

ORIGINAL ARTICLE

Oncogenic B-Raf signaling in melanoma cells controls a network of microRNAs with combinatorial functions

KL Coutts¹, EM Anderson², MM Gross², K Sullivan² and NG Ahn^{1,3}

Over two-thirds of melanomas have activating mutations in B-Raf, leading to constitutive activation of the B-Raf/MKK/ERK signaling pathway. The most prevalent mutation, B-RafV600E, promotes cancer cell behavior through mechanisms that are still incompletely defined. Here, we used a sensitive microarray profiling platform to compare microRNA (miRNA) expression levels between primary melanocytes and B-RafV600E-positive melanoma cell lines, and between melanoma cells treated in the presence and absence of an MKK1/2 inhibitor. We identified a network of > 20 miRNAs deregulated by B-Raf/MKK/ERK in melanoma cells, the majority of which modulate the expression of key cancer regulatory genes and functions. Importantly, miRNAs within the network converge on protein regulation and cancer phenotypes, suggesting that these miRNAs might function combinatorially. We show that miRNAs augment effects on protein repression and cell invasion when co-expressed, and gene-specific latency and interference effects between miRNAs were also observed. Thus, B-Raf/MKK/ERK controls key aspects of cancer cell behavior and gene expression by modulating a network of miRNAs with cross-regulatory functions. The findings highlight the potential for complex interactions between coordinately regulated miRNAs within a network.

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INTRODUCTION

Oncogenic mutations in the protein kinase, B-Raf, occur in 50–70% of sporadic melanomas.^{1,2} The most frequent mutation, B-RafV600E, elevates kinase activity and constitutively activates the MKK/ERK signaling pathway, which promotes tumor progression and metastasis through enhanced cell proliferation, survival, motility and invasion.^{3,4} Although the prevalence of the B-RafV600E mutation and cellular responses have been intensively studied, molecular targets of this pathway in melanoma are still incompletely understood.

MicroRNAs (miRNAs) are short, non-coding RNAs that provide feedback mechanisms to maintain stable protein expression and confer robustness in development and normal cell homeostasis.⁵ miRNAs also modulate protein levels and cell phenotypes in response to environmental stress, and are often mutated or deregulated in human diseases. Widespread miRNA deregulation has been reported in cancers,⁵ and certain miRNAs have been classified as oncogenes or tumor suppressors across multiple cancer types. Although changes in miRNA levels are not typically sufficient for promoting tumorigenesis, miRNAs are necessary for controlling cancer cell properties and maintaining disease.^{7–9}

Several oncogenic signaling pathways have been shown to support tumorigenesis by modulating miRNAs. Altered transcription factor expression is one mechanism that has been linked to the deregulation of cancer-associated miRNAs. For example, c-Myc induces the oncogenic miR-17~92 cluster and represses other miRNAs, including let-7, miR-22, miR-26a and miR-34a, in B-cell lymphomas.¹⁰ In neuroblastomas, the related transcription factor, MYCN, promotes oncogenesis by inducing miR-17~92

and miR-221.¹¹ Two studies have investigated the correlation between B-Raf mutational status and miRNA expression in melanomas, and only one was able to link three miRNAs to B-RafV600E.^{12,13} Other studies have reported large numbers of differentially expressed miRNAs in melanomas vs normal melanocytes, but little agreement was observed between them.^{14–16} As a result, the miRNAs that are deregulated in melanomas, and specific targets of oncogenic B-Raf, have yet to be established.

In this study, we used a highly sensitive microarray profiling platform to identify miRNAs regulated by oncogenic B-Raf signaling in melanoma cells. We show that a network of >20 miRNAs are controlled by B-Raf/MKK/ERK signaling, encompassing more than half of the miRNAs which are deregulated in melanoma cells compared with primary melanocytes. Cell treatment with miRNA mimics and inhibitors reveals that the majority of these miRNAs control cell proliferation and/or invasion. Target prediction algorithms suggested that many cancer-associated genes are targets for convergence by miRNAs within the network. This was validated at the level of protein expression and 3'UTR activity, raising the possibility that miRNAs functionally interact with one another. Both cell invasion and protein repression were augmented upon combinatorial miRNA expression, and more complex interactions between miRNAs, such as interference and latency effects, were observed for protein expression. Altogether, our findings establish a network of miRNAs under the control of oncogenic B-Raf in melanoma and highlight the complexity of cross-regulatory interactions between miRNAs in modulating cancer cell properties.

¹Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO, USA; ²Dharmacon, Inc., a wholly owned subsidiary of Thermo Fisher Scientific, Inc., Lafayette, CO, USA and ³Howard Hughes Medical Institute, University of Colorado, Boulder, CO, USA. Correspondence: Dr NG Ahn, Department of Chemistry and Biochemistry, HHMI, University of Colorado, Boulder, CO 80309-0215, USA.

E-mail: natalie.ahn@colorado.edu

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RESULTS

MicroRNA profiling in primary melanocytes and established melanoma cell lines

Microarray profiling was used to identify miRNAs both deregulated in melanoma cells and responsive to signaling through B-Raf/MKK/ERK. We analyzed miRNA expression levels in two primary melanocyte cell lines (NHEM2493 and HEM-LP) and compared these with six melanoma cell lines established from human primary vertical growth phase tumors (WM115, WM278 and WM793) or metastatic tumors (WM239A, WM1617 and 1205Lu). To distinguish miRNAs responsive to constitutive B-Raf/MKK/ERK signaling, we carried out an independent experiment comparing the six melanoma lines treated with and without the MKK1/2 inhibitor, U0126 (Figure 1a). All melanoma cell lines carried activating B-Raf-V600E or -V600D mutations^{1,17,18} and expressed elevated levels of phospho-ERK1/2, which was attenuated by U0126 treatment (Supplementary Figure S1).

Biological replicates were analyzed using microarrays containing triplicate probe sets of 470 miRNAs (Thermo Fisher Scientific, Lafayette, CO, USA; miRBase 9.0). A miRNA reference probe set was added to each sample, allowing better comparison of features within a single array and across many arrays. In total, 18800 measurements were generated and normalized for variability in labeling efficiency, hybridization and baseline fluorescence (Supplementary Table 1). Of the miRNAs surveyed, 171 were excluded from further analysis based on their score of 'present' ($P < 0.05$) in fewer than 10 of the 40 samples (Supplementary Table 2).

Averaged intensity measurements identified >30 miRNAs which differed between melanocyte and melanoma cell lines by 1.5-fold or more ($P < 0.05$, Figure 1b), as well as an additional 24 miRNAs which differed by at least 1.3-fold ($P < 0.05$, Supplementary Figure S2). Quantification of several miRNAs was confirmed by real-time qRT-PCR. The results showed excellent agreement with microarray experiments across all cell lines (Figures 2a,c), validating the magnitude and directionality of deregulation for each miRNA.

B-Raf/MKK/ERK regulates a network of cancer-associated miRNAs. To distinguish which miRNAs are targets of B-Raf/MKK/ERK signaling, we examined changes in miRNA expression following treatment with MKK1/2 inhibitor (Figure 1b). More than half of miRNAs altered by ≥ 1.5 -fold in melanoma cells vs melanocytes also showed consistent, reciprocal changes in expression after U0126 treatment. Several family and cluster members of these miRNAs were also regulated by U0126 but were only weakly regulated (1.3-fold or less) in melanoma vs melanocyte cells (Supplementary Figure S2). Although U0126 elicited relatively small changes in miRNA expression, the measurements were validated by qRT-PCR analysis of cells treated with U0126 as well as CI-1040, another MKK1/2 inhibitor which also decreases phospho-ERK1/2 levels in melanoma cells (Supplementary Figure S1b). Both U0126 and CI-1040 demonstrated consistent changes in miRNA expression compared with microarray measurements (Figures 2b and d). A previous study in HeLa cells showed that miR-17 and miR-20a were increased, and let-7 decreased each by 20% in response to U0126,¹⁹ consistent with their responses to drug in our study.

