Received Date : 27-Mar-2015

Revised Date : 31-Jul-2015

Accepted Date : 20-Aug-2015

Article type : Short Communication

Manuscript Category: Signaling & Cell biology

MITF is a critical regulator of the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) in malignant melanoma

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pcmr.12414

Summary:

The multifunctional Ig-like carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is neo-expressed in the majority of malignant melanoma lesions. CEACAM1 acts as a driver of tumor cell invasion and its expression correlates with poor patient prognosis. Despite its importance in melanoma progression, how *CEACAM1* expression is regulated is largely unknown. Here, we show that *CEACAM1* expression in melanoma cell lines and melanoma tissue strongly correlates with that of the microphthalmia-associated transcription factor (MITF), a key regulator of melanoma proliferation and invasiveness. MITF is revealed as a direct and positive regulator for *CEACAM1* expression via binding to an M-box motif located in the *CEACAM1* promoter. Taken together, our study provides novel insights into the regulation of *CEACAM1* expression and suggests an MITF-CEACAM1 axis as a potential determinant of melanoma progression.

Significance:

CEACAM1 has been identified to act as a prognostic marker for the progression of malignant melanoma; yet, the exact regulatory mechanisms of *CEACAM1* expression remain elusive. Here we provide a novel role for the master regulator of melanocyte differentiation and melanoma oncogene MITF as a direct regulator of *CEACAM1* expression. These novel mechanistic insights into the regulation of *CEACAM1* expression might help to decipher new targets for the development of innovative therapeutic strategies for the treatment of malignant melanoma.

Keywords: microphthalmia-associated transcription factor (MITF), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), M-box motif, melanoma.

Running title: MITF regulates *CEACAM1*

Main Text:

Malignant melanoma is one of the most aggressive types of skin cancer with a continuously rising incidence (Eggermont et al., 2014). Although substantial advances towards an effective anti-melanoma therapy have been made through the use of targeted therapies, resistance is common dormancy and relapse a long time after the removal of the cutaneous tumor poses a major clinical challenge.

The progression of melanoma is a complex multi-step process orchestrated by a variety of cellular factors (Bastian, 2014). Although, loss or reduced levels of the multi-functional carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) expression have been detected in several tumor types, including colon (Neumaier et al., 1993), prostate (Luo et al., 1999) and breast cancer (Riethdorf et al., 1997), neo-expression of *CEACAM1* acts as a driver of melanoma cell invasion, thereby favoring metastatic spread (Ebrahimnejad et al., 2004; Markel et al., 2010; Ortenberg et al., 2012; Thies et al., 2002; Ullrich et al., 2015).

Around 80% of all malignant melanoma lesions express CEACAM1 (Ortenberg et al., 2012; Ullrich et al., 2015), and, moreover its expression impacts on melanoma cell immunogenicity (Chen et al., 2011; Ullrich et al., 2015). Understanding the role of CEACAM1 and what drives its tightly controlled expression in melanoma is a key issue that may offer insights into the process of melanoma progression.

The microphthalmia-associated transcription factor (MITF) was first proposed to be a potential regulator of *CEACAM1* expression using a two-step DNA microarray strategy (Hoek et al., 2008). This prompted us to analyze the correlation of CEACAM1 and MITF mRNA which was revealed to be significant in 11 melanoma cell lines tested (Fig. 1A). Interestingly, all MITF-negative or -low cell lines analyzed (n = 4) were negative for CEACAM1 (data not shown). This correlation was further verified by single cell transcriptome analysis of both *in vitro* grown melanospheres of 501Mel cells (Pearson correlation of 0.67) as well as *in vivo* in xenograft tumors (Pearson correlation of 0.91) (Fig. 1B). Interestingly, the MITF/CEACAM1 correlation was comparable to that of MITF with its well-known target genes tyrosinase (TYR) and myelin basic protein (MBP) (Fig. 1B) (Hoek et al., 2008). In accordance with other reports, we found no positive correlation between MITF with the invasion-associated transcription factor ZEB1 (Fig. 1B), recently been proposed to be directly repressed by MITF (Denecker et al., 2014), as well as BIRC3 and GLI2 (Fig. 1B), whose expression was previously shown to inversely correlate with that of MITF (Javelaud et al., 2011). Together these results suggest a potential role of MITF in the regulation of *CEACAM1* expression. Next we used The Cancer Genome Atlas (TCGA) database to verify the *in situ* relevance of *CEACAM1* regulation by MITF in melanoma. Using a linear model, increased expression of *MITF* correlated with increased expression of *CEACAM1*. Although the model was highly significant, it explained only 10% of the variation in *CEACAM1* expression, suggesting that also other factors contribute to the regulation of *CEACAM1* (adjusted R-squared: 0.099, p-value = 1.767e-10, n = 385; Fig. S1). Additionally, we compared the *CEACAM1* expression of low and high *MITF* expressing samples. K-means clustering gave two clusters with sample sizes of 302 and 83 patients with a mean expression of 14.10 and 10.57, respectively (data not shown). Samples expressing higher amounts of *MITF*, also show higher expression of *CEACAM1* in a two-sided Wilcoxon rank sum test (p = 1.048e-12, Fig. 1C). Taken together, the analysis of the TCGA melanoma dataset underlines the importance of our *in vitro*

