

# Regulation of CEACAM1 Protein Expression by the Transcription Factor ETS-1 in BRAF-Mutant Human Metastatic Melanoma Cells<sup>1,2</sup>



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## Abstract

BRAF becomes constitutively activated in 50% to 70% of melanoma cases. CEACAM1 has a dual role in melanoma, including facilitation of cell proliferation and suppression of infiltrating lymphocytes, which are consistent with its value as a marker for poor prognosis in melanoma patients. Here we show that BRAF<sup>V600E</sup> melanoma cells treated with BRAF and MEK inhibitors (MAPKi) downregulate CEACAM1 mRNA and protein expression in a dose- and exposure time-dependent manners. Indeed, there is a significant correlation between the presence of BRAF<sup>V600E</sup> and CEACAM1 expression in melanoma specimens obtained from 45 patients. Vemurafenib-resistant cell systems reactivate the MAPK pathway and restore basal CEACAM1 mRNA and protein levels. These combined results suggest transcriptional regulation. Indeed, luciferase reporting assays show that CEACAM1 promoter (CEACAM1p) activity is significantly reduced by MAPKi. Importantly, we show that the MAPK-driven CEACAM1p activity is mediated by ETS1, a major transcription factor and downstream effector of the MAPK pathway. Phosphorylation mutant ETS1<sup>T38A</sup> shows a dominant negative effect over CEACAM1 expression. The data are consistent with independent RNAseq data from serial biopsies of melanoma patients treated with BRAF inhibitors, which demonstrate similar CEACAM1 downregulation. Finally, we show that CEACAM1 downregulation by MAPKi renders the cells more sensitive to T-cell activation. These results provide a new view on a potential immunological mechanism of action of MAPKi in melanoma, as well as on the aggressive phenotype observed in drug-resistant cells.

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## Introduction

Melanoma accounts for nearly 4% of all skin cancers, and it causes 75% of skin cancer–related deaths worldwide [1]. Disease progression and development of metastasis require stepwise acquisition of aggressive characteristics [2], including resistance to the immune system [3]. In the last years, the US Food and Drug Administration approved anti-CTLA4 mAb (ipilimumab), anti-PD-1 mAbs (nivolumab, pembrolizumab), selective BRAF<sup>V600E</sup> inhibitors (vemurafenib, dabrafenib), as well as MEK inhibitors (trametinib, cobimetinib) as monotherapies or in combination for the indication of metastatic melanoma. Although these drugs show proven benefit in overall

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survival [4–6], the treatment for melanoma is still far from being satisfactory.

Activating BRAF mutations appear early in melanoma development, mostly at the premalignant nevus [7], and cause constitutive activation of the MAPK pathway. Targeting of the MAPK pathway in BRAF-mutant patients yields high response rates with rapid kinetics, leading to an overall survival benefit [8–11]. This effect is mediated by shutdown of the pathway, as reflected by decreased pERK expression. Unfortunately, in almost all cases, pathway reactivation occurs in the face of the medications via a variety of resistance mechanisms [12,13], leading to treatment failure and rapid disease progression. Being such a dominant pathway, further understanding of how it is involved in the disease is still warranted.

CEACAM1 is a transmembrane glycoprotein that belongs to the carcinoembryonic antigen family and is encoded on chromosome 19 [14]. The gene gives rise to several alternative splice forms, including a long and short cytosolic tail. CEACAM1 interacts homophilically with CEACAM1 and heterophilically with CEACAM5 but not with other CEACAM proteins [15]. CEACAM1 is expressed on a variety of cells of epithelial and hematological origins, including melanoma and activated lymphocytes [14]. Many different functions have been attributed to the CEACAM1 protein, including antiproliferative properties in carcinomas of the colon and prostate, central involvement of CEACAM1 in angiogenesis and insulin clearance, as well as immune-modulation (reviewed in [14,16]).

CEACAM1 is deeply involved in the biology of melanoma. Indeed, the presence of CEACAM1 on primary cutaneous melanoma lesions strongly predicts the development of metastatic disease [17], and CEACAM1 expression predicts metastatic spread in melanoma xenograft models in immunodeficient mice [18]. We have previously shown that CEACAM1 is an immune checkpoint in activated NK cells [19–21] and melanoma-derived tumor-infiltrating lymphocytes [22] and that it is used as an adaptive immune resistance mechanism by melanoma cells [23]. Following these findings, we developed a novel anti-CEACAM1 mAb [24]. Moreover, we found that CEACAM1 expression increases along melanoma development and progression [25], and it directly facilitates the proliferation of melanoma cells [26]. It was also recently reported that CEACAM1 facilitates melanoma cell invasion and metastasis [27]. In addition, increased CEACAM1 expression on peripheral blood lymphocytes and concentration of soluble CEACAM1 in the serum has been observed in melanoma patients [28], with serum CEACAM1 potentially enabling monitoring melanoma patients treated with autologous vaccination [29] or with adoptive cell transfer therapy [30].

Here we report that CEACAM1 expression is associated with mutant BRAF and is regulated by the MAPK pathway at the transcriptional level via the ETS1 transcription factor.

