

An Oncogenic Role for *ETV1* in Melanoma

Judit Jané-Valbuena^{1,3}, Hans R. Widlund², Sven Perner⁵, Laura A. Johnson^{1,3,5,7}, Aurora C. Dibner^{1,3}, William M. Lin^{1,3,7}, Alissa C. Baker^{1,3}, Rosalynn M. Nazarian^{4,6}, Krishna G. Vijayendran^{1,3}, William R. Sellers⁸, William C. Hahn^{1,3,7}, Lyn M. Duncan^{4,6}, Mark A. Rubin⁵, David E. Fisher², and Levi A. Garraway^{1,3,7}

Abstract

Copy gains involving chromosome 7p represent one of the most common genomic alterations found in melanomas, suggesting the presence of “driver” cancer genes. We identified several tumor samples that harbored focal amplifications situated at the peak of common chromosome 7p gains, in which the minimal common overlapping region spanned the *ETV1* oncogene. Fluorescence *in situ* hybridization analysis revealed copy gains spanning the *ETV1* locus in >40% of cases, with *ETV1* amplification (>6 copies/cell) present in 13% of primary and 18% of metastatic melanomas. Melanoma cell lines, including those with *ETV1* amplification, exhibited dependency on *ETV1* expression for proliferation and anchorage-independent growth. Moreover, overexpression of *ETV1* in combination with oncogenic NRAS^{G12D} transformed primary melanocytes and promoted tumor formation in mice. *ETV1* overexpression elevated microphthalmia-associated transcription factor expression in immortalized melanocytes, which was necessary for *ETV1*-dependent oncogenicity. These observations implicate deregulated *ETV1* in melanoma genesis and suggest a pivotal lineage dependency mediated by oncogenic ETS transcription factors in this malignancy. *Cancer Res*; 70(5): 2075–84. ©2010 AACR.

Introduction

Recurrent tumor genomic alterations may pinpoint key drivers of tumorigenesis and offer avenues for rational therapeutic development. In some cancers, the importance of a gene to tumor maintenance is tightly linked to the presence of specific genetic alterations affecting that gene. In other cases, recurrent genomic changes illuminate mechanisms that are broadly operant, even in cancers where specific genetic alterations are not present at the “index” locus. Thus,

some cancer gene mutations may serve as signifiers for cardinal pathways that are commonly altered by multiple genetic and epigenetic mechanisms across many cancers.

Chromosomal copy number changes represent highly prevalent genomic aberrations in cancer. In principle, genes targeted by such events might be expected to underlie fundamental tumorigenic mechanisms that are deregulated by both genetic and other means in many tumor types, as described above. However, many chromosomal alterations (e.g., copy gains, losses, and loss of heterozygosity) involve broad, low-amplitude changes that may encompass an entire chromosome or chromosome arm. Dozens to hundreds of genes are altered by such genetic events; thus, characterizing the relevant effectors poses a significant challenge.

In melanoma, copy gains involving chromosome 7 are exceedingly common (up to 40% of cases; refs. 1–3). Recent melanoma genomic studies suggest that the “peaks” of statistically significant chromosome 7 copy gains localize to independent loci on 7p and 7q (2, 3). Chromosome 7q contains the *BRAF* oncogene, which commonly undergoes activating point mutations in melanoma (4). However, the target(s) of 7p gains in melanoma, although equally common, remain uncharacterized. We therefore sought to identify candidate target oncogenes of chromosome 7p in melanoma. The results herein suggest that *ETV1*, an ETS transcription factor known to undergo genetic deregulation in several cancer types (5–7), is targeted by 7p21 amplification events and exerts a mitogen-activated protein (MAP) kinase- and microphthalmia-associated transcription factor (MITF)-dependent tumor-promoting function in many melanomas. Thus, *ETV1* may represent a critical effector within an oncogenic module that is broadly operant in melanoma.

Authors' Affiliations: Departments of ¹Medical Oncology and ²Pediatric Oncology and ³Center for Cancer Genome Discovery, Dana-Farber Cancer Institute, Harvard Medical School; ⁴Department of Pathology, Harvard Medical School; ⁵Department of Pathology, Brigham and Women's Hospital; ⁶Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts; ⁷Broad Institute of M.I.T. and Harvard and ⁸Novartis Institutes for Biomedical Research, Cambridge, Massachusetts

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J. Jané-Valbuena and H.R. Widlund contributed equally to this work. Current address for S. Perner: Department of Pathology, University Hospital of Tübingen, Eberhard-Karls University of Tübingen, Liebermeisterstr. 8, Tübingen, Germany. Current address for M.A. Rubin: Department of Pathology, Weill Cornell Medical College, 1300 East 70th Street, New York, NY 10065. Current address for D.E. Fisher: Melanoma Program in Medical Oncology and Department of Dermatology, Massachusetts General Hospital, Harvard Medical School, Fruit Street, Boston, MA 02114. Current address for H.R. Widlund: Department of Dermatology, Harvard Institutes of Medicine, Brigham and Women's Hospital, 77 Avenue Louis Pasteur, Boston, MA 02115.

Corresponding Author: Levi A. Garraway, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115. Phone: 617-632-6689; Fax: 617-582-7880; E-mail: levi_garraway@dfci.harvard.edu.

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Materials and Methods

Microarray studies. High-density single nucleotide polymorphism (SNP) array and gene expression microarray data generation were done as described previously (3). A brief description is provided in Supplementary Methods.

Tissue microarray construction. The melanoma tumor progression array was generated through the collaborative efforts of three Skin Specialized Programs of Research Excellence (Harvard, M.D. Anderson, and U Penn) and contained 480 0.6-mm cores of tissues from 170 distinct clinical specimens, including 132 cores from 36 benign melanocytic nevi, 196 cores from 59 primary melanomas, and 150 cores from 75 metastatic lesions (8).

Fluorescence in situ hybridization. All bacterial artificial chromosome (BAC) clones were selected using the UCSC Genome Browser and obtained from the BACPAC Resource Center (CHORI). BAC probes preparation, labeling, and hybridization were done as described previously (9). To assess for *ETV1* amplification, a dual-color fluorescence *in situ* hybridization (FISH) assay was designed using an *ETV1* and a reference probe. To assess for *ETV1* rearrangement, a dual-color break-apart FISH assay was designed for the locus. Probes used and protocols followed are described in Supplementary Methods.

