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MicroRNA-211 modulates the DUSP6-ERK5 signaling axis to promote BRAF^{V600E}-driven melanoma growth *in vivo* and BRAF/MEK inhibitor resistance

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Abstract

Micro-RNAs are important post-transcriptional regulators of cell fate both in normal and disease states. miR-211 has previously been shown to be a direct regulator of metabolism in *BRAF*^{V600E}-mutant melanoma cells *in vitro*. Here we report that miR-211 expression promotes aggressive growth of *BRAF*^{V600E}-mutant melanoma xenografts *in vivo*. miR-211 promoted proliferation through post-transcriptional activation of ERK5 signaling, which has recently been implicated in BRAF and MEK inhibitor resistance. We therefore examined whether miR-211 similarly modulated melanoma resistance to the BRAF inhibitor vemurafenib and the MEK inhibitor cobimetinib. Consistent with this model, miR-211 expression increased melanoma cell resistance to both inhibitors and this resistance was associated with increased ERK5 phosphorylation.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

miR-211 mediates these effects by directly inhibiting the expression of DUSP6, an ERK5 pathway-specific phosphatase and now shown to be an miR-211 target gene. These results dissect the role of the miR-211-DUSP6-ERK5 axis in melanoma tumor growth and suggest a mechanism for the development of drug-resistant tumors and a target for overcoming resistance.

INTRODUCTION

Malignant melanoma has a high mortality burden if not detected early (Arrangoiz et al., 2016, Shain and Bastian, 2016). Since the majority of patients treated with MAPK inhibitors develop resistance and relapse (Sun et al., 2014), there is a need to identify molecular mechanisms of drug resistance and to develop novel drug targets,

Micro-RNAs have emerged as promising diagnostic, therapeutic, and theranostic candidates due to their emerging roles in melanoma hallmarks including proliferation, migration, apoptosis, immune responses, resistance to chemotherapy, and shaping the tumor niche (Aftab et al., 2014, Ross et al., 2018). We discovered a significant role for miR-211 in melanocyte and melanoma pathobiology, with subsequent studies confirming miR-211 as a critical regulatory molecule in melanoma (Babapoor Sankhiros et al., 2016, Bell et al., 2014, Boyle et al., 2011, Dai et al., 2015, Levy et al., 2010, Margue et al., 2013).

We previously showed that miR-211 is highly expressed in primary melanocytes but that expression is reduced in amelanotic melanoma cell lines (Mazar et al., 2010). Others have reported decreased miR-211 expression in clinical melanoma samples (Kozubek et al., 2013, Xu et al., 2012), driving efforts to use miR-211 as a clinical diagnostic test to discriminate melanomas from benign nevi (Babapoor S. et al., 2016). We also showed that miR-211 overexpression increases mitochondrial respiration in amelanotic melanoma cells, thereby inhibiting melanoma cell survival and invasion *in vitro* (Mazar et al., 2016).

Recent studies suggest that: (i) miR-211 expression and melanin biosynthetic pathways are induced by vemurafenib (a BRAF inhibitor) treatment and contribute to vemurafenib resistance (Vitiello et al., 2017); and (ii) miR-211 contributes to BRAF inhibitor resistance in melanoma via MAPK signaling (Diaz-Martinez et al., 2018). However, the precise miR-211 target pathways controlling vemurafenib resistance remain to be defined.

Here we interrogated the molecular mechanisms of miR-211 function *in vivo*. Ectopic miR-211 expression in $BRAF^{V600E}$-mutant melanoma cell lines promoted aggressive xenograft growth through the activation of the ERK5 signaling pathway, which is negatively regulated by the putative miR-211 target BIRC2 and DUSP family members. Finally, miR-211 conferred resistance to the BRAF inhibitor vemurafenib and MEK inhibitor cobimetinib, with corresponding increases in ERK5 phosphorylation. Taken together, these observations are consistent with a model in which miR-211 modulates melanoma proliferation and resistance to drugs that target the BRAF pathway by inducing signal transduction through the ERK5 pathway.

RESULTS AND DISCUSSION

miR-211 promotes tumor growth *in vivo*

The effects of ectopic expression of miR-211 on tumor phenotype were first assessed *in vivo*. The melanoma cell line A375, which has a constitutively activated MAPK pathway due to a homozygous $BRAF^{V600E}$ mutation and low basal miR-211 expression, and their stable miR-211-overexpressing counterparts (A375/211) (Mazar et al., 2016) (Supplementary Figure S1), were injected subcutaneously into SCID mice. Ectopic miR-211 expression significantly and rapidly increased tumor growth and weight (Figure 1a–c). An additional two independent stable clones (A375/211-c2 and A375/211-c3) behaved similarly (Supplementary Figure S2), excluding a clonal effect, and there were no significant differences between parental cells (A375) and vector-only control cells (A375/vo) (Supplementary Figure S2). A negative control miR-210 (known as a hypoxia-inducible miRNA and also a fine-tuner of the hypoxic response (Huang et al., 2009)) did not affect tumor growth when overexpressed in A375 (A375/210) cells (Supplementary Figure S3), suggesting that tumor formation by A375 cells expressing miR-211 was unrelated to hypoxia. To investigate whether the effect of miR-211 might be specific to A375 cells, we ectopically expressed miR-211 in additional cell lines, LOX-IMVI (LOX-IMVI/211) and HT-144 (HT-144/211), which also harbor $BRAF^{V600E}$ mutations (Hoeflich et al., 2006). Again, there were significant increases in tumor size and weight relative to tumors induced by the vector-only control cell lines *in vivo* (Supplementary Figure S4), suggesting that the tumor-promoting activity of miR-211 in xenografts is generalizable to other $BRAF^{V600E}$-mutant amelanotic melanomas.

