Mutated MITF-E87R in Melanoma Enhances Tumor Progression via S100A4

Alice Nordlinger1,8, Shani Dror2,8, Abdel Elkahloun3, Justine Del Rio1, Elisa Stubbs1, Tami Golan2, Hagar Malcov2, Todd D. Pricket6, Julia C. Cronin5, Shivang Parikh3, Sapir Labes6, Laetitia Thomas1, Gal Yankovitz2, Yuval Tabach6, Carmit Levy2, Yardena Samuels7 and Mehdi Khaled1

Melanoma, a melanocyte origin neoplasm, is the most lethal type of skin cancer, and incidence is increasing. Several familial and somatic mutations have been identified in the gene encoding the melanocyte lineage master regulator, MITF; however, the neoplastic mechanisms of these mutant MITF variants are mostly unknown. Here, by performing unbiased analysis of the transcriptomes in cells expressing mutant MITF, we identified calcium-binding protein S100A4 as a downstream target of MITF-E87R. By using wild-type and mutant MITF melanoma lines, we found that both endogenous wild-type and MITF-E87R variants occupy the S100A4 promoter. Remarkably, whereas wild-type MITF represses S100A4 expression, MITF-E87R activates its transcription. The opposite effects of wild-type and mutant MITF result in opposing cellular phenotypes, because MITF-E87R via S100A4 enhanced invasion and reduced adhesion in contrast to wild-type MITF activity. Finally, we found that melanoma patients with altered S100A4 expression have poor prognosis. These data show that a change in MITF transcriptional activity from repression to activation of S100A4 that results from a point mutation in MITF alters melanoma invasive ability. These data suggest new opportunities for diagnosis and treatment of metastatic melanoma.


INTRODUCTION

Malignant melanoma is a malignancy of melanocytes, the pigment-producing cells. Melanoma is the most aggressive and treatment-resistant of all human skin cancers and is responsible for increasing skin cancer mortalities worldwide (Dror et al., 2016; Horner et al., 2009). MITF, the melanocyte master regulator, is pivotal in regulating differentiation, growth, and survival of the melanocyte lineage (Bell and Levy, 2011). Additionally, MITF plays a crucial role in melanoma formation and progression (Bell and Levy, 2011; Levy et al., 2006). It was suggested that MITF serves as the key mediator of switching of melanoma cells from a slow-growing invasive phenotype to a fast-paced proliferative

MITF

MITF

phenotype (Cheli et al., 2011; Hoek and Goding, 2010). The rheostat model of MITF suggests that melanoma phenotype depends on MITF expression levels: high MITF levels result in cell cycle arrest, moderate MITF levels promote proliferation, and low MITF levels switch the cellular program into an invasive and metastatic phenotype (Carreira et al., 2006). The mechanism behind the phenotypic outcome of varied MITF activity is still unclear (Bell and Levy, 2011; Carreira et al., 2006).

Genetic alterations in the MITF locus, including genetic amplifications (Levy et al., 2006) and somatic mutations (Cronin et al., 2009; Yokoyama et al., 2011), are correlated with melanoma progression. Amplification of MITF is observed in 10–20% of melanoma patients (Garraway et al., 2005). The fact that MITF expression induces tumor growth suggests an oncogenic role for MITF in melanoma (Garraway et al., 2005). MITF somatic mutations detected in primary and metastatic melanoma samples (Cronin et al., 2009) were found to alter MITF post-translational modifications, such as SUMOylation (Miller et al., 2005; Yokoyama et al., 2011), affecting MITF transcriptional activity (Cronin et al., 2009; Yokoyama et al., 2011). Certain MITF mutants (E87R, L135V, G244R, and D380N) are capable of inducing TYR or TRPM1 expression (Cronin et al., 2009; Yokoyama et al., 2011) but not expression of P21 (Cronin et al., 2009). However, the mechanisms by which MITF-associated mutations drive melanoma progression are less known.