Genomic quantitative PCR. *ETV1* locus genomic quantitative PCR was done following standard protocols and using two *ETV1*-specific primers. Protocols and primer sequences are provided in Supplementary Methods.

Analysis of mRNA expression by quantitative reverse transcription-PCR. Quantitative reverse transcription-PCR (RT-PCR) was done following standard protocols. Primer sequences and protocols are provided in Supplementary Methods.

Molecular cloning and expression of *ETV1* and MITF. Human *ETV1* was cloned from total fetal brain RNA. Total cDNA was generated with the SuperScript III first strand synthesis kit and random hexamers (Invitrogen). *ETV1* cDNA was generated by PCR from the pool of fetal brain cDNA using primers containing *ETV1*-specific sequences flanked by a 5' *EcoRI* site and a 3' *XhoI* site to allow for cloning in to the retrovirus-blast vector pWZL (see Supplementary Methods for primer sequences). The pWZL-blast-HA-MITF vector used to overexpress melanocytic MITF was generated as described previously (10). All cDNAs were verified by Sanger sequencing.

Immunoblot analysis. Cell lysis and immunoblot analysis were done as described in Supplementary Methods.

Cell culture. Culture conditions for short-term cultures and cell lines used are described in Supplementary Methods.

Retroviral infections. Retroviruses were obtained by triple cotransfection of HEK293-ebna cells (Invitrogen) with the pWZL-blast vector (empty or containing the cDNA of interest) and the packaging and envelope plasmids pN8e-VSV-G and pN8e-GagPolΔS (kind gift from Dr. Jay Morgenstern, Millennium Pharmaceuticals, Cambridge, MA). P^{mel}* cells were infected with the retrovirus pBABE-zeo-NRAS^{G12D} or pBABE-zeo-BRAF^{V600E} and selected by growth factor deprivation

in Ham's F10 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin. P^{mel}*-BRAF^{V600E} and P^{mel}*-NRAS^{G12D} expressing cells were infected with the pWZL-blast-empty, pWZL-blast-*ETV1*, or pWZL-blast-HA-MITF retroviruses followed by blasticidin selection.

Lentivirally delivered short hairpin RNA. pLKO1-based lentiviral vector knockdown assays were done following standard protocols, described in Supplementary Methods.

Cell proliferation assays. Cells were plated in triplicate in 12-well plates at 20,000 per well in a final volume of 2 mL of medium. At each time point, cells were washed twice with PBS, trypsinized, and counted with a cell counter (Beckman Coulter).

Assessment of growth factor autonomy. Cells were plated on 24-well plates (7,000 per well) in Ham's F10 medium containing 50 ng/mL 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 1 mmol/L cyclic AMP (cAMP), L-glutamine, 0.1 mmol/L 1-methyl-3-isobutylxanthine, 1 μmol/L Na₃VO₄, 7% FBS, and penicillin/streptomycin [referred to as +growth factors (+GF) medium], or in Ham's F10 medium containing L-glutamine, 10% FBS, and penicillin/streptomycin [referred to as -growth factors (-GF) medium]. Growth factor dependence of cells was determined by counting surviving cells in +GF medium compared with -GF medium. At each time point, cells were washed with PBS, trypsinized, and counted on a hemacytometer.

Anchorage-independent assays. Cells (10,000 or 20,000 per well) suspended in medium (2 mL) containing 0.7% noble agar, 0.5% fungizone (Sigma-Aldrich), and marker drug (when applicable) were seeded into six-well plates containing a 0.5% noble agar layer (2 mL). Colony formation in soft agar was assayed in triplicate.

Xenograft tumor experiments. Animal experiments were conducted according to the Dana-Farber Cancer Institute Animal Care and Use Committee guidelines. Isogenic sets of transformed melanocytes were injected (5 × 10⁵ cells per site in 100 μL of Hanks buffered saline) at three flank positions (in cohorts of five animals) in 6-wk-old female NCR-Nu mice and tumor growth was monitored for 5 to 7 wk after injection. Animals were sacrificed before behavioral malaise followed by excision and analysis of resulting tumors.

Results

Amplification of the *ETV1* locus in melanoma. To identify candidate effector genes targeted by chromosome 7p gains in melanoma, we analyzed chromosomal copy number data from SNP arrays done on melanoma short-term cultures and cell lines. As shown in Fig. 1A, many samples contained broad regions of chromosome 7p copy gain, as expected; however, focal 7p21 amplification events were apparent in a few samples. Notably, the M25 short-term culture and the 501mel and WW94 cell lines harbored overlapping focal amplifications at the 7p21.3 locus (Fig. 1A, arrowheads). Inspection of genes common to the amplified regions identified *ETV1* (Fig. 1A), a known oncogene and a member of the ETS

transcription factor family (5–7). Quantitative genomic PCR confirmed *ETV1* amplification in these samples compared with primary melanocytes and to cell lines without focal amplification (SKMEL2 and SKMEL28; Fig. 1B). In addition, FISH analysis showed higher numbers of *ETV1*-probe signals in 501mel and WW94 relative to SKMEL2 and SKMEL28 (Supplementary Fig. S1). Moreover, quantitative RT-PCR and immunoblot analyses also showed increased *ETV1* mRNA and protein levels in 501mel and WW94, respectively (Fig. 1C), suggesting that the DNA amplification events resulted in increased *ETV1* expression.

To confirm the presence of *ETV1* amplification in clinical specimens, we performed FISH analysis on an assembled melanoma tissue microarray (TMA) containing 170 evaluable nevi, primary, and metastatic melanoma specimens (Table 1). The FISH results were segregated based on the quantity of *ETV1* probe signals detected relative to the reference probe (see Materials and Methods). Detection of two *ETV1* copies per nuclei was considered to indicate no amplification; between >2 and ≤ 6 *ETV1* copies indicated “low-level” amplification; and >6 *ETV1* copies represented “high-level” amplification. Although no *ETV1* copy gains were detected in any of the nevi examined, low-level *ETV1* gains were detected at $\sim 40\%$ frequency in all melanomas examined, regardless of stage. These results were consistent with SNP array and comparative genomic hybridization results reported here and elsewhere (2, 3). Notably, high-level *ETV1* copy gains occurred in 13% of primary samples and in 18% of metastatic melanomas present on the TMA (Fig. 1D; Table 1). These results suggest that DNA amplifications involving the *ETV1* locus occur frequently in melanoma.