Proliferation marker, ki67, was examined in xenograft tissues and the Ki67 staining intensity of A375/211 tumors was significantly greater than in A375 tumors (~1.3 fold; $p \leq 0.01$; Figure 1d). CD31 immunofluorescence to assess blood vessel density revealed that angiogenesis was markedly enhanced in A375/211 tumors compared to A375 tumors (Figure 1d). Using pimonidazole (Hypoxyprobe™) to evaluate tumor hypoxia (Aguilera and Brekken, 2014), A375/211 xenografts contained more hypoxic areas compared to A375 xenografts, suggesting that A375/211 tumors rapidly outgrew their blood supplies even with enhanced angiogenesis (Figure 1d). We previously established a link between miR-211 and metabolism; miR-211 overexpression increases mitochondrial respiration by targeting PDK4 (Mazar et al., 2016) and, consistent with this, PDK4 expression was reduced in A375/211 cells both at the transcript (Supplementary Figure S5) and protein levels (Figure 1d). Collectively, these data established a general oncogenic role for miR-211 by enhancing proliferation and angiogenesis.

In vivo tumor growth induced by miR-211 is not fully explained by metabolic changes

Our previous report showed that inhibition of PDK4 by miR-211 overexpression in A375 cells *in vitro* increased the oxygen consumption rate (OCR), indicating enhanced mitochondrial activity (Mazar et al., 2016). To determine whether PDK4 regulation affects tumor growth by modulating metabolism and whether this effect persists *in vivo*, we performed targeted metabolite analysis (Supplementary Figure S6). Glycolysis intermediates were not significantly affected by miR-211 overexpression (Supplementary

Figure S6b). Similarly, TCA cycle intermediates were generally unaffected except for fumarate (Supplementary Figure S6c), which was significantly reduced by miR-211 overexpression. Amino acid levels were almost uniformly decreased in A375/211 xenografts (Supplementary Figure S6d). Overall, although miR2–11 acts as a metabolic switch *in vitro* (Mazar et al., 2016) and in that context might be expected to dictate the tumor phenotype, miR-211's effects on metabolic pathways appear to be largely "buffered" within the physiological environment *in vivo*. These results prompted us to investigate whether miR-211 acts via an additional pathway in mouse xenografts.

ERK5 signaling is downregulated in miR-211-expressing melanoma cells

To identify the mechanistic basis of miR-211-driven tumorigenesis, we subjected A375 and A375/211 cells and its xenografts to RNA-seq and analyzed the transcriptomic network. We found that miR-211 expression was associated with the activation of four independent pathways (ERK5 signaling, cyclins and cell cycle regulation, hypoxia signaling, and telomerase signaling; all z-scores ≥ 2.0) and also the inactivation of five human-specific pathways (cAMP-mediated signaling, interferon signaling, PPARα/RXRα activation, Gαi signaling, and Gαq signaling; z-score ≤ −2.0) in xenografts (Figure 2a and Supplementary Table S1).

To gain a further insights into which biological pathways contribute to A375/211 xenograft growth, we performed gene set enrichment analysis (GSEA) on the human-specific upregulated transcripts (log2 fold-change ≥ 1.0, $p \leq 0.05$). Epithelial-to-mesenchymal transition (EMT) was the most significantly enriched gene set (Supplementary Table S2), consistent with previous reports of EMT pathway activation in *BRAF*-activated melanomas (Caramel et al., 2013, Mitchell et al., 2016a, Mitchell et al., 2016b). In addition, miR-211 expression was associated with alterations in several EMT-related pathways including early (FDR = 1.52×10^{-2}) and late (FDR = 8.33×10^{-4}) estrogen response genes, genes upregulated in hypoxia (FDR = $4.09E \times 10^{-23}$), and genes responding to ultraviolet (UV) radiation (up (FDR = $2.15E \times 10^{-4}$) and down (FDR = 2.41×10^{-6})) (Hudson et al., 2007, Lamouille et al., 2014). Several lipid metabolism-related gene sets including cholesterol homeostasis, adipogenesis, and fatty acid metabolism were also upregulated (FDR = 2.54×10^{-6}, 1.63×10^{-4}, and 2.15×10^{-4}, respectively).

ERK5 signaling was, however, the highest significantly altered candidate pathway associated with miR-211 overexpression (Figure 2a). ERK5 was recently shown to be activated in melanomas (Tusa et al., 2018), and we reasoned that negative regulators of the ERK5 pathway could be miR-211 targets. Using TargetScan (Lewis et al., 2005), microRNA.org (Betel et al., 2008), and miRDB (Wong and Wang, 2015), we identified putative miR-211 targets in the ERK5 signaling pathway, which included baculoviral IAP repeat containing 2 (*BIRC2*) and several dual specificity phosphatases (DUSPs) including *DUSP3*, *DUSP4*, *DUSP6*, and *DUSP19*. BIRC2 is an ubiquitin ligase and its family member, BIRC4, is a known regulator of MEKK2, which lies upstream of MAPK kinase kinase in the ERK5 pathway (Takeda et al., 2014). DUSP proteins are possible phosphatases of ERK5 (Moncho-Amor et al., 2019) and would be expected to inhibit ERK5 signaling activation (Figure 2b), and *DUSP4* was previously predicted as a miR-211 target in melanoma (Couts et al., 2013).