Most B-Raf-responsive miRNAs identified in our screen have not been extensively studied in melanoma, but show oncogenic or tumor suppressor roles in other cancers (Table 1). Among the best studied is miR-17~92, which is elevated in many cancers and associated with increased proliferation and tumorigenesis.²⁰ Conversely, miR-34a and let-7 family members are tumor suppressors in cancers, and inhibit proliferation by modulating key cell-cycle regulatory genes.^{21,22} Together, the results reveal a

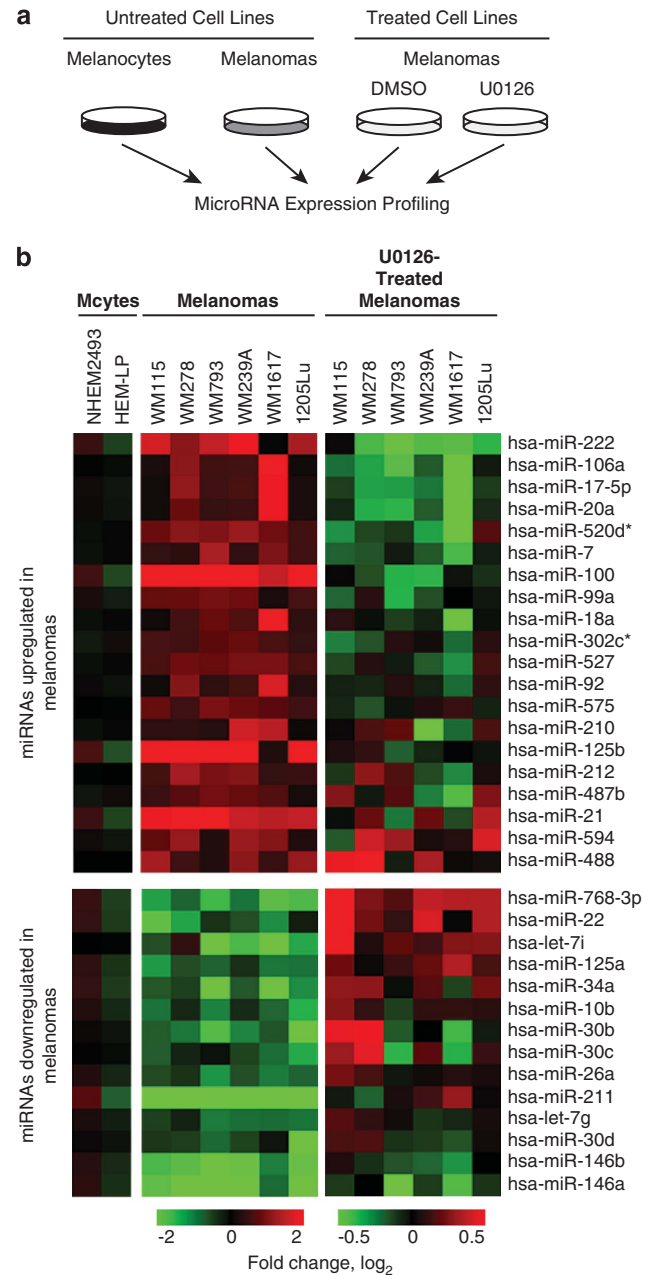


Figure 1. B-Raf/MKK/ERK regulates miRNAs differentially expressed between melanocyte and melanoma cells. **(a)** Strategy for identifying B-Raf/MKK/ERK-regulated miRNAs in melanoma cells. **(b)** Heat map indicating fold changes in miRNA expression between melanocytes ('Mcytes') vs melanomas, and dimethyl sulfoxide (DMSO)- vs U0126-treated cells. Signal intensities averaged between biological replicates for each melanoma cell line were normalized to the averaged signal between two melanocyte cell lines. Averaged signal intensities of U0126-treated cells were normalized to the average intensity of the corresponding DMSO-treated cells. Data are represented on a log₂ scale.

network of cancer-associated miRNAs that are controlled by oncogenic B-Raf signaling in melanoma.

B-Raf/MKK/ERK-regulated miRNAs control cell proliferation and invasion

To investigate cellular functions of miRNAs responsive to B-Raf/MKK/ERK, we examined effects on cell proliferation and invasion.

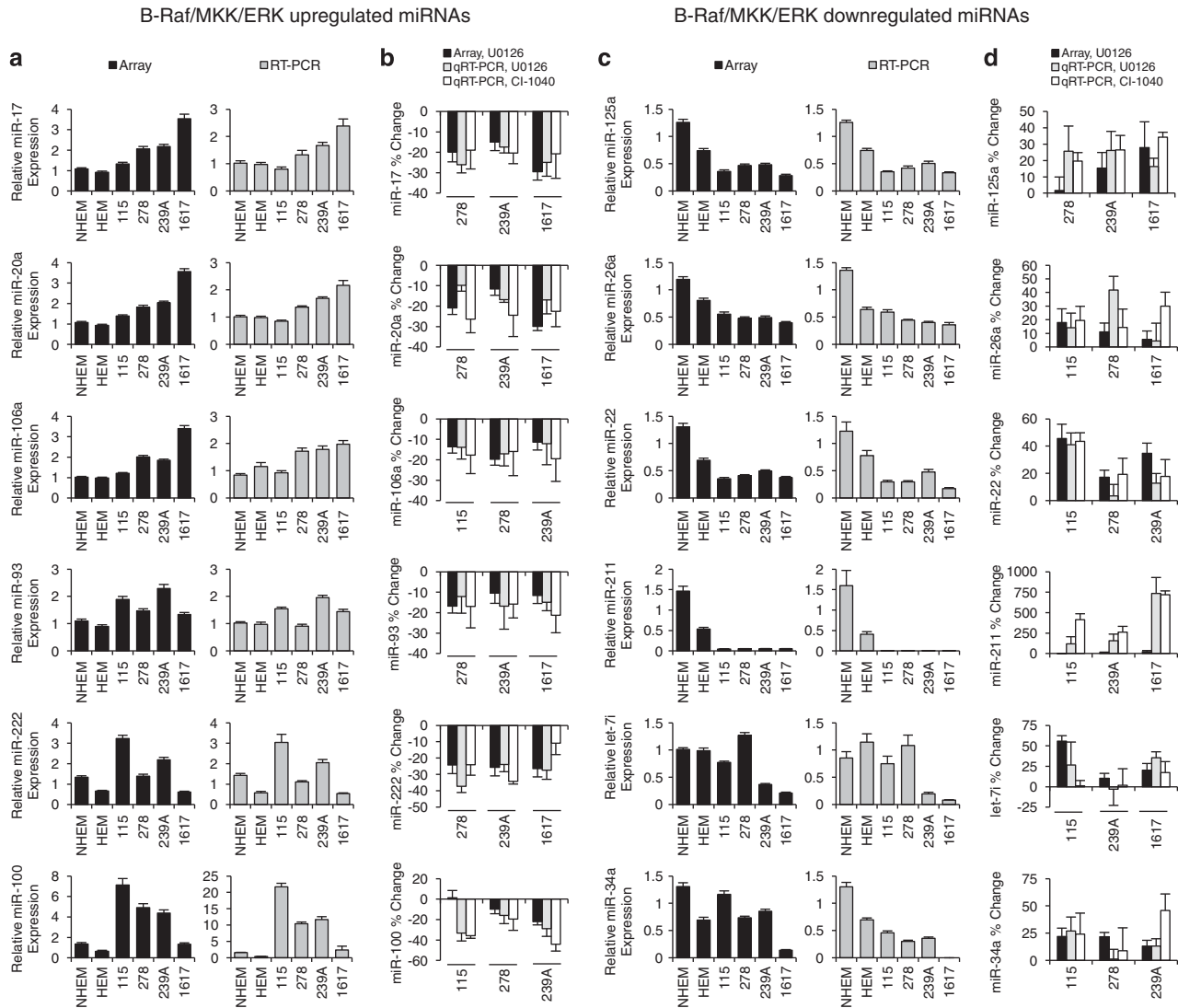


Figure 2. Real-time PCR validation of microarray data. Quantitative RT-PCR for selected miRNAs was performed and compared with microarray results. **(a, c)** miRNA expression across cell lines from microarray and qRT-PCR analyses. Expression in each cell line was normalized to the averaged signal from two melanocyte cell lines. **(b, d)** Percent change in signal after U0126 treatment from microarray and qRT-PCR analyses, and CI-1040 treatment from qRT-PCR analyses. Values represent average \pm s.e.m. from two combined biological replicates (microarray) or three technical replicates (qRT-PCR). The qRT-PCR experiment was performed independently using samples prepared separately from the microarray experiment.