## Materials and Methods

### Cell Lines and Tissue Culture

The human melanoma lines 526mel and 624mel (obtained from Dr. S.A. Rosenberg, NCI, USA) bear BRAF<sup>V600E</sup>. BRAF<sup>WT</sup> 04mel and 076mel were established from surgically removed specimens, as described previously [31]. SKmel-5 and SKmel-2 (ATCC, USA) bear BRAF<sup>V600E</sup>NRAS<sup>WT</sup> and BRAF<sup>WT</sup>NRAS<sup>Q61R</sup>, respectively. All melanoma cultures were cultured in standardized supplemented RPMI medium as described previously [31]. Primary melanoma patient-derived tumor-infiltrating lymphocytes culture (TIL14) was

established from a surgically excised melanoma specimen (Israel Ministry of Health approval no. 3518/2004) and cultured as previously described [32].

### BRAFi- and MEKi-Resistant Cell Lines

526mel and 624mel cells were cultured in the chronic presence of a BRAF<sup>V600E</sup> inhibitor vemurafenib (PLX4032) or ERK1/2 inhibitor selumetinib (AZD6244, ARRY-142886). The inhibitors were initially added to the culture in  $\times 0.01$  IC<sub>50</sub>. Once a week, the concentration was doubled up to  $\times 10$  IC<sub>50</sub>. Cells with acquired resistance are maintained in 310 nM vemurafenib or 140 nM selumetinib.

### Antibodies

MRG1 is a home-made mouse monoclonal antibody specific to human CEACAM1 [24]. Other antibodies used were anti-phospho-p44/42 MAPK (ERK1/2) Thr 202/Tyr 204 (Cell Signaling), anti-p44/42 MAPK (ERK1/2) (Cell Signaling), and anti-ETS1 antibody [1G11] 10936 (Abcam). FITC-conjugated goat anti-mouse polyclonal antibodies were used as secondary reagent in FACS assays (Jaxon Immunoresearch, USA).

### Flow Cytometry and Immunoblotting

A total of 100,000 cells were stained using standard extracellular and intracellular flow cytometry staining protocols [24]. Cells were analyzed with FACSCalibur instrument (BD Biosciences) and FlowJo software. Lysates of  $5 \times 10^6$  cells were washed with PBS, lysed in RIPA lysis buffer (Sigma Aldrich), and incubated with protease inhibitor cocktail (Roche) and Phosphatase Inhibitor Cocktail (PMSF 1 mM, sodium orthovanadate 1mM, beta-glycerophosphate 2.5 mM, NaF 5mM, DTT 50 mM), where applicable. Standard immunoblotting protocols were used with specific antibodies and visualized with by standard ECL reaction [33]. Band quantification was determined by densitometry using Image-J software.

### RNA Isolation and Reverse Transcription

Total RNA was isolated from Trizol-homogenized cells using Tri Reagent (Sigma Aldrich) extraction method. Integrity of the RNA was determined by spectrophotometry and electrophoresis. The cDNA pools were generated with a high-capacity reverse transcriptase kit (Applied Biosystems) using random hexamer primers.

### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Primers were designed using Primer-Express software guidelines (Applied Biosystems) and manufactured by Sigma Aldrich (Supplementary Table 1). The qRT-PCRs were run on LightCycler 480 (Roche) in triplicates. Transcripts were detected using 2 $\times$  SYBR Green Master Mix (Roche) according to manufacturer's instructions and were normalized to GAPDH. The list of primers appears in Supplementary Table 1.

### Cloning and Mutagenesis

ETS1 isoform 2 was PCR-amplified from cDNA of melanoma cells and cloned into pQCXIP vector (Clontech laboratories, Mountain View, CA) using enzyme restriction sites NotI and PacI (New England Biolabs, MA). The promoter of CEACAM1 (CEACAM1p) cloned into the pGL1.4 luciferase reporting vector was generated previously [26]. ETS1 point mutation at position 38 (ETS1<sup>T38A</sup>) and deletions of the sequences GGGGGATCCTCCTCCCCT on the negative strand and GCGTTCCTG on the positive strand (putative

ETS1 binding sites) from CEACAM1p to create CEACAM1p-ΔETS1(−) and CEACAM1p-ΔETS1(+), respectively, or both were done using QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), according to manufacturer's protocol. The list of primers appears in Supplementary Table 1. All cloned inserts were fully sequenced (Hylabs Laboratories, Israel).

### Quantification of Promoter Activity with Luciferase Assay

To measure the effect of BRAF or MEK inhibitors on CEACAM1 promoter activity, pGL4.14 empty vector, CEACAM1p or CEACAM1p-ΔETS1 constructs were co-transfected with pRL *Renilla* Luciferase Reporter Vector (Promega, Madison, WI) into melanoma cells in a 50:1 ratio using TurboFect Transfection Reagent (Fermentas, Burlington, Canada) according to manufacturer's instructions. Cells were incubated with 1 μM of vemurafenib or selumetinib for 48 hours. After 48 hours, cells were lysed, and luciferase activity was measured. To measure the effect of ETS1 on CEACAM1 promoter activity, 293T cells were co-transfected with 10 ng pGL4.14 empty vector or CEACAM1p, together with 100 ng ETS1 or ETS1<sup>T38A</sup> or mock (pQCXIP), along with 0.4 ng pRL *Renilla* Luciferase Reporter Vector using TurboFect Transfection Reagent according to manufacturer's instructions. After 48 hours, cells were lysed and luciferase activity was measured. All assays were measured by Dual Luciferase Reporter Assay System (Promega) using GlowMax microplate reader (Promega) and normalized to the *Renilla* signal.