To determine which candidates were directly regulated by miR-211 *in vivo*, we next used an antibody targeting argonaute-2 (Ago2) for affinity immuno-purification under conditions in which the bound mRNAs are captured and their identity determined by RNA-seq (Hendrickson et al., 2008, Malmevik et al., 2015). The bound RNA sequences were computationally filtered by miR-211-binding sites to identify putative miR-211 targets. Compared to the enrichment of *BIRC2* and DUSPs in A375/211 cells, their enrichment was considerably lower in A375/vo cells (Supplementary Table S3). Both *BIRC2* and DUSP family members were enriched (1.8–6.6-fold compared to input) by Ago2 immuno-purification (Figure 2c and Supplementary Table S3), consistent with these mRNAs being direct targets of miR-211.

We next performed a miRNA target cleavage assay (luciferase reporter assay) to further confirm that *BIRC2, DUSP3, DUSP4, DUSP6*, and *DUSP19* were direct miR-211 targets (Figure 2d–e and Supplementary Figure S7). When reporter plasmids were co-transfected with miR-211-expressing plasmids into HEK-293T cells, the wild-type reporter showed reduced luciferase activity, while the mutant one did not (Figure 2d). Further, when a reporter carrying luciferase with the 3'UTR of DUSP6 was transiently transfected into A375 or A375/211 cells, luciferase activity reduced in A375/211 cells compared to A375 cells (Figure 2e). To determine which DUSPs specifically dephosphorylate ERK5, A375 cells were treated with siRNAs against *DUSPs* and ERK5 phosphorylation was assessed. As shown in Figure 3a and Supplementary Figure S8, knockdown of each DUSP except *DUSP3* increased ERK5 phosphorylation. Furthermore, when DUSP proteins were ectopically expressed in A375/211 cells, DUSP6 decreased ERK5 phosphorylation, indicating DUSP6 may act specifically on ERK5 (Figure 3b). DUSP6 protein levels were lower in A375/211 cells and corresponded to higher ERK5 phosphorylation than in A375 cells (Figure 3c), and the stable expression of DUSP6 in both cell lines (A375/DUSP6 and A375/211/DUSP6) reduced ERK5 phosphorylation (Figure 3c). We did not observe an effect with *BIRC2* knockdown or overexpression on ERK5 phosphorylation (Supplementary Figure S9). ERK5 has multiple phosphorylation sites including major sites in the kinase domain and autophosphorylation sites at the C-terminal (Honda et al., 2015). The phospho-ERK5 antibody used here detects the major kinase domain phosphorylation sites (Thr218 and Tyr220). Depending on total ERK5 phosphorylation status, we often observed several bands; we considered the doublet appearing at ~140 kDa as a major form of phospho-ERK5. The increase in pERK5 and decrease in DUSP6 were also observed in other miR-211-overexpressing melanoma cell lines LOX-IMVI/211, HT-144/211, and WM1346/211 (Figure 3d). The same effect of miR-211 overexpression on the ERK5 pathway was also observed in the *BRAF* wild type melanoma cell line, WM1346, which has am $NRAS^{Q61L}$ mutation (Smalley et al., 2008). As mentioned in materials & methods, bands were quantified using Image J. pERK5 band was normalized with ERK5 and GAPDH (or TUBULIN) and DUSP6 band was normalized with TUBULIN. Bar graph is showing relative intensity compared to control (Figure 3).

Next, we injected A375/DUSP6 and A375/211/DUSP6 cells into the flanks of SCID mice, with A375 and A375/211 cells used as controls. DUSP6 re-expression significantly reduced pERK5 levels (Figure 3c) and A375/211 tumor growth *in vivo* (Figure 3e). DUSP6-overexpressing tumors were significantly smaller than A375/211 xenografts harvested the

same day (Figure 3f; $p \leq 0.001$), while A375/DUSP6 tumors were not significantly different to the A375 tumors. Given these results, we further investigated the role of miR-211 overexpression in melanoma cells through the DUSP6-ERK5 axis.

miR-211 overexpressing cells are more susceptible to pharmacological inhibition of the ERK5 pathway

Phospho-ERK5 (pERK5) expression was lower in A375 cells than in A375/211 cells, and stable overexpression of *DUSP6* consistently decreased phospho-ERK5 levels (Figure 3c), whereas its levels increased upon siRNA-mediated *DUSP6* knockdown (siDUSP6) (Figure 4a and Supplementary Figure S10). ERK5 is activated upon ERK1/2 inhibition in intestinal epithelial cells and colorectal cancer cell lines (de Jong et al., 2016). To establish whether ERK5 was also activated by ERK1/2 pathway inhibition in melanoma, A375 cells were treated with PD0325901, which inhibits the ERK1/2 kinase MEK1/2. PD0325901 efficiently abrogated ERK1/2 phosphorylation and significantly increased ERK5 phosphorylation, suggesting that the ERK5 pathway could be an escape pathway in melanoma (Figure 4a, **lane 2**). As expected, ERK5 phosphorylation was further increased upon siDUSP6 knockdown and PD0325901 treatment (Figure 4a, **lane 4**).

