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Direct Early Growth Response-1 (EGR1) Knockdown Decreases Melanoma Viability Independent of Mitogen-Activated Extracellular Signal-Related Kinase (MEK) Inhibition

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Abstract

Objective: To investigate downstream molecular changes caused by mitogen activated protein kinase (MEK) inhibitor treatment and further explore the impact of direct knockdown of early growth response-1 (*EGR1*) in melanoma cell culture.

Methods: RNA-sequencing (RNA-Seq) was performed to determine gene expression changes with MEK inhibitor treatment. Treatment with MEK inhibitor (trametinib) was then assessed in two cutaneous (MEL888, MEL624) and one conjunctival (YUARGE 13-3064) melanoma cell line. Direct knockdown of *EGR1* was accomplished using lentiviral vectors containing shRNA. Cell viability was measured using PrestoBlueHS™ Cell Viability Reagent. Total RNA and protein were assessed by qPCR and SimpleWestern.

Results: RNA-Seq demonstrated a profound reduction in *EGR1* with MEK inhibitor treatment, prompting further study of melanoma cell lines. Following trametinib treatment of melanoma cells, viability was reduced in both cutaneous (MEL888 26%, $p<0.01$; MEL624 27%, $p<0.001$) and conjunctival (YUARGE 13-3064 33%, $p<0.01$) melanoma compared with DMSO control, with confirmed *EGR1* knockdown to 0.04-, 0.01-, and 0.16-fold DMSO-treated levels (all $p<0.05$) in MEL888, MEL624, and YUARGE 13-3064, respectively. Targeted *EGR1* knockdown using shRNA reduced viability in both cutaneous (MEL624 78%, $p=0.05$) and conjunctival melanoma (YUARGE-13-3064 67%, $p=0.02$).

Conclusion: RNA-Sequencing in MEK inhibitor-treated cells identified *EGR1* as a candidate effector molecule of interest. In a malignant melanoma cell population, MEK inhibition reduced viability in both cutaneous and conjunctival melanoma with a profound downstream reduction in *EGR1* expression. Targeted knockdown of *EGR1* reduced both cutaneous and conjunctival melanoma cell viability independent of MEK inhibition, suggesting a key role for *EGR1* in melanoma pathobiology.

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Conflicts of Interest: None Declared

Keywords

Mitogen Activated Protein Kinase (MEK); Early Growth Response-1 (EGR1); RNA-sequencing (RNA-Seq)

Introduction

Melanoma is an aggressive and devastating disease with increasing incidence, especially in the Caucasian population.[1] In the United States, melanoma is the fifth most common cancer, with over 320% increase in incidence since 1975, and metastatic melanoma accounts for approximately 80% of all skin cancer-related deaths.[2, 3] The 5-year overall survival for stage IV metastatic melanoma is less than 30%.[3]

Upregulation of the mitogen-activated protein kinase (MAPK) pathway is a key factor in melanoma proliferation.[4] The MAPK pathway, also known as the RAS/RAF/MEK/ERK pathway, involves the interaction of Ras (rat sarcoma), Raf (rapidly accelerated fibrosarcoma), MEK (mitogen-activated protein kinase kinase), and ERK (extracellular signal-regulated kinase).[5, 6] In this pathway, activated MEK phosphorylates ERK, which translocates to the nucleus and activates multiple downstream transcription factors involved in cell growth, proliferation, differentiation, and survival.[6, 7] Mutations causing unchecked activation of the MAPK pathway, such as those seen in melanoma, lead to uncontrolled cellular proliferation, increased cell survival, genomic instability, and evasion of apoptosis.[6]

MEK inhibitors are therapeutics that bind to and inhibit MEK1 and/or MEK2.[5] Several MEK inhibitors, such as trametinib, have been approved to treat metastatic cutaneous melanoma.[5, 6, 8] Despite good initial efficacy, the impact on normal cells can lead to dose-limiting toxicities,[5, 6] and a large proportion of patients will develop acquired treatment resistance within 6–7 months.[4, 9, 10] Thus, further efforts to adopt more targeted approaches to treatment could improve patient outcomes. In our laboratory, we have identified a molecule of interest, early growth response-1 (*EGR1*). *EGR1* is a downstream effector of the MAPK pathway that can behave as either an oncogene or a tumor suppressor depending on the cellular context.[11–16] Herein, we explore the effect of MEK inhibition on *EGR1* expression and further investigate the impact of targeted *EGR1* knockdown on melanoma cell viability.

Materials and Methods

RNA-sequencing

RNA-Sequencing was performed in a benign retinal pigment epithelial (RPE) cell population, which showed no viability reduction when treated with MEK inhibitors. As previously described, 006 Biotr0001 CL1 induced pluripotent stem cells (iPSC) were differentiated to RPE cells.[17] Additional 006 Biotr0001 CL1 iPSC-RPE cells were purchased from LAgen Laboratories. Lines were maintained in RPEM media (LAgen Laboratories) containing 1 or 2% fetal bovine serum and 1X antibiotic/antimycotic (ThermoFisher Scientific, 15240) and incubated at 37°C, 5% CO_2 under humidified

conditions. The iPSC-derived RPE were positive for RPE65, bestrophin, MERTK, and CRALBP proteins when analyzed by western blot, and had been cultured for three to four months prior to being used in an assay. iPSC-RPE monolayers were dosed apically and basally daily for 96 hours with three concentrations of the MEK inhibitor trametinib (180 nM, 18 nM or 1.8 nM) or selumetinib (1400 nM, 140 nM, or 14 nM). These concentrations corresponded to 100 X, 10 X or 1X IC_{50} according to the product data sheet. To harvest RNA, iPSC-RPE monolayers were rinsed two times with 1X DPBS without calcium and magnesium. The filter was removed from the support and transferred to TRIzol® Reagent (Ambion, 15596-026), and total RNA was purified using RNA Clean and Concentrator-5 kit (Zymo Research, R1013). Total RNA was treated with DNAse I using RNAse-free DNAse I (Sigma, 4716728001) according to the manufacturer's protocol. cDNA libraries for each sample were prepared from total RNA according to the manufacturer's instructions for the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA). Paired end sequencing was done on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit version 3 and HCS version 2.0.12.0 data collection software. Base-calling was performed using Illumina's RTA version 1.17.21.3. Reads were analyzed using the Mayo Analysis Pipeline for RNA-Seq.[18] Reads were aligned and mapped to the HG38 genome assembly.

Melanoma cell lines

MEL888 and MEL624 (BRAFV600E mutant cutaneous melanoma cell lines) were obtained from Richard Vile, PhD (Mayo Clinic, Rochester, MN), and YUARGE 13-3064 (BRAFV600E wild-type conjunctival melanoma cell line) was obtained from the Yale SPORE in Skin Cancer. Cell lines were maintained in Dulbecco's Modified Eagles Medium (DMEM, Gibco, 11960-044) containing 4.5 g/L glucose, supplemented with 10% fetal bovine serum, 1X antibiotic/antimycotic (ThermoFisher Scientific, 15240) and 2 mM L-glutamine, and incubated at 37°C in 5% CO_2 under humidified conditions. Short tandem repeat (STR) testing was performed. All cell lines were confirmed to be negative for mycoplasma prior to study initiation. Cells were maintained in culture for no more than 60 days or 15 passages from thaw.

