative flow sorts showing the proportion of CEACAM1+ and CEACAM1- cells obtained following SOX10 expression in YUMM1.7 cells.

(B) qPCR analysis for Sox10 and the Ceacam1 isoforms in sorted YUMM1.7 pBABE-SOX10 cells shown in (A).

(C) In vivo growth comparison between the pBABE-SOX10 (n = 5), CEACAM1- (n = 5) and CEACAM1+ (n = 5) YUMM1.7 cell lines in C57 immune-competent mice.

(D) Flow cytometry analysis of the CD4+ and CD8+ T-cell populations within the pBABE-SOX10, CEACAM1- and CEACAM1+ tumors at endpoint.

(E) Quantitation of primary and secondary sphere formation in the YUMM1.7 pBABE-SOX10, CEACAM1- and CEACAM1+ cells.

(F) Representative images of both primary and secondary sphere assays from (E) (scale bar = 100μM).
SOX10-deficient YUMM1.7 cells following transduction with pBABE-Sox10. Following qPCR, exogenous SOX10 expression induced both Ceacam1 isoform groups in YUMM1.7 cells (Figure 4D). Total CEACAM1 upregulation was further confirmed via flow cytometry (Figure 4E). To determine whether SOX10 directly activates Ceacam1, we cloned multiple 1kb fragments of the Ceacam1 promoter region in pGL3-P, a luciferase backbone. The overlapping fragments allowed for coverage of up to -5kb from the Ceacam1 transcriptional start site. Interestingly, we identified two negative regulatory regions (−1525/+5 and −5305/+4045) that showed decreased luciferase activity only in the YUMM1.7 cell lines lacking both CEACAM1 and SOX10 (Figure S4D). As expected, we identified one major fragment at approximately -3kbps from the transcriptional start site (−4135/−2785) that displayed a 5-fold increase in luciferase activity in SOX10 + YUMM1.1 cells (Figure 4F). Scanning of the −4135/−2785 fragment revealed only one potential SOXE binding site containing the (A/T)(A/T)CAA(A/T)G binding consensus sequence. Previous studies have shown that SOX10 can bind to distal promoter and/or enhancer regions through a variant site with a requirement for a CAA(A/T) sequence. Using the CAA(A/T) motif, we identified eight potential regions with four bearing a single nucleotide mismatch of the conventional SoxE consensus region. To determine whether SOX10 is bound to these consensus regions we used YUMM1.1 cells for ChiP analysis. Chromatin pull downs with anti-SOX10 antibodies showed significant enrichment above IgG in 3 of the 8 regions within the −4135/−2785 fragment boundary by SOX10, suggesting that it is a direct regulator of CEACAM1 (Figure 4G).

**CEACAM1 decreases CD8⁺ T-Cell infiltration and promotes tumor growth**

Since CEACAM1 has been shown to be expressed as multiple isoforms we tested whether the longest or shortest isoforms had any effect on tumor growth and CSC properties. Isoform 1 contains the complete extra and intra cellular domains of CEACAM1 whereas isoform 4 has a truncated extracellular and intracellular domain while still allowing for ligand binding. These two isoforms were cloned into a retroviral backbone and transduced into YUMM1.7 cells. Expression was confirmed using flow cytometry (Figure 4A). We then tested the CSC properties of these cells using tumor sphere assays as above. Similar to SOX10, CEACAM1 expression decreased the secondary sphere formation of these cells (Figure 5B). Although both isoforms showed a downward trend, only isoform 4 showed a significant reduction (Figures 5B and 5C). To test whether this decrease in CSC properties correlated with overall growth, we injected YUMM1.7 cells expressing the different isoforms subcutaneously into NCG mice. Surprisingly, although isoform 4 expression decreased CSC properties, we found that it conferred a marked growth advantage in vivo as previously reported (Figure 5D). Interestingly, isoform 1 expression had no effect on tumor growth in vivo (Figure 5D). To test the effect of CEACAM1 on immune infiltration we injected the cells in immune-competent C57 mice. Although isoform 4 conferred a similar growth advantage, flow cytometry showed that both CEACAM1 isoforms increased CD4⁺ T-cell and decreased CD8⁺ T-cell infiltration compared to controls (Figures 5E and 5F). Together these data show a direct functional correlation between CEACAM1 and SOX10 at repressing CD8⁺ T-cell infiltration and decreasing CSC properties in BRAF + melanomas.