Since the ERK5 pathway was activated in A375 cells upon PD0325901 treatment, we hypothesized that the ERK5 pathway may compensate for loss of ERK1/2 signaling to drive hyperproliferation in melanomas. Consistent with this, A375/211 cells were more sensitive to the ERK5 inhibitor XMD8–92 than parental cells (Figure 4b and c). The IC_{50} for XMD8–92 in A375/211 cells was substantially lower than in A375 cells (Figure 4c). Since XMD8–92 has been reported to have off-target activity on bromodomain-containing proteins (Lin et al., 2016), we also examined the effect of ERK5 knockdown on cellular proliferation. ERK5 siRNA knockdown directly inhibited A375/211 cell growth and had a lesser effect on A375 cell proliferation (Supplementary Figure S11), suggesting that the reduction in cellular proliferation caused by XMD8–92 was not due to off-target effects (at the 10 μM concentration used here). To further confirm the dependence of A375/211 cells on the ERK5 pathway, we also determined whether inhibition of MEK5, an upstream regulator of ERK5, also reduced proliferation (Tatake et al., 2008). As expected, BIX02189 decreased cellular proliferation in a dose-dependent manner, with A375/211 cells more sensitive than A375 cells (Figure 4d, A375/vo $IC_{50} = 41.0 \pm 2.5$ μM, A375/211 $IC_{50} = 26.9 \pm 1.8$ μM, student t-test p=0.0013). Both XMD8–92 and BIX02189 efficiently abrogated ERK5 phosphorylation (Supplementary Figure S12). Other miR-211-overexpressing cells were also more sensitive to BIX02189, whereas the *BRAF* wild type cell line, WM1346/211, was insensitive (Supplementary Figure S13). Although pERK5 levels increased in WM1346/211 cells, ERK1/2 pathway activation by activated $Nras^{Q61L}$ seemed to drive proliferation pathway in WM1346/211 cells, resulting in insensitivity to BIX02189. Lastly, we determined the effect of AX15836, an XMD8–92 derivative with no significant off-target activity (Lin et al., 2016), on A375/211 cell proliferation. The IC_{50} of AX15836 in A375 cells (Supplementary Figure S14a) was higher than that in A375/211 cells grown as 2D cultures. In 3D cultures (colony-forming assay), the IC_{50} reduced from 62 μM to 6.7 μM (Supplementary Figure S14b) in A375/211 cells. Unfortunately, the IC_{50} of A375 cells could not be determined due to inefficient 3D growth. These important results suggest that A375/211 cells are highly

sensitive to ERK5 inhibition, and ERK5 signaling might therefore be as important as the ERK1/2 pathway in human melanomas (Balmanno and Cook, 2009, Nazarian et al., 2010).

miR-211 confers melanoma drug resistance through activation of ERK5 signaling

Interestingly, miR-211 expression increases in melanomas treated with vemurafenib and may contribute to vemurafenib resistance (Diaz-Martinez et al., 2018, Lunavat et al., 2017, Vitiello et al., 2017). We therefore hypothesized that miR-211-regulated circuits contributed to BRAF/MEK inhibitor resistance in melanoma cells. Consistently, miR-211 levels were increased in A375, LOX-IMVI, and HT-144 cells treated with vemurafenib and the MEK inhibitor cobimetinib (Supplementary Figure S15). We did not observe any induction of miR-211 by vemurafenib in the *BRAF* wild type cell line WM1346 (Supplementary Figure S15). In addition, miR-211 expression increased vemurafenib resistance 10.4-fold compared to A375 cells (Figure 5a). This increase in resistance was associated with increases in phosphorylated ERK5 and a reduction in DUSP6 levels (Figure 5b). The increase in vemurafenib resistance was also observed in LOX-IMVI/211 and HT-144/211 but not WM1346/211 cells (Supplementary Figure S16). Next, to show that the miR-211/DUSP6/ERK5 axis plays a role in vemurafenib resistance, we knocked down ERK5 in A375/211 cells, which restored vemurafenib sensitivity. Since *ERK5* knockdown in A375/211 reduced proliferation independently of vemurafenib, we treated cells with various concentrations of vemurafenib to determine the optimal drug concentration (Supplementary Figure S17). *ERK5* knockdown restored sensitivity to vemurafenib in A375/211 cells at low concentrations (Figure 5c). Thus, despite the reduction in proliferation due to *ERK5* knockdown, cells were able to grow in the presence of vemurafenib at concentrations that would normally be inhibitory. However, high concentrations of vemurafenib significantly reduced proliferation, therefore masking any observable effects of *ERK5* knockdown on vemurafenib resistance. As anticipated, the sensitivity of A375/vo negative control cells was not affected by *ERK5* knockdown across the whole range of drug concentrations tested (Supplementary Figure S17). These results suggest a role for the miR211/DUSP6/ERK5 axis in vemurafenib resistance.