Here, we performed an unbiased gene expression analysis in melanoma cells harboring (WT) and mutant MITF. In mutant MITF melanoma cell lines, the expression of S100A4 was significantly up-regulated. Serum S100 levels have been shown to be a clinical marker for metastatic melanoma progression (Henze et al., 1997). The S100 protein family

Tumor Progression via S100A4

Mutated MITF-E87R in Melanoma Enhances
consists of at least 21 different members; all bind calcium, and 12 members are expressed in melanoma cells (Bresnick et al., 2015). We discovered that, whereas MITF-WT transcriptionally represses expression of S100A4, mutant MITF-E87R activates it. Cells that carry mutated MITF or that were engineered to overexpress S100A4 had significantly higher invasive ability and decreased adhesion compared with WT cells. Furthermore, we showed that high S100A4 expression levels are significantly correlated with poor patient prognosis. Our data place S100A4 as a downstream target of MITF and provide evidence for the neoplastic mechanism of mutated MITF.

RESULTS AND DISCUSSION
Melanoma cell lines harboring mutations in MITF have increased S100A4 expression
To test whether there is a common neoplastic mechanism for the MITF mutations (Cronin et al., 2009), we used various cell lines expressing the identified mutations in a global gene expression analysis. We chose four cell lines derived from metastatic sites (4T, 85T, 13T, and 21T) harboring MITF mutations and five cell lines expressing MITF-WT (23T, 51T, 52T, 3T, and 1T), also derived from metastatic sites (Cronin et al., 2009). mRNA from these cell lines were analyzed using Illumina (San Diego, CA) (see Supplementary Table S1 online) and Qiagen (Hilden, CA) (see Supplementary Table S2 online) microarray platforms. We selected the top 5% up-regulated genes in the mutated lines compared with WT lines and restricted the gene list to those that were up-regulated in at least three out of four cell lines expressing mutant MITF, resulting in a list of 17 genes (see Supplementary Table S3 online). Next, we identified among this list all genes associated with metastasis in melanoma using the Gene Cards Gene Ontology catalog (see Supplementary Table S3). This analysis yielded five genes, S100A4, CLU, CLDN1, MVP, and ADM, that were up-regulated in mutant MITF lines compared with the WT cells. S100A4 was highly up-regulated and strongly correlated with tumor progression (Figure 1a, and see Supplementary Figure S1a) microarray platform. We selected the top 5% up-regulated genes in the mutated lines compared with WT lines and restricted the gene list to those that were up-regulated in at least three out of four cell lines expressing mutant MITF, resulting in a list of 17 genes (see Supplementary Table S3 online). Next, we identified among this list all genes associated with metastasis in melanoma using the Gene Cards Gene Ontology catalog (see Supplementary Table S3). This analysis yielded five genes, S100A4, CLU, CLDN1, MVP, and ADM, that were up-regulated in mutant MITF lines compared with the WT cells. S100A4 was highly up-regulated and strongly correlated with tumor progression (Figure 1a, and see Supplementary Table S3). It was previously shown that ectopic expression of S100A4, in the MITF-WT human melanoma xenograft M21 model, enhances tumor growth and vascularization, placing this gene as an important regulator of tumor progression (Hernandez et al., 2013).

To further understand the relationship between MITF and S100A4 in melanoma, we evaluated publicly available mRNA expression data from a set of 88 melanoma samples (Lin et al., 2008). We found that the levels of MITF and genes known to be regulated by MITF (MLANA, SILV, and TYR) (Levy et al., 2006) were inversely correlated with levels of S100A4 (\( R^2 = -0.29 \)) (Figure 1b). Because MITF is mutated in only a small proportion of melanomas (Cronin et al., 2009; Yokoyama et al., 2011), we assumed that most of the melanoma cell lines used in this analysis express MITF-WT.