Targeted *ETV1* gene disruption in melanoma. As *ETV1* undergoes translocation in some tumor types (6, 7, 11), we sought to determine if *ETV1* gene rearrangements were evident in melanoma. Accordingly, we performed a FISH break-apart assay on the melanoma TMA using flanking telomeric and centromeric *ETV1* probes (Supplementary Fig. S2). All *ETV1* translocations described to date show 5' coding exons replaced by an ectopic promoter and, in some cases, by 5'-end exons of the partner gene. These rearrangements result in a truncated product that retains the ETS DNA binding domain but whose expression is controlled by the fusion partner upstream regulatory promoter elements. Interestingly, the *ETV1* FISH assay exhibited a “break-apart” pattern in two lymph node metastases (from a total of 41 lymph and visceral metastases analyzed), suggestive of targeted gene disruption or possible translocation of the *ETV1* locus (Supplementary Fig. S2). The *ETV1* locus remained intact in the antecedent primary tumors (not shown). Interestingly, one of the lymph node metastases contained more signals corresponding to the telomeric probe (green) than to the centromeric probe (red; Supplementary Fig. S2, right). Because *ETV1* is positioned with its 5' end nearest to the centromere, this pattern could reflect loss of the centromeric region after translocation or of selective amplification of a translocation product containing the (telomeric) 3' end of *ETV1*. These results raised the possibility that *ETV1* might

undergo rare chromosomal translocation events in addition to the more common amplification events in melanoma.

***ETV1* expression is elevated in melanoma.** In some cancers, the oncogenicity of ETS transcription factors derives primarily from deregulated gene expression (5, 7, 11). To examine *ETV1* expression in melanoma, we analyzed microarray data from a collection of metastatic short-term melanoma cultures and cell lines. *ETV1* mRNA levels were elevated in most of the samples relative to primary melanocytes (Fig. 2A). As expected, two cell lines harboring the focal amplification (501mel and WW94) were among the highest *ETV1*-expressing samples (Fig. 2A and B). On average, the presence of chromosome 7p copy gains correlated with a slightly higher *ETV1* expression compared with samples with no 7p gains (Fig. 2A), although this correlation did not reach statistical significance. Immunoblot analysis confirmed the increased levels of *ETV1* protein compared with normal melanocytes in melanoma samples with and without 7p gains (Fig. 2B). Thus, elevated *ETV1* expression was observed in melanoma samples relative to normal melanocytes regardless of 7p copy gains. Although we identified *ETV1* as a melanoma oncogene based on genetic criteria, these results raised the possibility that *ETV1* dysregulation might be achieved in melanoma by both genetic and nongenetic mechanisms.

***ETV1* oncogene dependency in melanoma.** To determine whether melanoma samples are dependent on *ETV1* and whether *ETV1* dependency correlates with *ETV1* amplification, we studied the consequences of RNAi-mediated suppression of *ETV1* expression on melanoma cell proliferation and colony formation. Here, we used two independent lentivirally delivered short hairpin RNA (shRNA) constructs that effectively reduced *ETV1* mRNA and protein levels (Fig. 3A), as well as a control hairpin against the green fluorescent protein (shGFP). We tested the effects of *ETV1* knockdown in 501mel and WW94, two cell lines with focal *ETV1* amplification, as well as in SKMEL2 and SKMEL28 (Fig. 1B). To eliminate the possibility that the effects we observed were the consequence of off-target effects of the *ETV1*-specific shRNAs, we also tested HeLa cells, which do not express *ETV1* (Supplementary Fig. S3).

Suppression of *ETV1* resulted in a marked reduction of proliferation in both 501mel and WW94 cells (*ETV1* amplified; Fig. 3B). Moreover, silencing of *ETV1* suppressed anchorage-independent growth in 501mel cells, whereas control shRNA had no effect (Fig. 3C). The effect of *ETV1* knockdown was more variable in melanoma cell lines that lacked chromosome 7p copy gains. The proliferation of SKMEL28 cells was substantially inhibited following *ETV1* suppression; however, SKMEL2 cells were largely unaffected (Fig. 3B). These results suggest that *ETV1* amplification induces an *ETV1* dependency in melanoma, while supporting the hypothesis that *ETV1* dependency also exists in some (but not all) melanoma cells lacking *ETV1* amplification.

***ETV1* overexpression cooperates with oncogenic NRAS and BRAF to transform immortalized human melanocytes.** To test the hypothesis that *ETV1* functions as an oncogene in melanoma, we overexpressed *ETV1* in genetically modified primary melanocytes (hereafter referred to as p'mel* cells).