We further explored the association between miR-211 and drug resistance. First, we examined whether vemurafenib resistance was mediated by the miR-211-ERK5 axis in drug resistant cell lines. As expected, pERK5 levels were elevated in vemurafenib-resistant cell lines (Figure 5d). Drug resistance in these cell lines has previously been explained by ERK1/2 pathway reactivation (Vitiello et al., 2017). Indeed, while ERK1/2 phosphorylation was significantly increased in resistant cell lines compared to parental A375 cells, ERK5 phosphorylation was also increased, suggesting that ERK5 signaling might also contribute to acquired drug resistance in melanoma (Figure 5d). In agreement with this hypothesis, miR-211 expression was also significantly increased in these cell lines (Figure 5e). To investigate whether this observation was cell line-specific or generalizable, miR-211 gene expression was quantified in 39 melanoma cell lines (Supplementary Table S4). miR-211 expression (low and high) and vemurafenib resistance partitioned melanoma cell lines into two groups (Supplementary Figure S18a), with the mean −ΔCt values between the two groups exhibiting at least a 5.4-fold difference in miR-211 expression (Supplementary Figure S18b). Vemurafenib resistance and miR-211 expression were correlated with the

miR-211 low but not the miR-211 high group (Figure 5f), which is important because a majority of metastatic melanomas exhibit low miR-211 expression (Mazar et al., 2016). In addition, we observed that DUSP6 expression was inversely correlated with both vemurafenib resistance and miR-211 expression only in miR-211 low-expressing melanoma cell lines (Figure 5f and Supplementary Figure S19). We note that miR-211 plays a greater role in drug resistance in *BRAF*-mutant melanoma cells (Supplementary Table S4) compared to *BRAF* wild type cells. Among the miR-211 low group, there was a significant correlation between miR-211 expression and vemurafenib resistance in cell lines with *BRAF* mutations (Supplementary Figure S20). DUSP6 expression showed an inverse correlation with drug resistance in *BRAF*-mutant melanoma cell lines (Supplementary Figure S21).

In conclusion, miR-211 expression in $BRAF^{V600E}$-driven melanomas inhibits the DUSP6 protein phosphatase, which activates the ERK5 signaling pathway to promote tumorigenesis *in vivo* and contribute to the resistance of melanoma cells to BRAF/MEK inhibitors. These data add to the growing evidence that ERK5 pathway activation is an escape mechanism for cancer cells to overcome BRAF/MEK inhibition (Benito-Jardon et al., 2019) and that miR-211 is a critical node in the development of acquired resistance in patients with melanoma (Diaz-Martinez et al., 2018). Future clinical studies should examine the value of miR-211 as a resistance biomarker and determinant.

MATERIAL AND METHODS

The detailed protocols are described in the Supplementary Materials.

Cell lines

A375 and HT-144 were obtained from the ATCC (American Type Culture Collection, Manassas, VA). LOX-IMVI was purchased from the National Cancer Institute (NCI). WM1346 was a generous gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). A375-derived vemurafenib resistant cell lines (Vitiello et al., 2017) A375-P1, A375-P2, A375-C2, and A375-C3 were generously provided by Dr. Laura Poliseno at the Institute of Clinical Physiology (Pisa, Italy). The generation of A375/211 cells was described in a previous study (Mazar et al., 2010). Other cell lines overexpressing miR-211 were generated using lentiviruses harboring the miR-211 gene (Sahoo et al., 2017). Cell lines were authenticated by short tandem repeat profiling.

RNA immunopurification and sequencing (RIP-Seq)

RIP assays in A375/211 and A375/vo cell extracts were performed as described previously (Hendrickson et al., 2008, Zisoulis et al., 2012). Eluted RNAs were subjected to high-throughput sequencing on a HiSeq 2500 (Illumina) with 50 base-pair (bp) read lengths.

Mouse xenografts

Five-week-old severe combined immunodeficiency (SCID) mice were subcutaneously injected with 5.0×10^6 cells/site into their lower left flanks. Tumor size was monitored with digital calipers and tumor volume in mm^3 was calculated by the formula: volume = $(\text{width})^2 \times \text{length}/2$. Mice were euthanized and weights and pictures of excised tumors were

taken. All mice experiments were performed in accordance with institutional guidelines under Johns Hopkins University IACUC approval.

Statistical analysis

The date represent mean±SD values calculated at least two independent experiments or stated in the figure legends. p-values > 0.05 were considered not significant. Two datasets were compared with the unpaired Student's t-test. GraphPad Prism 8.0 (GraphPad, La Jolla, CA) was used for all statistical analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY

All xenografts RNA-seq and Ago2 RIP-seq datasets have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE125836.