We then used an in situ approach to further study the relationship between MITF and S100A4. A melanoma tissue array, which includes tissue samples from melanoma patients at various stages of disease (see Supplementary Table S4 online) was evaluated by immunofluorescence for MITF and S100A4 (Figure 1c). A double-blind analysis of signal intensities across the tissue samples showed an inverse correlation between MITF and S100A4 (\( R^2 = -0.19 \)). Again, we assumed here that MITF is WT in most samples across the tissue array. Therefore, our data suggest that S100A4 expression is inversely correlated with MITF expression when the latter is WT but that this inverse correlation is lost in cell lines where MITF is mutated.

MITF-E87R leads to S100A4 upregulation, whereas MITF-WT has the opposite effect
To identify potential MITF binding sites in the S100A4 genomic region, we searched for the well-defined MITF DNA-binding sites, Ebox sequences (CATGTG, CACGTG, and CACATG), using a bioinformatics approach (Bentley et al., 1994; Hemesath et al., 1994; Yasumoto et al., 1994). We found three potential MITF binding sites within the transcribed region of S100A4 (Figure 2a). All are conserved among primates (see Supplementary Figure S1a online), suggesting that MITF may bind to these elements.

Cronin et al. (2009) identified several MITF mutations in melanoma patient samples. To evaluate MITF gene occupancy, we performed chromatin immunoprecipitation (ChiP) analyses of cells that harbor MITF-WT (51T and 1T) and mutated MITF (13T, 85T, and 4T) (Figure 2b). MITF-WT bound strongly to S100A4 in the second Ebox region. Of the mutant forms, MITF-E87R (from cell line 4T) bound to S100A4 to the highest extent (Figure 2c).

We next sought to directly determine the effects of MITF-WT and MITF-E87R on S100A4 expression. Overexpression of MITF-WT in melanoma lines with low endogenous levels of MITF and low adhesion potential and high invasion abilities (WM3314 and WM1716) (Golan et al., 2015b) (see Supplementary Figure S1b) resulted in decreased (\( P < 0.05 \)) levels of mature and pre-mRNA encoding S100A4 compared with control cells transfected with empty vector (Figure 2d). This observation indicates that MITF-dependent regulation of S100A4 expression is at the transcriptional level. MITF-E87R resulted in the opposite effect on S100A4 expression: MITF-E87R expression resulted in a significant increase in S100A4 expression compared with control cells (Figure 2e).

To further assess the regulation of S100A4 by MITF, UACC62 melanoma cells (WT for MITF) were co-transfected with a luciferase reporter driven by S100A4 promoter and either MITF-WT or MITF-E87R. We observed that overexpression of MITF-WT failed to stimulate luciferase activity, whereas MITF-E87R enhanced the activity by 3-fold (Figure 2f). These results indicate that MITF-E87R has opposite effect on S100A4 transcription from that of MITF-WT. This is in contrast to the effect of other reported MITF mutants, which have transcriptional effects similar to MITF-WT for genes investigated such as TYR and TRPM1 (Cronin et al., 2009; Yokoyama et al., 2011). This finding is not surprising because MITF is necessary but not sufficient to regulate its target (Gaggioli et al., 2003) and relies on cofactors for its transcriptional specificity (Laurette et al., 2015; Sato et al., 1997; Schepsky et al., 2006).

To determine which element of S100A4 promoter is important for its regulation by MITF, we mutated each of the three Eboxes individually and in combination. The luciferase activity of each of the mutants showed that Ebox1 and 2 are
Figure 1. Melanoma cell lines harboring mutations in MITF have increased S100A4 expression. (a) Venn diagram showing overlap between the top 5% upregulated genes in at least three of the lines harboring mutant MITF in both Illumina (San Diego, CA) and Qiagen (Valencia, CA) arrays with melanoma metastasis-related genes (http://www.genecards.org/). The final five gene list included regulators genes in at least three of the lines harboring mutant MITF in both Illumina (San Diego, CA) and Qiagen (Valencia, CA) arrays with melanoma metastasis-related genes. (b) Pearson correlation for mRNA expression levels of each gene relative to MITF. mRNA levels were calculated based on data from 88 melanoma cell lines (Lin et al., 2008). MITF and MITF-related genes are marked in green; S100A4 is marked in red (MITF: R² = 1, P = 0; TYR: R² = 0.94, P = 6.12 × 10⁻¹⁰; TYROSINASE: R² = 0.89, P = 2.58 × 10⁻²²; S100A4: R² = −0.29, P = 1.9 × 10⁻⁶; P-values are corrected for false discovery rate). (c) Representative immunofluorescence analysis of two malignant melanoma patient samples (left and right columns) for MITF (red) and S100A4 (green). Lower panels show DAPI and MITF overlay of corresponding samples. Scale bar = 50 μm.