**Co-expression of SOX10-CEACAM1 markedly decreases the cancer stem cell pool and CD8⁺ T-cell infiltration in melanoma**

CEACAM1 has been shown to play contradictory roles within different cancer types. To gain insight into the SOX10-CEACAM1-driven phenotypes in melanoma, we sorted our YUMM1.7 pBABE-Sox10 cells into their CEACAM1⁺ and CEACAM1⁻ populations (Figure 6A). These populations remained stable using flow cytometry for up to 17 days post sorting (Figure S5A). The YUMM1.7 CEACAM1⁺ cells were found to be enriched for both the short and long isoforms (Figure 6B) and displayed a significant increase in both Sox10 mRNA and protein levels, supporting the observation that Ceacam1 is a SOX10 target (Figures 6B and S5B). In agreement with this, the CEACAM1⁺ population had lower levels of SOX10 compared to the pBABE-Sox10 and CEACAM1⁻ cells (Figure S5B). To test whether SOX10-CEACAM1 expression could enhance tumor growth, we injected our YUMM1.7 CEACAM⁺, CEACAM⁻, and parental pBABE-Sox10 cells subcutaneously into immune-competent mice. Unexpectedly, we found that the
CEACAM1+ cells grew the slowest while the pBABE-Sox10 parental line grew significantly faster (Figure 6C). Following flow cytometry, we found no major differences in CD4+ T cell content between all groups but an increase in CD8+ T cell infiltration in the CEACAM1+, suggesting that SOX10-driven CEACAM1 expression suppresses CD8+ T cell recruitment (Figure 6D).

One possibility for the observed reduced tumor growth in vivo is the potential depletion of the CSC pool within both the YUMM1.7 SOX10-CEACAM1+ and SOX10-CEACAM1-populations. To test this, we performed primary and secondary sphere assays in vitro. Supporting our hypothesis, we found that the CEACAM1+ cells had significantly reduced primary and secondary sphere-forming potential, suggesting a reduction in CSC content and self-renewal capacity (Figures 6E and 6F). Interestingly, both SOX10+ populations showed a reduction in self-renewal capacity compared to the parental YUMM1.7 cells (Figure 2B), suggesting that SOX10 expression is sufficient to reduce the CSC content of those cells.

We retested the CSC potential of those cells using limiting dilution assays in vivo. We injected 10-fold dilutions of each group into the flanks of NCG mice and allowed tumors to develop for 4 weeks. Supporting the melanosphere data, CEACAM1+ populations showed a markedly reduced tumor size and take rate upon dilution (Figures 6G and 6H). The CSC frequency in CEACAM1-cells was estimated to be approximately 11-fold higher than the CEACAM1+ population (Figure 6I). These data suggest that SOX10-driven CEACAM1 expression can negatively modulate the CSC pool but enhance immune evasion by suppressing CD8+ T cell infiltration.

**DISCUSSION**

The role of SOX10 in melanoma is well documented. To gain further insight into the role of SOX10 in melanoma progression, we screened a panel of YUMM lines for SOX10 expression. Western blot analysis showed high SOX10 expression in YUMM1.1 cells with moderate and very low levels in YUMM2.1 and 1.7, respectively (Figure 1B). Our data show that high SOX10 expression in melanoma confers a tumor growth advantage in immune-competent mice (Figure 3A). Surprisingly this growth advantage was lost in immune compromised mice, supporting a role for SOX10 in immunomodulation (Figures 3C and S2C). Due to the high intra-tumor heterogeneity in melanomas, we tested the CSC potential of SOX10 + YUMM1.1 cells. Unexpectedly, we found that SOX10 decreased the self-renewal capacity in those cells (Figures 2E and S1A). To further investigate the mechanisms underlying these phenotypes, we tested a panel of immune ligands expressed by tumor cells. Those studies revealed that CEACAM1 is a direct gene target of SOX10 (Figure 4G). When co-expressed, we also find that the SOX10-CEACAM1 axis is detrimental to tumor growth by depleting the CSC pool (Figures 6G and 6H).