### LDH Cytotoxicity Assays

Cytotoxicity assays were performed by measuring lactate dehydrogenase (LDH) release using CytoTox-96 (Promega). Briefly, target cells were co-incubated for 18 hours with effector cells at an E:T ratio of 5:1 in a 96-well plate. Wells with target cells only were lysed prior to readout to obtain maximum LDH release. Plates were centrifuged, and 50 μl of supernatants was transferred to a new 96-well plate. Fifty microliters of LDH substrate mix was added to each well, and plates were incubated covered at room temperature for 30 minutes, followed by 50 μl of stop buffer to each well. Optic density was estimated at a wavelength of 490 nm (GlowMax). All experiments were performed in triplicate wells. Percent of specific lysis was calculated using the equation (Experiment-Effector spontaneous – Target spontaneous) / (Target maximum – Target spontaneous) × 100.

### Statistics

Significance of effects of specific treatments compared to control was determined by Student's *t* test or analysis of variance (ANOVA). Association between two binary parameters was tested with Fisher's exact test. In all graphs, bars represent standard error.

## Results

### Correlation of CEACAM1 Expression with BRAF-V600E Mutation in Melanoma

The association of BRAF mutation genotype and CEACAM1 expression status, as determined by flow cytometry or qPCR, was tested in 24 low-passage primary cultures of metastatic cutaneous melanoma cell lines [32] and by immunohistochemistry in other 21 metastatic cutaneous melanoma specimens (Table 1). Remarkably, almost all of the CEACAM1-negative melanoma cultures or histological specimens were among the BRAF WT cases (Table 1). These observations could suggest that CEACAM1 expression is controlled by the constitutively activated MAPK pathway.

### The Effect of MAPK Inhibition on CEACAM1 Expression

BRAF<sup>V600E</sup> melanoma cells (526mel and 624mel) and BRAF<sup>WT</sup> melanoma cells (076mel) were incubated in the presence of BRAF-V600E inhibitor vemurafenib or the MEK1/2 inhibitor selumetinib. Expectedly, vemurafenib and selumetinib significantly reduced pERK expression among the BRAF<sup>V600E</sup> melanoma cells (Figure 1A). While vemurafenib had no functional effect on BRAF<sup>WT</sup> cells and in some cases even resulted in a paradoxical increase in pERK, selumetinib reduced pERK expression in these cells as well (Figure 1A).

Melanoma cells were exposed to the inhibitors for 2, 24, or 48 hours using concentrations of 0.1 μM or 1 μM. Importantly, CEACAM1 expression was downregulated among the BRAF<sup>V600E</sup> melanoma lines in response to both inhibitors in dose- and exposure time-dependent manners (Figure 1B). Notably, in line with the reduction in pERK expression following exposure to selumetinib (Figure 1A), CEACAM1 was also downregulated in BRAF<sup>WT</sup> melanoma cells in response to selumetinib only (Figure 1B). A similar decrease was also observed in intracellular staining, arguing against altered intracellular trafficking as a potential explanation (Supplementary Figure 1). The response to the inhibitors was similarly evident at the mRNA level (Figure 1C). There was no preferential effect on certain CEACAM1 cytoplasmic tail splice variants, suggesting that splicing is unaffected (Figure 1C). Similar results were observed with additional BRAF mutant or wild-type melanoma cells (Supplementary Figure 2). CEACAM1 downregulation was observed also using Western blotting (Supplementary Figure 3). CEACAM1 expression was downregulated in NRAS mutant (Q61R) SK-mel2 cells only following treatment with selumetinib, as vemurafenib had little or no effect (Supplementary Figure 4). Collectively, these results indicate that CEACAM1 expression is controlled by the MAPK pathway at the transcriptional level.

### Resistance to MAPK Pathway Inhibitors Restores CEACAM1 Expression

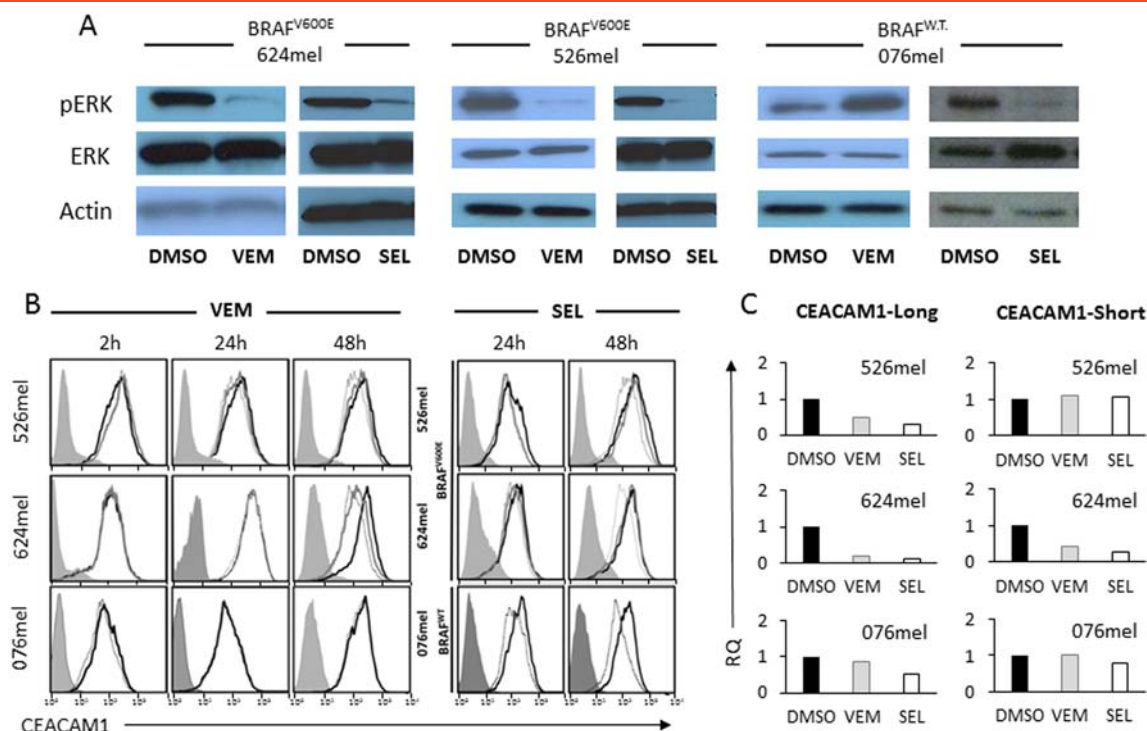
526mel and 624mel cells with acquired resistance to vemurafenib or selumetinib (526<sup>VEM</sup>, 526<sup>SEL</sup>, 624<sup>VEM</sup>, and 624<sup>SEL</sup>, respectively) were generated by prolonged culturing in increasing concentrations of vemurafenib or selumetinib, as detailed in Materials and Methods. While acute exposure to vemurafenib or selumetinib reduces pERK (Figure 1), its expression was restored or increased in all cells resistant to vemurafenib or selumetinib (Figure 2A). The mechanisms of resistance here are undefined, but the restoration of the MAPK pathway activity was confirmed, which is in line with the outcome of the majority of resistance mechanisms to BRAF or MEK inhibitors [13]. Remarkably, CEACAM1 expression increased in all cells resistant to vemurafenib or selumetinib at the protein level using flow cytometry (Figure 2B) or Western blot (Supplementary