MITF-E87R promotes melanoma invasion via S100A4

To show how the MITF-S100A4 axis influences melanoma progression, we investigated how these two proteins are correlated with melanoma cell invasion and adhesion abilities and with patient survival. Invasion assay using Boyden chambers coated with Matrigel (BD Biosciences, San Jose, CA) showed that overexpression of MITF-E87R or S100A4 in poorly invasive WM3682 melanoma cells (Levy et al., 2010a) significantly increased the invasiveness potential compared with overexpression of MITF-WT (Figure 3a). Additionally, inhibition of S100A4 expression in highly invasive WM3314 melanoma cells strongly decreased their invasiveness potential (Figure 3b, and see Supplementary Figure S2a and S2f online). S100A4 needs to bind calcium to interact with its targets (Boye and Maelandsmo, 2010). Therefore, we tested the invasiveness potential of WM3682 melanoma cells transfected with MITF-E87R and grown in regular or calcium-depleted media. We observed that calcium is required for the mutant to stimulate invasion, possibly by allowing S100A4 activity (see Supplementary Figure S2g).

The mechanism of cancer cell invasion and dissemination involves loss of adhesive contacts with neighboring cells (Bell et al., 2013). We therefore investigated whether MITF-E87R and S100A4 mediate melanoma invasion through disruption of cellular adhesion by the examination of the actomyosin cytoskeleton during detachment (Bell et al., 2013). MITF-E87R overexpression resulted in morphological changes relative to control cells from an elongated to a rounded structure because of loss of stress fibers and polymerized actin (Figure 3c upper panels, and see Supplementary Figure S2b, lower panel). The numbers of attached cells were examined every 30 seconds after EDTA treatment. MITF-E87R expression significantly decreased the number of attached cells (Figure 3c, lower panels). Moreover, S100A4 overexpression enhanced the formation of rounded cells and significantly reduced the number of adherent cells after EDTA treatment (Figure 3d, lower panels, and see Supplementary Figure S2c, lower panel). In addition, reduction in S100A4 levels by treatment with a small interfering RNA (siRNA) (see Supplementary Figure S2d, upper panels, and e, left panel) resulted in a phenotype similar to that of control cells and reduced cell detachment upon EDTA treatment (see Supplementary Figure S2d, lower panels, and e, lower panel). Taken together, our data show that MITF-E87R mediates melanoma invasion and adhesion via its downstream target S100A4.