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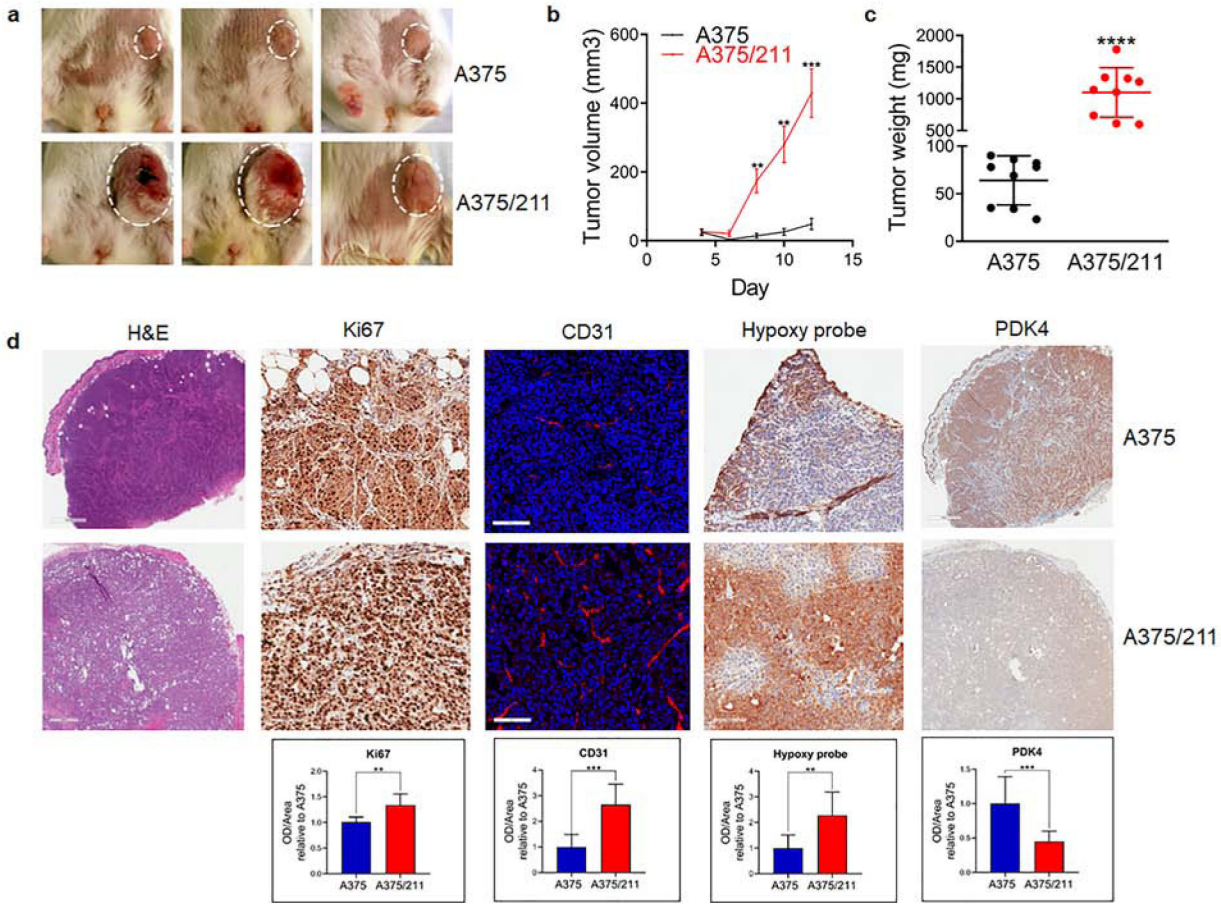

Figure 1. miR-211 promotes melanoma tumor growth *in vivo*.

(**a**) Photographs of subcutaneous tumor xenografts at day 12. (**b**) A375 and A375/211cells were injected subcutaneously into the flanks of SCID mice (n=9 per group) and tumor volume measured with electronic calipers. (**c**) Tumors were excised and weighed at day 12. (**d**) Photomicrographs of A375 and A375/211 xenografts showing tumor morphology (H&E; scale bar 500 μm); proliferation in A375/211 xenografts (Ki67 IHC; scale bar 100 μm); blood vessel density in A375/211 xenografts (CD31 IF; scale bar 100 μm); areas of hypoxia in A375/211 xenografts (hypoxy probe; scale bar 100 μm); and PDK4 expression in A375/211 xenografts (PDK4 IHC; scale bar 500 μm). Student *t*-test, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