Our data finds that SOX10-null cells have increased CSC properties (Figures 2E and S1A). This observation is in stark contrast to a large number of reports identifying the SoxE family as CSC-inducing. For example, SOX10 has been shown to induce stem/progenitor activity in mammary epithelial cells while also increasing the CSC pool in HER2+ breast cancer. One reason for this opposing function of SOX10 in epithelial cells when compared to melanoma could be due to the mutual exclusivity of SOX10 and SOX9 expression in melanoma. Interestingly, SOX9 regulates SOX10 expression by direct binding to SOX10 upstream sequences and reducing transcription. Furthermore, SOX9 is believed to be a major marker for CSCs where it has been shown to confer multiple CSC properties in various cancer subtypes, including glioma, lung and breast cancer. Similarly, SOX9 can directly activate SOX10 gene expression in breast cancer to induce a more stem cell-like phenotype. As SOX9 is usually not expressed in SOX10 + melanoma populations, SOX9 deficiency may lead to a decrease in potential CSC properties. Although SOX9 and SOX10 expression is mutually exclusive in melanoma, understanding the specific phenotypic changes imparted by these factors will shed light into potential SoxE-specific therapeutic strategies.

Here, we have shown that CEACAM1 expression in melanoma is dependent on SOX10 (Figures 3E and 3F). Furthermore, we find that SOX10 can directly bind to a CEACAM1 distal enhancer region and induce its expression in melanoma (Figure 4G). Surprisingly, SOX9 has been shown to repress CEACAM1 in melanoma, further supporting an antagonistic function between SOX9 and SOX10. Although SOX9 and SOX10 share very similar binding sites, SOX9 seems to function more on a CEACAM1 proximal promoter element with no evidence of SOX10 binding within the same region (Figure 4F). Interestingly, we find that not all SOX10+ cells express CEACAM1, suggesting that SOX10 may require additional co-factor(s) to fully activate CEACAM1 (Figures 3E and 3F). This has been previously observed for SOX10.
Indeed, SOX10-dependent activation of MITF requires Pax3 expression and TYPR1 induction has been shown to be mediated through direct interaction between SOX10 and BRG1.60,61 Interestingly, we do observe that SOX10 activates both subsets of CEACAM1 isoforms (long and short), suggesting that it does not affect isoform selectivity (Figures 4C and 4D). The regulation of the SOX10-CEACAM1 axis is still not fully understood and in-depth studies should identify potential SOX10 co-factors critical in the activation of CEACAM1 gene expression.

Although SOX10 promotes tumor formation and was identified as a regulator of the proliferative state in melanoma, the mechanistic details about how it regulates this are not fully understood.4–6,23 We show that SOX10 has the ability to regulate T-cell infiltration, primarily CD8+ T cells (Figure 3B). This is in agreement with previous work showing that SOX10 can confer tumor growth partially through blocking CD8+ T-cells infiltration.56 Although various immune subtypes can infiltrate the tumor microenvironment, we find that patients with high CD8+ T-cell infiltration have a better outcome (Figure S2E). For this reason, CD8+ T cells have been the primary target of current immunotherapies. Immune checkpoint inhibition, the most common form of immunotherapy, blocks inhibitory receptor interaction on cytotoxic lymphocytes such as CD8+ T-cells, thereby activating the immune response. Current ligands/receptors targets include PDL1/PD1, CTLA4, and TIM3 blockers, currently in clinical trials. Whether SOX10high melanomas can be halted by single checkpoint blockers or in combination is still unclear. Nevertheless, our studies and others36 have identified SOX10 as a potential biomarker of tumor immunity.

CEACAM1 can be found on both tumor and immune cells where it can participate in homophilic or heterophilic interactions. CEACAM1 is alternatively spliced and is expressed as 12 human and 4 murine isoforms.40,62 Interestingly, blocking CEACAM1 homophilic interactions in melanoma sensitized the cells to CD8+ T-cell cytotoxicity.63 Here we have shown that isoform 4 had a significantly bigger effect on reducing CSC properties as well as CD8+ T-cell infiltration in melanoma (Figures 5B and 5E). Albeit missing the ITIM domains, the shorter isoform 4 can still activate downstream signaling.64 It has been shown that, although truncated at the N-terminus, the shorter isoforms such as 3 and 25 (human and mouse respectively) still retain their homophilic and heterophilic interactions.65 It is then likely that the differential expression between the various CEACAM1 isoforms or the isoform ratio (Figure 5A) plays a critical role in the penetrance of the immune phenotype.