To clinically examine the relevance of S100A4 levels, we performed Kaplan-Meier analysis on melanoma patient survival using the cBioPortal for Cancer Genomics (Gao et al., 2018).
Figure 2. Mutated MITF-E87R up-regulates S100A4, whereas MITF-WT has the opposite effect. (a) Schematic of the S100A4 gene promoter with MITF DNA binding sites (Eboxes) indicated in red. Nucleotide positions are marked relative to the transcription start site (TSS) at position +1. (b) Schematic of MITF-M protein with activating domain (AD) and basic helix-loop-helix leucine zipper domain (bHLHZip). Arrows indicate MITF mutations. (c) Chromatin immunoprecipitation analysis performed on melanoma lines: MITF-WT (1T and 51T), MITF-E87R (4T), MITF-L135V (13T), and MITF-D380N (85T). Protein-chromatin crosslinked complexes were precipitated with indicated antibodies. MITF occupancy at the three Eboxes in S100A4 is plotted (n = 3). (d) Levels of mature (spliced) S100A4 mRNA and pre-mRNA in melanoma cells expressing MITF cDNA or an empty vector (control). Data were normalized to ACTB and plotted relative to levels in control cells (n = 4). (e) Levels of mature S100A4 mRNA in melanoma cells expressing MITF-WT, MITF-E87R cDNA or an empty vector (control). Data were normalized to ACTB and plotted relative to levels in control cells (n = 3). (f) UACC62 cells were transiently co-transfected with either pBV luciferase vector without promoter (Vector Luc) or pBV-S100A4 promoter (S100A4 prom Luc) together with MITF-WT or MITF-E87R. At 24 hours after transfection, firefly luciferase activity was measured and normalized to the renilla luciferase control. Histograms represent the mean of three experiments performed in triplicate. In all panels, error bars represent ± standard error of the mean. *P < 0.05. b, base pair; kb, kilo base pair; MUT, mutated; WT, wild type.
Figure 3. MITF-E87R promotes melanoma cells invasion via S100A4. (a) Number of invading cells of poorly invasive WM3682 cells upon MITF-E87R or S100A4 expression. Below each bar is a representative image. DAPI nuclear staining is shown in white. Error bars represent ± standard error of the mean.
There was a significant difference in the survival between the group of patients with no amplification of S100A4 (blue) and those with amplification (Figure 3e, \( P = 0.00865 \)). Finally, S100A4 has been described to drive cancer metastasis by up-regulating MMP9 expression (Zhang et al., 2013, 2014). We therefore tested the dependency of MMP9 expression on S100A4 in 4T melanoma cells. We observed that siRNA-mediated inhibition of S100A4 expression led to a strong decrease in MMP9 protein expression (Figure 3f).

In conclusion, by performing unbiased analysis of the transcriptomes in cells harboring mutant MITF, we identified S100A4 as downstream target of MITF. Whereas MITF-WT represses S100A4 expression, MITF-E87R activates its transcription. These opposing effects result in different cellular phenotypes: MITF-E87R enhanced invasion and reduced adhesion, and we showed that this effect was mediated by S100A4. The poor prognosis of melanoma patients with amplification of S100A4 supports our hypothesis that the change in MITF transcriptional activity from repression to activation of S100A4 that resulted from mutation of a single residue of MITF alters the cellular gene expression program and enhances melanoma invasion. Our data suggest new opportunities for diagnosis and treatment of metastatic melanoma.

**MATERIALS AND METHODS**

**Cell culture**

Patient-derived melanoma metastases lines wild-type for MITF (23T, 51T, 52'T, 3T and 1T) and mutated for MITF (13T, 85T, 21T and 4T) were previously described (Cronin et al., 2009). WM3314, WM3314, WM3314, WM3314, WM3314 and 451LU melanoma cells were kind gifts of Levi A. Garraway (Dana-Farber Cancer Institute, Boston, MA). MITF status was verified by Sanger sequencing for all cell lines. Plasmids for expression of MITF-WT or MITF-E87R controlled by a S100A4 promoter residue of MITF alters the cellular gene expression program and enhances melanoma invasion. Our data suggest new opportunities for diagnosis and treatment of metastatic melanoma.

**Plasmids**

Plasmids for expression of MITF-WT or MITF-E87R controlled by a cytomegalovirus promoter were generated by our laboratory as described (Cronin et al., 2009). Human S100A4 cDNA in pEGFP-N3 expression plasmid (House et al., 2011) was kindly provided by Anne R. Brensnick from Albert Einstein College of Medicine (New York, NY). A 1.1-kilo base pair portion of S100A4 promoter containing three Eboxes was cloned by PCR using genomic DNA as the template. The fragment was digested with EcoR1 and EcoRV and inserted into pBV luciferase vector. Site-directed mutagenesis for each was performed using the QuickChange method from Stratagene (San Diego, CA) according to the supplier's recommendations.

