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## An oncogenic role for *ETV1* in melanoma

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### 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 (FISH) analysis revealed copy gains spanning the *ETV1* locus in >40% of cases, with *ETV1* amplification 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, over-expression of *ETV1* in combination with oncogenic NRAS<sup>G12D</sup> transformed primary melanocytes and promoted tumor formation in mice. *ETV1* overexpression elevated MITF 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.

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## Keywords

melanoma; amplification; ETV1; oncogene; ETS factors

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## 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 (LOH)) 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) (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—though 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 MAP kinase- and 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.

## MATERIALS AND METHODS

### Microarray studies

High-density SNP array and gene expression microarray data generation were performed 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 SPORES (Harvard, MD Anderson and U Penn), and contains 480 0.6mm 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 (FISH)

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 was performed as described previously (9). To assess for *ETV1* amplification, a dual-color 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 (Q-PCR)

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

### Analysis of mRNA expression by quantitative RT-PCR

Quantitative RT-PCR was performed 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 sequence 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 performed 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 co-transfection 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). P<sup>+</sup>mel\* cells were infected with the retrovirus pBABE-zeo-NRAS<sup>G12D</sup> or -BRAF<sup>V600E</sup> and selected by growth-factor deprivation in Ham's F10 media supplemented with 10% FBS, L-Glutamine and P/S. p<sup>+</sup>mels\*-BRAF<sup>V600E</sup> and -NRAS<sup>G12D</sup> expressing cells were infected with the pWZL-blast -empty, -ETV1 or -HA-MITF retroviruses followed by blasticidin selection.

### Lentivirally delivered short hairpin RNA (shRNA)

pLKO1-based lentiviral vector knockdown assays were performed following standard protocols described in Supplementary Methods.

### Cell Proliferation assays

Cells were plated in triplicate in 12-well plates at 20,000 cells/well in a final volume of 2 ml of media. At each time point, cells were washed twice with PBS, trypsinized and counted with a cell counter (Beckman Coulter, Fullerton, CA).

### Assessment of growth factor autonomy

Cells were plated on 24-well plates (7000 cells/well) in Ham's F10 media containing 50ng/ml TPA, 1mM cAMP, L-Glutamine, 0.1mM IBMX, 1uM  $\text{Na}_3\text{VO}_4$ , 7% FBS and P/S (referred to as + Growth factors (+GF) media), or in Ham's F10 media containing L-Glutamine, 10% FBS and P/S (referred to as - Growth factors (-GF) media). Growth factor dependence of cells was determined by counting surviving cells in +GF media compared to -GF media. At each time point, cells were washed with PBS, trypsinized and counted (hemacytometer).

### Anchorage independent assays

Cells (10,000 or 20,000/well) suspended in media (2 ml) containing 0.7% noble agar, 0.5% fungizone (Sigma-Aldrich) and marker drug (when applicable) were seeded into 6 well plates containing a 0.5% noble agar layer (2ml). Colony formation in soft agar was assayed in triplicate.

### Xenograft tumor experiments

Animal experiments were conducted according to Dana-Farber Cancer Institute Animal Care and Use Committee guidelines. Isogenic sets of transformed melanocytes were injected ( $5 \times 10^5$  cells per site in 100uL Hank's buffered saline) at three flank positions (in cohorts of 5 animals) in 6 week old female NCR-Nu and tumor growth was monitored for 5–7 weeks post-injections. 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 single nucleotide polymorphism (SNP) arrays performed on melanoma short-term cultures (STCs) 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 to primary melanocytes and to cell lines without focal amplification (SKMEL2 and SKMEL28) (Fig. 1B). In addition, FISH analysis demonstrated higher numbers of *ETV1*-probe signals in 501mel and WW94, relative to SKMEL2 and SKMEL28 (Supplementary Fig. 1). 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 2 *ETV1* copies per nuclei was considered to indicate no amplification; between >2 and  $\geq 6$  *ETV1* copies indicated “low-level” amplification; and >6 *ETV1* copies represented “high-level” amplification. While no *ETV1* copy gains were detected in any of the nevi examined, low-level *ETV1* gains were detected at ~40% frequency in all melanomas examined, regardless of stage. These results were consistent with SNP array and CGH 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 occurred 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 a centromeric *ETV1* probes (Supplementary Fig. 2). 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. 2). 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. 2; right panel). Since *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 (Figs. 2A–B). On average, the presence of chromosome 7p copy gains correlated with a slightly higher *ETV1* expression compared to 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 to 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 non-genetic 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 utilized 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 3).