Synthetic oligonucleotide miRNA mimics were transfected into WM239A cells, and proliferation was quantified using a colorimetric assay. Of the 25 miRNAs tested, 14 altered cell proliferation in a manner consistent with their direction of regulation by signaling (Figure 3a). Seven miRNAs that were inhibited by B-Raf/MKK/ERK in melanoma cells suppressed proliferation, including let-7i, miR-34a and miR-22, which repressed proliferation in other cell systems (Table 1). Likewise, seven miRNAs induced by B-Raf/MKK/ERK increased proliferation, including miR-17 and miR-92/25 family members, which enhanced proliferation and tumorigenicity in other cancers (Table 1). Although the magnitudes of proliferative responses in the 96-well assays were modest, transfection by miRNAs in each case led to qualitative differences in cell confluency compared with control treated cells, after several days (Figure 3b).

We next examined the network of miRNAs for effects on cell invasion using a three-dimensional spheroid assay. Spheroids were formed from melanoma cells transfected with miRNA mimics and embedded into collagen, allowing growth and invasion to be

monitored in an extracellular matrix with composition similar to that of human dermis. Striking phenotypes were observed, where miRNAs induced by B-Raf/MKK/ERK significantly enhanced invasion into matrix (Figure 3c; Supplementary Figure S3). On the other hand, miRNAs that were downregulated by B-Raf/MKK/ERK showed mixed effects. Some inhibited invasion (let-7i, miR-211 and miR-22; Figure 3d; Supplementary Figure S3), suggesting that their repression by B-Raf signaling promotes invasion in melanomas. Others increased the extent of cell invasion (miR-34a and miR-26a; Figure 3d; Supplementary Figure S3), suggesting that they targeted genes controlling mechanisms inhibiting invasion as well as mechanisms promoting cell proliferation. Although let-7i, miR-211 and miR-22 decreased cell proliferation, which might have accounted for the reduced spheroid invasion, these miRNAs also reduced cell motility in transwell migration assays (data not shown) suggesting that loss of spheroid invasion was not solely due to decreased cell number. Because B-Raf/MKK/ERK often co-regulated members of the same miRNA family (for example, let-7i/7g, miR-17/20/106/93, miR-92/25 and miR-221/222), functional

Table 1. miRNAs regulated by B-Raf/MKK/ERK in melanoma cells appear in other cancers

miRNAs	Deregulation in cancer ^a	Associated functions ^a	Known targets ^b
(Downregulated)			
let-7	Decreased in lung, breast, prostate, gastric cancer, hepatocellular carcinoma	Suppresses proliferation and metastasis ³² Promotes apoptosis	CCND1, NRAS, KRAS, HMGA2
miR-34	Decreased in lung, pancreatic cancer, neuroblastoma	Suppresses proliferation, tumorigenesis Promotes apoptosis, senescence	CDK6, E2F3, CCNE2/BCL2 ³³
miR-22	Decreased in breast cancer ³⁴	Suppresses proliferation ³⁴	ER α ³⁴
miR-26	Decreased in thyroid cancer	Suppresses proliferation	EZH2, PTEN ³⁵
miR-211	Increased in gliomas ³⁵	Promotes apoptosis	Unknown
miR-125	Increased in pancreatic cancer	Unknown	Unknown
miR-125	Decreased in breast cancer, neuroblastoma	Suppresses proliferation, migration, invasion	ERBB2, ERBB3
miR-30	Decreased in anaplastic thyroid carcinoma ³⁶	Suppresses invasion ³⁶	TGFBR1/SMAD2 ³⁶
miR-10	Increased in breast cancer and glioblastoma	Promotes invasion, metastasis	HOXD10, HOXA1
(Upregulated)			
miR-221/222	Increased in pancreatic, prostate, cancer, papillary thyroid carcinoma, glioblastoma	Promotes and suppresses proliferation	KIT, p27, p57
miR-17~92	Increased in lung, colon cancer, B-cell lymphoma, glioblastoma	Promotes proliferation, tumorigenesis, angiogenesis	E2F1, p21, AIB1, PTEN
miR-106a~363	Increased in T-cell leukemia	Promotes anchorage-independent growth ³⁷	p21, RB1, ARID4B
miR-106b~25	Increased in prostate cancer, ³⁸ hepatocellular carcinoma ³⁹	Promotes proliferation, anchorage-independent growth ³⁹	p21, E2F1/BIM ³⁹
miR-100	Increased in pancreatic cancer, Decreased in ovarian cancer ⁴⁰	Unknown	mTOR ⁴⁰

^aInformation from review by Lee and Dutta⁶ unless otherwise referenced. ^bConfirmed targets identified using TarBase database⁴¹ unless otherwise referenced.

screens used miRNA mimics to avoid complications from simultaneously inhibiting multiple family members. However, cells transfected with miRNA inhibitors confirmed opposite effects on spheroid invasion compared with miRNA mimics (Supplementary Figure S4). Together, the results suggest that miRNAs which are coordinately upregulated in response to B-Raf/MKK/ERK promote the cancer phenotype by enhancing cell proliferation and/or invasion, while miRNAs inhibited in response to signaling cause derepression of these functions. The convergence of co-regulated miRNAs on cancer cell functions raised the possibility that they interact to mediate oncogenic control of tumor growth and invasion.

miRNAs regulated by B-Raf/MKK/ERK cooperate in repressing invasion

To test the hypothesis that coordinately regulated miRNAs with shared functions might cooperate in augmenting phenotypic responses, spheroid invasion was assayed in WM239A cells transfected with miRNA mimics that individually repressed invasion and proliferation (let-7i, miR-211 and miR-22; Figures 3a and c), as well as pairwise combinations of these miRNA mimics. The combinations of let-7i + miR-22, let-7i + miR-211 and let-7i + miR-22 + miR-211 significantly augmented the effects of each miRNA alone by further repressing spheroid cell invasion (Figures 4a and b). However, no differences were observed with other miRNA pairs, indicating that combinatorial repression of spheroid invasion was not due to non-specific toxicity (Supplementary Figure S5). The results show that miRNA co-regulation by B-Raf/MKK/ERK may augment the effects of individual miRNAs and suggest that miRNAs work combinatorially to modulate cancer phenotypes.

miRNAs converge on cancer regulatory genes

We next considered gene products which might be controlled by B-Raf/MKK/ERK-regulated miRNAs, examining cases where a single miRNA targets multiple genes and cases where miRNAs converge on one gene. TargetScan and miRanda prediction algorithms^{23,24} were used to identify potential targets based on seed sequence matches, conservation between species and other criteria.

However, predicted miRNA targets often include many false positives. Therefore, we filtered predicted gene target lists by matching them against B-Raf/MKK/ERK-responsive genes identified by DNA microarray analysis of the six melanoma cell lines treated with U0126.²⁵ By filtering predicted targets against mRNAs responsive to U0126, candidate genes were identified with known roles in melanoma proliferation, adhesion/invasion, signaling, survival and melanogenesis (Figure 5a).

The results predicted that gene targets are controlled by multiple network miRNAs. Most candidate genes contained seed match binding sites for more than one miRNA regulated in the same direction, as well as opposite directions, in response to B-Raf/MKK/ERK. For example, cyclins D and E were predicted targets of miRNAs that were downregulated (let-7, miR-34, -211, -26 and -30) as well as upregulated (miR-17/93 and miR-92/25 families) by B-Raf/MKK/ERK signaling. This suggested that while inhibiting miRNAs individually may induce cyclin, the effect of many pathway-regulated miRNAs might be more complex. We therefore asked whether co-regulated and oppositely regulated miRNAs within the B-Raf/MKK/ERK network converge on genes.