Viability assays

Viability was measured with PrestoBlueHS™ Cell Viability Reagent (ThermoFisher Scientific, P50201), a resazurin-based solution which can quantitatively measure proliferating cells via the reducing power of mitochondrial output. Briefly, the reagent was added to the experimental and control wells and incubated at 37°C in 5% CO_2 under humidified conditions for 30–60 minutes. Fluorescence was measured at 560/590nm using a SpectraMax i3 multi-mode plate reader (Molecular Devices, CA). Experimental samples were compared to vehicle control to establish percent viability.

Sensitivity of melanoma lines to MEK inhibitors

Cell response to trametinib (Selleckchem, S1008) was determined by titration as follows: Melanoma cells were plated at a density of 2000–4000 cells/cm^2, incubated overnight, and treated daily with 1.8 nM, 18 nM or 180 nM trametinib dissolved in DMSO. These concentrations corresponded to 1X, 10X or 100X IC_{50} according to the manufacturer's product data sheet. Independent wells were treated with DMSO and served as vehicle

controls. Following 3 days of treatment, cell viability was assessed with PrestoBlueHS™ Cell Viability Reagent (ThermoFisher Scientific, P50201).

cDNA synthesis and qPCR

Treated melanoma cells were scraped into centrifuge tubes and washed two times with 1X DPBS without calcium and magnesium. Pellets were resuspended and divided into 2 samples. One pellet aliquot from each sample was resuspended in TRI Reagent (Zymo Research, R2050-1-50), and total RNA was purified using RNA Clean and Concentrator-5 kit (Zymo Research, R1013). Total RNA was treated with RNAse-free DNAse I (Sigma, 4716728001) and RNasin® Plus RNAse Inhibitor (Promega, N2615) according to the manufacturer's protocol. cDNA was synthesized from total RNA using SuperScript IV VILO Master Mix (Thermo Fisher, 117660050) and treated with RNase H (New England BioLabs, M02975). Additional genes were selected for analysis by qPCR. Primer pairs targeting *EGR1*, *TP53*, *PTEN*, *TOE1*, *NAB1*, *NAB2*, and *TGFB3* were designed using Primer-BLAST software (Supplemental Table 1) and purchased from Integrated DNA Technologies (Coralville, IA).[19] At least one primer in each pair spanned an exon junction. PCR reactions were prepared with 10 ng of input cDNA, 25 μM of each primer, and PowerUp Sybr Green Master Mix (Applied Biosystems, A25742). PCR amplification was performed in quadruplicate on an Applied Biosystems QuantStudio 5 qPCR instrument using the following conditions: 50°C, 2 min, 95°C, 2 min, 1 cycle; 95°C, 15 sec, 55°C, 15 sec, 72°C, 30 sec, 40 cycles; 95°C, 15 sec, 55°C, 1 min, 95°C, 15 sec until recovery, 1 cycle. Data analysis was performed using QuantStudio Design and Analysis software v 1.4 (Applied Biosystems). Expression was calculated using the ddCt method relative to expression levels of DMSO vehicle or non-targeted cells. *GAPDH* served as the internal control.

Simple western blotting

The second pellet aliquot from each sample (described above) was lysed in RIPA buffer containing 0.1% SDS supplemented with PhosSTOP (Sigma, 4906845001), cOmplete Mini (Sigma, 4693159001), 10 mM PMSF (Sigma, P7626), and Roche IV Protease Inhibitor Cocktail Set III, EDTA-Free - Calbiochem (Sigma, 539134). Total protein was quantified using Pierce Rapid Gold BCA Protein Assay Kit (Thermoscientific, A53226). Western blots were performed using a WES SimpleWestern automated capillary electrophoresis and immunoblot system (ProteinSimple, San Jose, California, USA) using either a 25-capillary or 13-capillary 12-230 kDa Wes Separation Module (ProteinSimple, SMW004). Antibodies to EGR1 (Cell Signaling Technology, 4153) and GAPDH (Novusbio, NB300-328SS) were diluted 1:50 and 1:1,000, respectively. Antibodies to p44/42 MAPK (Erk1/2) (Cell Signaling Technology, 9102) and phospho-p44/42 MAPK (pErk1/2) (Cell Signaling Technology, 9101) were diluted 1:50. Following protein separation, luminescence intensity data from individual capillaries were indexed to each other by comparison to standard size markers included in the Master Mix and compared to a set of molecular mass markers run simultaneously in a separate capillary. Luminescent peaks and peak areas were determined using Compass SW version 4.0.1 software. Quantitative assessment of protein expression was performed by comparing the peak area EGR1 to GAPDH relative to DMSO control.

For comparisons of inhibition of ERK phosphorylation, total ERK served as the reference relative to DMSO control.

Preparation of lentivirus particles

Four separate lentivirus transfer plasmids containing GFP-tagged 29-mer shRNA sequences directed toward *EGR1* as well as a non-targeting shRNA control (Supplemental Table 1) were purchased from Origene (TL313277, TR30021V5). The shRNA were co-transfected with lentiviral packaging plasmids pMD2.G (Addgene plasmid #12259) and psPAX2 (Addgene plasmid #12260) into HEK293T17 cells (ATCC CRL-11268). Lentiviral packaging plasmids pMD2.G and pSPAX2 were a gift from Didier Trono. Supernatants containing lentiviruses were harvested daily for 48 hours and purified via ultracentrifugation through a 20% sucrose cushion. Titers were determined by transducing the purified lentiviral particles in HEK293T17 cells, harvesting the cells 48 hours post transduction, and counting green cells on a Cellometer (Nexcelom Auto 2000).

EGR1 shRNA knockdown

MEL624 and YUARGE 13-3064 cells were plated at 2000–4000 cells/cm^2 and incubated overnight. The following day, cells were transduced with lentivirus containing the four pooled shRNA viral particles targeting EGR1 (MOI 10 and MOI 50 for MEL 624 and YUARGE 13-3064, respectively). All lentiviral shRNA experiments included a lentiviral non-targeting control shRNA of an equal MOI. Cells were harvested 48 hours post-induction of the targeting shRNAs.

Statistical analysis

For RNA-sequencing, changes in gene expression levels were calculated using DESeq2[20] with the default parameters and a FDR of 0.1 (10%). Differentially expressed genes were considered if the log 2-fold change in gene expression was 1.5 or greater with $p \leq 0.05$ and the change in expression exhibited a dose response to trametinib. Continuous data were compared using the Student's t-test to compare experimental conditions to vehicle control. Results were considered significant if $p \leq 0.05$.

Results

MEK inhibition reduces EGR1 expression

While investigating MEK inhibitor toxicity on RPE cells, our laboratory discovered that MEK inhibition produced a profound reduction in *EGR1* gene expression in benign RPE cells with no change in cell viability. Specifically, 18 nM trametinib treatment was associated with a 39-fold decrease in *EGR1* gene expression (Fig 1). Given this robust effect and the known opposing roles of *EGR1* as a tumor suppressor or an oncogene, we sought to investigate the interaction between MEK inhibition and *EGR1* expression in malignant melanoma.