**Oligonucleotide transfection**

siS100A4 and siControl siRNA were transfected into the cells using HiPerFect (Qiagen) according to the manufacturer’s protocols. Cells were transfected twice with 100 pmol of siRNA per well at 24-hour intervals. Transfected cells were assayed 24 hours after the second transfection. MITF-WT, MITF-E87R, and the S100A4 expression plasmids were transfected into cells using Lipofectamine-2000 (Thermo Fisher Scientific, Wallham, MA) according to the manufacturer's instructions. Cells were assayed 24 hours after transfection.

**Chromatin immunoprecipitation (ChIP)**

ChIP was performed as described previously (Levy et al., 2010b). The immunoprecipitations were performed with MITF rabbit polyclonal antibody (kindly provided by David E. Fisher, Harvard Medical School, Boston, MA) and normal rabbit IgG (sc-2027; Santa Cruz Biotechnology, Dallas, TX) as control antibody. Quantitative real-time reverse transcriptase—PCR was carried out using primers listed in Supplementary Table S5 online.

**RNA purification and quantitative real-time reverse transcriptase—PCR**

Total RNA was purified using Trizol (Ambion, Austin, TX) according to the manufacturer's instructions. RNA was quantified by measuring optical density at 260 and 280 nm. RNA was subjected to qScript cDNA synthesis (Quanta, Houston, TX) and FastStart Universal SYBR Green FastMix (Quanta). Relative expression was normalized to ACTB. Means plus and minus standard errors are presented. All primer sequences are shown in Supplementary Table S5.

**Adhesion assay**

Cell attachment was analyzed 48 hours after transfection. Cells were cultured in DMEM medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/-glutamine. Minimal media was made with DMEM medium supplemented with 0%–1% fetal bovine serum and 1% penicillin/streptomycin/-glutamine.

*P < 0.05. n = 3. (b) Relative number of invading cells of melanoma WM3314 cells with high invasion abilities upon treatment with siS100A4 expression or siControl. Error bars represent ± standard error of the mean, n = 3. (c) Graph plots are representing the number of adhering cells over time (n = 3). Scale bar = 20 \( \mu \)m. (d) Detachment analysis of WM3314 melanoma cells with high adhesion potential and low invasion abilities upon MITF-E87R or an empty vector (control) expression. Cells stained with phalloidin (red) and DAPI (blue). Graph plots are representing the number of adhering cells over time (n = 3). Scale bar = 20 \( \mu \)m. (e) Kaplan-Meier survival plot of melanoma patients, no amplification (blue) and with S100A4 genetic amplification (red). \( P = 0.00865 \). In all panels, error bars represent ± standard error of the mean. *P < 0.05. The survival curve of patient with amplification of S100A4 does not reach the x-axis because all patients left the trial. (f) Protein extract from 4T melanoma cells transfected with indicated siRNA for 48 hours before being subjected to Western blot analysis using antibodies against MMP9 and actin. sec, second; si, small interfering; WT, wild-type.
Invasion assays
Cells were transfected as described above. At 48 hours after transfection, cells were serum-starved overnight. Cells (5 x 10^5) were added to invasion chambers coated with Matrigel. Cells were allowed to invade for 9 hours toward media containing 10% fetal bovine serum. Cells remaining on the top side of the membrane were counted and then removed using a cotton swab. Invading cells were fixed and stained with DAPI. Samples were analyzed in duplicate, 10 fields per insert were photographed, and scored SEM was measured. The number of invaded cells was normalized to the number of total seeded cells.