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, while the 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 suggested 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). 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 (RB) and p53 pathway inactivation, which occur commonly in this malignancy. Although immortalized, p'mel\* cells require TPA and cyclic AMP (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*<sup>G12D</sup> oncoprotein in p'mel\* cells (Fig. 4B). When expressed alone, *NRAS*<sup>G12D</sup> 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*<sup>V600E</sup> oncogene in these cells (10). In contrast, combined expression of both *ETV1* and *NRAS*<sup>G12D</sup> 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*<sup>V600E</sup> expression also conferred soft agar growth to p'mel\* cells, albeit with lower efficiency (Supplementary Fig. 4). Thus, *ETV1* overexpression is able to cooperate with both *NRAS*<sup>G12D</sup> and *BRAF*<sup>V600E</sup> 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*<sup>G12D</sup> alone or in combination with *ETV1* were injected subcutaneously into immunodeficient mice and monitored for tumor formation. P'mel\* cells expressing *NRAS*<sup>G12D</sup> alone failed to form tumors (0/15 mice examined). In contrast, p'mel\* cells expressing both *NRAS*<sup>G12D</sup> and *ETV1* formed tumors in 67% of cases (10 tumors/15 injections) (Fig. 4D). Together with the soft agar findings above, these results suggested that the *ETV1* transcription factor can function as an oncogene in melanoma.

### **ETV1 overexpression induces MITF up-regulation in p'mel\*-NRAS<sup>G12D</sup> cells**

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

To test this hypothesis, we performed immunoblot analysis of lysates derived from p\*mel\*-NRAS<sup>G12D</sup> cells expressing empty vector, ETV1, or HA-MITF. Interestingly, ETV1 overexpression strongly induced endogenous MITF protein up-regulation in p\*mel\*-NRAS<sup>G12D</sup> 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; however, 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<sup>G12D</sup> 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<sup>G12D</sup> cells. Here, we suppressed *MITF* expression using a *MITF*-specific shRNA in p\*mel\*-NRAS<sup>G12D</sup> cells that overexpressed ETV1. As a control, we performed similar experiments in p\*mel\*-NRAS<sup>G12D</sup> 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 cells, which do not express MITF (Supplementary Fig. 5). Thus, MITF is required for ETV1-dependent oncogenicity in this setting.

Since 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 down-regulate endogenous MITF levels. Indeed, RNAi-mediated suppression of ETV1 resulted in a measurable albeit modest reduction in endogenous MITF in 501mel cells, which harbor *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. 6). In contrast to the studies of p\*mel\*-NRAS<sup>G12D</sup> cells above, we did not detect a corresponding decrease in MITF mRNA following ETV1 knockdown (Fig. 5C, right). These results suggested 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, non-focal 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. Tissue microarray analyses also identified *ETV1* amplification in 13%–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. While 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), ~40 additional ETS factors have been identified (reviewed in (15)). Multiple members of the ETS family undergo oncogenic dysregulation in cancer, often through chromosomal translocation (reviewed in (15) and in (16)). In Ewing Sarcoma (EWS) (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 over 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* up-regulation 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 to studies of *MITF* dependency in melanoma: focal amplifications targeting *MITF* are found in only 10–15% 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 ((24,25) and reviewed in (15)). Interestingly, a recent study of oncogenic MAP kinase output in melanoma showed that *ERK* also regulates the transcriptional expression of several ETS factors, including *ETV1* (26). In this report, *ETV1* mRNA levels in *BRAF*<sup>V600E</sup> melanoma cell lines were dramatically reduced following pharmacologic MAP kinase inhibition using a MEK 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 up-regulation of *MITF* mRNA and protein levels. This effect, though 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