Trametinib decreases melanoma cell viability

To investigate the effect of the MEK inhibitor trametinib on melanoma cell viability, three melanoma cell lines (MEL888, MEL624, YUARGE 13-3064) were treated with three different trametinib concentrations (1.8, 18 and 180 nM) (Fig 2A). All melanoma cell lines showed a dose dependent reduction in cell viability, demonstrating a toxic effect of MEK inhibitor treatment on melanoma cells. Trametinib at 18 nM reduced cell viability compared to DMSO-treated controls in all three cell lines (MEL888 26%, p=0.001, MEL624 27%, p<0.001, YUARGE 13-3064 33%, p=0.003) (Fig 2B). This concentration was used in further studies.

Trametinib reduces EGR1 expression in melanoma cells

qPCR was performed to determine whether the EGR1 knockdown effect of MEK inhibition that had been observed in RPE was reproducible in melanoma cells. Trametinib reduced *EGR1* mRNA in each of the three melanoma cell lines compared to vehicle-treated controls (MEL888 0.04-fold, p<0.001, MEL624 0.01-fold, p<0.01, YUARGE 13-3064 0.16-fold, p=0.01) (Fig 2C). Similarly, EGR1 protein expression was reduced in each cell line compared to DMSO vehicle control levels (MEL888 0%, p<0.001, MEL624 0%, p<0.001, YUARGE 13-3064 5% p=0.001.) (Fig 2D, E).

Trametinib increases expression of tumor suppressors

An additional panel of genes was selected for investigation based on reported tumor suppressor function and/or MEK pathway involvement. qPCR analysis of melanoma cell lines treated with trametinib revealed increased expression of *TP53*, *PTEN*, *TGFβ3*, *TOE1*, and *NAB2* in all three cell lines with MEL888: 4.2, 6.3, 14.3, 6.4, 6.6 fold increase, respectively, all p≤0.045 (Fig 3A), MEL624: 23.6, 10.4, 1.8, 2.8, 2.6 fold increase, respectively (Fig 3B), all p≤0.027, and YUARGE 13-3064: 6.0, 4.7, 7.1, 2.1, 3.4 fold increase, respectively, all p≤0.045 (Fig 3C).

Trametinib decreases pERK protein expression in melanoma cell lines

To confirm the downstream effects of MEK inhibition, we evaluated pERK protein expression in trametinib-treated melanoma cells. Treatment with trametinib reduced pERK in MEL888, MEL624, and YUARGE 13-3064 to 7% (p<0.001), 16% (p=0.01), and 20% (p=0.009) of basal pERK expression levels, respectively, confirming the inhibitory capacity of trametinib (Fig 4).

Direct knockdown of *EGR1* decreases melanoma cell viability

To investigate the importance of *EGR1* in melanoma cell viability and specifically to determine whether targeting this downstream effector would reduce viability in cancer cells, we explored the effects of direct *EGR1* knockdown in two melanoma cell lines. Targeted *EGR1* knockdown resulted in reduction of melanoma cell viability in both cutaneous (MEL624: 78%, p=0.05) and conjunctival (YUARGE 13-3064: 67%, p=0.02) melanoma compared to non-targeting control, suggesting a key role for *EGR1* in melanoma pathogenesis (Fig 5A).

Direct knockdown of *EGR1* decreases *EGR1* RNA and protein levels

To confirm direct *EGR1* knockdown in our cell lines, we investigated *EGR1* RNA and protein expression with targeted knockdown. Overall, *EGR1* mRNA (Fig 5B) and protein (Fig 5C, D) expression were reduced to 0.61-fold and 42%, respectively, in MEL624 (n=4: p=0.15 and n=3: p=0.006) and 0.50-fold and 32%, respectively, in YUARGE 13-3064 (n=4: p=0.05 and n=4: p=0.01), compared to non-targeting control.

Direct knockdown of *EGR1* has variable effects on tumor suppressor genes

Analysis of a panel of tumor suppressor genes and molecules associated with *EGR1* showed variable changes in expression following knockdown of *EGR1* (Fig 6) in the cutaneous versus conjunctival melanoma cells despite consistent reduction in cell viability. Although not statistically significant at these modest *EGR1* knockdown levels, expression of the tumor suppressor *TGFβ3* consistently increased in both the cutaneous (MEL624: 1.29-fold, p=0.24) and conjunctival melanoma cells (YUARGE 13-3064: 1.51-fold, p=0.15), suggesting a potential role for *TGFβ3* as a downstream effector related to melanoma toxicity with *EGR1* knockdown. The tumor suppressor *PTEN* appeared to increase in the cutaneous melanoma cell line MEL624 (1.72-fold, p=0.16) but not in the conjunctival melanoma cell line YUARGE 13-3064 (0.64-fold, p=0.15). Other tumor suppressors and targets of *EGR1* (*TP53*, *TOE1*, and *NAB1*) showed minimal change in MEL624 (1.1-fold, p=0.16, 0.96-fold, p=0.45, 0.78-fold, p=0.08, respectively) and YUARGE 13-3064 (0.93-fold, p=0.69, 1.08-fold, p=0.72, 0.96-fold, p=0.83, respectively).

Discussion

MEK inhibitors have been used clinically to treat a variety of cancers,[5, 6, 8, 21–23] with high clinical utility for melanoma. Despite widespread use, these medications have a limited therapeutic index and development of treatment resistance is common.[5, 6, 24, 25] Thus, ongoing investigation of other molecules which regulate melanoma pathogenesis is worthwhile to develop more effective treatments.

While attempting to study a drug side effect of MEK inhibitors on RPE cells, our laboratory noticed a profound reduction in *EGR1* gene expression with MEK inhibition and sought to further investigate whether this molecular change also occurred in malignant melanoma cells. Using the MEK inhibitor trametinib in three distinct melanoma cell lines, we confirmed that MEK inhibition reduced melanoma cell viability and consistently reduced *EGR1* gene and protein expression in all three melanoma cell lines. After confirming *EGR1* reduction with MEK inhibition in malignant cells, we investigated direct inhibition of *EGR1* expression using shRNA in melanoma. Curiously, a reduction in melanoma cell viability was observed with direct *EGR1* knockdown in the absence of MEK inhibition in both cutaneous and conjunctival melanoma cell lines.

EGR1 is a member of the zinc finger transcription factor family[26, 27] that plays a key role in cell growth, proliferation, differentiation, and apoptosis.[28] *EGR1* can behave as either an oncogene or a tumor suppressor depending on the cellular context.[11–16] For example, *EGR1* is suppressed in breast cancer, non-small-cell lung cancer, glioma, sarcoma,

and hematopoietic malignancies. In these cancers, restoration of *EGR1* expression prevents cellular growth, slowing down the disease process.[15, 16] In contrast, prostate cancer expresses elevated levels of *EGR1*, while *EGR1* suppression inhibits prostate cancer cell proliferation.[12–14] In The Cancer Genome Atlas (TCGA) cohort of uveal melanoma samples from patients requiring enucleation, elevated *EGR1* expression was associated with higher risk for metastatic disease,[29] but less is known about the role of *EGR1* in more common melanoma subtypes.