To explore this question, we monitored the effect of different miRNA mimics on eight proteins, each confirmed a target of B-Raf/MKK/ERK based on responses to two MKK1/2 inhibitors, U0126 and CI-1040 (Figure 5b). The eight genes had 29 predictions for targeting by B-Raf/MKK/ERK miRNAs, of which 22 (~75%) accurately predicted protein repression. Thus, filtering against cDNA microarray measurements yielded reasonably accurate target identification. Proteins were coordinately repressed by members of seed-matched miRNA families, such as miR-17/20a/106a/106b/93 and let-7i/7g (Figure 5b), consistent with inhibitory mechanisms involving shared 3'UTR seed sequences. Five proteins (CCND1, p21, ITGB3, LEF1 and FOSL1) were repressed by two or more predicted miRNAs that were downregulated by B-Raf/MKK/ERK, confirming that co-regulated miRNAs converge on proteins. Additionally, all six genes containing 3'UTR seed sites corresponding to oppositely regulated miRNAs were indeed repressed by at least one downregulated and one upregulated miRNA. Examination of luciferase reporters containing 3'UTR sequences from CCND1, CCNE2 and ITGB3 confirmed that each gene was directly

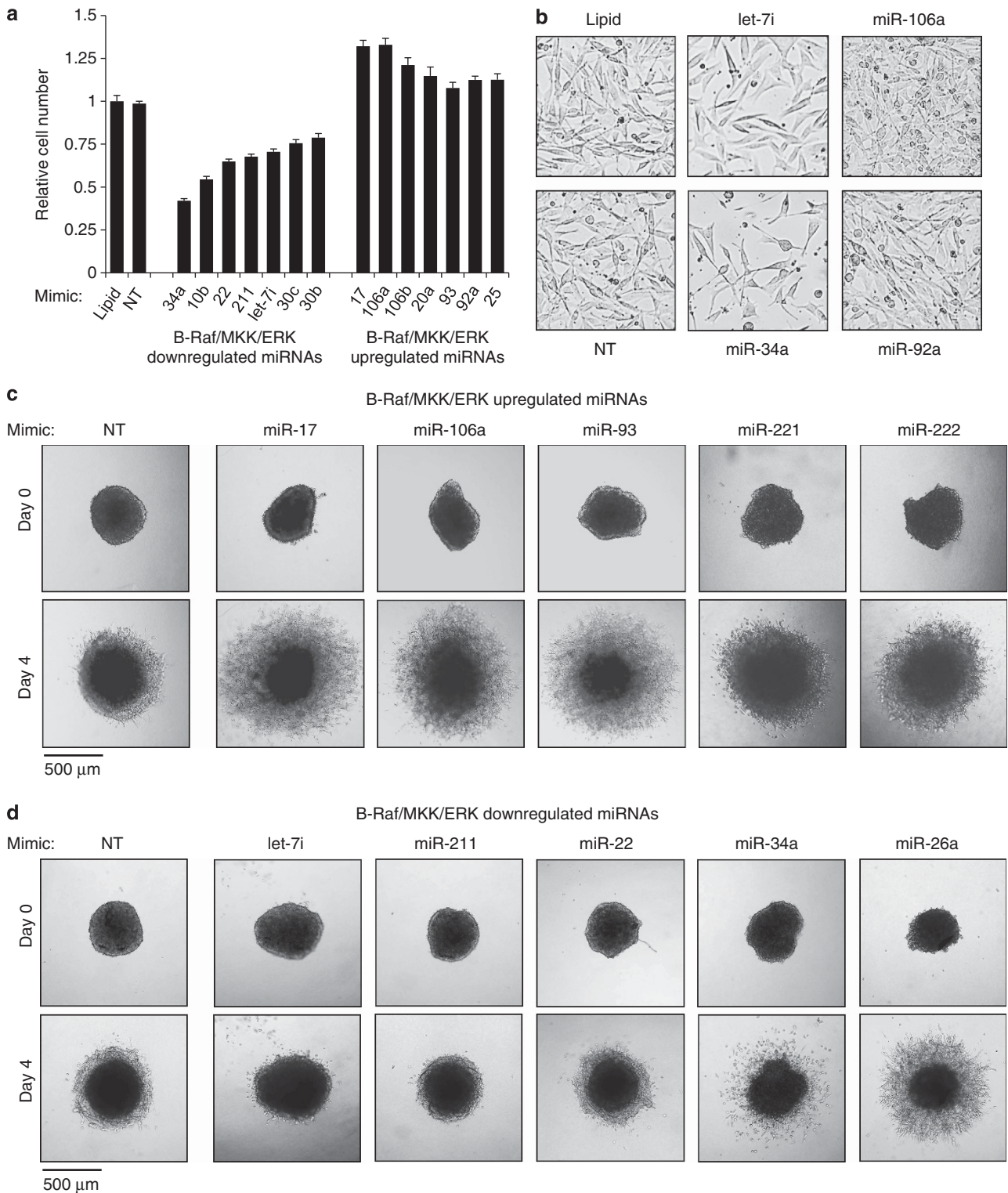


Figure 3. miRNAs regulated by B-Raf/MKK/ERK control proliferation and 3D invasion of melanoma cells. **(a)** WM239A cells transfected with 100 nM miRNA were assayed for cell proliferation using WST-1. Values 3 days after plating were normalized to values at the time of plating, and then normalized to DMRIE-C-treated control cells. NT: negative control (non-targeting) miRNA. Error bars represent s.e.m. ($n = 3$). **(b)** Cell confluency after miRNA transfection. Phase microscopy images were collected on cells transfected as in **(a)**, after 4 days. **(c, d)** Spheroid growth and invasion following transfection of miRNAs **(c)** upregulated or **(d)** downregulated by B-Raf/MKK/ERK.

targeted by miRNAs that were repressed as well as induced by B-Raf/MKK/ERK (Figure 5c). Together, the results show that multiple network miRNAs converge on 3'UTRs, suggesting that they might interact to control protein repression.

Combinatorial miRNA expression reveals complex effects on protein expression
 Given the evidence for coordinate regulation of multiple proteins, we asked how B-Raf/MKK/ERK regulated miRNAs work in