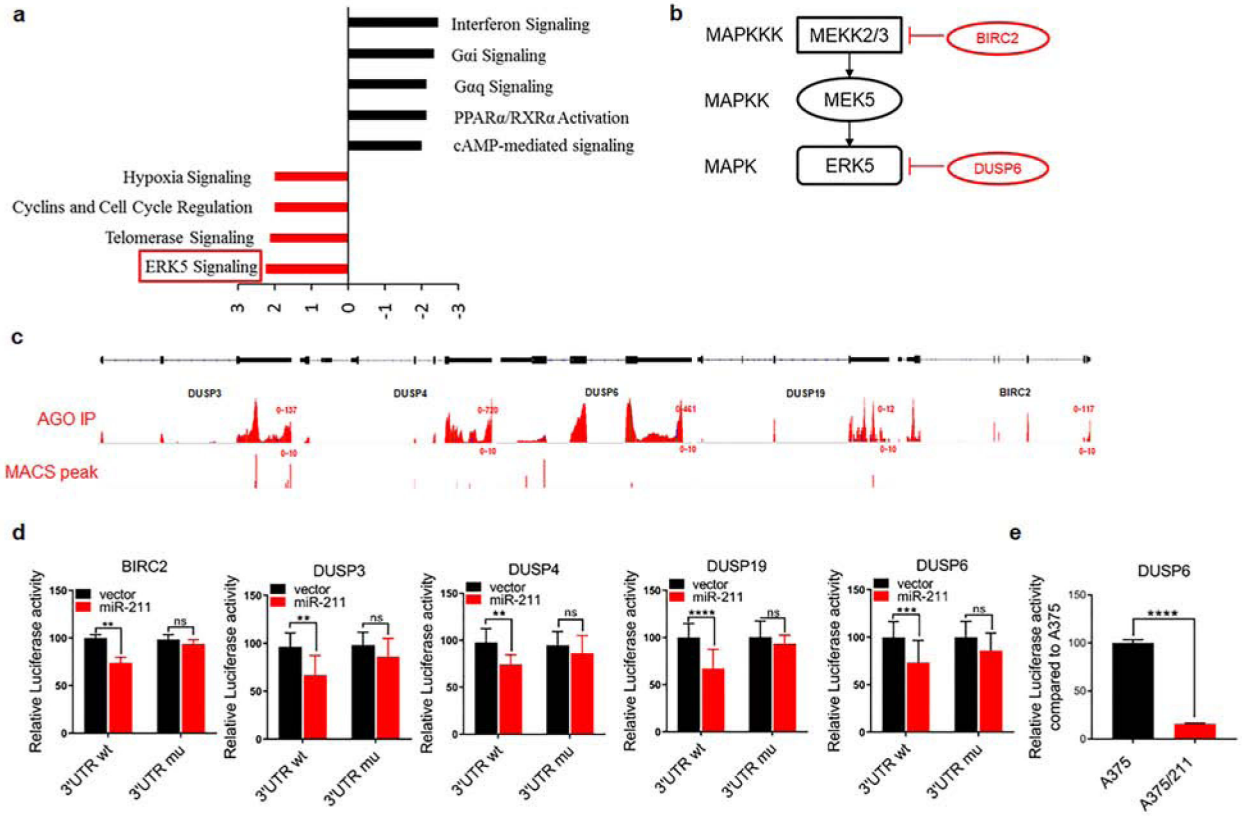

Figure 2. miR-211 targets negative regulators of ERK5 signaling.

(**a**) IPA analysis of differentially expressed genes in xenografts (A375 vs A375/211). (**b**) Schematic of the ERK5 pathway. BIRC2 and DUSP6 are miR-211 targets that negatively regulate ERK5 signaling. (**c**) MACS peaks show enriched regions compared to input. (**d**) Luciferase reporter assays with wild-type 3'UTR or mutant in miR-211 seed. The data shown represent the mean ± SD from two independent experiments. (**e**) Luciferase reporter assay with 3' UTR of DUSP6 in transiently transfected A375 and A375/211 cells. The data represent the mean ± SD of three replicates from one experiment. Student *t*-test, ns: not significant, ** $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

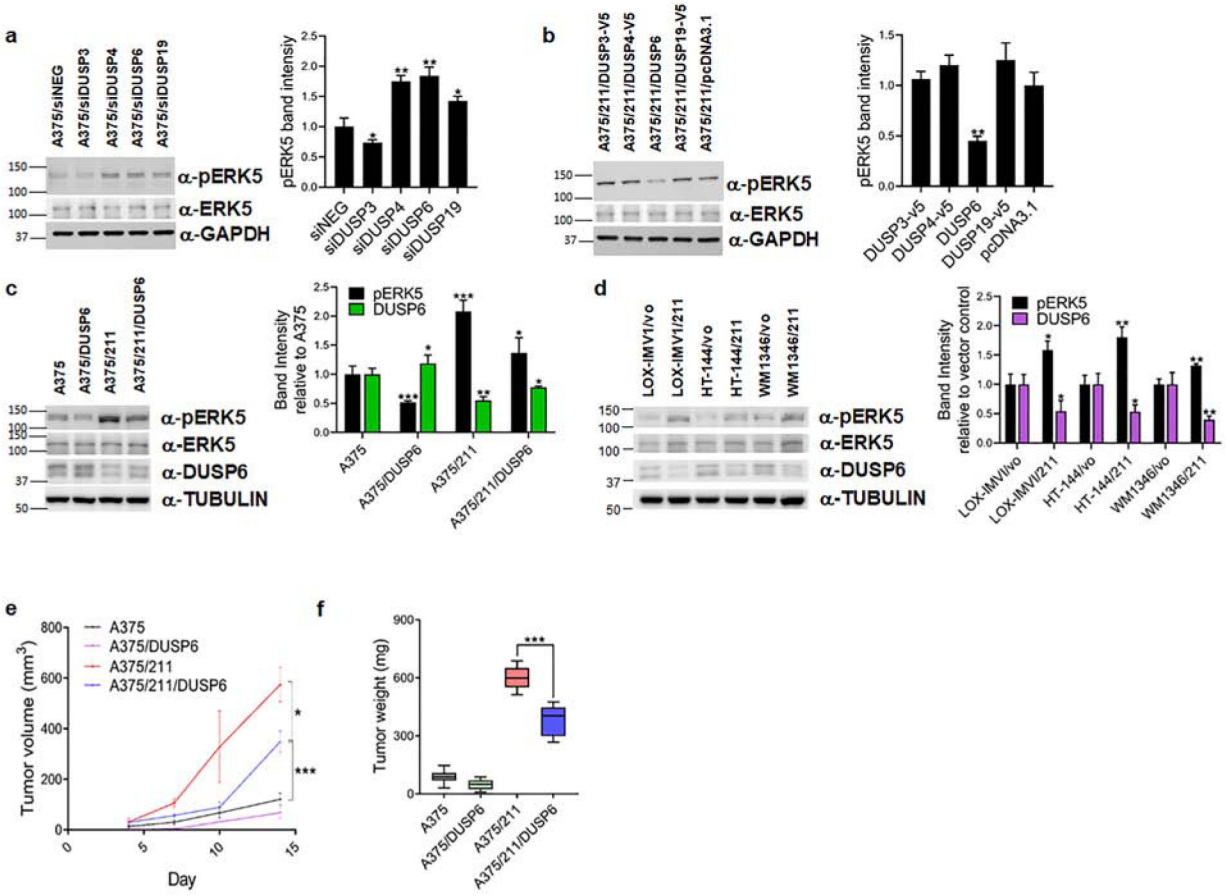

Figure 3. miR-211 targets DUSP6 to regulate ERK5 and associated with tumor growth.