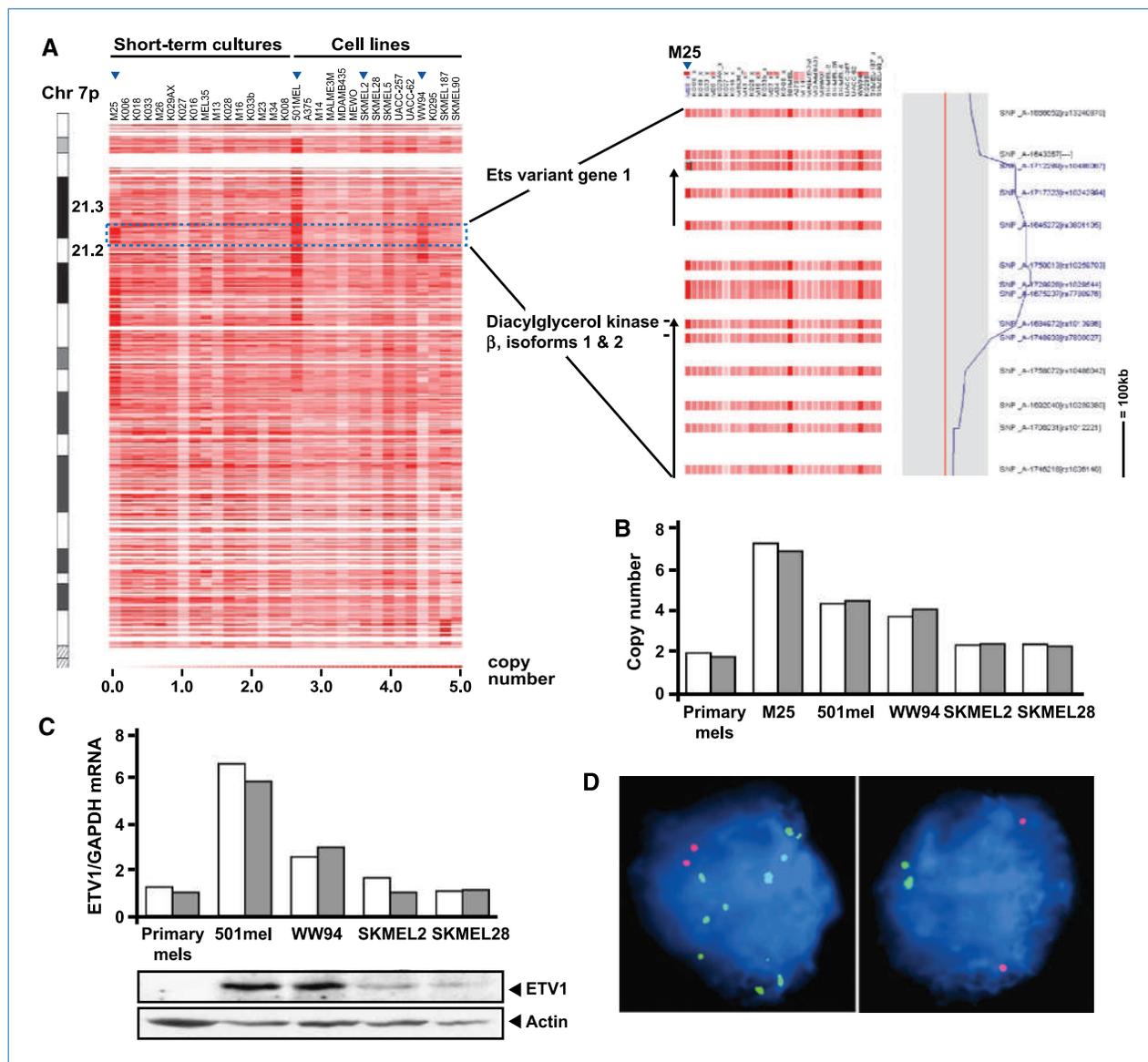


Figure 1. Amplification of the *ETV1* locus in melanoma. **A**, SNP array-based copy number analysis of chromosome 7p (left; cytobands indicated) is shown for a collection of melanoma short-term cultures and cell lines. Increasing red intensity denotes increasing copy number (legend; bottom). The region containing the *ETV1* locus is indicated (blue box) and a closer view is shown (right). *ETV1* and the neighboring diacylglycerol kinase- β genes are indicated. The copy number at the *ETV1* locus in sample M25 is shown graphically (right). **B**, quantitative genomic PCR assessment of *ETV1* amplification is shown for M25, 501mel, WW94, SKMEL2, and SKMEL28 melanoma lines. Two sets of *ETV1* locus-specific primers were used (white and gray columns). **C**, relative *ETV1* mRNA and protein expression of the melanoma cell lines analyzed in **B** and in primary melanocytes were determined by quantitative RT-PCR using two different *ETV1*-specific primers (white and gray columns) and by immunoblot analysis using an α -*ETV1* antibody. **D**, FISH analysis is shown for illustrative melanoma samples from a TMA. Green- and red-labeled BAC probes detect the *ETV1* and reference loci, respectively. Left, a diploid case; right, a case of *ETV1* amplification.

As described previously (10), p^{mel}* cells were immortalized through ectopic expression of hTERT, p53DD (dominant-negative p53), and CDK4(R24C) (INK-resistant CDK4). These factors facilitated telomere stabilization as well as retinoblastoma and p53 pathway inactivation, which occur commonly in this malignancy. Although immortalized, p^{mel}* cells require TPA and cAMP agonists for survival, a hallmark of non-transformed melanocytes.

Overexpression of ectopic *ETV1* alone did not overcome the TPA and cAMP growth factor requirement in p^{mel}* cells (Fig. 4A). As expected (10), similar results were obtained following ectopic expression of MITF, a known oncogenic transcription factor in melanoma. However, most melanomas also harbor activating *NRAS* or *BRAF* mutations that result in aberrant MAP kinase pathway activation (4, 12, 13). Toward this end, we expressed *NRAS*^{G12D} oncoprotein in

Table 1. ETV1 FISH analysis of melanoma tissue samples

Sample type	Samples on array	Analyzable samples*	% of samples		
			No amp	Low amp [†] (≤6)	High amp [‡] (>6)
Thin nevus	21	10	100	0	0
Thick nevus	15	10	100	0	0
Thin primary	39	16	56.2	31.3	12.5
Thick primary	20	15	46.7	40.0	13.3
Lymph node mets	39	17	23.6	58.8	17.6
Visceral mets	46	23	43.5	39.1	17.4

Abbreviation: mets, metastases.

*Samples that rendered an informative result.

[†]Samples presenting three to six ETV1-probe signals.

[‡]Samples presenting more than six ETV1-probe signals.

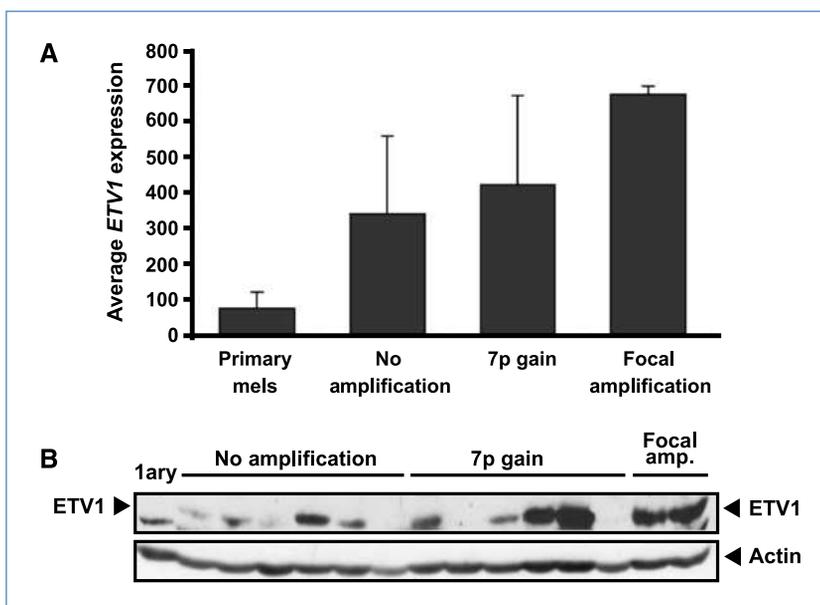
p'mel* cells (Fig. 4B). When expressed alone, NRAS^{G12D} activated MAP kinase signaling (Fig. 4C) but failed to confer robust anchorage-independent growth (Fig. 4D). This result is reminiscent of prior studies using the BRAF^{V600E} oncogene in these cells (10). In contrast, combined expression of both ETV1 and NRAS^{G12D} strongly induced anchorage-independent growth in p'mel* cells, with a phenotype that resembled the effect of ectopic MITF expression (Fig. 4D). Combined ETV1 and BRAF^{V600E} expression also conferred soft-agar growth to p'mel* cells, albeit with lower efficiency (Supplementary Fig. S4). Thus, ETV1 overexpression is able to cooperate with both NRAS^{G12D} and BRAF^{V600E} to transform immortalized human melanocytes.