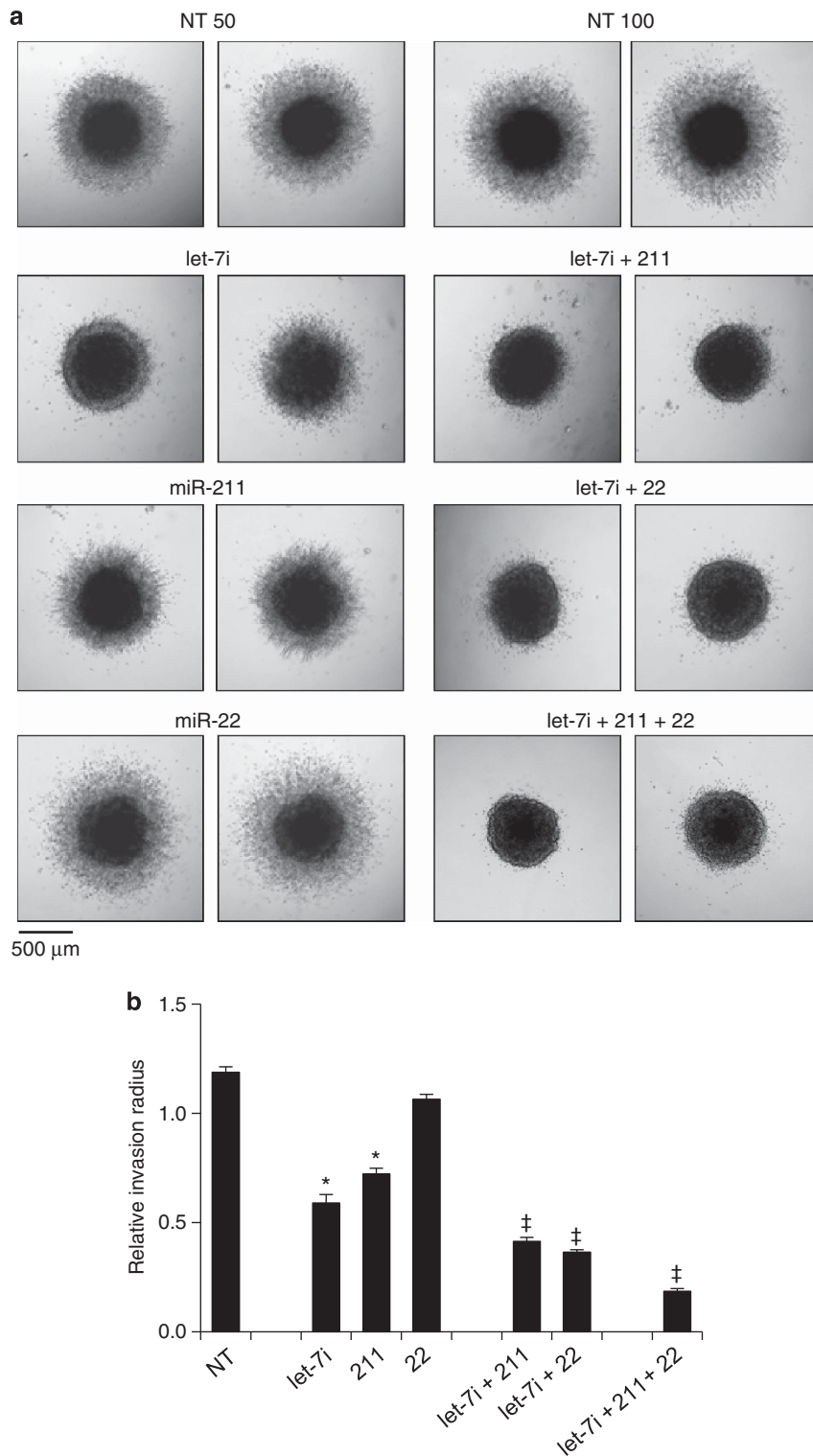


Figure 4. Co-expression of miRNAs enhances suppression of cell invasion. **(a)** Spheroid invasion following transfection of miRNAs individually and in combination. Cells were transfected with 50 nM of each miRNA mimic and imaged for cell invasion 3 days after collagen implantation. Duplicate spheroids after 3 days in collagen are shown. **(b)** Cell invasion was quantified by the difference between the outer (R2) and inner (R1) spheroid radii (Supplementary Figure S3, panel a), and normalized to control spheroids transfected with negative control (NT: non-targeting) miRNA. Asterisks indicate individual miRNAs differing significantly from NT (P -value < 0.003). Double crosses (‡) designate miRNA combinations that differ significantly from each individual miRNA (P -value < 0.05). Error bars represent s.e.m. ($n = 3$).

combination to control protein expression. Four genes were selected for analysis, based on their miRNA binding sites separated by varying distances (Figure 6a). ITGB3, p21, LEF1 and FOSL1 were

examined in cells transfected with individual miRNA mimics or combinations of two miRNA mimics, where one, both or neither miRNA in the combination contained a direct seed match to the

a

B-Raf/MKK/ERK upregulated genes	B-Raf/MKK/ERK downregulated miRNAs	B-Raf/MKK/ERK upregulated miRNAs	B-Raf/MKK/ERK downregulated genes	B-Raf/MKK/ERK downregulated miRNAs	B-Raf/MKK/ERK upregulated miRNAs
<u>Cell Cycle/Proliferation</u>			<u>Cell Cycle/Proliferation</u>		
CCND1	let-7, 34, 211	17	CDKN1A (p21)	let-7, 22	17
CCNE2	34, 26, 30	17, 92a	CDKN1B (p27)	34	222
CCNF	let-7, 22, 211, 30	17, 7	CDKN1C (p57)	34, 26	222, 92a
CDC6	26, 10	92a	CHES1	let-7, 34, 22, 211	17, 92a, 18
NR4A2	34, 211, 30	17	EZH1	let-7, 22	17, 222, 18
<u>Adhesion/Invasion</u>			<u>Adhesion/Invasion</u>		
EPHA2	let-7, 26, 10		ANGPTL2	let-7, 211, 26	222, 92a
HMGA2	let-7, 26, 211	17	CYLD	let-7, 34, 26, 30	17, 222, 92a, 19
ITGB3	let-7, 22, 211, 30	222, 92a	EFBN2	211	17, 222, 92a, 19
ITGA10	let-7, 211	17, 222	PTEN	22, 26, 10	17, 92a, 18, 19
THBS1	let-7, 211	222, 18, 19	TIMP2	22, 211, 30,	17, 222, 18, 19
<u>Signaling</u>			<u>Signaling</u>		
CD200	34, 211, 26	17	DKK3	let-7	92a, 19
DUSP4	let-7, 211		ETS1	26, 10	222, 92a, 19
LEF1	34, 22, 26	17	IRS2	let-7, 30	92a
MYCN	let-7, 34,	17, 19	MARCKS	34, 211, 30, 10	222
MAP3K1	let-7, 26a, 30	17, 18	PIK3R1	211	17, 222, 92a
<u>Survival</u>			<u>Melanogenesis</u>		
MCL1	26, 211	17, 92a	ADCY9	let-7	17, 92a, 19
FOSL1	34, 22	17, 19	EDNRB	30	92a, 19
LIF	22, 26, 211, 10	17, 18, 19	KIT	34	17, 222, 18, 19
IER3	26		MLANA	26	92a
IGF1R	let-7, 22, 211, 30, 10	17, 19	MLPH	34	17

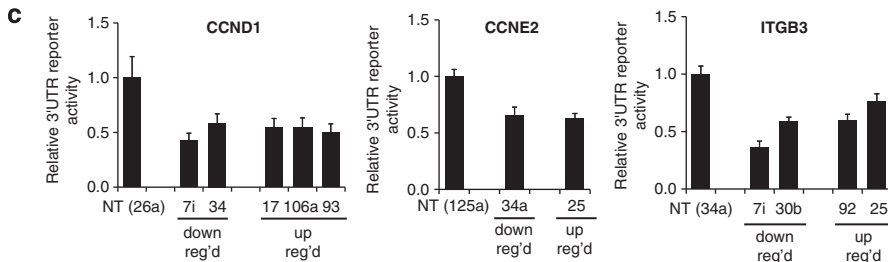
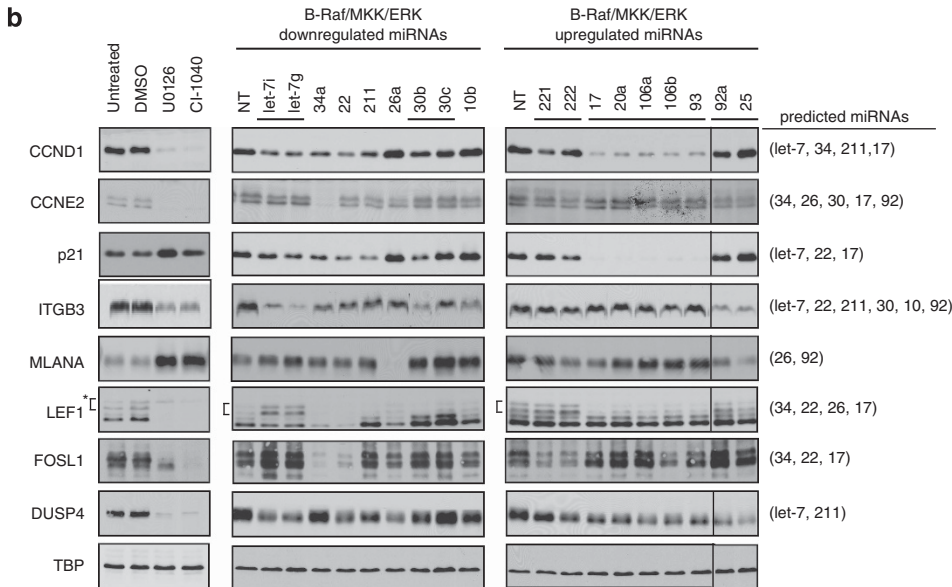


Figure 5. miRNAs within the B-Raf/MKK/ERK network cross-regulate cancer regulatory proteins. **(a)** Cancer genes controlled by B-Raf/MKK/ERK were matched to gene targets of miRNAs predicted by either TargetScan (conserved and non-conserved) or miRanda. **(b)** Western blots show repression of the proteins corresponding to selected cancer genes, following WM239A transfection with miRNA mimics (50 nM) for 48 h. TBP served as the loading control. Underlined miRNAs designate family members with similar seed sequence, which are therefore likely to share targets. Asterisk indicates two upper bands of LEF1 which represent longer variants that contain the 3'UTR in the transcript. The lower two bands represent variants lacking the 3'UTR. Vertical lines indicate where lanes have been removed from the blots for clarity. **(c)** Luciferase reporter assays validate CCND1, ITGB3 and CCNE2 as direct targets of selected miRNAs. 3'UTR luciferase activity was measured 48 h after reporters were co-transfected with 20 nM mimic. Values were normalized first to reporters treated with a B-Raf/MKK/ERK miRNA mimic with no binding site in the 3'UTR (NT) and then to cells transfected in parallel with GAPDH-3'UTR reporter. Error bars represent s.e.m. of triplicates.