**Table 1.** Association between CEACAM1 Expression and BRAF Mutation Status

|          | Cell Cultures |      | Histopathology |      |
|----------|---------------|------|----------------|------|
|          | Wild Type     | V600 | Wild Type      | V600 |
| Positive | 3             | 18   | 6              | 9    |
| Negative | 3             | 0    | 5              | 1    |
| P value  | 0.009         |      | 0.06           |      |

CEACAM1 expression status was tested in cell cultures using RT-PCR and flow cytometry, and with immunohistochemistry in tissue specimens. BRAF genotyping was performed by sequencing. Fisher's exact test was used to determine the statistical significance of the association.



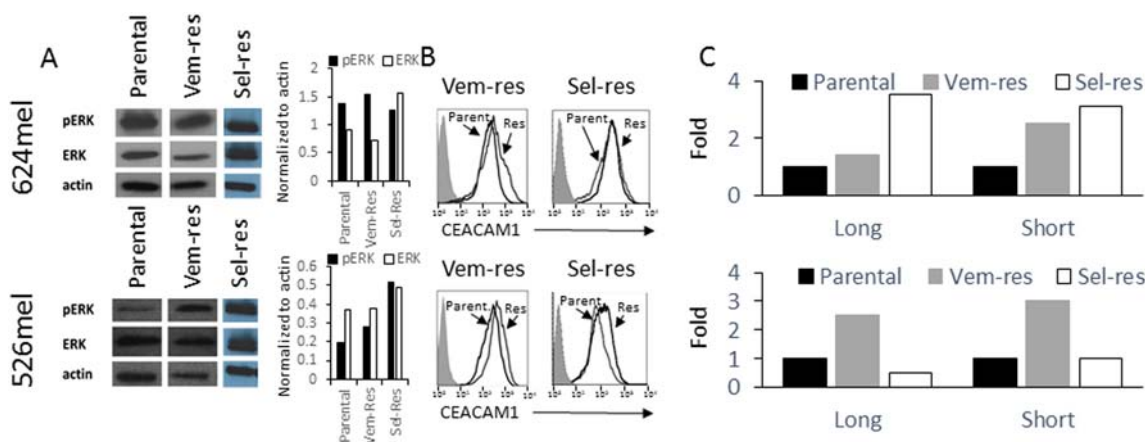


**Figure 1.** Inhibition of MAPK pathway downregulates CEACAM1 expression. The indicated BRAF mutant or wild-type (WT) melanoma cells were incubated with vemurafenib (VEM), selumetinib (SEL), or control (DMSO). (A) The effect of each treatment on pERK. (B) The effect of different doses of each treatment on CEACAM1 expression, as tested by flow cytometry, in each of the melanoma cell lines, in the indicated time points. Shaded histograms represent staining with secondary reagent only. Black histograms represent treatment with DMSO. Gray and dotted histograms represent treatment with 0.1  $\mu$ M or 1  $\mu$ M, respectively, of VEM or SEL. (C) The effect of each treatment on CEACAM1 isoform expression (long, short) using RT-PCR. Results are depicted as fold change (RQ) of the DMSO control. Figure shows a representative experiment out of three performed.