ETV1 overexpression promotes melanoma tumor formation in vivo. We next investigated the effect of ETV1 overexpression on melanoma genesis in a xenograft model. Here,

p'mel* cells expressing NRAS^{G12D} alone or in combination with ETV1 were injected s.c. into immunodeficient mice and monitored for tumor formation. P'mel* cells expressing NRAS^{G12D} alone failed to form tumors (0 of 15 mice examined). In contrast, p'mel* cells expressing both NRAS^{G12D} and ETV1 formed tumors in 67% of cases (10 tumors/15 injections; Fig. 4D). Together with the soft-agar findings above, these results suggest that the ETV1 transcription factor can function as an oncogene in melanoma.

ETV1 overexpression induces MITF upregulation in p'mel*-NRAS^{G12D} cells. In previous work, our group showed that the MITF transcription factor, a master regulator of melanocyte lineage development and survival, cooperates with BRAF^{V600E} to function as a melanoma oncogene (10). Consistent with these earlier observations, overexpression of HA-tagged melanocytic MITF in p'mel*-NRAS^{G12D} cells also

Figure 2. ETV1 expression in relation to chromosome 7p copy gains. A, normalized ETV1 expression levels in cultured primary melanocytes and melanoma lines. Average expression values from subgroups stratified by ETV1 locus amplification are indicated (focal amplification: 501mel and WW94 lines). B, immunoblot analysis using α -ETV1 and α -actin antibodies is shown for a subset of melanoma cell lines and short-term cultures analyzed in A.



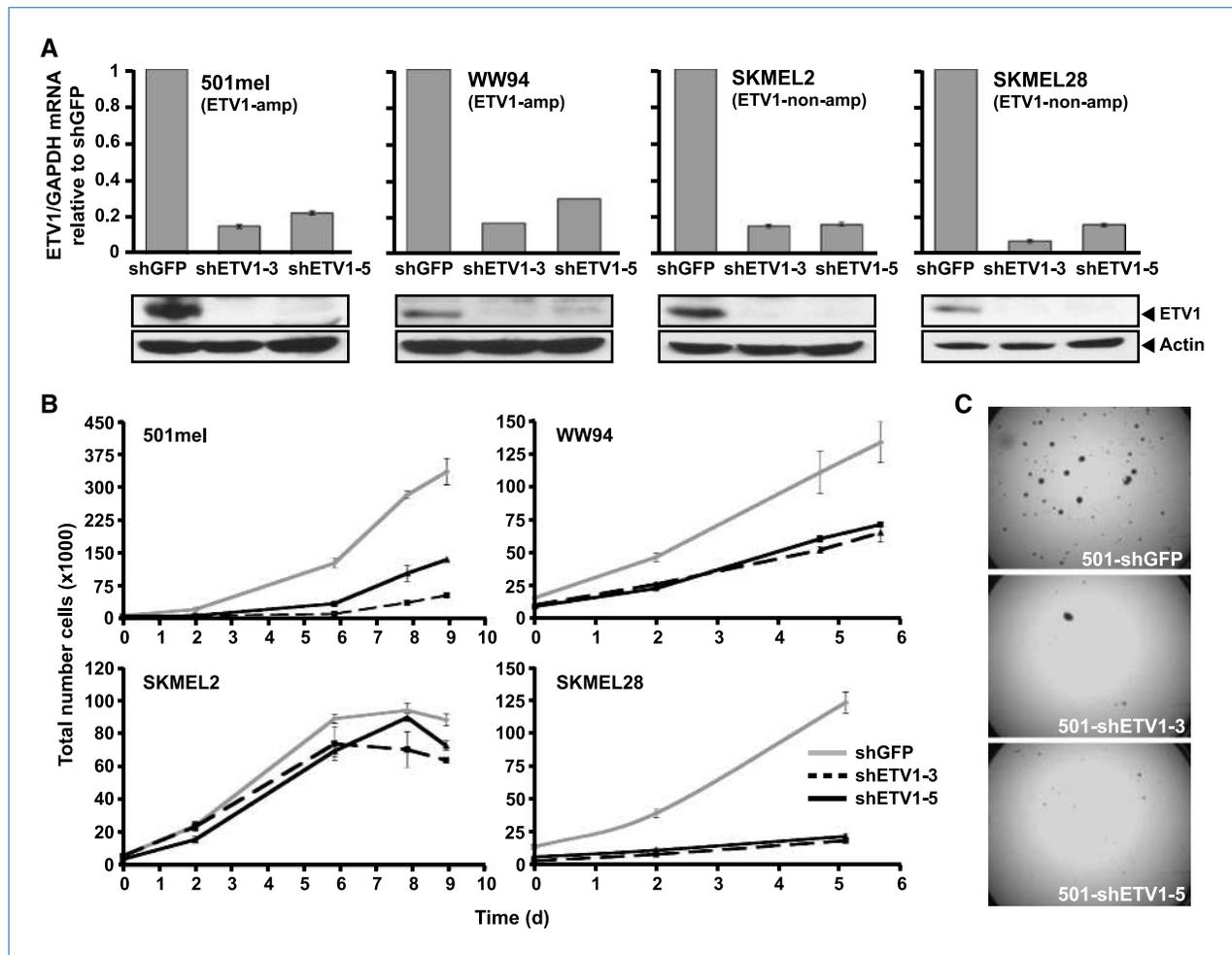


Figure 3. Phenotypic effects of suppression of *ETV1* expression in melanoma cell lines. A, *ETV1* mRNA levels following infection with lentivirus containing *ETV1*-specific shRNAs (shETV1-3 and shETV1-5) relative to infection with control *GFP*-specific shRNAs (shGFP) in representative melanoma cell lines with (501mel and WW94) or without (SKMEL2 and SKMEL28) *ETV1* amplification (top). Corresponding immunoblot studies of *ETV1* protein are also shown (bottom). B, proliferation curves are shown for the melanoma cell lines in A after introduction of shRNAs against *ETV1* (solid and dashed black lines) or *GFP* (control; gray lines). C, anchorage-independent growth of 501mel cells (*ETV1* amplified) infected with lentivirus containing *ETV1*-specific or *GFP*-specific shRNAs.

resulted in robust colony formation (Fig. 4D). These results indicated that MAP kinase pathway activation by either NRAS or BRAF mutation could cooperate with MITF to transform p'mel* cells. Together with the observation that *ETV1* transforms p'mel* cells in the same genetic context, these findings also raised the possibility that a mechanistic relationship might exist between *ETV1* and MITF with regard to melanocyte transformation.