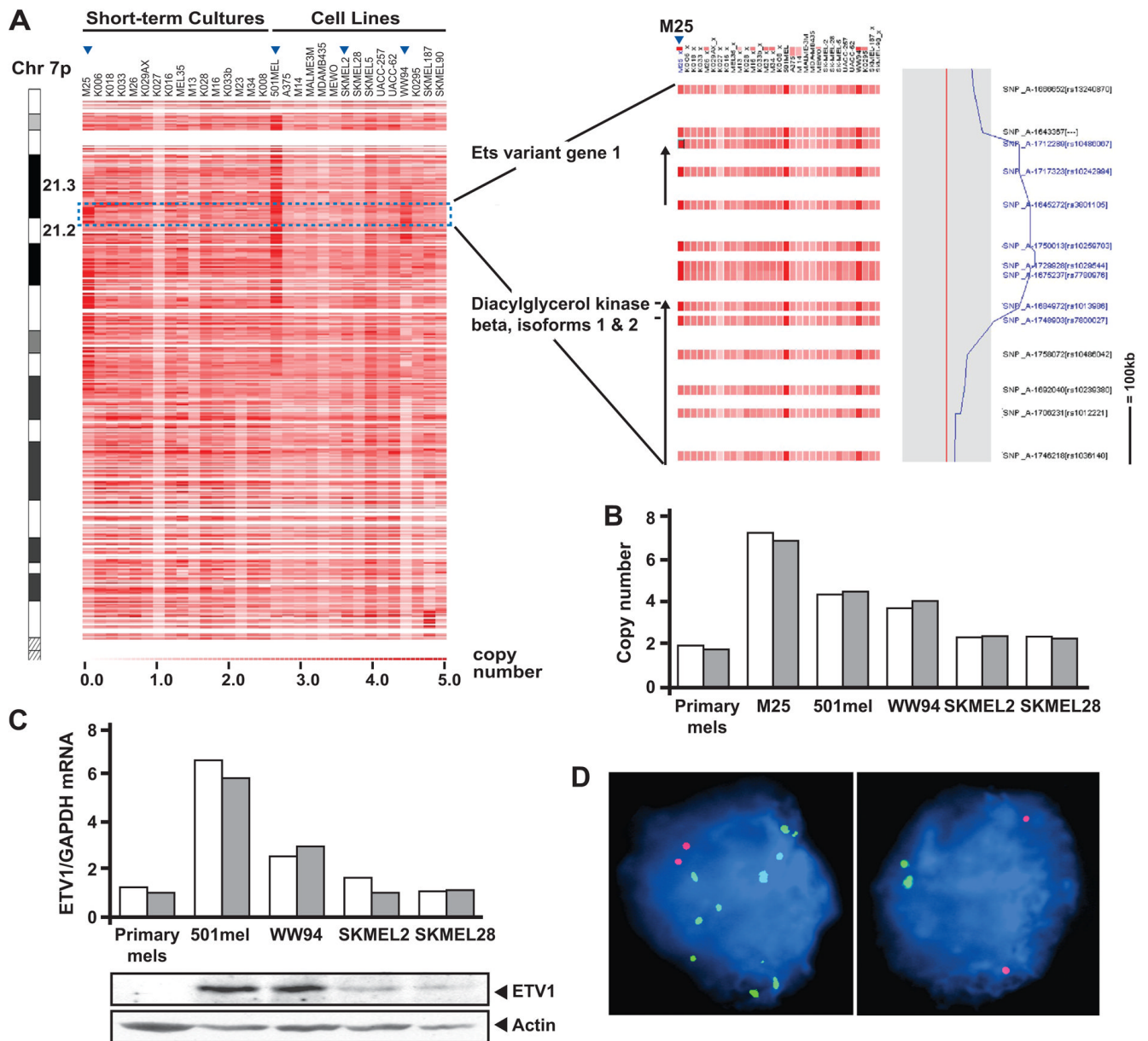
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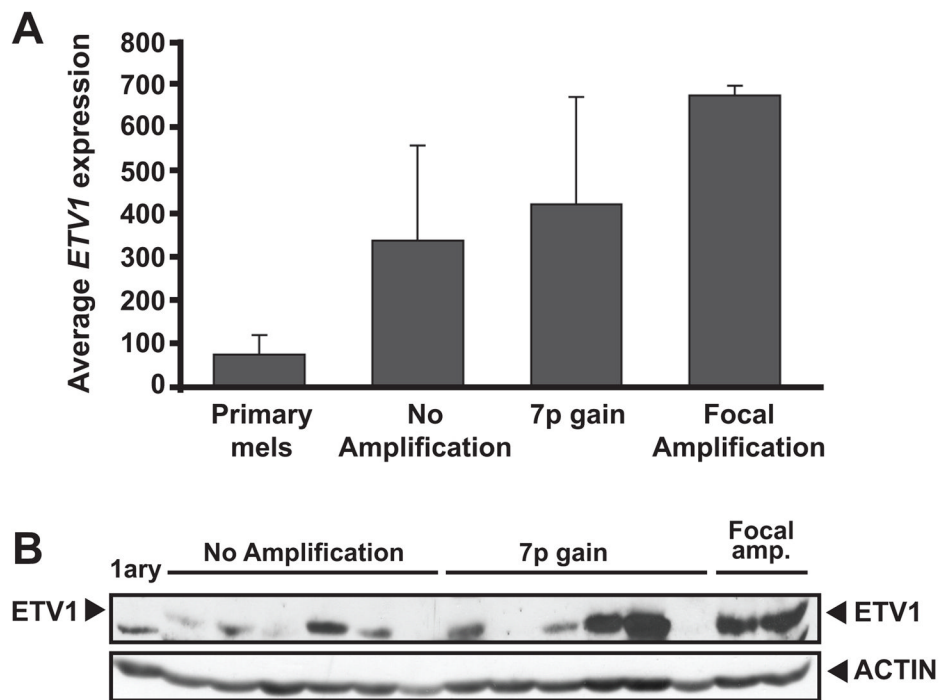
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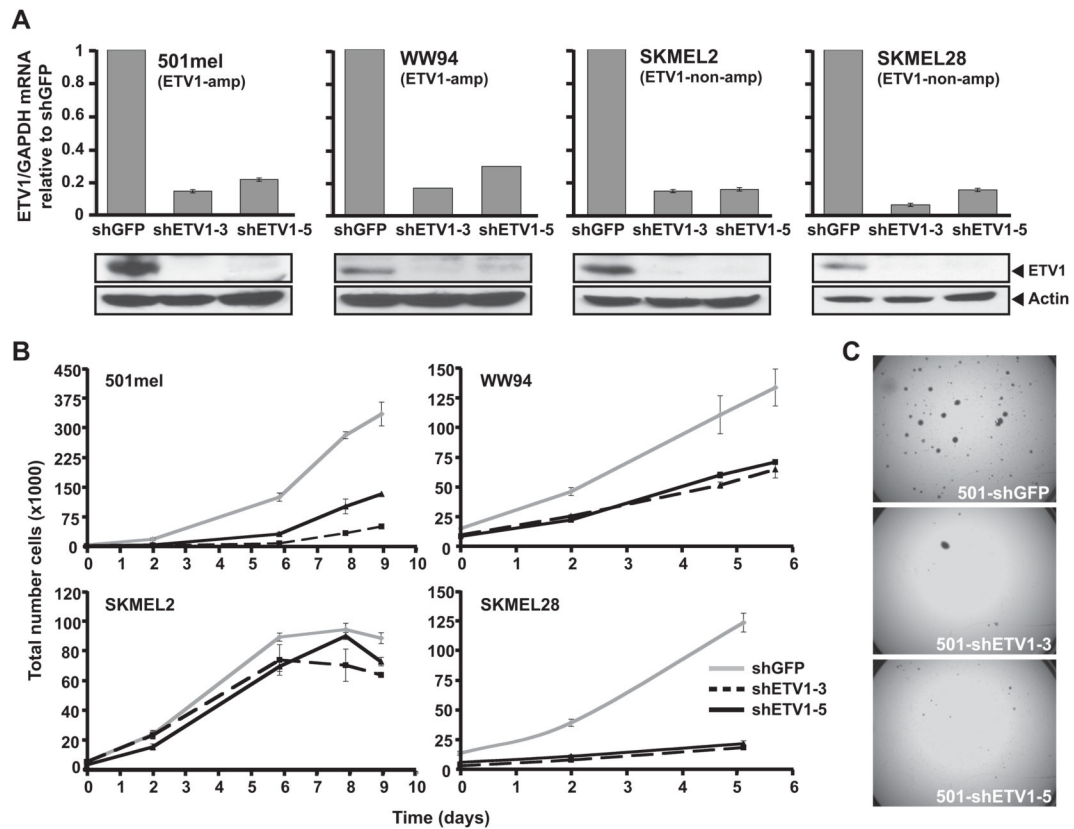
**Figure 1.** Amplification of the *ETV1* locus in melanoma. **A**, SNP array-based copy number analysis of chromosome 7p (left panel; cytobands indicated) is shown for a collection of melanoma short-term cultures and cell lines. Increasing red intensity denotes increasing copy number (legend; bottom). The region containing the *ETV1* locus is indicated (blue box), and a closer view is shown (right panel). *ETV1* and the neighboring diacylglycerol kinase beta 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 bars). **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 bars), and by immunoblot analysis using an  $\alpha$ -*ETV1* antibody. **D**, FISH analysis is shown for illustrative melanoma samples from a tissue