CEACAM1 can also interact with TIM3, a receptor found on CD8+ T-cells usually during exhaustion.65 Current TIM3 blocking antibodies have shown great potential at reactivating immune function and inhibiting tumor immunity.66 It will be of great interest to determine whether CEACAM1 on tumors can induce CD8+ T-cell exhaustion by promoting TIM3-dependent responses. In addition, a greater understanding of the role of the various CEACAM1 isoforms on melanoma progression is required.

Melanomas typically present as heterogeneous populations with gene expression that is non-uniform throughout the tumor.67 Our data show that co-expression of SOX10 and CEACAM1 imparts a marked growth advantage to melanoma tumors (Figure 6C). As it was shown that CEACAM1 expression in melanoma was strongest at invading fronts,68 it is possible that the co-expression of SOX10/CEACAM1 occurs at the periphery of tumors. This would suggest that CEACAM1 may be expressed primarily on the outer edge of the tumor or areas of vascularization. Overall, our data identify SOX10 is a direct regulator of CEACAM1. This SOX10-CEACAM1 axis functions in both a pro- and anti-tumorigenic fashion by decreasing CD8+ T-cell infiltration but by also modulating the CSC properties in melanoma (Figures 6D and 6E-6I respectively). We believe future studies will identify other potential immune cellular subtypes regulated by this pathway as well as potential immune markers for the development of specific melanoma therapies.

Limitations of the study
In our study, we identified that SOX10 directly regulates CEACAM1 expression. One limitation of this study is that SOX10 binding was validated in a murine cell line. Although conservation exists, the exact location of SOX10 binding on the human CEACAM1 promoter has still been unidentified. Another limitation of our study was the use of CEACAM1 isoforms 1 and 4. Additional isoforms exist, namely the intermediate mouse isoforms (Iso 2 & 3) and to fully comprehend CEACAM1’s capabilities at regulating the CSC potential and the immune system within melanoma a more in-depth study should compare all isoforms.
STAR METHODS
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SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
Conceptualization, J.A.H. and L.A.S.; Methodology, J.A.H., J.J.H. and M.M.; Investigation, J.A.H., J.J.H., C.S., B.G., C.L., C.G., S.D. and J.P.; Visualization, J.A.H.; Supervision, J.A.H. and L.A.S.; Writing - Original Draft, J.A.H. and L.A.S.; Writing - Review & Editing, J.A.H., L.A.S., J.J.H., and C.L.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| SOX10               | New England BioLabs | Cat# 89356; Clone D5V9L; RRID:AB_2792980 |
| Beta-Actin          | Sigma-Aldrich | Cat# A5316; Clone AC-74; RRID:AB_476743 |
| Histone H3          | New England BioLabs | Cat# 4620; Clone D2B12; RRID:AB_1904005 |
| Mouse: CEACAM1 BV421 | BioLegend | Cat# 134531; Clone Mab-CC1; RRID:AB_2687363 |
| Human: CEACAM1 AF647 | R&D Systems | Cat# FAB2244R; Clone 283340 |
| PDL-1 BV786         | BD Biosciences | Cat# 741014; Clone MIH5; RRID:AB_2740636 |
| PDL-2 PE            | BD Biosciences | Cat# 557796; Clone TY25; RRID:AB_3966874 |
| CD80 BV510          | BD Biosciences | Cat# 740130; Clone 16-10A1; RRID:AB_2739887 |
| CD86 BV650          | BD Biosciences | Cat# 564200; Clone GL1; RRID:AB_2738665 |
| CD45 Pe Texas Red   | BioLegend | Cat# 103146; Clone 30-F11; RRID:AB_2564003 |
| CD45 AF700          | BioLegend | Cat# 103127; Clone 30-F11; RRID:AB_493714 |
| CD3 PeCy5           | BioLegend | Cat# 100310; Clone 145-2C11 RRID:AB_312675 |
| CD4 FItc            | BioLegend | Cat# 100510; Clone RM4-5 RRID:AB_312713 |
| CD8 BV650           | BioLegend | Cat# 100742; Clone 53-6.7; RRID:AB_2563056 |
| CD8 BV786           | BioLegend | Cat# 100750; Clone 53-6.7; RRID:AB_2562610 |
| Zombie NIR APC Cy7  | BioLegend | Cat# 423106 |
| Rat Anti-Mouse CD16/CD32 | BD Biosciences | Cat# 553142; Clone 2.4G2; RRID:AB_394665 |
| Normal Rabbit IgG   | New England BioLabs | Cat# 1031062 |
| BV650 Rat IgG2a Isotype control | BD Biosciences | Cat# 563236; Clone R3S-95; RRID:AB_2869472 |
| BV510 Hamster IgG2 Isotype control | BD Biosciences | Cat# 563202; Clone 881-3; RRID:AB_2869469 |
| PE Rat IgG2a Isotype control | BD Biosciences | Cat# 553930; Clone R3S-95; RRID:AB_479724 |
| BV786 Rat IgG2a Isotype control | BD Biosciences | Cat# 563335; Clone R3S-95; RRID:AB_2869486 |
| Alexa Fluor 647 Mouse IgG2b Isotype control | BD Biosciences | Cat# 557903; Clone 27-35; RRID:AB_396928 |
| BV421 Mouse IgG1 Isotype control | BD Biosciences | Cat# 562438; Clone X40; RRID:AB_11207319 |
| Dnk pAb to Rb IgG AF488 | Abcam | Cat# ab150073; RRID:AB_2636877 |
| Goat Anti-Rabbit IgG (H+L)-HRP Conjugate | BIO-RAD | Cat# 1706515; RRID:AB_2617112 |
| Goat Anti-Mouse IgG (H+L)-HRP Conjugate | BIO-RAD | Cat# 1706516; RRID:AB_2921252 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| iTaq Universal SYBR Green Supermix | Bio-Rad | Cat# 1725124; |
| Lipofectamine 3000 transfection Reagent | ThermoFisher | Cat# L3000008 |
| Mammary Epithelial Growth Supplement (MEGS) | ThermoFisher | Cat# S0155 |
| **Critical commercial assays** | | |
| eBioscience™ Foxp3/Transcription Factor Staining Buffer Set | ThermoFisher Scientific | Cat# 00-5523-00; |
| Dual-Luciferase® Reporter Assay System | Promega | Cat# E1980; |
| SimpleChIP® Enzymatic Chromatin IP Kit | New England BioLabs | Cat# 9003; |
| **Experimental models: Cell Lines** | | |
| Mouse: Yale University Mouse melanoma cell line | Dr. William Damsky, Yale University | YUMM1.1, YUMM1.7, YUMM1.9, YUMM2.1 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Human: OHRI-13 melanoma cell line | Dr. Carolina Ilkow; University of Ottawa | OHRI-13 |
| Human: SKMEL2 | American Type Culture Collection (ATCC) | Cat# HTB-68 |
| Human: SKMEL28 | Dr. John Bell; University of Ottawa | N/A |
| Human: M14 | Dr. John Bell; University of Ottawa | N/A |
| Human: HEK293T | American Type Culture Collection (ATCC) | Cat# CRL-3216; 293T |