observations and suggests a corresponding regulatory mechanism of *CEACAM1* expression in patients with malignant melanoma.

Consequently, we used siRNA-mediated knock-down (using two different MITF siRNA sequences, si#1 and si#2) of endogenous MITF to test whether manipulation of MITF levels affects *CEACAM1* expression. As expected, silencing of MITF resulted in a significant reduction of CEACAM1 on transcript and protein level in all three tested melanoma cell lines (Fig. 2A and B; shown for MITF si#2). Data were confirmed by a second siRNA (MITFsi#1, data not shown). Consistent with MITF regulating *CEACAM1* expression, overexpression of MITF in Ma-Mel-63a cells (Fig. 2C) as well as the tetracycline-induced expression of MITF in 501Mel cells (Fig. 2D) resulted in an enhanced CEACAM1 protein levels. Moreover, we detected increased CEACAM1 levels as a consequence of forskolin treatment, a cAMP-elevating agent, known to be a key stimulator of MITF expression (Fig. 2E).

To assess whether MITF directly affects *CEACAM1* transcription by binding to regulatory elements within the *CEACAM1* promoter, we performed Chromatin-IP followed by high-throughput sequencing (Chip-seq) of 501mel cells expressing 3-HA-tagged MITF. Using this method, we could identify three sites where MITF was binding to the *CEACAM1* locus (Fig. 3A). Since the two distal MITF binding sites do not correlate with H3K27 acetylation in human foreskin melanocytes (HFM), a characteristic of active enhancer elements, we asume that these binding sites play a minor role in the regulation of *CEACAM1* expression. Interestingly, one binding site revealed a high level of H3K27 acetylation in the melanocyte lineage, suggesting a potential binding site for MITF. The examination of this MITF occupied site revealed a M-box motif (TCATGTG) thus representing potential binding sites for MITF (Fig. 3A). This finding is especially intriguing since MITF has been shown to regulate the expression of tyrosinase, tyrosinase-related protein 1 (Tyrp1) and dopachrome tautomerase (DCT), enzymes involved in melanogenesis, via binding to M-box motifs (Lowings et al., 1992). Hence, we asked if endogenous MITF can bind M-box motifs located within the *CEACAM1* promoter. For that purpose, we cloned 1000 bp upstream of the *CEACAM1* coding region into a luciferase reporter construct. In order to investigate the importance of the M-box motif, we further mutated (M-box Mut; TCATtcG) as well as deleted (ΔM-box) the M-box motif and transfected these constructs into UKVR-Mel-15a cells (MITF/CEACAM1-positive cells). Whereas mutation of the M-box motif showed only little impact on promoter activity (Fig. 3B), most likely due to reduced binding ability of MITF, its complete deletion resulted in a significant reduction in reporter gene activation compared to wild type controls (Fig. 3B).

To evaluate if that is caused by a failure of MITF to bind the *CEACAM1* promoter, we transfected the luciferase reporter construct along with MITF-coding and control plasmids into low MITF-expressing A375 cells. As predicted, this approach revealed a significant increase of promoter activity only in the presence of both the wild type promoter together with MITF expression constructs (Fig. 3C). These results imply *CEACAM1* as a direct target gene of MITF in melanoma cells. We note that although the

two upstream MITF binding sites identified by ChIP-seq are not correlated with high H3K27 acetylation in melanocytes, H3K27 acetylation has not been examined in melanoma. Thus, upstream MITF binding sites may well play a key role in regulation of the endogenous gene and operate synergistically with the promoter M-box. Thus, the relatively weak effects of the M-box mutation in the context of the luciferase reporter assay may not reflect its potential role in the genomic context.

In summary, we provide evidence that *MITF* and *CEACAM1* share an exclusive expression pattern in malignant melanoma and we identified MITF as a novel and critical regulator of *CEACAM1* expression. This study not only improves our knowledge on the regulatory events that are involved in the melanoma-specific regulation of *CEACAM1* expression, but may potentially serve as a prognostic factor for melanoma progression.