microarray (TMA). Green- and red- labeled BAC probes detect the *ETVI* and reference loci, respectively. A diploid case (left) and a case of *ETVI* amplification (right) are shown.



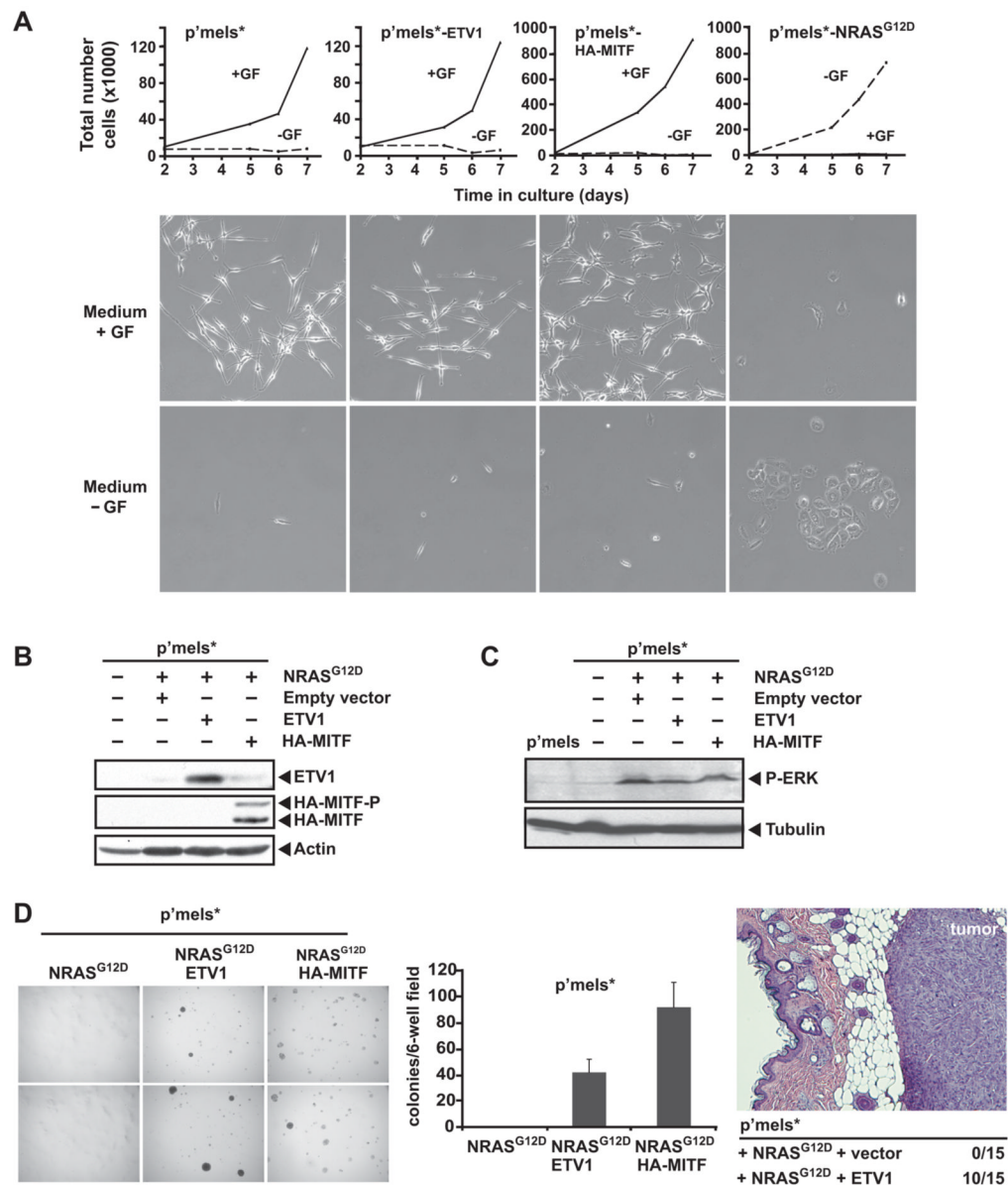
**Figure 2.** *ETV1* expression in relation to chromosome 7p copy gains. *A*, normalized *ETV1* expression levels in cultured primary melanocytes and melanoma lines are shown. Average expression values from subgroups stratified by *ETV1* locus amplification are indicated (focal amplification: 501mel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