To test this hypothesis, we performed immunoblot analysis of lysates derived from p'mel*-NRAS^{G12D} cells expressing empty vector, *ETV1*, or HA-MITF. Interestingly, *ETV1* overexpression strongly induced endogenous MITF protein upregulation in p'mel*-NRAS^{G12D} cells (Fig. 5A, left). Quantitative RT-PCR using MITF primers revealed a concomitant increase in *MITF* mRNA levels (Fig. 5A, right). The melanocytic MITF promoter contains several four-base GGAA/T sequences characteristic of the ETS factor consensus binding site; how-

ever, *ETV1* did not augment MITF promoter activity in luciferase reporter assays (data not shown). Thus, *ETV1* expression induced *MITF* mRNA and protein expression markedly but indirectly in p'mel*-NRAS^{G12D} cells.

MITF is necessary for *ETV1* oncogenicity in melanoma. Given its oncogenic properties in MAP kinase-driven melanomas, we next examined whether MITF is required for *ETV1*-mediated transformation in p'mel*-NRAS^{G12D} cells. Here, we suppressed *MITF* expression using a *MITF*-specific shRNA in p'mel*-NRAS^{G12D} cells that overexpressed *ETV1*. As a control, we performed similar experiments in p'mel*-NRAS^{G12D} cells overexpressing MITF (Fig. 5B, left). Silencing of MITF greatly diminished the anchorage-dependent growth phenotype induced by both *ETV1* and MITF overexpression in these cells (Fig. 5B, right). To eliminate the possibility that the effects we observed were the consequence of off-target effects of the *MITF*-specific shRNA, we also tested MCF7