**Experimental models: Organism/strains**

| Mouse: C57BL/6 | Jackson Laboratory | Cat# 000664; RRID: IMSR_JAX:000664 |
| Mouse: CB17.Cg-PrkdcsildHhrhr/lcrCr/ | Charles River | SCID |
| Mouse: B6.129S7-Rag1tm1Mom/J | Jackson Laboratory (Bred in house; Dr. Michele Ardolino) | Rag1 KO |
| Mouse: NOD-Prkdcs26Cd5l2tgem26Cd22/NjuCr/ | Charles River (Bred in house; Dr. Michele Ardolino) | NCG |

**Oligonucleotides**

- siRNA SOX10-1: Dharmaco Cat# J-049957-09
- siRNA SOX10-2: Dharmaco Cat# J-049957-10
- siRNA CTN: Dharmaco Cat# D-001810-01

**qPCR Primer:**

- Sox10 forward: AGGTGTGCTAAAGCAGAAAGGTGAC
- Sox10 reverse: CCGAGGTGCTACTGTCGAGCC

**ChIP Primer:**

- Ceacam1 forward [-4000]: GTAGAACCAACCTAGATCTCCA
- Ceacam1 reverse [-3820]: CTGTAAACTTTGTGATTCATCT

**Recombinant DNA**

- pUMVC: Addgene Cat# 8449; RRID:Addgene_8449
- pCMV-dR8.2 dvpr: Addgene Cat# 8455; RRID:Addgene_8455

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Luc Sabourin (lsabourin@ohri.ca).