Immunofluorescence
The melanoma tissue arrays prepared from biopsy samples collected with written informed consents from the donors and their relatives were purchased from US BioMax, Inc (ME1003; Rockville, MD). Melanoma tissue arrays were deparfumized in xylene, hydrated in a graded series of ethanol, and subjected to microwave EDTA antigen retrieval. Samples were blocked with 5% bovine serum albumin, 0.5% Tween-20 in phosphate buffered saline. Slides were incubated with MITF (C5, kindly provided by David E. Fisher) and S100A4 (ab27957; Abcam, Cambridge, UK) antibodies. Staining was performed by incubating with Alexa-488- or Alexa-594-conjugated secondary antibodies (Life Technologies, Waltham, MA). Images were obtained at x40 magnification using fluorescence and bright light microscopy (Leica Sp8; Wetzlar, Germany). Images were scored from 1 to 3 for MITF and S100A4 expression by two investigators for each sample. After exclusion of inconsistent data, the correlations and the statistics were calculated.

Kaplan-Meier analysis
The information analyzed was taken from the Cancer Genome Atlas database of skin melanoma patients, which contains data on 281 patients. These data were generated by the TCGA Research Network (http://cancergenome.nih.gov/). The Kaplan-Meier graph was generated by using the “survival” tab of cBioPortal for Cancer Genomics (Cerami et al., 2012, Gao et al., 2013). Patients were divided into two groups (with and without amplifications of S100A4).

Microarray analysis
Seven cell lines were examined in this project: 4T, 21T, 85T, and 13T harboring MITF mutations and 23T, 51T, 52T, 3T, and 1T harboring MITF-WT (Cronin et al., 2009). RNA extracted from each cell line was replicated on three oligonucleotide arrays. Two hybridizations were against the same pooled WT RNA sample (control), two of the hybridizations were identical, and the other two used dye-swap labeling protocol between the experiment and control pair. The Lowess normalized ratios from each array were log transformed (log2), and the average log ratios for each cell line over three replicates were calculated.

Transfection and dual luciferase reporter assay
UACC62 melanoma cells were seeded onto 24-well dishes and transfected the following day in 1.5 µl of Fugene 6 (Promega, Madison, WI) with 0.1 µg from each of pBV, pS100A4, and p5100A4 mutants, pCMV MITF-WT, and pCMV MITF-E87R. Test plasmids were transfected with pGL2SV40 renilla luciferase reporter to control for the variability in transfection efficiency. After 24 hours of transfection, cell lysates were prepared, and the activity of firefly and renilla luciferase was measured using the Dual Luciferase Kit (Promega) according to the manufacturer’s recommendations.

Gel electrophoresis and immunoblotting
Melanoma cells were cultured in six-well dishes before being transfected with small interfering RNA as indicated in each figure. Subsequently, the cells were lysed in buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, proteases, and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Samples (30 µg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and then exposed to the appropriate antibodies: anti--MMP9 (Cell Signaling Technology, Danvers, MA), anti-actin (Sigma-Aldrich), and anti-MITF (C5). Proteins were visualized using the ECL system (GE Healthcare) using horseradish peroxidase-conjugated anti-rabbit or antioimmune secondary antibody. Western blot assays shown are representative of at least three experiments.

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
Authors would like to thank Meenhard Herlyn and Levi Garraway for supplying melanoma lines for these studies. MK is supported by ATIP-AVENIR and ANR-MMO research grants and by Natisix. YS is supported by the European Research Council (SG-335377), the European Research Council under the European Union’s Horizon 2020 research and innovation program (grant agreement 677645). CL gratefully acknowledges grants from The Israel Centers for Research Excellence (I-CORE no. 41/11), The Israel Science Foundation, Israel Cancer Research Fund, US-Israel Binational Science Fund, and Fritz Thyssen Stiftung foundation.
AUTHOR CONTRIBUTIONS
AN and SD helped designed the experimental approach and performed most of the experiments. AE computationally analyzed the microarray. ES performed S100A4 expression analysis. TP and JC experimentally validated the microarray results. HM and SP performed the tissue array analysis. SL and YT generated the survival analysis. CL developed the hypothesis, performed the ChIP experiment and analysis, and helped write the paper. YS developed the hypothesis and performed the microarray analysis. MK developed the hypothesis, designed the experimental approach, coordinated the project, and wrote the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.03.1524.

REFERENCES