Acknowledgements:

We thank Eirikur Steingrimsson (Reykjavik) for providing a piggy-bac vector containing the mouse MITF cDNA and Céline Keime for the bioinformatics analysis. We gratefully acknowledge Anja Heinemann (Sydney) for supporting the luciferase experiments and Ivo Bendix (Essen) providing access to the Tecan luminescence reader. We thank Antje Sucker (Essen) for providing the luciferase reporter plasmids. The results shown here are in part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/.

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Figure legends

Figure 1. Expression of *MITF* and *CEACAM1* correlates in melanoma cell lines and tissue.

(A) Pearson correlation of *CEACAM1* and *MITF* expression in 11 melanoma cell lines. Gene expression was analyzed by qRT-PCR. (B) Pearson correlation of the expression of indicated genes in single 501Mel cells isolated from *in vitro* grown melanoshperes and *in vivo* grown xenograft tumors. The correlation values are color coded and indicated numerically. (C) TCGA data analysis reveals a correlation of *MITF* and *CEACAM1* expression in melanoma tissues. The TCGA cohort was classified into MITF low (mean, 10.57) and high (mean, 14.10) expressing samples by K-means clustering. *CEACAM1* expression between these groups was statistically compared by a two-sided Wilcoxon rank sum test. ***$p \leq 0.001$.

Figure 2. MITF is a direct and positive regulator for *CEACAM1* expression**.**

(A + B) The influence of siRNA-mediated MITF silencing (si#2) on *CEACAM1* expression was analyzed by (A) qRT-PCR and (B) Western blotting of indicated cell lines (72 hours post transfection). Graphs are presented as mean ± SEM (n = 4), * $p \leq 0.05$; ** $p \leq 0.01$ (C) Western blot analysis of MITF over-expression and its effect on CEACAM1 protein level in Ma-Mel-63a cells (72 hours post transfection). (D) Western blot analysis of tetracycline-induced (Tet; 2 µg/ml) expression of MITF in 501Mel cells (48 hours after treatment). (E) Western blot analysis of forskolin (Fsk; 40 µM)-induced expression of endogenous MITF in Ma-Mel-63a cells (24 hours after treatment). All panels with Western blot analysis show representative blots of at least two independent experiments. Actin was used as a loading control.

Figure 3. MITF directly regulates *CEACAM1* expression via binding to the M-box motif**.**

(A) Chromatin-IP followed by high-throughput sequencing (Chip-seq) was performed using 501Mel cells stably expressing 3-HA-tagged MITF. Figure shows UCSC genome browser view of ChIP-seq data at the *CEACAM1* locus. MITF occupied sites are indicated by arrows. The H3K27ac Histone-modification of human foreskin melanocytes (HFM) as well from the encode consortium is shown in the lower part. (B + C) Luciferase reporter assays to assess *CEACAM1* promoter activity. (B) Luciferase promoter plasmids containing the wild type (WT), M-Box mutated (M-Box Mut) or M-Box deleted (ΔM-Box) *CEACAM1* promoter were transfected in UKVR-Mel-15a cells (MITF/CEACAM1 expressing cells) (n = 7). (C) *CEACAM1* WT and M-box deleted promoter constructs were co-transfected either with MITF construct or vector control (mock) into A-375 cells (low MITF expressing cells) (n = 3). Graphs in C and D are presented as mean ± SEM. *$p \leq 0.05$; **$p \leq 0.01$.

Figure S1. Expression of *MITF* and *CEACAM1* correlates in melanoma tissue.

Correlation analysis for *MITF* and *CEACAM1* expression in melanoma tissue based on TCGA data (R^2 = 0.099, p = 1.767e-10).