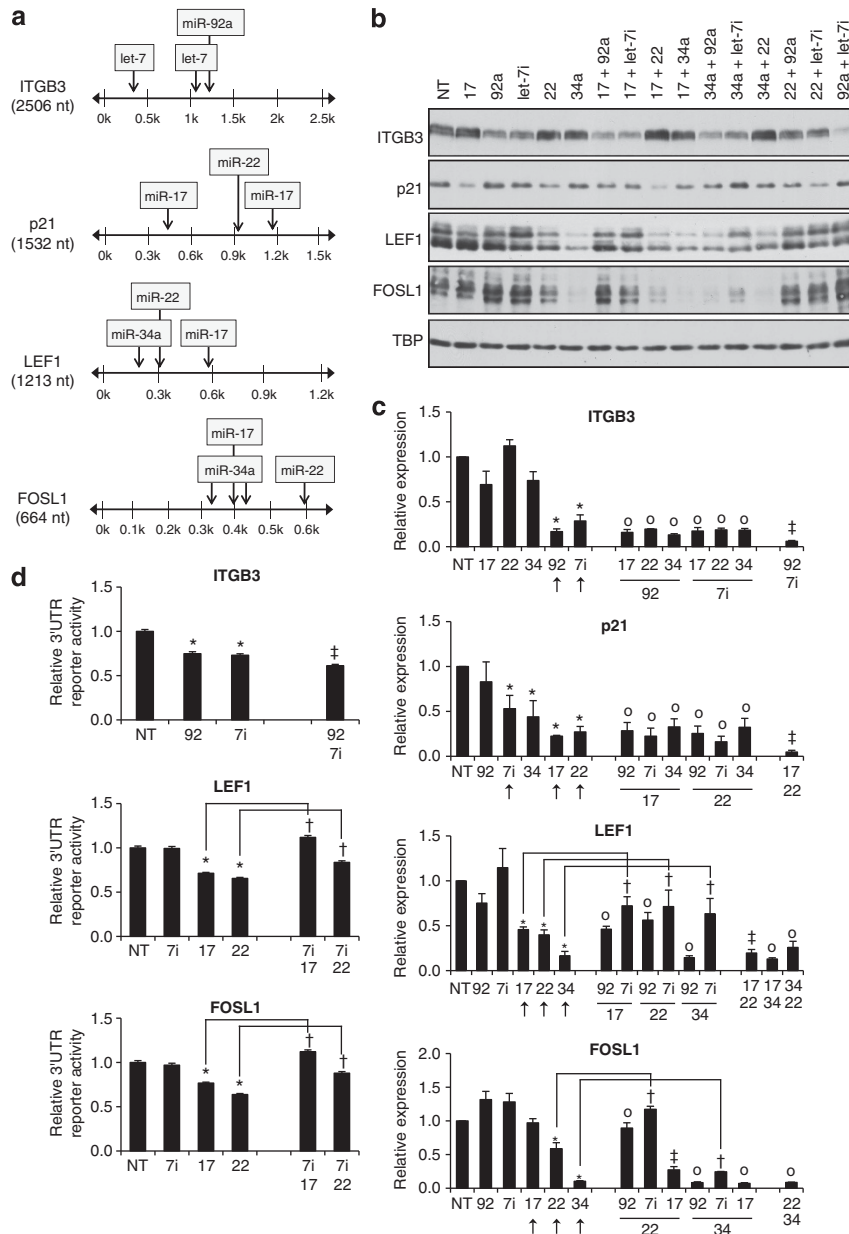


Figure 6. Combinatorial effects on protein repression by miRNAs within the B-Raf/MKK/ERK network. **(a)** Predicted miRNA binding sites within the 3'UTR of four genes. **(b)** Western blots show protein expression following treatment with miRNA mimics, individually and in combination. WM239A cells were transfected with 15 nM of each miRNA and/or negative control (NT: non-targeting) miRNA (30 nM total). qRT-PCR analyses of WM239A cells transfected with individual miRNAs show fold increases in expression of ~40–200, verifying that the miRNA mimics increase the levels of mature miRNA (data not shown). **(c)** Quantification of western blots in **(b)**, from cells transfected in biological triplicates, normalized to TBP and then NT control mimic. Data show mean and s.e.m. ($n = 3$). Arrows (↑) indicate miRNAs predicted to bind 3'UTRs. **(d)** Luciferase reporter assays for miRNA combinations. 3'UTR luciferase activity was measured 48 h after WM239A cells were co-transfected with luciferase reporter constructs and 20 nM total miRNA mimics (10 nM each in combination, 10 nM each plus 10 nM non-targeting for individual miRNAs). Luciferase activity for each gene was normalized to a control reporter (lacking a 3'UTR) treated in parallel, and then to non-targeting control treatment. For **(c)** and **(d)**, asterisks (*) specify proteins or 3'UTR activity repressed by individual miRNAs vs non-targeting control (P -value < 0.05). Single crosses (†) indicate miRNA combinations differing from individual targeting miRNAs (P -value < 0.07). Double crosses (‡) indicate miRNA combinations differing from both individual miRNAs (P -value < 0.05).

gene 3'UTR (Figure 6b). In general, individual miRNAs repressed only proteins containing at least one 3'UTR seed sequence match (Figure 6c, arrows and asterisks) and did not affect proteins without seed matches. Exceptions included the lack of FOSL1 repression by seed-matched miR-17, and the observed p21 repression by miR-34a although no seed matches are present (Figures 6a and c).

Protein expression analysis after co-transfection with two different miRNA mimics revealed varied interactions (Figure 6c; Supplementary

Figure S6). Generally, co-expressing two 'direct targeting' miRNAs (meaning each miRNA has a matched seed sequence and repressed protein expression) resulted in augmented protein repression compared with individual miRNAs. For example, ITGB3 was further repressed by the let-7i + miR-92a combination compared with let-7i or miR-92a alone, and LEF-1 was further repressed by miR-17 + miR-22 compared with miR-17 or miR-22 alone (Figure 6c, double crosses ‡). On the other hand, no further repression of LEF1 or FOSL1 was

observed when miR-34 was combined with other direct targeting miRNAs (Figure 6c), as if the repression of LEF1 and FOSL1 by miR-34a alone was already saturating.

Combinations of a direct targeting miRNA and a 'non-targeting' miRNA (meaning did not repress protein expression) showed complex effects. In most cases, protein repression resembled the direct targeting miRNA alone (Figure 6c, combinations denoted by 'o'). For example, ITGB3 repression by the combination of any non-targeting miRNA with either let-7i or miR-92a equaled that of let-7i or miR-92a alone. Likewise, p21 repression by the combination of any non-targeting miRNA with miR-17 or miR-22 equaled that of miR-17 or miR-22 alone. Thus, most non-targeting miRNAs yielded no interactions. However, we noted exceptions to this rule. Although miR-17 individually did not affect FOSL1, it augmented repression beyond that seen with miR-22 alone when the two miRNAs were combined (Figure 6c). This suggests that certain miRNAs show a 'latency' effect, where protein repression is observed only when co-expressed with another miRNA. Unexpectedly, we also observed interference by a non-targeting miRNA toward targeting miRNAs (Figure 6c, single crosses '+'). For example, the non-targeting let-7i derepressed LEF1 and FOSL1 proteins when combined with direct-targeting miRNAs (miR-17, -22, -34a for LEF1; miR-22, -34a for FOSL1). Augmentation and interference effects between miRNAs were also observed for 3'UTR activity upon co-transfection of luciferase reporters and miRNA combinations (Figure 6d), suggesting that these interactions may occur at the level of the 3'UTR. Altogether, the results demonstrate that protein expression is influenced by complex rules that govern miRNA-specific and gene-specific interactions between coordinately regulated miRNAs.

Knockdown of gene targets phenocopies miRNA effects on cell proliferation and invasion

Finally, we asked whether protein targets regulated by B-Raf/MKK/ERK miRNAs could contribute to miRNA effects on cell proliferation or invasion. FOSL1 (miR-34a target) and ITGB3 (let-7i target) expression was reduced using siRNA treatment (Figure 7a) and cell proliferation and/or invasion was analyzed. Both FOSL1 and ITGB3 siRNA repressed cell proliferation, similarly to levels seen with miR-34a or let-7i (Figure 7b). Additionally, ITGB3 knockdown also phenocopied let-7i invasion effects (Figure 3d; Supplementary Figure S3) by repressing spheroid invasion to comparable levels (Figures 7c and d). The results suggest that complex regulation of ITGB3 and FOSL1 by B-Raf/MKK/ERK miRNAs may contribute to the widespread influence of the network miRNAs on oncogenic melanoma cell behaviors.