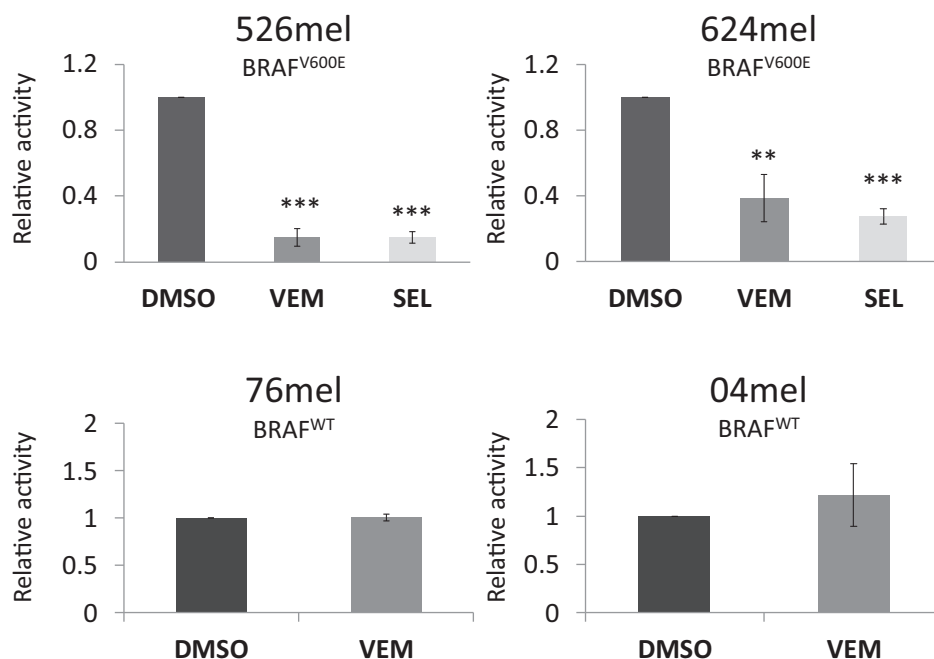
Figure 3) and the mRNA level (Figure 2C). There were no significant differences between long and short CEACAM1 isoforms (Figure 2C). These results substantiate the control of CEACAM1 expression by the MAPK pathway at the transcription level.

### *Inhibition of MAPK Pathway Reduces CEACAM1 Promoter Activity in BRAF<sup>V600E</sup> Melanoma Cells*

The promoter of CEACAM1 was cloned upstream to a firefly luciferase reporter gene. Empty vector served as control. Each construct was transiently transfected into BRAF<sup>V600E</sup> 526mel or



**Figure 2.** Resistance to inhibitors of the MAPK pathway restores CEACAM1 expression. Vemurafenib-resistant (Vem-Res) and selumetinib-resistant (Sel-Res) sublines of 624mel and 526mel cells were tested. (A) The restored expression of pERK. The graph shows the ratio of each indicated protein as normalized according to actin using densitometry. (B) The restored expression of CEACAM1 using flow cytometry. Shaded histograms represent staining with secondary reagent only. Parental (Parent.) and resistant (Res) histograms are indicated in each panel. (C) The effect of each treatment on CEACAM1 isoform expression (long, short) using RT-PCR. Results are depicted as fold change (RQ) of the parental cell control. Figure shows a representative experiment out of three performed.

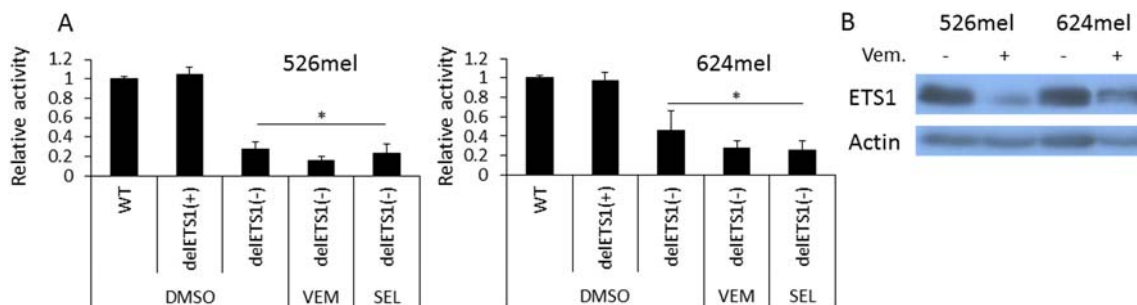


**Figure 3.** Inhibition of MAPK pathway downregulates the activity of the CEACAM1 promoter. CEACAM1 promoter was cloned upstream to firefly luciferase and co-transfected into the indicated melanoma cell lines together with a normalizing construct of Renilla luciferase. Empty vector served as negative control. Cells were treated for 2 days with DMSO, or 1  $\mu$ M vemurafenib (VEM) or selumetinib (SEL). Relative promoter activity was calculated relative to the control (cells transfected with an empty vector and treated with DMSO). Figure shows the average results of four independent experiments. Significance was tested with ANOVA, \*\* and \*\*\* depict *P* value of <.01 and <.001, respectively.