EGR1 is an important downstream target of the MAPK pathway. However, as a transcription factor, *EGR1* interacts with multiple other pathways, which could make it a candidate molecule to inhibit melanoma progression via a complementary mechanism to MEK inhibition. In particular, Schmidt et al. studied the interaction between *EGR1*, the androgen receptor, and the long non-coding *RNA SLNCR*.[30] Noting that cutaneous melanoma is more common and presents at more advanced stages in men, the authors found that *SLNCR* recruits the androgen receptor to chromatin-bound *EGR*1, switching physiologic EGR1-mediated p21 activation to oncogenic EGR1-mediated p21 repression.[30] Thus, the authors suggested a critical role for *EGR1* in melanoma pathobiology. Our study similarly suggests that *EGR1* plays an important role in melanoma pathogenesis, with viability reduction proportionate to the degree of *EGR1* knockdown.

From a clinical perspective, Kappelmann-Fenzl et al. investigated EGR1-stained tissue sections, finding that EGR1 could be detected in most melanoma cells.[31] The authors further noted an absence of EGR1 protein expression in benign melanocytes, weak cytoplasmic staining in melanoma cells from primary tumor, and intense nuclear staining in melanoma metastasis, suggesting an association between increasing EGR1 expression and melanoma progression.[31] Kanemaru et al. similarly found increased EGR1 expression in tumor tissues from patients with invasive melanoma compared to tissues of non-invasive melanoma.[32] Investigating the rare subtype of uveal melanoma, Yu et al. found that EGR1 was responsible for upregulation of angiogenesis and accelerated tumorigenesis mediated by nuclear programmed cell death protein ligand 1 (nPD-L1).[33] They confirmed their findings *in vivo*, demonstrating that restoration of EGR1 rescued tumor growth in an orthotopic xenograft mouse model.[33] Despite these data supporting the role for EGR1 in more advanced stages of cutaneous melanoma and unfavorable prognosis in uveal melanoma,[29] *in vivo* EGR1-related prognostic information in cutaneous melanoma is lacking.

In addition to changes in *EGR1* in our experiments, we demonstrated a consistent increase in expression of the tumor suppressor *TGFβ3* with both MEK inhibition and direct *EGR1* knockdown in melanoma cell lines, albeit the increase did not reach statistical significance at the more modest levels of direct *EGR1* knockdown induced. Interestingly, *TGFβ3* plays a complex role in cancer pathobiology, acting as a tumor suppressor in early stages of malignant transformation but subsequently promoting metastatic spread in later stages.[34–37] In fact, studies have investigated inhibition of *TGFβ* to treat or prevent cancer progression, including a study by Kodama et al. demonstrating that progression of melanoma was suppressed by combination targeting of *TGFβ1*, *TGFβ2*, and *TGFβ3*.[38, 39] Additional studies have identified *TGFβ3* as a critical factor for

maintenance of epidermal differentiation and suggest that low epidermal *TGFβ3* levels could be associated with cutaneous melanoma formation.[40, 41] These studies suggest that external application of *TGFβ3* could prevent melanomagenesis.[35] However, other studies showed that more aggressive and metastatic melanoma becomes resistant to *TGFβ*-mediated growth inhibition.[42] Future studies are needed to elucidate the relationship between *EGR1* and *TFGβ3* in the context of tumor initiation and progression in melanoma. Furthermore, given the inconsistent impact of *EGR1* knockdown on *PTEN*, which increased in the BRAFV600E mutant cutaneous melanoma cell line but decreased in the BRAFV600E wild-type conjunctival melanoma cell line, further investigation is warranted to explore whether BRAF status or other molecular distinctions between melanoma subtypes impact *EGR1* downstream interactions. In contrast to treatment with MEK inhibition, minimal impact was observed with targeted *EGR1* knockdown on other tumor suppressors or targets of *EGR1* in either cell line (*TP53*, *TOE1*, and *NAB1*).

Limitations of our study included limited exploration of only three melanoma cell lines, of which two were derived from cutaneous melanoma and one was derived from conjunctival melanoma. Although cutaneous and conjunctival melanoma share many of the same driving mutations, we acknowledge that these melanoma subtypes can have distinct features. In our experiments, we saw consistent results in all three utilized cell lines treated with MEK inhibitor, and we investigated one cutaneous and one conjunctival melanoma cell line in direct *EGR1* knockdown experiments. We did not investigate the impact of *EGR1* knockdown on other rare melanoma subtypes, such as uveal melanoma, in this series of experiments due to the molecularly distinct nature of these cancers. We also acknowledge the possibility of off-target effects when inducing *EGR1* knockdown via lentiviral vector with shRNA. We found the melanoma cell lines available in our laboratory to be particularly difficult to transfect, which made other mechanisms of direct *EGR1* knockdown, such as siRNA, challenging. Future experiments could utilize other techniques such as CRISPR-Cas9, to produce reliable *EGR1* knockdown.

In summary, our data demonstrate that reduction of *EGR1* is associated with reduced melanoma cell viability, suggesting a key role for *EGR1* in melanoma pathobiology. Future studies are needed to confirm these findings in common and rare melanoma subtypes. Better defining the molecular events downstream of *EGR1* in melanoma could elucidate new avenues for targeted melanoma treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

MEL888 and MEL624 (BRAFV600E mutant cutaneous melanoma cell lines) were obtained from Richard Vile, PhD (Mayo Clinic, Rochester, MN), and YUARGE 13-3064 (BRAFV600E wild-type conjunctival melanoma cell line) was obtained from the Yale SPORE in Skin Cancer.Lentiviral packaging plasmids pMD2.G and psPAX2 were a gift from Didier Trono (Addgene plasmid #12259 and 12260).