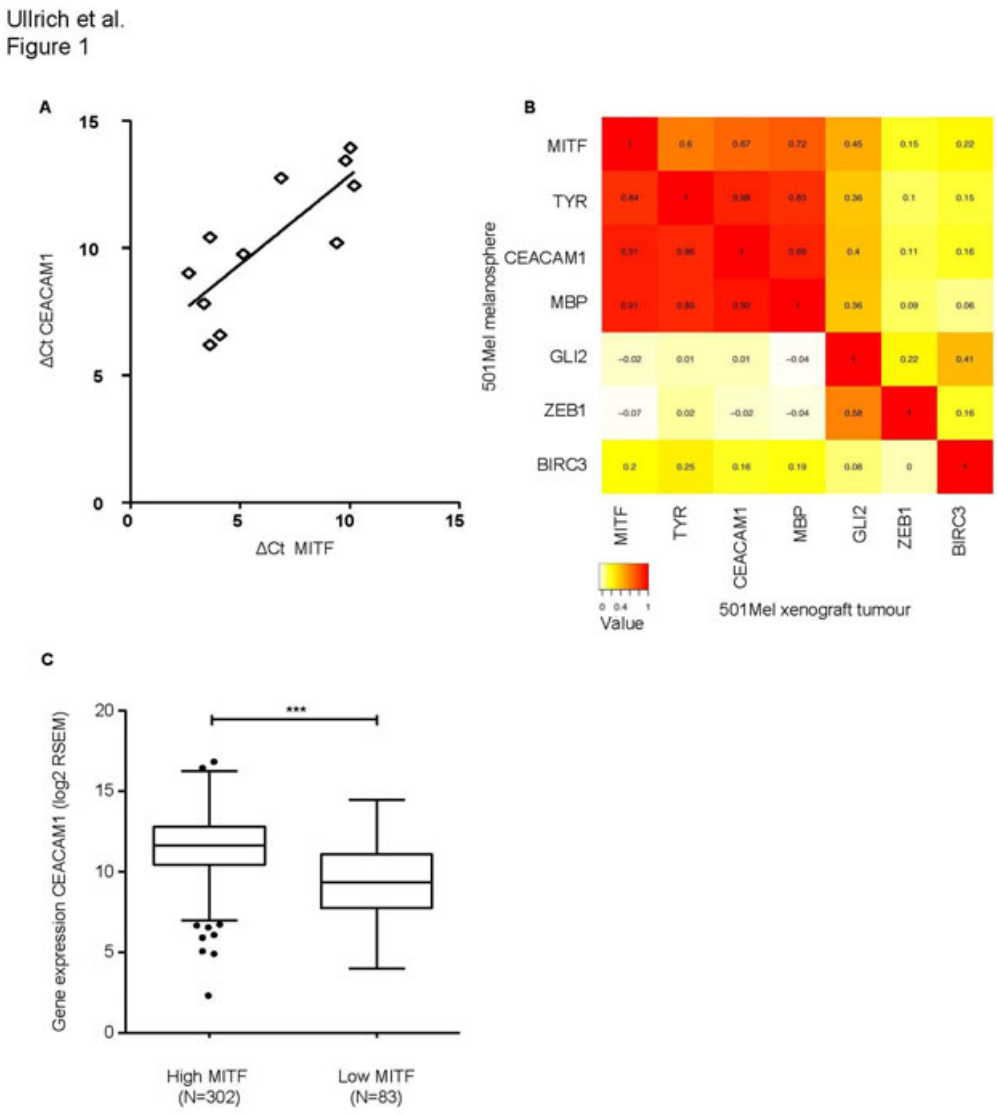

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Figure 2

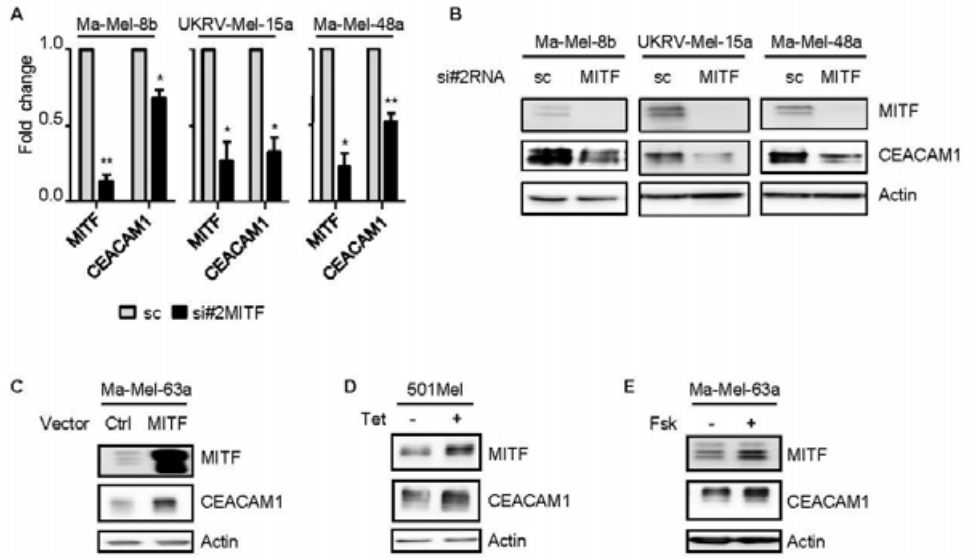

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Figure 3