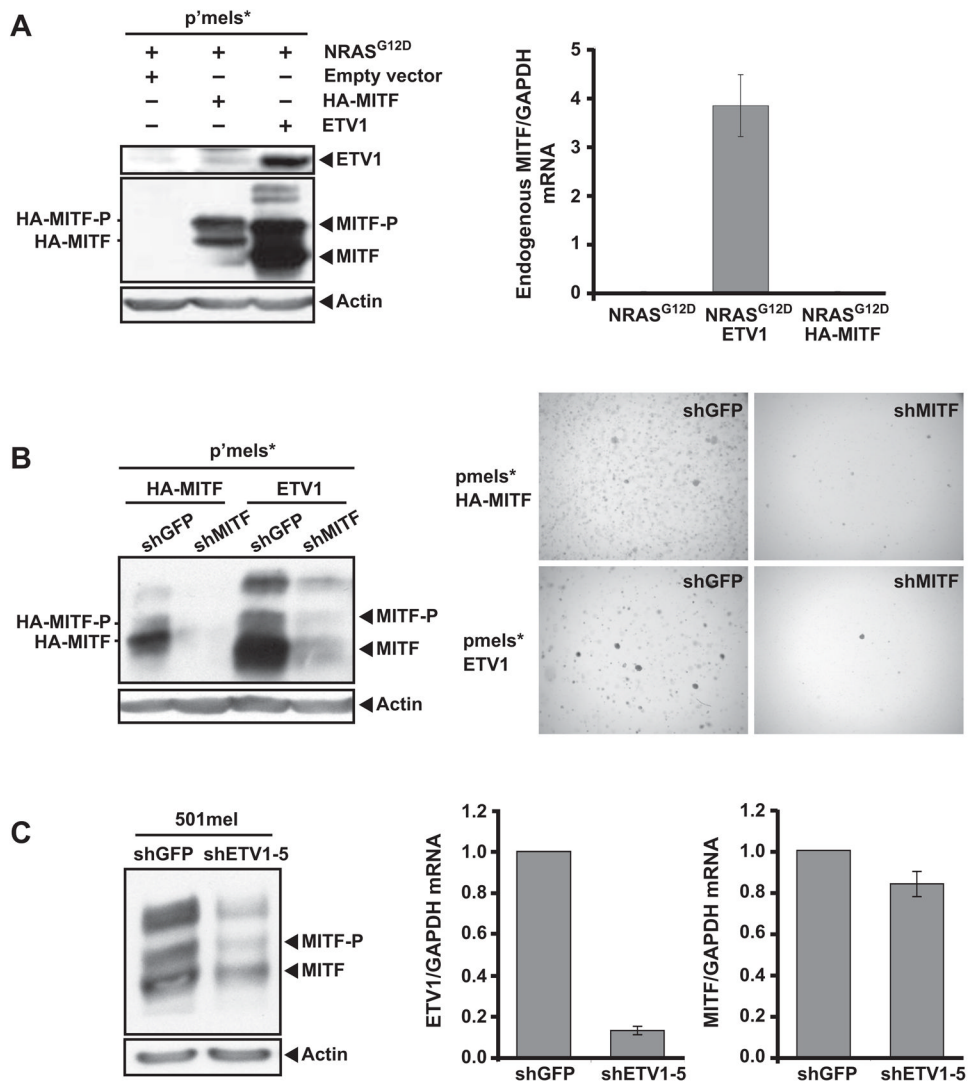
**Figure 3.**

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 -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 panels). Corresponding immunoblot studies of *ETV1* protein are also shown (bottom panels). *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.

**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\* melanocytes expressing ETV1, HA-MITF or NRAS<sup>G12D</sup> cultured in F10 media in the presence (+GF) or absence (-GF) of TPA and cAMP growth factors. Cells were photographed after 5 days in culture. **B**, **C**, immunoblot analyses of p'mel\* and p'mel\*-NRAS<sup>G12D</sup> cells infected with empty vector, ETV1 or HA-MITF retroviruses are shown. Antibodies recognizing ETV1, HA epitope, actin, phospho-ERK and tubulin were used. HA-MITF-P denotes the phosphorylated form of HA-MITF. **D**, (left) anchorage-independent growth of p'mel\* cells expressing NRAS<sup>G12D</sup> alone or in combination with ETV1 or HA-MITF is shown (photographs from two independent assays are shown for each cell type). (middle) Colony counts from these experiments are also indicated. (right) p'mel\*-NRAS<sup>G12D</sup> cells infected with empty- or ETV1-retroviruses were injected subcutaneously in mice. Tumor formation following injection is indicated

numerically, and a histological section of a representative ETV1-dependent tumor is shown (stained with hematoxylin and eosin).