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References

1. Jemal A, et al. , Cancer statistics, 2007. CA Cancer J Clin, 2007. 57(1): p. 43–66. [PubMed: 17237035]
2. Paluncic J, et al. , Roads to melanoma: Key pathways and emerging players in melanoma progression and oncogenic signaling. Biochim Biophys Acta, 2016. 1863(4): p. 770–84. [PubMed: 26844774]
3. Saginala K, et al. , Epidemiology of Melanoma. Med Sci (Basel), 2021. 9(4).
4. Amaral T, et al. , MAPK pathway in melanoma part II-secondary and adaptive resistance mechanisms to BRAF inhibition. Eur J Cancer, 2017. 73: p. 93–101. [PubMed: 28162869]
5. Cheng Y and Tian H, Current Development Status of MEK Inhibitors. Molecules, 2017. 22(10).
6. Degirmenci U, Wang M, and Hu J, Targeting Aberrant RAS/RAF/MEK/ERK Signaling for Cancer Therapy. Cells, 2020. 9(1).
7. Friday BB and Adjei AA, Advances in targeting the Ras/Raf/MEK/Erk mitogen-activated protein kinase cascade with MEK inhibitors for cancer therapy. Clin Cancer Res, 2008. 14(2): p. 342–6. [PubMed: 18223206]
8. Subbiah V, Baik C, and Kirkwood JM, Clinical Development of BRAF plus MEK Inhibitor Combinations. Trends Cancer, 2020. 6(9): p. 797–810. [PubMed: 32540454]
9. Patel A, et al. , Targeting p63 Upregulation Abrogates Resistance to MAPK Inhibitors in Melanoma. Cancer Res, 2020. 80(12): p. 2676–2688. [PubMed: 32291316]
10. Long GV, et al. , Increased MAPK reactivation in early resistance to dabrafenib/trametinib combination therapy of BRAF-mutant metastatic melanoma. Nat Commun, 2014. 5: p. 5694. [PubMed: 25452114]
11. Mohamad T, et al. , EGR1 interacts with TBX2 and functions as a tumor suppressor in rhabdomyosarcoma. Oncotarget, 2018. 9(26): p. 18084–18098. [PubMed: 29719592]
12. Eid MA, et al. , Expression of early growth response genes in human prostate cancer. Cancer Res, 1998. 58(11): p. 2461–8. [PubMed: 9622090]
13. Baron V, et al. , Inhibition of Egr-1 expression reverses transformation of prostate cancer cells in vitro and in vivo. Oncogene, 2003. 22(27): p. 4194–204. [PubMed: 12833142]
14. Gregg J and Fraizer G, Transcriptional Regulation of EGR1 by EGF and the ERK Signaling Pathway in Prostate Cancer Cells. Genes Cancer, 2011. 2(9): p. 900–9. [PubMed: 22593802]
15. Huang RP, et al. , Egr-1 negatively regulates human tumor cell growth via the DNA-binding domain. Cancer Res, 1995. 55(21): p. 5054–62. [PubMed: 7585551]
16. Huang RP, et al. , Decreased Egr-1 expression in human, mouse and rat mammary cells and tissues correlates with tumor formation. Int J Cancer, 1997. 72(1): p. 102–9. [PubMed: 9212230]
17. Johnson AA, et al. , Autosomal Recessive Bestrophinopathy Is Not Associated With the Loss of Bestrophin-1 Anion Channel Function in a Patient With a Novel BEST1 Mutation. Invest Ophthalmol Vis Sci, 2015. 56(8): p. 4619–30. [PubMed: 26200502]
18. Kalari KR, et al. , MAP-RSeq: Mayo Analysis Pipeline for RNA sequencing. BMC Bioinformatics, 2014. 15: p. 224. [PubMed: 24972667]
19. Ye J, et al. , Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics, 2012. 13: p. 134. [PubMed: 22708584]
20. Love MI, Huber W, and Anders S, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol, 2014. 15(12): p. 550. [PubMed: 25516281]
21. Proietti I, et al. , Mechanisms of Acquired BRAF Inhibitor Resistance in Melanoma: A Systematic Review. Cancers (Basel), 2020. 12(10).
22. Fujimura T, et al. , Treatment of Advanced Melanoma: Past, Present and Future. Life (Basel), 2020. 10(9).

23. Czarnecka AM, et al. , Targeted Therapy in Melanoma and Mechanisms of Resistance. Int J Mol Sci, 2020. 21(13).
24. Mergener S, Siveke JT, and Peña-Llopis S, Monosomy 3 Is Linked to Resistance to MEK Inhibitors in Uveal Melanoma. Int J Mol Sci, 2021. 22(13).
25. Steeb T, et al. , How to MEK the best of uveal melanoma: A systematic review on the efficacy and safety of MEK inhibitors in metastatic or unresectable uveal melanoma. Eur J Cancer, 2018. 103: p. 41–51. [PubMed: 30205280]
26. Cao XM, et al. , Identification and characterization of the Egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals. Mol Cell Biol, 1990. 10(5): p. 1931–9. [PubMed: 2109185]
27. Ahmed MM, Regulation of radiation-induced apoptosis by early growth response-1 gene in solid tumors. Curr Cancer Drug Targets, 2004. 4(1): p. 43–52. [PubMed: 14965266]
28. Papanikolaou NA, et al. , A systems approach identifies co-signaling molecules of early growth response 1 transcription factor in immobilization stress. BMC Syst Biol, 2014. 8: p. 100. [PubMed: 25217033]
29. Robertson AG, et al. , Integrative Analysis Identifies Four Molecular and Clinical Subsets in Uveal Melanoma. Cancer Cell, 2017. 32(2): p. 204–220.e15. [PubMed: 28810145]
30. Schmidt K, et al. , The lncRNA SLNCR Recruits the Androgen Receptor to EGR1-Bound Genes in Melanoma and Inhibits Expression of Tumor Suppressor p21. Cell Rep, 2019. 27(8): p. 2493–2507.e4. [PubMed: 31116991]
31. Kappelmann-Fenzl M, et al. , Molecular Changes Induced in Melanoma by Cell Culturing in 3D Alginate Hydrogels. Cancers (Basel), 2021. 13(16).
32. Kanemaru H, et al. , A mechanism of cooling hot tumors: Lactate attenuates inflammation in dendritic cells. iScience, 2021. 24(9): p. 103067. [PubMed: 34541473]
33. Yu J, et al. , Nuclear PD-L1 promotes EGR1-mediated angiogenesis and accelerates tumorigenesis. Cell Discov, 2023. 9(1): p. 33. [PubMed: 36977660]
34. Massagué J, TGFbeta in Cancer. Cell, 2008. 134(2): p. 215–30. [PubMed: 18662538]
35. Laverty HG, et al. , TGF-beta3 and cancer: a review. Cytokine Growth Factor Rev, 2009. 20(4): p. 305–17. [PubMed: 19656717]
36. Lebrun JJ, The Dual Role of TGFβ in Human Cancer: From Tumor Suppression to Cancer Metastasis. ISRN Mol Biol, 2012. 2012: p. 381428. [PubMed: 27340590]
37. Pardali K and Moustakas A, Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta, 2007. 1775(1): p. 21–62. [PubMed: 16904831]
38. Kim BG, et al. , Novel therapies emerging in oncology to target the TGF-β pathway. J Hematol Oncol, 2021. 14(1): p. 55. [PubMed: 33823905]
39. Kodama S, et al. , Progression of melanoma is suppressed by targeting all transforming growth factor-β isoforms with an Fc chimeric receptor. Oncol Rep, 2021. 46(3).
40. Schmid P, et al. , TGF-beta s and TGF-beta type II receptor in human epidermis: differential expression in acute and chronic skin wounds. J Pathol, 1993. 171(3): p. 191–7. [PubMed: 8277368]
41. Rodeck U, et al. , Transforming growth factor beta production and responsiveness in normal human melanocytes and melanoma cells. Cancer Res, 1994. 54(2): p. 575–81. [PubMed: 8275496]
42. Elliott RL and Blobe GC, Role of transforming growth factor Beta in human cancer. J Clin Oncol, 2005. 23(9): p. 2078–93. [PubMed: 15774796]