**Materials availability**

Materials generated in this study can be made available upon request to the lead contact.

**Data and code availability**

This paper did not generate any original code. TCGA data used for correlation analysis is publicly available from [https://www.cbioportal.org/](https://www.cbioportal.org/). TCGA data used for immune infiltration within skin cutaneous melanoma is publicly available from [http://timer.cistrome.org/](http://timer.cistrome.org/). Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Mice**

All mice experiments were approved and in accordance with our Animal care and veterinary committee standard at the University of Ottawa. For our immune competent mouse experiments, C57BL/6J were purchased from Jackson Laboratory and bred in house. For our tumor studies male mice 8–10 weeks old were matched and divided blindly within the different tumor groups. For our various subcutaneous tumor growth models all studies were done at 1 × 10^6 cells in 100μL of PBS. 12–15 days post-injection mice were palpatated and checked for tumor growth using a caliper and tumor size was noted every 3 days until endpoint of 1700mm^3.
For the immune compromised studies, $1 \times 10^5$ YUMM cells in 100µL were administered subcutaneously to the right flank of SCID, RAG1 and NCG mice ranging within the age of 8–12 weeks. For SCID and NCG mice, tumors were checked every 3 days up to 12 days post injection and tumor sizes were measured using a caliper. Mice were euthanized once one animal in the study reached the endpoint set at 1700mm$^3$. For RAG1 tumors, mice were all euthanized on day 24 post injection and tumor sizes were compared.

For limiting dilution assays, NCG mice were injected twice, once in each flank with various cell concentrations ($1 \times 10^2$, $1 \times 10^3$, $1 \times 10^4$, $1 \times 10^5$) ($n = 4$ per group). 28 days post injection, mice were euthanized and assessed for tumor growths from the various cell concentrations. Images were taken of each respective tumor identified.

**Cell lines**

All Yale University Mouse Melanoma (YUMM) cell lines were a kind gift from Dr. William Damsky (Yale University). SKMEL-28 and M14 cells were a kind gift from Dr. John Bell (University of Ottawa). SKMEL-2 was obtained from ATCC. A human melanoma cell line OHRI-13 was isolated and provided by Dr. Carolina Ilkow (University of Ottawa). All mouse melanoma cell lines (YUMMs) were maintained in DMEM supplemented with 10% fetal bovine serum, 1X penicillin/streptomycin, 2mM L-glutamine and 1X nonessential amino acids. SKMEL-2, SKMEL-28 and M14 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1X penicillin/streptomycin, 2mM L-glutamine. The OHRI-13 was maintained in RPMI supplemented with 10% fetal bovine serum, 1X penicillin/streptomycin, 2mM L-glutamine. All cells were grown at 37°C with 5% CO₂ in humidified chambers.

**METHOD DETAILS**

**Protein collection and western blotting**

Cultured cells were all collected within 75–95% confluency and rinsed twice with 4mL of 1X ice-cold DPBS. Cells were scraped and collected at 8000G for 3 min. Cells were than lysed with RIPA lysis buffer (RIPA buffer containing 6m NaVO₃, 10µg/mL pepstatin and 10µM PMSF) and spun down to clear for 10 min at 12000G. For western blots pertaining to tumor lysates, mice were euthanized, tumors were isolated and minced into small pieces and further lysed within RIPA lysis buffer using a hand held homogenizer. Protein lysates were once again cleared for 10 min at 12000G. All lysates were quantified using a Bradford assay. Samples were set to a concentration of 1 µg/µL supplemented with 4X Laemmli buffer and denatured at 95°C for 5 min. Samples were loaded in 8–10% tris-glycine SDS-PAGE gels, transferred to PVDF membranes and analyzed for various protein expression.

**qPCR**

Total RNA was isolated using TRIzol (ThermoFisher). Cell culture media was removed and 1mL of TRIzol was added to each dish. Dishes were scraped and the TRIzol was transferred to a 1.5mL Eppendorf. 200µL of chloroform was added to each tube and shaken vigorously for 15 s, and left to rest for 3 min. Samples were then spun at 12000 g at 4°C for 15 min and the top clear layer was transferred to a fresh 1.5mL eppendorf. 500µL of isopropanol was added to the new tubes, vortexed and left to rest. After 10 min the samples were spun at 12000 g at 4°C for 10 min and the supernatant was removed, keeping only the white pellet. 1mL of 75% ethanol was added to each tube and spun at 7500G at 4°C for 5 min. Ethanol was removed and the pellet was left to air dry for 10 min before re-suspending in 100µL of water.