DISCUSSION

Using a sensitive miRNA array platform, we established a network of >20 miRNAs regulated by oncogenic B-Raf signaling in melanoma cells. Importantly, the majority of miRNAs controlled melanoma cell growth, invasion and gene expression. Interactions between co-expressed miRNAs augmented repression of cancer-associated proteins and cell invasion from spheroids. We also observed unexpected, complex interactions between co-expressed miRNAs. Altogether, our findings reveal that molecular targets of oncogenic B-Raf include an miRNA network, which controls cancer cell properties, and network members in combination show different cross-regulatory interactions.

The miRNAs controlled by B-Raf/MKK/ERK in our screen were consistent with those reported in melanoma tumors and cell lines. For example, miR-211 and let-7i, which were repressed across melanoma cells in our study, were also repressed in melanoma tumors compared with melanocytes.^{12,15} Likewise, our results agreed with reports that miRs-10b, 34a, 34b, 22, 125a and 132 are significantly downregulated in primary and metastatic melanoma

cell lines.¹² We also observed increased miR-221 and miR-222 expression, which was previously reported for melanoma tumors.²⁶ Finally, six miR-17~92 family and cluster members (miR-17-5p, miR-20a, miR-106b, miR-106a, miR-106b and miR-92b) were significantly elevated in our melanoma cells, consistent with the upregulation of family members in melanoma tissues.^{15,27} Our results demonstrate that miRNAs previously shown to be deregulated in melanomas are controlled by oncogenic B-Raf/MKK/ERK signaling.

It was intriguing that a large number of miRNAs were co-regulated in response to one signaling pathway. Significantly, we found that most miRNAs repressed by B-Raf/MKK/ERK inhibited cell proliferation and invasion, while miRNAs induced by this pathway enhanced these responses. Thus, the directionality of miRNA regulation by oncogenic B-Raf/MKK/ERK signaling was consistent with promoting cancer phenotypes. This raised the question of whether miRNAs coordinately repressed or induced by oncogenic signaling are redundant, or whether they interact to augment cancer cell behavior. The latter hypothesis was corroborated by experiments demonstrating cooperative interactions between let-7i, miR-22 and miR-211 in suppressing spheroid cell invasion. It appears that cell invasiveness in melanoma can be augmented by combinatorial interactions between coordinately regulated miRNAs within the network.

Another interesting finding was that coordinately regulated miRNAs often converge on the same gene target, which allowed us to explore combinatorial miRNA effects on protein expression for several genes. In most cases, co-transfecting two miRNAs that had 3'UTR seed matches and individually repressed protein levels resulted in further repression. This was consistent with prior studies demonstrating multiplicative effects on transcript abundance when 3'UTR regions contained multiple seed matches to the same or different miRNAs.^{28,29} We observed novel, more complex interactions upon co-transfection with direct-targeting and non-targeting miRNAs. The augmented repression in the combination of miR-17 + miR-22 revealed a latency effect by miR-17 that was gene specific, because unlike FOSL1, miR-17 targeted both p21 and LEF1 when added alone and in combination with miR-22 (Supplementary Figure S6). We also observed interference effects, where the ability of one miRNA to repress protein was blocked by another. To our knowledge, this is the first demonstration that miRNAs without overlapping binding sites are able to cancel the effects of others. Conceivably, interference between miRNAs could be explained by competition for cell uptake or binding the Ago-RISC complex. However, we believe this unlikely, because the miRNAs showed interference toward some proteins but not others in the same co-transfected samples. Altogether, our results reveal unexpected combinatorial interactions between components of an oncogene-regulated miRNA network. The interactions are specific for miRNAs and proteins; thus, miRNA regulatory mechanisms are complex and rules for combinatorial regulation cannot be assumed.

Our findings show that B-Raf/MKK/ERK may have far-reaching effects in regulating cancer cell behavior through the control of miRNA expression. Each miRNA within the network targets many genes which could contribute to the cancer phenotype (Figure 8). Importantly, our study uncovers new combinatorial interactions between miRNAs. Coordinate regulation of an miRNA network by a signaling pathway may lead to unpredictable effects on proteins when combinatorial effects are considered, and further exploration of the rules for such interactions are needed. Finally, while our study focuses on only one signaling pathway, most cancers (including melanoma) harbor many deregulated pathways. To the extent that different signaling pathways each control different miRNA networks, an important question for future investigation is whether these networks interact combinatorially to relay the effects of multiple pathways on the cell proteome.

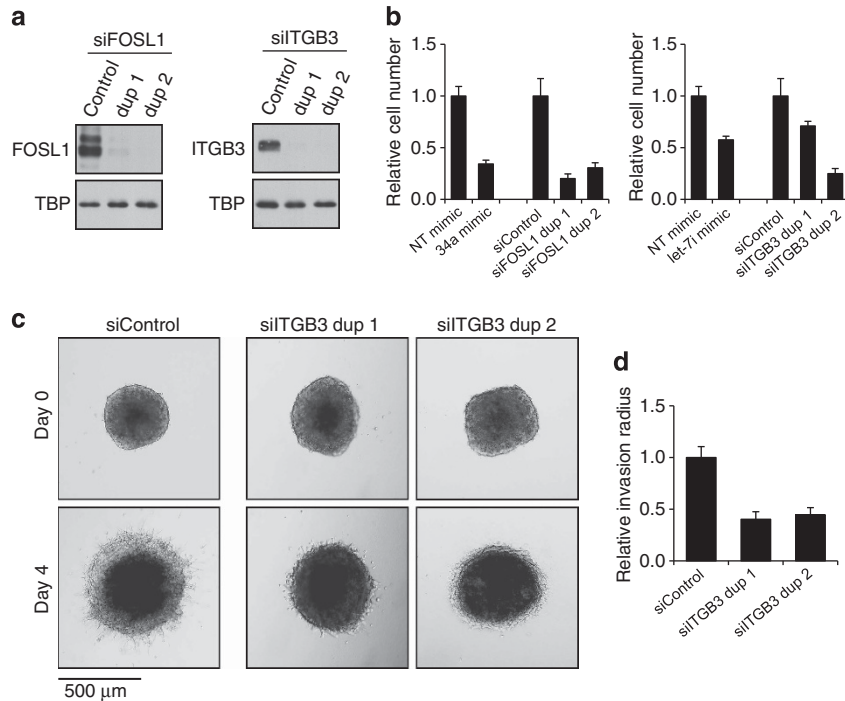


Figure 7. Knockdown of FOSL1 and ITGB3 phenocopy their targeting miRNAs. (a) Western blots show repression of FOSL1 and ITGB3 expression 48 h after WM239A cells were transfected with 50 nM siRNA. Two individual duplexes (dup 1, dup 2) were used for each gene. TBP serves as loading control. (b) Cell count proliferation assays for WM239A cells transfected with 50 nM miRNA mimics or siRNAs. In all, 100 000 cells were plated in triplicate on 6-well plates and counted 5 days after plating. Cell counts were normalized to cell concentrations obtained using the WST-1 assay at the time of plating. (c) Spheroid invasion was analyzed for WM239A cells treated with 50 nM control siRNA or ITGB3 siRNA 48 h before spheroid formation. (d) Spheroid invasion was quantified by the difference between the outer (R2) and inner (R1) spheroid radii, and normalized to control spheroids transfected with control, non-targeting siRNA. Error bars represent s.e.m. for three spheroids.

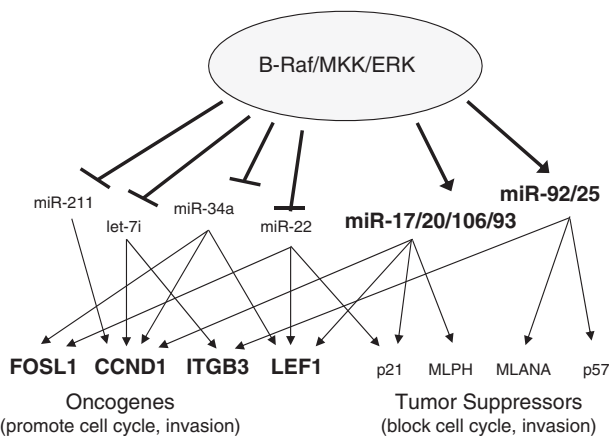


Figure 8. Model illustrating part of the miRNA network regulated by B-Raf/MKK/ERK in melanoma cells. B-Raf/MKK/ERK induces or represses sets of miRNAs, each which target melanoma-associated oncogenes or tumor suppressors. Multiple co-regulated miRNAs may converge on and cross-regulate each gene, allowing oncogenic B-Raf to exert variable regulatory effects on melanoma genesis.