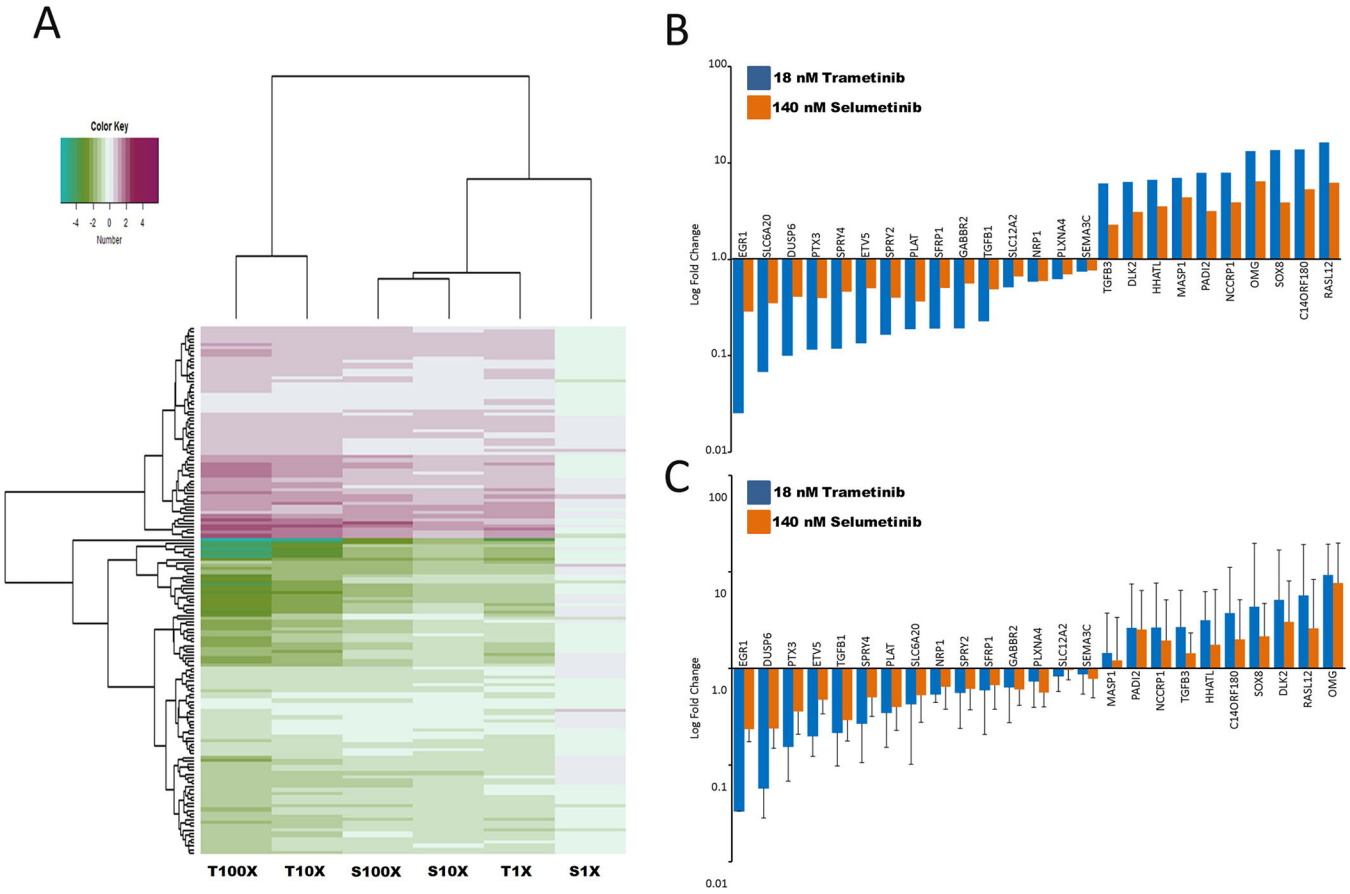

Fig 1: Gene Expression analysis of iPSC-RPE treated with MEK Inhibitors.

(A) Heat map of the top 161 genes that had log 2-fold change in gene expression ≥1.5 with $p \leq 0.05$. T is trametinib, S is selumetinib. For trametinib the dosages were 100X IC_{50} 180 nM, 10X IC_{50} 18 nM, and 1X IC_{50} 1.8 nM. For selumetinib dosages were 100X IC_{50} 1400 nM, 10X IC_{50} 140 nM, and 1X IC_{50} 14 nM. (B) A panel of 25 differentially expressed genes selected for further analysis. The log 2-fold change is based on 10X IC_{50} of each drug from the RNA-Seq dataset. (C) qPCR validation of the 25 gene panel.

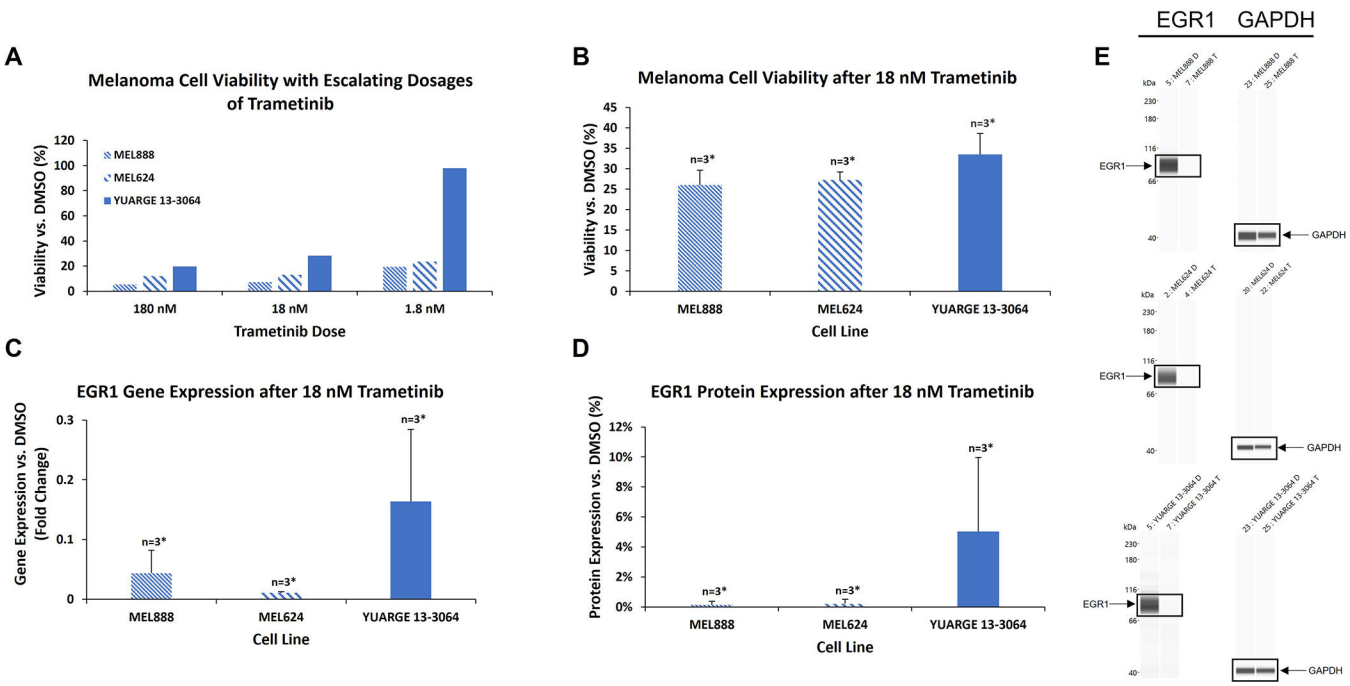

Fig 2: Viability and *EGR1* expression in melanoma cell lines treated with MEK inhibitor.