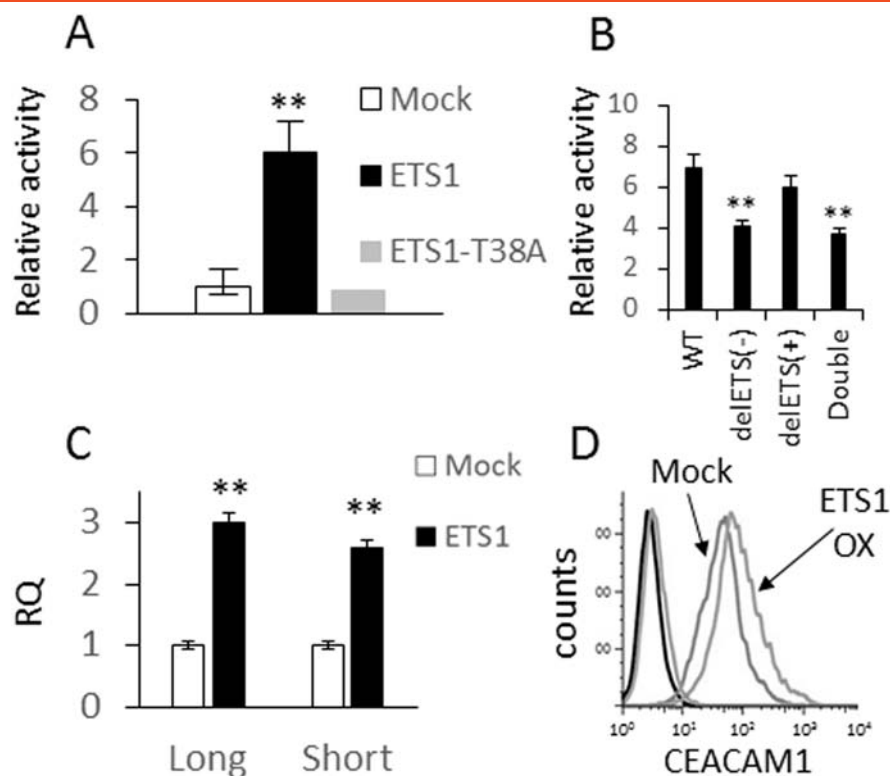
624mel cells, or into BRAF<sup>WT</sup> 076mel or 04mel cells. Activity was standardized by co-transfection with Renilla luciferase under a constitutive promoter. The different transfectants were exposed to vemurafenib or selumetinib (1  $\mu$ M, for 48 hours) or to 0.01% DMSO as control. A significant reduction in CEACAM1 promoter activity following treatment with MAPK inhibitors as compared to control treatment was observed exclusively in BRAF<sup>V600E</sup> cells but not in BRAF<sup>WT</sup> cells (Figure 3). These experiments confirm that CEACAM1 is controlled by the MAPK pathway at the level of transcription.

#### CEACAM1 Promoter Is Controlled by the MAPK Pathway Via ETS1

Bioinformatics prediction with MAPPER tool [34] points to a putative binding site for ETS1 on the negative strand, from which CEACAM1 is transcribed, as well as on the positive strand (Supplementary Figure 5A). ETS1 was recently reported as a potential effector of the MAPK pathway [35] and has known oncogenic roles in various types of cancer, including melanoma [36,37]. There are no previous reports on the regulation of CEACAM1 by ETS1.



**Figure 4.** CEACAM1 expression is controlled by the MAPK pathway via the ETS1 transcription factor. (A) CEACAM1 promoter with a deletion in the putative ETS1 binding site on the negative strand (delETS1(-)), positive strand (delETS1(+)), or the wild-type sequence (WT) was cloned upstream to firefly luciferase and co-transfected into the indicated melanoma cell lines together with a normalizing construct of Renilla luciferase. Empty vector served as negative control. Cells were treated for 2 days with DMSO, or 1  $\mu$ M vemurafenib (VEM) or selumetinib (SEL). Relative promoter activity was calculated relative to the control (cells transfected with an empty vector and treated with DMSO). Figure shows the average results of four independent experiments. (B) ETS1 expression in the indicated melanoma lines in the presence or absence of vemurafenib. Figure shows a representative experiment out of four independent experiments. Significance was tested with ANOVA, \* depicts *P* value of <.05.

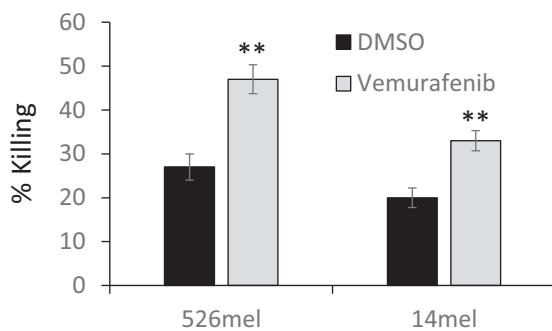


**Figure 5.** ETS1 upregulates the expression of CEACAM1. (A) CEACAM1 promoter was cloned upstream to firefly luciferase and co-transfected with a normalizing construct of Renilla luciferase, together with a vector encoding for ETS1 (ETS1), mutated ETS1 (ETS1-T38A), or an empty vector (Mock). Relative promoter activity was calculated relative to the effect of transfection with Mock vector. (B) Wild type (WT) or deletions in the putative ETS1 binding site in the negative strand (delETS1(-)), positive strand (delETS1(+)), or both (double) of the CEACAM1 promoter were cloned upstream to firefly luciferase and co-transfected with a normalizing construct of Renilla luciferase, together with a vector encoding for ETS1 (ETS1) or an empty vector (Mock). Data shown are normalized to Mock. (C) The effect of ETS1 (ETS1) compared to an empty vector (Mock) on CEACAM1 isoform (long, short) expression following transfected into melanoma was tested at the mRNA level using RT-PCR. (D) CEACAM1 expression was tested at the protein level using flow cytometry. The histograms of each of the transfectants are indicated in the figure. (A-C) The average results of three independent experiments. Significance was tested with Student's *t* test, \*\* depicts *P* value of <.01.