MATERIALS AND METHODS

Cell culture

Primary melanocyte cell lines NHEM693 and NHEM2493 (Cambrex Bio Sciences, Walkersville, MD, USA) were cultured in Medium154 plus Human Melanocyte Growth Supplement (Cascade Biologics, Portland, OR, USA). Melanoma cell lines were a gift from Meenhard Herlyn, Wistar Institute,³⁰ and were grown in RPMI (Invitrogen, Carlsbad, CA, USA) supplemented

with 10% FBS (Gemini, West Sacramento, CA, USA) and penicillin-streptomycin (100 U/ml). All cell lines were maintained at 37 °C and 5% CO₂.

miRNA microarray profiling

Melanocyte and melanoma cell lines were plated on 6 cm plates to achieve ~60% confluency after 48 h, at which time cells were harvested in Buffer RLT Plus (Qiagen, Germantown, MD, USA) and stored at -80 °C. For melanoma cell drug treatments, 10 μM U0126 (Promega, Madison, WI, USA) or 3 μM CI-1040 (Pfizer, Inc., New York, NY, USA) in 0.1% (v/v) dimethyl sulfoxide or 0.1% dimethyl sulfoxide carrier was added 24 h before harvesting. Biological replicates were generated from different cell passages, each ≥2 passages removed. Total RNA was purified using the RNeasy Plus kit (Qiagen) according to manufacturer's Supplementary Protocol 1. MicroRNAs were quantified using the Thermo Scientific Dharmacon microRNA expression profiling platform, which uses two-color high-density eight-plexed slides containing probes to 470 mature human miRNAs. Total RNA (500 ng) was combined with spike-in control to monitor labeling efficiency, then fluorescence dye-labeled with pCp-Cy5 using T4 RNA ligase (Promega). A control oligonucleotide was added to measure recovery, and the sample desalted through a P6 Biospin column (Bio-Rad, Hercules, CA, USA). The miRIDIAN Synthetic miRNA Reference Probe Set (Thermo Fisher Scientific), 2 × HI-RPM hybridization buffer (Agilent, Palo Alto, CA, USA) and 25 × fragmentation buffer were added to each sample and hybridized to eight 15K custom miRNA microarrays (miRBase 9.0, probes in triplicate) (Agilent). Hybridizations were performed in a rotating incubator at 12 r.p.m. and 53 °C for 20 h. Slides were washed and scanned on an Agilent Microarray Scanner (Model G2505B), and data were extracted using Agilent Feature Extraction 9.5.1 software. Triplicate probe signals were averaged and adjusted for background, and the Cy5 (sample) channel was normalized to Cy3 (synthetic reference) for each probe. For each array, the normalized Cy5 signals were scaled against spike-in controls to adjust for global labeling bias. 'Is-Present' P-values were calculated for signals in the Cy5 channel using a set of background (non-

specific hybridizing) probes and the Gaussian error function. A set of 264 high-confidence probes, defined as those with $P < 0.05$ in 20 of 40 samples, was used as the basis feature set for inter-array normalization, using a mean-centering scaling. The normalized signals were transformed to \log_{10} and analyzed using Microsoft Excel.

Real-time quantitative RT-PCR

Cell lysates were prepared as described for microarray analysis, and real-time RT-PCR by absolute quantification was performed by contract (Asuragen, Inc., Austin, TX, USA). Briefly, total RNA was isolated using a column-based method and quality was assessed with an Agilent 2100 Bioanalyzer (Agilent). In all, 1 ng of total RNA was reverse transcribed using ABI Taqman miRNA assays (Applied Biosystems, Austin, TX, USA) and 3 μ l of the product was used in subsequent Taqman PCR amplification, performed in triplicate on a validated real-time thermocycler (ABI 7900HT). C_t values were compared with standard curves generated with synthetic miRNAs to obtain absolute copy number per ng RNA, and values were normalized using stably expressed RNU44 small nucleolar RNA.

siRNA, miRNA oligonucleotide transfections

Synthetic oligonucleotide siGENOME siRNAs and miRIDIAN miRNA Mimics and Hairpin Inhibitors (Thermo Fisher Scientific) were reconstituted in 1 \times sterile siRNA buffer (60 mM KCl, 6 mM HEPES-pH 7.5, 0.2 mM MgCl₂) to a stock concentration of 20 μ M. Cells were transfected in suspension using DMRIE-C (Invitrogen) and incubated for 5 h before replacing the media with RPMI + 10% FBS.

WST-1 cell proliferation assay

Cells were transfected with miRNA mimic or DMRIE-C lipid only. After 24 h, 5000 cells were plated in triplicate on duplicate 96-well plates. Three hours before reading, a standard curve of 500–50 000 cells was added. Plates were centrifuged at 1000 r.p.m. for 5 min before replacing the media with 100 μ l media containing WST-1 reagent at 60 μ l/ml (Clontech, Mountain View, CA, USA). Plates were incubated at 37 °C for 1 h and absorbances quantified at 440 nm.

Spheroid invasion assays

Spheroids were grown in collagen matrix as described.³¹ Twenty-four hours after miRNA transfection, cells were plated in triplicate on 1.5% Noble agar coated 96-well plates, then incubated for 1–2 days. Spheroids were collected and centrifuged, resuspended in 2.5 mg/ml PureCol bovine collagen-I (INAMED, Leimuiden, Netherlands) containing RPMI + 10% FBS, and overlaid on a pre-solidified layer of the same collagen solution in 96-well plates. The collagen/spheroid suspension was solidified for 1 h, then overlaid with RPMI + 10% FBS. Microscopy images of spheroid growth and invasion were quantified using Image J software (NIH, Bethesda, MD, USA).

Western blotting

Cells were lysed in 20 mM Tris pH 8.0, 2% SDS, 10 mM dithiothreitol and boiled for 10 min. Total protein was determined using the DC Protein Assay (Bio-Rad). Membranes were blocked with 5% (w/v) non-fat dry milk and washed in phosphate-buffered saline + 0.1% (v/v) Tween-20. Primary antibodies were diluted in 5% BSA + phosphate-buffered saline/0.1% Tween-20 and recognized ERK1/2, LEF-1, CCNE2, p21, FOSL1 (Cell Signaling Technology, Danvers, MA, USA), MLANA, ITGB3, DUSP4, TBP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-ERK1/2 (Sigma, St Louis, MO, USA) and CCND1 (Upstate, Charlottesville, VA, USA). Horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were diluted 1:5000 in phosphate-buffered saline + 0.1% Tween-20 with 5% non-fat milk and visualized using enhanced chemiluminescence (Perkin-Elmer, Waltham, MA, USA).

3'UTR luciferase reporter assays

Melanoma cells were co-transfected with miRNA mimics and 3'UTR luciferase reporters (Switchgear Genomics, Menlo Park, CA, USA, individual miRNA assays; or GeneCopoeia, Rockville, MD, USA, combinatorial miRNA assays) using DharmaFECT Duo (Thermo Fisher Scientific). In all, 20 000 cells/well were plated onto opaque 96-well plates (Becton Dickinson, Franklin Lakes, NJ, USA), incubated for 24 h, and transfected with 100 ng (Switchgear Genomics) or 50 ng (GeneCopoeia) 3'UTR luciferase reporter

plus 5–20 nM mimic. Luminescence was analyzed after 24 h by replacing media with 75 μ l Steady-Glo luciferase reagent (Promega), and measuring luminescence after 30 min (Beckman Coulter DTX 880, Fullerton, CA, USA).

CONFLICT OF INTEREST

Dr Emily Anderson, Maren Mayer and Kevin Sullivan are employed by and own stock in Dharmacon, Inc., a wholly owned subsidiary of Thermo Fisher Scientific, Inc. Remaining authors declare no conflict of interest.

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