(A) MEK inhibitor treatment with 1.8 nM, 18 nM or 180 nM trametinib reduced cell viability in a dose-dependent fashion compared to DMSO vehicle control. (B) MEK inhibitor (MEKi) treatment with 18 nM trametinib on melanoma cell lines MEL888, MEL624, and YUARGE 13-3064 reduced melanoma cell viability compared with DMSO control. MEK inhibitor treatment with 18 nM trametinib on melanoma cell lines (MEL888, MEL624, YUARGE 13-3064) resulted in reduced *EGR1* (C) gene and (D) protein expression [shown on (E) representative blots]. Asterisk indicates statistical significance of $p \leq 0.05$.

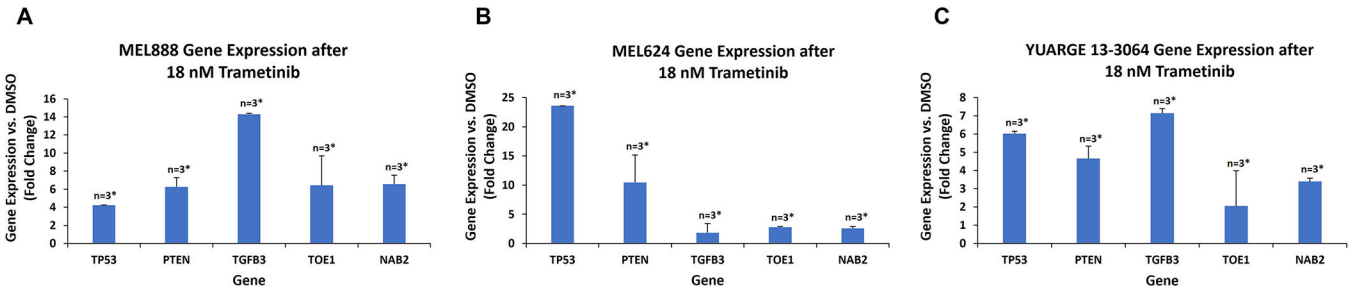

Fig 3: Downstream gene expression changes in melanoma cell lines treated with MEK inhibitor.

MEK inhibitor treatment with 18 nM trametinib on melanoma cell lines (MEL888, MEL624, YUARGE 13-3064) resulted in increased expression of tumor suppressor genes, including *TP53*, *PTEN*, *TGFβ3*, *TOE1*, and *NAB1.* Asterisk indicates statistical significance of $p \leq 0.05$.

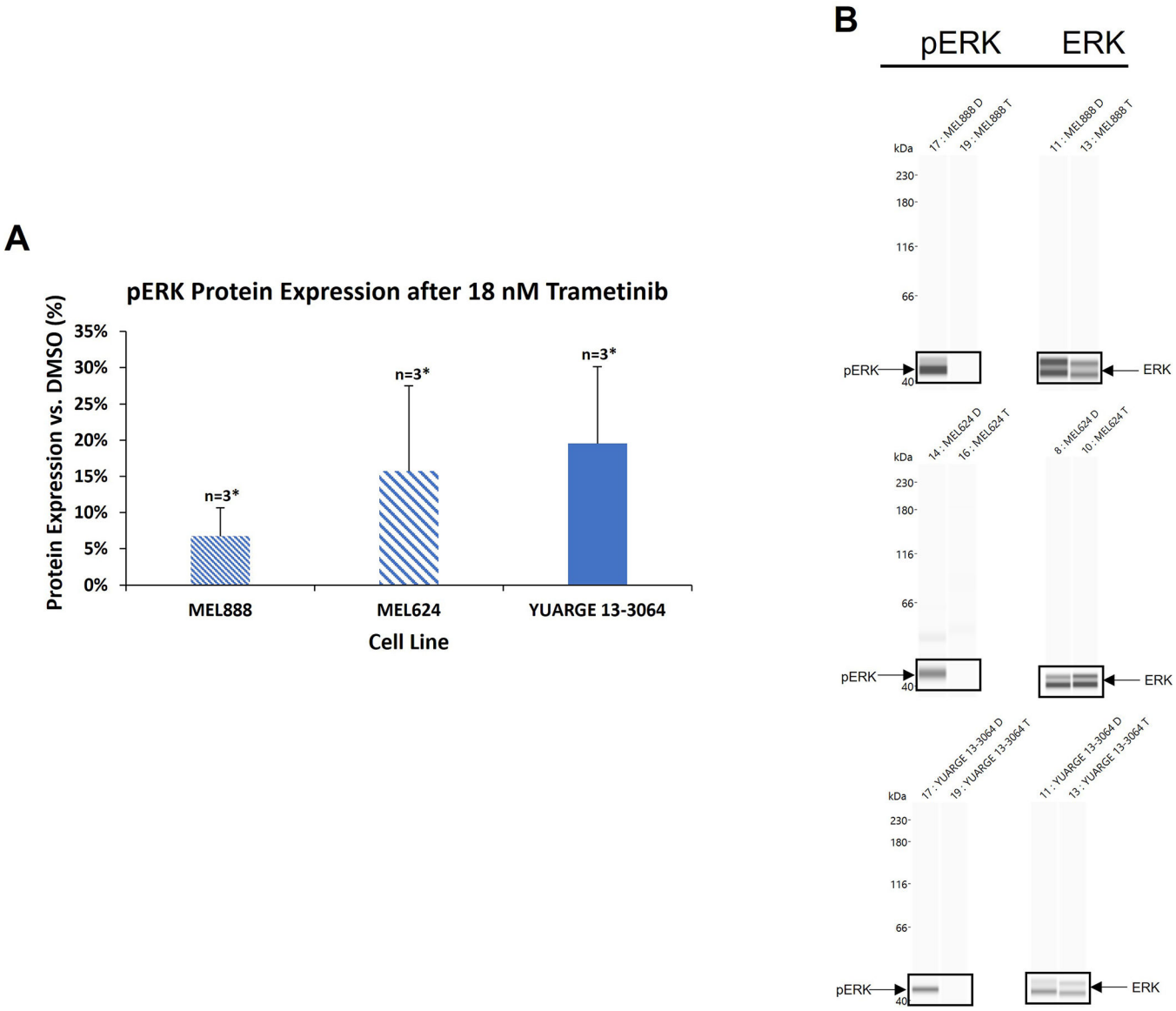

Fig 4: pERK expression in melanoma cell lines treated with MEK inhibitor.

MEK inhibitor treatment with 18 nM trametinib on melanoma cell lines (MEL888, MEL624, YUARGE 13-3064) reduced expression of pERK protein (A) to 7%, 12%, and 20%, respectively [shown on (B) representative blots]. Asterisk indicates statistical significance of $p \leq 0.05$.