**Figure 5.** ETV1 regulation of MITF expression. *A*, (left) immunoblot analyses of p'mel\*-NRAS<sup>G12D</sup> cells infected with empty vector, ETV1 or HA-MITF retroviruses are shown. Antibodies recognizing ETV1, HA epitope, and actin were used. MITF-P and HA-MITF-P denote the 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<sup>G12D</sup> cells overexpressing HA-MITF or ETV1 following infection with lentivirus containing shRNAs against *MITF* (shMITF) or *GFP* (shGFP). (right) Anchorage-independent growth of 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.

Table 1

ETV1 FISH analysis of melanoma tissue samples

Sample type	Samples on array	Analyzable Samples*	No Amp	% of samples		
				Low Amp <sup>†</sup> (<6)	High Amp <sup>‡</sup> (>6)	
Thin Nevus	21	10	100	0	0	0
Thick Nevus	15	10	100	0	0	0
Thin Primary	39	16	56.2	31.3	12.5	12.5
Thick Primary	20	15	46.7	40.0	13.3	13.3
Lymph Node Mets	39	17	23.6	58.8	17.6	17.6
Visceral Mets	46	23	43.5	39.1	17.4	17.4

\* Samples that rendered an informative result

<sup>†</sup> Samples presenting 3 to 6 ETV1-probe signals<sup>‡</sup> Samples presenting more than 6 ETV1-probe signals