RNA was quantified using a nanodrop and 250ng of RNA was moved into PCR tubes. cDNA synthesis was achieved using Superscript III Reverse Transcriptase (ThermoFisher) according to the manufacturer’s protocol where 5X First Strand buffer, oligo(dT), random primers, and dNTP mix were all added to the 250ng of RNA. cDNAs were synthesized (25°C at 5 min, 55°C 60 min, 70°C 15 min and finally 37°C at 20 min) and samples were diluted 1/10 for qPCR. qPCRs were run on a 7500 Fast Real-Time PCR system using the standard ΔΔCT program with melt curve.

**Tumor sphere assay**

For primary spheres, YUMM cells were disaggregated with trypsin, counted and pushed through a 25 gauge needle three times to produce a single cell suspension. $2 \times 10^5$ cells were seeded per well into a 24 well ultralow attachment plate in 500µL of 1:1 DMEM/F12 supplemented with 1x B27 supplement and 1x MEGS (ThermoFisher). Each group was seeded in technical duplicates and imaged after 5–7 days s (depending on the cell group). Only spheres greater than or equal to 50µm were counted for total sphere
efficiency. To seed secondary spheres, primary spheres were collected, rinsed in 1XPBS, treated with trypsin, ran through a 25 gauge needle three times and counted. $2 \times 10^3$ cells were once again seeded in the same process as primary spheres and the quantification process was repeated.

Flow cytometry
Cells were collected and rinsed with 1X DPBS, stained with Zombie NIR viability dye (Biolegend) for 20 min and rinsed with 1X DPBS with 1% BSA (flow buffer). Cells were further stained with FC block (Biolegend) for 20 min and stained with antibodies for ligands found at the surface of cells (antibodies used are found in The Resource table). For intracellular flow, the Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher) was used following extracellular staining. In short, cells were fixed with Foxp3 Fixation/Permeabilization for 60 min, rinsed with 1X Permeabilization Buffer and stained with anti-rabbit SOX10 (Cell Signaling 1:50) for 60 min. Cells were rinsed once again with 1X Permeabilization Buffer and stained with Goat anti-Rabbit IgG Alexa Fluor 488 secondary for 60 min. All tumor samples were minced into small tumor pieces (no larger than 2mm$^3$) homogenized using the GentleMACS Octo Dissociator and incubated with ammonium chloride for 3 min to remove all red blood cells. Samples were then stained for extracellular and/or intracellular markers using the above methods. Data was collected using FACS DIVA on a BD LSR Fortessa and analyzed using FlowJo.

Cloning, viral production and siRNA transfection
CEACAM1 luciferase fragments were generated using BAC DNA (RP23-404A12 and RP23-64F12). In short various oligonucleotides were synthesized to span 1kbp of the CEACAM1 promoter region and PCR amplimers were cloned into pCloneJet. The CloneJet plasmids were digested with KpnI and Xhol and the inserts were moved over to the luciferase backbone pGL3-P. For pBABE-CEACAM1 Isoform 1 and 4 constructs, cDNAs from origene were amplified and cloned into pCloneJet. Using BamHI and EcoRI, the inserts were subcloned into the pBABE retroviral backbone. Sox10 guides targeting the N-terminal region of Sox10 were created using ChopChop (ChopChop.com), and oligos sgSox10-1 Forward: CACCGTGTGGATTTAGGCGAGTGTG, sgSox10-1 Reverse: AAACTCCGGACTACAAGTACCAACC and sgSox10-18 Forward: CACCGACAAGTACCAACCTCGGCGG, sgSox10-18 Reverse: AAACCCGCCGAGTGTGGATTTAGGCGAGTGTG were annealed using a thermocycler at 37°C for 30 min, 95°C for 5 min and temperature was ramped down 5°C/min to 25°C. pLKO H2B-mRFP-2A puro (a kind gift from Dr. Daniel Schramek, University of Toronto) was digested with BsmBI and the annealed oligos were subcloned into the digested vector.