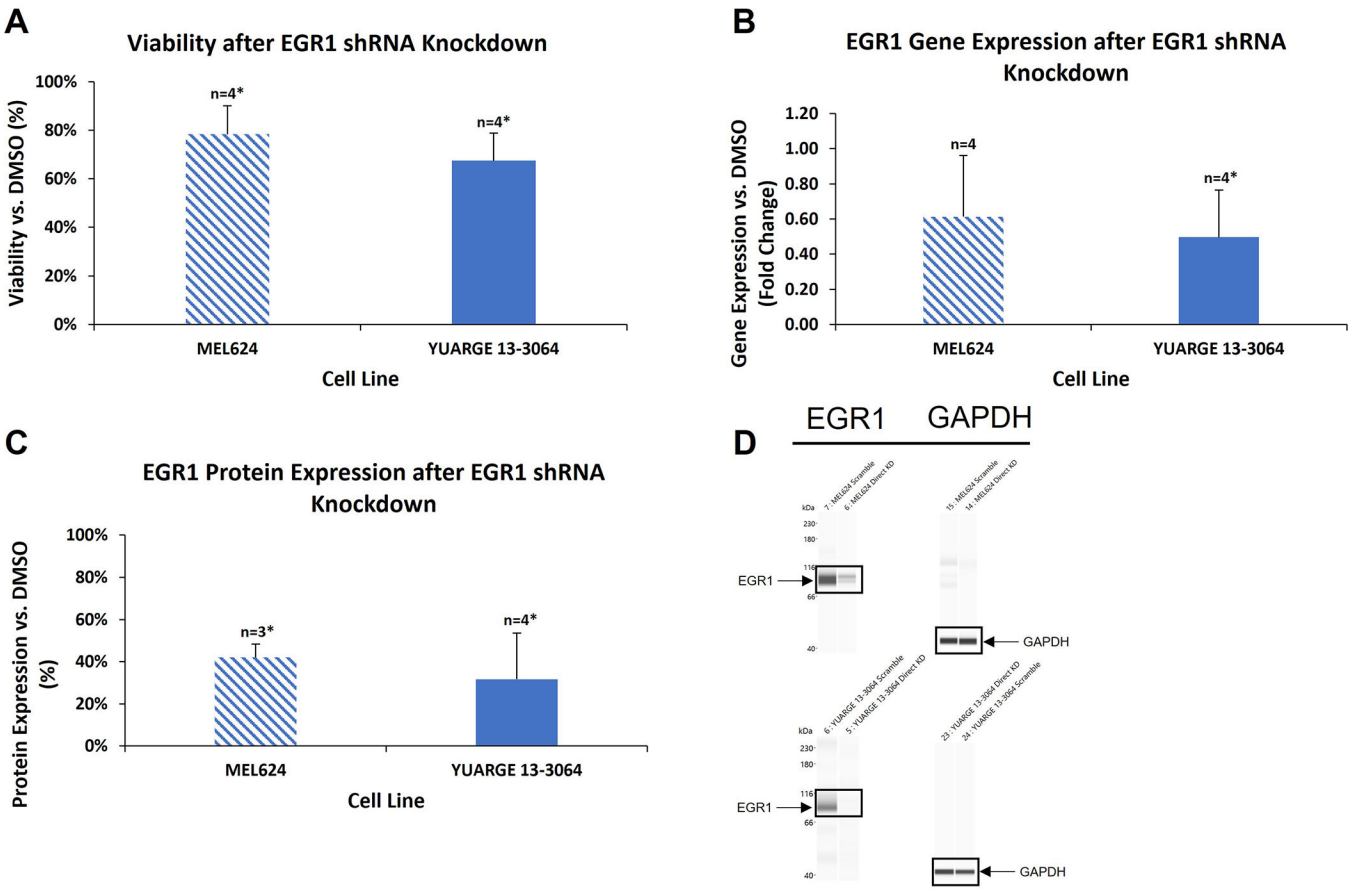

Fig 5: Viability and *EGR1* expression in melanoma cell lines with *EGR1* knockdown.

Targeted *EGR1* knockdown in cutaneous (MEL624) and conjunctival (YUARGE 13-3064) melanoma cell lines reduced (A) melanoma cell viability to 78% and 67% compared to non-targeting control (p=0.05 and p=0.02). *EGR1* (B) gene expression was reduced 0.61- and 0.50-fold (p=0.015 and p=0.01), and (C) protein expression was reduced to 42% and 32% (p=0.006 and p=0.01) compared to non-targeting control [shown on (D) representative blots]. Asterisk indicates statistical significance of $p \leq 0.05$.

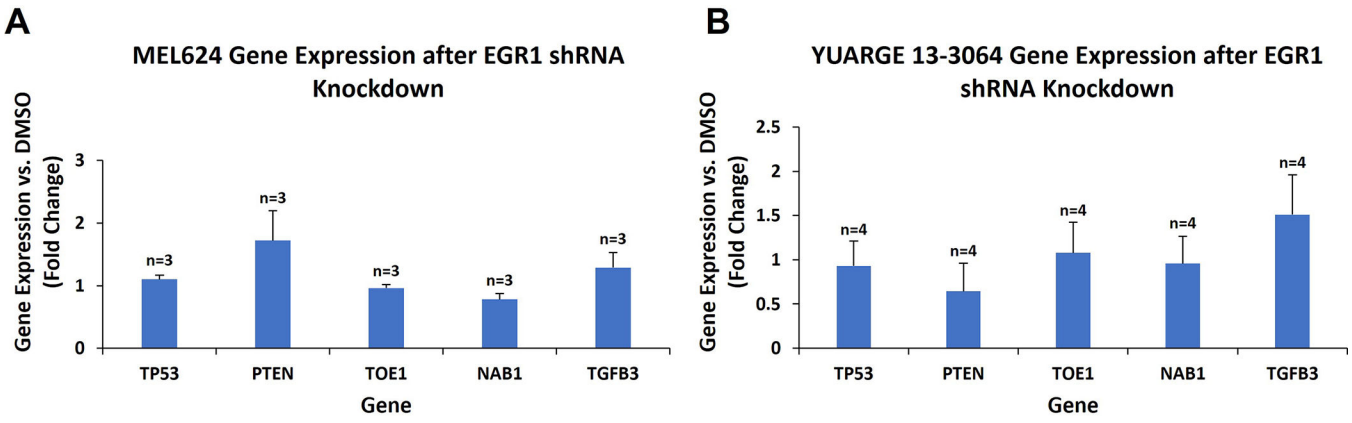

Fig 6: Downstream gene expression changes in melanoma cell lines treated with *EGR1* knockdown.

Targeted *EGR1* knockdown in cutaneous (MEL624) and conjunctival (YUARGE 13-3064) melanoma cell lines resulted in a non-significant increase in expression of the tumor suppressor *TGFβ3*, with variable changes in expression of other tumor suppressors and targets of *EGR1*. The tumor suppressor *PTEN* appeared to increase in the cutaneous melanoma cell line MEL624 but not in the conjunctival melanoma cell line YUARGE 13-3064.