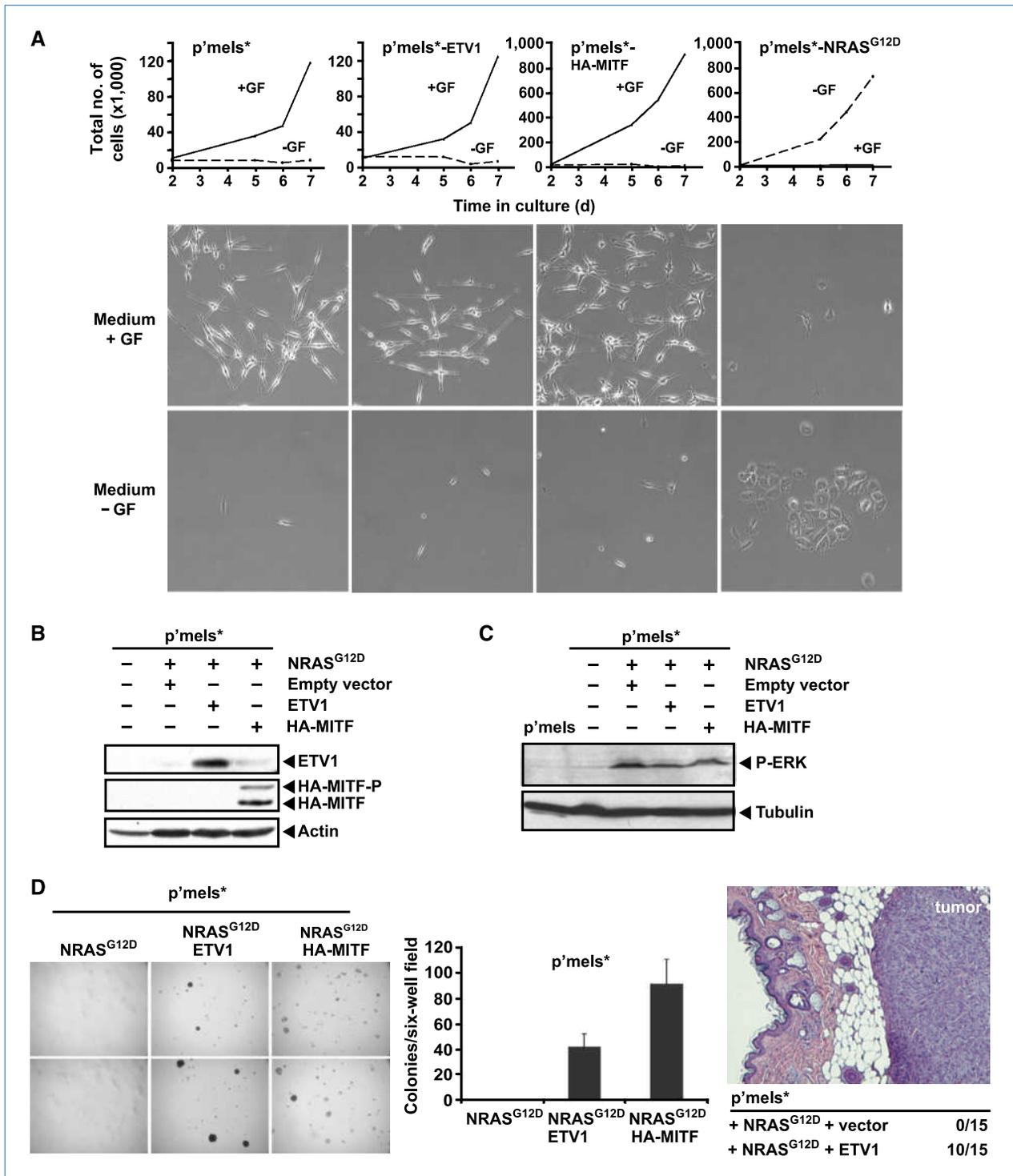


Figure 4. Effects of ETV1 overexpression on melanocyte transformation. A, proliferation curves are shown for p'mel* cells (immortalized human melanocytes described in the text) and p'mel* cells expressing ETV1, HA-MITF, or NRAS^{G12D} cultured in F10 medium in the presence (+GF) or absence (-GF) of TPA and cAMP growth factors. Cells were photographed after 5 d in culture. B and C, immunoblot analyses of p'mel* and p'mel*-NRAS^{G12D} cells infected with empty vector, ETV1, or HA-MITF retroviruses. Antibodies recognizing ETV1, HA epitope, actin, phospho-ERK, and tubulin were used. HA-MITF-P, phosphorylated form of HA-MITF. D, left, anchorage-independent growth of p'mel* cells expressing NRAS^{G12D} alone or in combination with ETV1 or HA-MITF (photographs from two independent assays are shown for each cell type). Middle, colony counts from these experiments are also indicated. Right, p'mel*-NRAS^{G12D} cells infected with empty or ETV1 retroviruses were injected s.c. in mice. Tumor formation following injection is indicated numerically and a histologic section of a representative ETV1-dependent tumor is shown (stained with H&E).

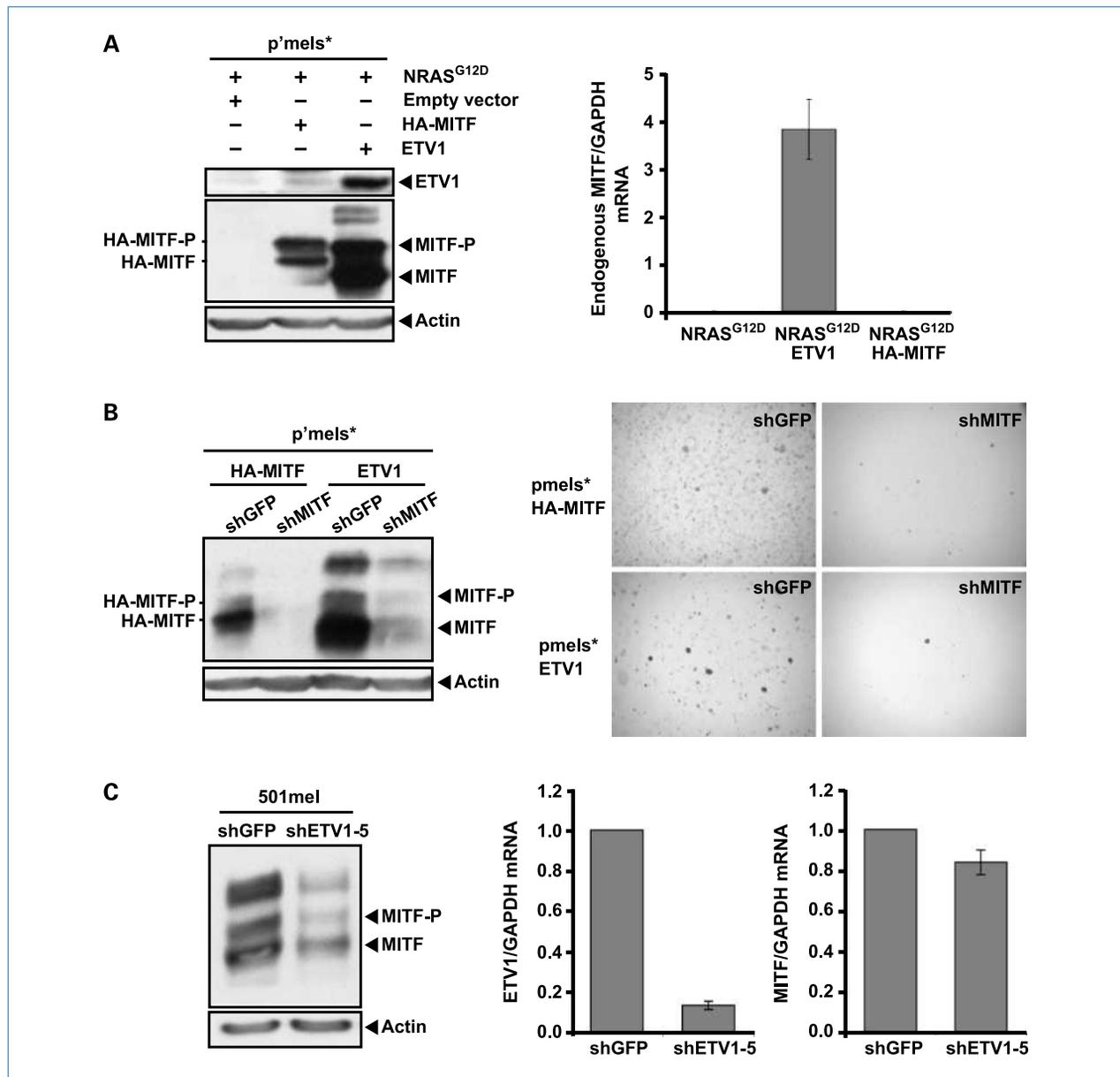


Figure 5. ETV1 regulation of MITF expression. A, left, immunoblot analyses of p'mel^{*}-NRAS^{G12D} cells infected with empty vector, ETV1, or HA-MITF retroviruses. Antibodies recognizing ETV1, HA epitope, and actin were used. MITF-P and HA-MITF-P, phosphorylated forms of endogenous MITF and HA-MITF, respectively. Right, relative mRNA expression levels of endogenous melanocytic *MITF* are shown for the cell types analyzed at left as determined by RT-PCR. B, left, suppression of MITF expression is shown for p'mel^{*}-NRAS^{G12D} cells overexpressing HA-MITF or ETV1 following infection with lentivirus containing shRNAs against *MITF* (shMITF) or *GFP* (shGFP). Right, anchorage-independent growth of the cells analyzed at left. C, left, immunoblot analysis of MITF levels following suppression of *ETV1* expression using *ETV1*-specific shRNAs in 501mel cells. Right, *ETV1* and *MITF* mRNA levels are shown for the cells analyzed at left, as determined by RT-PCR (in triplicate) and normalized to *GAPDH*.

cells, which do not express MITF (Supplementary Fig. S5). Thus, MITF is required for ETV1-dependent oncogenicity in this setting.