The putative ETS1 binding site was deleted in the CEACAM1p/pGL4.14 promoter construct from the negative strand (CEACAM1p  $\Delta$ ETS1(-)), the positive strand (CEACAM1p  $\Delta$ ETS1(+)), or both (CEACAM1p- $\Delta$ ETS1(double)). The wild-type CEACAM1p,  $\Delta$ ETS1(-),  $\Delta$ ETS1(+), or mock/pGL4.14 constructs were transiently transfected into BRAF<sup>V600E</sup> cells (526mel or 624mel) or the BRAF<sup>WT</sup> cells (076mel). Activity was standardized by co-transfection with Renilla luciferase under a constitutive promoter. A significant reduction in the basal activity of CEACAM1p- $\Delta$ ETS1(-) but not of CEACAM1p- $\Delta$ ETS1(+) was evident in BRAF<sup>V600E</sup> cells as compared to wild-type CEACAM1p activity. This observation suggests that ETS1 positively regulates the promoter activity of CEACAM1 via the binding site on the negative strand (Figure 4A). Some decrease in the basal activity of CEACAM1p- $\Delta$ ETS1(-) as compared to wild-type CEACAM1p was observed also in BRAF<sup>WT</sup> 076mel cells, probably reflecting the effect of the endogenous ETS1 in these cells (Supplementary Figure 5B). Remarkably, treatment of BRAF<sup>V600E</sup> cells with MAPK inhibitors (1 $\mu$ M for 48h) did not further decrease the promoter activity of CEACAM1p- $\Delta$ ETS1(-) (Figure 4A). Western blot shows that blocking of the MAPK pathway downregulates the expression of ETS1 in these cells (Figure 4B). These collective results suggest that the MAPK pathway regulates the activity of the CEACAM1 promoter via ETS1 by controlling ETS1 expression levels.

### ETS1 Activates the Promoter of CEACAM1

ETS1 isoform analysis in five primary low-passage metastatic melanoma cultures shows that isoform 2 (known as p51/p54) is the dominant form in melanoma (Supplementary Figure 5C), and therefore, it was cloned for subsequent mechanistic studies. The threonine-38 residue, which is important for the Ras-responsive transcriptional activity of ETS1 [38], was mutated to alanine (ETS1-T38A). Co-transfection of WT CEACAM1p into 293T cells with ETS1, but not with ETS1-T38A or with an empty vector, dramatically increased the promoter activity of CEACAM1p (Figure 5A). This observation points to the regulation of CEACAM1 promoter by ETS1 in a way that depends on active phosphorylation of threonine-38. Further, the activity of the CEACAM1p- $\Delta$ ETS1(-) or CEACAM1p- $\Delta$ ETS1(double) constructs is substantially less responsive to co-transfection with ETS1 as compared to WT CEACAM1p or CEACAM1p- $\Delta$ ETS1(+) (Figure 5B). This suggests that ETS1 regulates CEACAM1p only through the putative binding site in the negative DNA strand. The fact that ETS1 still increases to a certain degree the activity of CEACAM1p- $\Delta$ ETS1 (Figure 5B) suggests that there are additional indirect mechanisms. In line with the promoter experiments, overexpression of ETS1 in melanoma cells moderately but consistently induces CEACAM1 expression at both the mRNA



**Figure 6.** Vemurafenib enhances the cytotoxic effect by T cells. 526mel and 14mel cells were treated with vemurafenib (VEM) or DMSO control and then co-cultured for 18 hours with cognate T cells in an E:T ratio of 5:1. Cytotoxicity was measured using LDH release assay. Figure shows the average of three independent experiments. Significance was tested with Student's *t* test, \*\* depicts *P* value of <.01.

and the protein levels (Figure 5C and D). These collective results show that ETS1 controls CEACAM1 expression at the transcription level.

#### Enhanced Elimination of Vemurafenib-Treated Melanoma Cells by Specific T Cells

526mel and mel14 cells were used as target cells for bulk tumor-infiltrating lymphocyte culture obtained from melanoma patient (TIL14). TIL14 are late effector T cells comprised of more than 97% CD8(+) T cells. They were derived from the same patient as mel14, and we have previously shown that it specifically recognizes 526mel through HLA-A2 [22,23]. Melanoma cells were exposed to vemurafenib (1  $\mu$ M; 48 hours) or volume equivalent DMSO and co-cultured with TIL14 for 18 hours in an effector-to-target ratio of 5:1. Remarkably, vemurafenib-treated melanoma cells were significantly more sensitive to T cells than the DMSO-treated cells (Figure 6). This is in line with the reduction in CEACAM1 expression induced by vemurafenib (Figure 1). These experiments suggest that indeed BRAF inhibitors could mediate a local, transient, enhancement of T-cell activity following reduction of immune checkpoint ligands such as CEACAM1.

#### Discussion

CEACAM1 holds a key role in the pathogenesis of metastatic melanoma. It is not expressed on normal melanocytes but becomes upregulated during melanoma development and progression, and is eventually found on the majority of metastatic melanoma cases [25,26]. Functionally, CEACAM1 protects melanoma cells from both activated NK and T cells by inhibiting their cytotoxic activity [19,21–23], and in parallel, it enhances melanoma cell proliferation [26]. These probably account for its prognostic association with poor survival in melanoma [17]. Understanding the regulation of CEACAM1 expression in melanoma is therefore important but still mostly unknown. Here we show for the first time the mechanistic link between the extensively investigated MAPK pathway and the expression of the CEACAM1 protein.

We observed an association between the presence of activating BRAF mutation and the expression of CEACAM1 (Table 1). Selective BRAF<sup>V600</sup> inhibition with vemurafenib leads to CEA-

CAM1 downregulation in dose- and time-dependent manners (Figure 1), establishing the mechanistic regulation of CEACAM1 by the MAPK pathway. Similar results were also observed following downstream inhibition of MEK1/2 with selumetinib (Figure 1). This phenomenon was demonstrated with several detection methods and in several melanoma lines of different mutational status (Figure 1 and Supplementary Figures 1–4) to solidify its validity. Interestingly, both CEACAM1 expression on the primary tumor [17] and BRAF V600 mutations [39] are considered as markers of poor prognosis, particularly once the first metastasis is diagnosed.