For viral transduction $5 \times 10^5$ 293T cells were transfected with 10μg of plasmid vector, 8μg of pCMV-dR8.2 dvPR (Addgene) or pUMVC (Addgene) packaging plasmid and 2μg of pCMV-VSV-G (Addgene). Media was changed 12 h post transfection and collected 72 h later. The media was filtered and used to infect the various YUMM lines. 48 h post infection, cells were either sorted for RFP (for guide transduction), treated with puromycin for 9 days at 4μg/mL (pBABE transductions) or blastidin at 5μg/mL for 10 days (for Cas9 selection). Specifically for knockout cells, YUMM cells were first transduced with pLenti-Cas9-Blast and selected for. Following Cas9 selection, cells were then transduced with pLKO H2B-mRFP-2A-Puro encoding the guide RNA targeting Sox10. Cells were sorted for RFP 48 h post infection and knockdown was confirmed by western and qPCR analysis.

Dharmacon-designed siRNA specifically targeting mouse Sox10 was used for short term Sox10 knockdown. Briefly, 200nM of each siRNA was transfected using Lipofectamine 3000 and samples were collected 72 h post transfection.

Luciferase assay
In vitro cell cultures were transfected with 1μg of pGL3P, pGL3P Ceacam1[-1525/+5], pGL3P Ceacam1[-2875/-1435], pGL3P Ceacam1[-4135/-2785] or pGL3P Ceacam1[-5305/-4045] with 0.2μg of pRL-CMV used as the transfection efficiency control. 48hrs post transfection media was aspirated, cells were washed and 200μL 1X passive lysis buffer (provided by vendor) was placed in each well and left for 15 min with vigorous rotation. 20μL of lysed cells were placed in triplicate in a 96-well plate with 100μL of Luciferase assay buffer II and bioluminescence was read using a Biotek Cytation 5 imaging reader. Following the reading 100μL of Stop and Glow buffer was added and the plate was read once gain using Biotek Cytation 5 imaging reader to determine Renilla activity. All individual cell samples were seeded in triplicate and averaged as a single biological replicate. All experiments were done in biological triplicates.
Chromatin immunoprecipitation
For ChIP the SimpleChIP protocol (New England BioLabs) was used. In short, $1 \times 10^6$ YUMM1.1 cells were seeded in a 15cm dish and allowed to grow for 48 h. Following 48 h, four 15cm dishes containing the YUMM1.1 cells were cross-linked using formaldehyde, inactivated using glycine and collected and pooled according to the manufacturer’s instructions. Nuclei were isolated and chromatin was digested by treating samples with MNase for 20 min at 37°C followed by sonication on the Misonix sonicator 3000 using 30s pulses at the third setting for a total of three times. Input samples were collected at this point and used downstream in our PCR for normalization. Anti-SOX10 (1:50) was used and mixed with 10μg of digested chromatin while also using total Histone and rabbit IgG as positive and negative controls, respectively. Samples were left on a rotor overnight at 4°C and the next day 30μL of magnetic beads were added to each tube and left on the rotor for an additional 2 h. Beads were rinsed multiple times according to the manufacturer’s instructions and chromatin pulled downs were eluted. Chromatin was further cleaned using the DNA spin columns provided and concentration of samples was determined using Qubit 3.0. PCRs were run on various regions of the CEACAM1 promoter while also using RPL30 Intron 2 primers as positive and negative controls. IgG samples were used as our normalization control to assess background noise.

QUANTIFICATION AND STATISTICAL ANALYSIS
Correlation and immune infiltration analysis
For percent survival between high and low CD4+ and CD8+ T cell infiltration in melanoma, data was processed and analyzed by Timer2.0 (http://timer.cistrome.org/).69 The top 25% and bottom 25% of T cell infiltrates were used in the Timer2.0 analysis.

The correlation data between SOX10 and CEACAM1 mRNA in melanoma, was retrieved on cBioPortal (https://www.cbioportal.org/),70,71 and re-plotted using GraphPad Prism software. The Skin Cutaneous Melanoma TCGA Firehose Legacy dataset was used to retrieve the data. Z score threshold of ±2 was used to determine correlation between Sox10 and various genes.

Statistical analysis
All in vitro experiments were performed in three biological replicates while all mice work was done once with animal numbers indicated in the figure legends. All analysis was done through GraphPad Prism software and data is represented as the SEM(±SEM). T tests, one way and two ANOVA were used for statistical analysis of various experiments (statistical significance and tests used is specified in figure legends).