Because ETV1 overexpression augmented MITF expression (at least indirectly) as described above, we next investigated whether reduction of ETV1 expression in established melanoma cell lines might downregulate endogenous MITF levels. Indeed, RNAi-mediated suppression of ETV1 resulted

in a measurable albeit modest reduction in endogenous MITF in 501mel cells, which harbored *ETV1* amplification (Fig. 5C, left). Moreover, shRNAs directed against MITF reduced both proliferation (data not shown) and colony formation in soft agar in 501mel cells (Supplementary Fig. S6). In contrast to the studies of p'mel^{*}-NRAS^{G12D} cells above, we did not detect a corresponding decrease in MITF mRNA following ETV1 knockdown (Fig. 5C, right). These results suggest that

the oncogenic function of ETV1 may derive, at least in part, through regulation of MITF protein levels in human melanoma cells.

Discussion

The systematic characterization of target or driver genes enacted by recurrent genomic aberrations offers a means to elucidate mechanisms of tumorigenesis. However, many prevalent chromosomal copy number aberrations consist of large, low-amplitude events that do not provide sufficient resolution to pinpoint driver genes definitively. Rare focal genetic alterations may enrich for driver cancer genes that are also enacted by highly recurrent, nonfocal events spanning the same locus. In this study, we identified three focal amplifications encompassing 7p21.3, which nominated the ETS transcription factor *ETV1* as a candidate driver gene targeted by chromosome 7p gains in melanoma. TMA analyses also identified *ETV1* amplification in 13% to 18% of melanomas, with occasional evidence of targeted gene disruption (e.g., translocation) involving this locus. Subsequent studies indicated that ETV1 expression is required in several melanoma contexts, including cell lines harboring *ETV1* amplification. Ectopic ETV1 overexpression in the context of aberrant MAP kinase pathway activation transformed immortalized human melanocytes. Although these findings do not exclude the possibility that other genes located at chromosome 7p21 or elsewhere on 7p may be targeted by these same copy gains, our results strongly suggest that *ETV1* functions as a melanoma oncogene.

Recent findings have drawn increasing attention to the role of ETS transcription factors in cancer. Since the original ETS sequence was discovered in an avian erythroblastosis virus (14), approximately 40 additional ETS factors have been identified (reviewed in ref. 15). Multiple members of the ETS family undergo oncogenic dysregulation in cancer, often through chromosomal translocation (reviewed in ref. 15 and in ref. 16). In Ewing's sarcoma (EWS; ref. 6), *EWS:ETV1* translocations result in highly transforming chimeric ETS fusion proteins (17, 18). More recently, chromosomal translocations involving *ETV1* and other ETS genes were found in more than 40% of prostate cancers (7, 19). Most commonly, these translocations interpose the promoter and 5' coding exons of the *TMPRSS2* gene upstream of an ETS factor gene (*ERG*, *ETV1*, *ETV4*, or *ETV5*), resulting in androgen-dependent regulation and elevated expression of these genes (7, 19); results described herein add malignant melanoma to the growing list of cancers where ETS transcription factors in general and *ETV1* in particular exert critical tumor dependencies.

In melanoma, ETV1 dependency may also extend beyond cases of 7p21 amplification/copy gain. Analyses of gene expression microarray data showed significantly higher *ETV1* mRNA levels in melanoma relative to primary melanocytes, yet we did not observe a clear correlation between *ETV1* mRNA expression and chromosome 7p copy gains per se. Thus, chromosomal copy changes represent only one mechanism of ETV1 upregulation in melanoma. Moreover, melanoma cell viability was reduced following ETV1 knockdown

in two cell lines with *ETV1* amplification (and high ETV1 expression), but at least one melanoma cell line lacking 7p copy gain also exhibited a reliance on ETV1 for viability. These results are reminiscent of studies on MITF dependency in melanoma: Focal amplifications targeting *MITF* are found in only 10% to 15% of melanomas (10), yet MITF dependency is broadly operant and not exclusive to *MITF*-amplified tumors. Thus, *ETV1* amplifications and copy gains may highlight a widely relevant ETV1 dependency in melanoma.

In our hands, the ability of deregulated ETV1 to transform immortalized human melanocytes is dependent on constitutive MAP kinase pathway activation by gain-of-function BRAF or NRAS mutations. This result is reminiscent of studies in prostate and mammary transgenic mouse models, where ERG or ETV1 expression alone was insufficient to drive tumorigenesis (19–23). Our findings also accord well with studies indicating that ETS factors become activated by MAP kinase-mediated phosphorylation (refs. 24, 25; reviewed in ref. 15). Interestingly, a recent study of oncogenic MAP kinase output in melanoma showed that extracellular signal-regulated kinase (ERK) also regulates the transcriptional expression of several ETS factors, including ETV1 (26). In this report, ETV1 mRNA levels in *BRAF^{V600E}* melanoma cell lines were dramatically reduced following pharmacologic MAP kinase inhibition using a MEK kinase inhibitor (26). Together with published evidence, our findings support a model wherein deregulated ETV1 expression in melanoma (by genomic amplification or other means) elaborates an oncogenic signal that is both enabled and potentiated by concomitant MAP kinase pathway activation.

Ectopic ETV1 expression in immortalized human melanocytes results in upregulation of *MITF* mRNA and protein levels. This effect, although indirect, is necessary for ETV1-mediated melanocyte transformation. These results indicate that MITF expression may be necessary to elaborate a full ETV1 dependency in melanoma. ETV1 also regulates the expression of other proteins with tumorigenic functions, including hTERT (27). Our observations imply that MITF, which itself is activated by MAP kinase (28), may participate in a melanocytic transcriptional program that includes ETV1 and possibly other factors. This program may become deregulated at multiple points during melanoma genesis or progression.

In conclusion, this study suggests that deregulated ETV1 underpins a melanoma oncogene dependency involving both MAP kinase and MITF activation. The high prevalence of chromosome 7p gains together with activating MAP kinase mutations (e.g., *BRAF* or *NRAS* mutation) in melanoma may offer an optimal setting for *ETV1*-driven oncogenicity. Given the abundance of genetically characterized *in vitro* models for this malignancy, melanoma may offer a robust context for elucidating downstream oncogenic mechanisms linked to ETV1 and possibly other ETS factors in many human cancers.

Disclosure of Potential Conflicts of Interest

W.C. Hahn, D.E. Fisher, and L.A. Garraway received sponsored research grants and consulting fees from Novartis, Inc. The other authors disclosed no potential conflicts of interest.

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Judit Jané-Valbuena, Hans R. Widlund, Sven Perner, et al.

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