It should be noted, however, that analysis of CEACAM1 according to BRAF mutational status of the RNAseq data of the 468 tumors in the TCGA collection shows nonstatistically significant trends of a) higher CEACAM1 levels among primary BRAF-mutant melanoma as compared to BRAF-WT and b) an increase in CEACAM1 levels in metastasis as compared to primary tumors in BRAF-WT melanoma but not in BRAF-mutant melanoma cells (data not shown). The lack of conclusive evidence from the TCGA indicates the complexity of CEACAM1 regulation. Indeed, it is regulated by SOX9 [40], AP-2 [41], and IFN $\gamma$  [23,42]. Importantly, independent *in vivo* support for CEACAM1 decrease following acute exposure to BRAFi was obtained from a recently published RNAseq database of serial melanoma biopsies before and during response to vemurafenib, dabrafenib, or combined BRAF and MEK inhibitors [43]. Indeed, CEACAM1 was downregulated in 50% of the patients by at least two-fold, and in 43% of them, a concomitant downregulation in ETS1 mRNA by at least two-fold was observed [43]. Reestablishment of MAPK signaling in different BRAFi- or MEKi-resistant melanoma lines is coupled with restored CEACAM1 expression (Figure 2). Albeit the exact resistance mechanism to BRAFi and MEKi in our melanoma cells has not been determined, reestablishment of MAPK signaling is visible by pERK upregulation (Figure 2). In the independent RNAseq data, CEACAM1 mRNA was still downregulated upon disease progression but to a lesser extent than during response [43]. The differences between this data set and our *in vitro* results may be explained by tumor heterogeneity in biopsies versus *in vitro* straightforward cell line data. Another possibility is the difference between mRNA and protein measurements, as the effect of mRNA quantities on protein quantities cannot be easily extrapolated.

Functionally, BRAF inhibition has a dominant and direct effect on cell survival and proliferation, which probably overshadows the effect of reduced CEACAM1 on proliferation. Nevertheless, we show that acute exposure of melanoma cells to BRAF inhibitors renders melanoma cells more sensitive to cognate T cells (Figure 6), concurring with the downregulation in CEACAM1 levels (Figure 1) and its known T-cell-suppressive effect [21–24]. This observation is in line with previous reports that BRAF inhibition may result in immune sensitization, albeit transient [44]. The CEACAM1 downregulation observed in the RNAseq data in MAPK inhibitor-treated patients supports this direction [43]. It should be noted that the expression of PD-L1 in melanoma cells is variably regulated following treatment with BRAF inhibitors [45]. As melanoma cells hardly express PD-L1 *in vitro* (unpublished data) unless stimulated with interferons, it does not seem plausible that the enhanced immune sensitivity observed here can be accounted for by PD-L1 downregulation. Importantly, it was recently demonstrated that disease progression on MAPK inhibitors is associated in at least half of the cases with CD8(+) T-cell deficiency. RNAseq demonstrated reduced antigen presentation, cytolytic function, and exhaustion markers on T-cell subset [43]. It would be interesting to



study in the future the expression of CEACAM1 on these cells in such specimens. Taken together, it seems that CEACAM1 downregulation following patient therapy with BRAF inhibitors may contribute to a transiently facilitated immune-mediated effect against the melanoma cells; however, in the face of a frequent subsequent T-cell depletion, further studies are needed to determine a scientific rationale for combination therapies.

We show that CEACAM1 expression is controlled by the MAPK pathway at the transcriptional level (Figure 3) and provide evidence that this is mediated by ETS1 transcription factor. ETS1 is an important oncogenic factor in various types of cancer [36], including melanoma [37,46–48]. It was previously published that ETS1 phosphorylation at T38 by ERK1/2 increases its transcriptional activity [49]. Here we show that deletion of the putative ETS1 binding site within the CEACAM1 promoter abrogates the effect of MAPK inhibition (Figure 4). In line with the previous report [49], mutation at the critical phosphorylation site T38 within ETS1 eliminates its ability to induce CEACAM1 promoter activity (Figure 5). In addition, we show that inhibition of the MAPK pathway downregulates ETS1 expression (Figure 4). This suggests that the MAPK pathway controls ETS1-mediated effects at both the expression and function levels. ETS1 similarly induces both long and short isoforms of CEACAM1 (Figure 5), suggesting no effect on splicing of the transcribed mRNA. This is in agreement with the effects of MAPK inhibitors on CEACAM1 isoform expression (Figures 1 and 2).

We have previously published that the rare, highly linked germline alleles of SNPs rs8103285 and rs8102519 within the promoter of CEACAM1 dramatically enhance the activity of CEACAM1 promoter and are associated with melanoma (allelic OR of 2.05), and that homozygosity to these alleles confers an increased risk to melanoma (RR of 1.35, 95% CI: 1.01–1.81,  $p=0.05$ ) [26]. Strikingly, this genotype generates a new putative binding site for ETS1, which could explain the promoter hyperactivity. Taking into account that CEACAM1 facilitates melanoma proliferation [26] and as the activating BRAF mutation is acquired along melanoma transformation at the premalignant stage, it may particularly increase the risk for melanoma among individuals with this SNP genotype. Further genetic analyses are required to establish the clinical link between this genotype and BRAF mutation, but as it is a rare genotype, this can only be tested in large patient cohorts.

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