(**a**) pERK5 level in A375 cells. Relative pERK5 intensity to siNEG is shown in the bar graph. (**b**) pERK5 level in A375/211 cells. Relative pERK5 intensity to vector control was shown. (**c**) Western blot of pERK5 and DUSP6 in A375, A375/DUSP6, A375/211, and A375/211/DUSP6 cells. (**d**) Western blot showing pERK5 and DUSP6 levels in miR-211 overexpressing melanoma cell lines. All western blots (**a-d**) were performed at least twice. (**e**) A375, A375/DUSP6, A375/211, and A375/211/DUSP6 tumor volumes (n=5 per group). (**f**) Tumors weight. The whiskers show the min and max values and line shows the median value. Student *t*-test, * $p \leq 0.05$, *** $p \leq 0.001$.

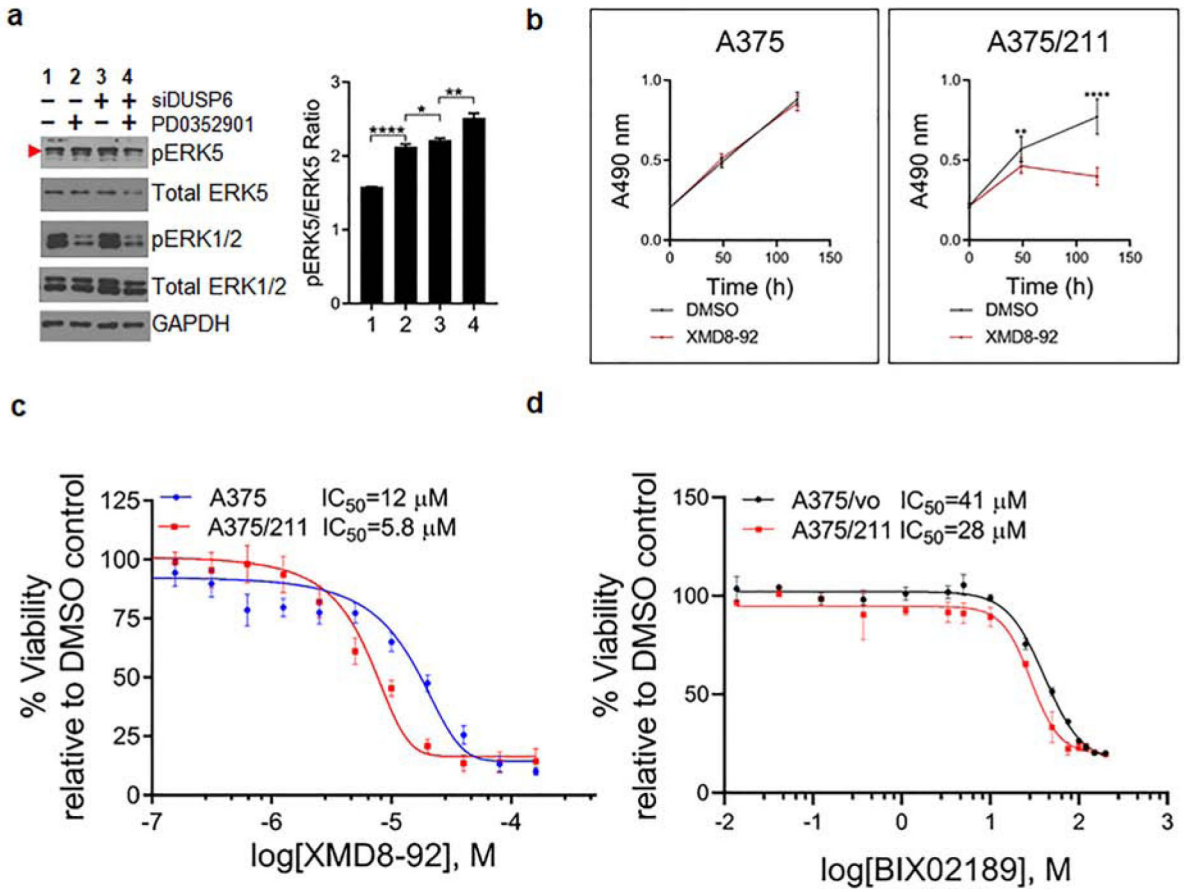

Figure 4. miR-211 activates the ERK5 pathway via DUSP6.

(**a**) Western blot of A375 cells treated with siDUSP6 or PD0325901 (100 nM, 24 h), a MEK1/2 inhibitor. Bar graph shows pERK5 normalized to ERK5 from two independent experiments. (**b**) Cell viability assays upon treatment of ERK5 inhibitor XMD8–92 (10 μM). The data represent the means of two independent experiments with standard deviations. (**c**) IC_{50} of XMD8–92 for A375 and A375/211 cells. Data are shown from two independent experiments. (**d**) IC_{50} of BIX02189 for A375 and A375/211 cells. Data are shown from two independent experiments. Student *t*-test, * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

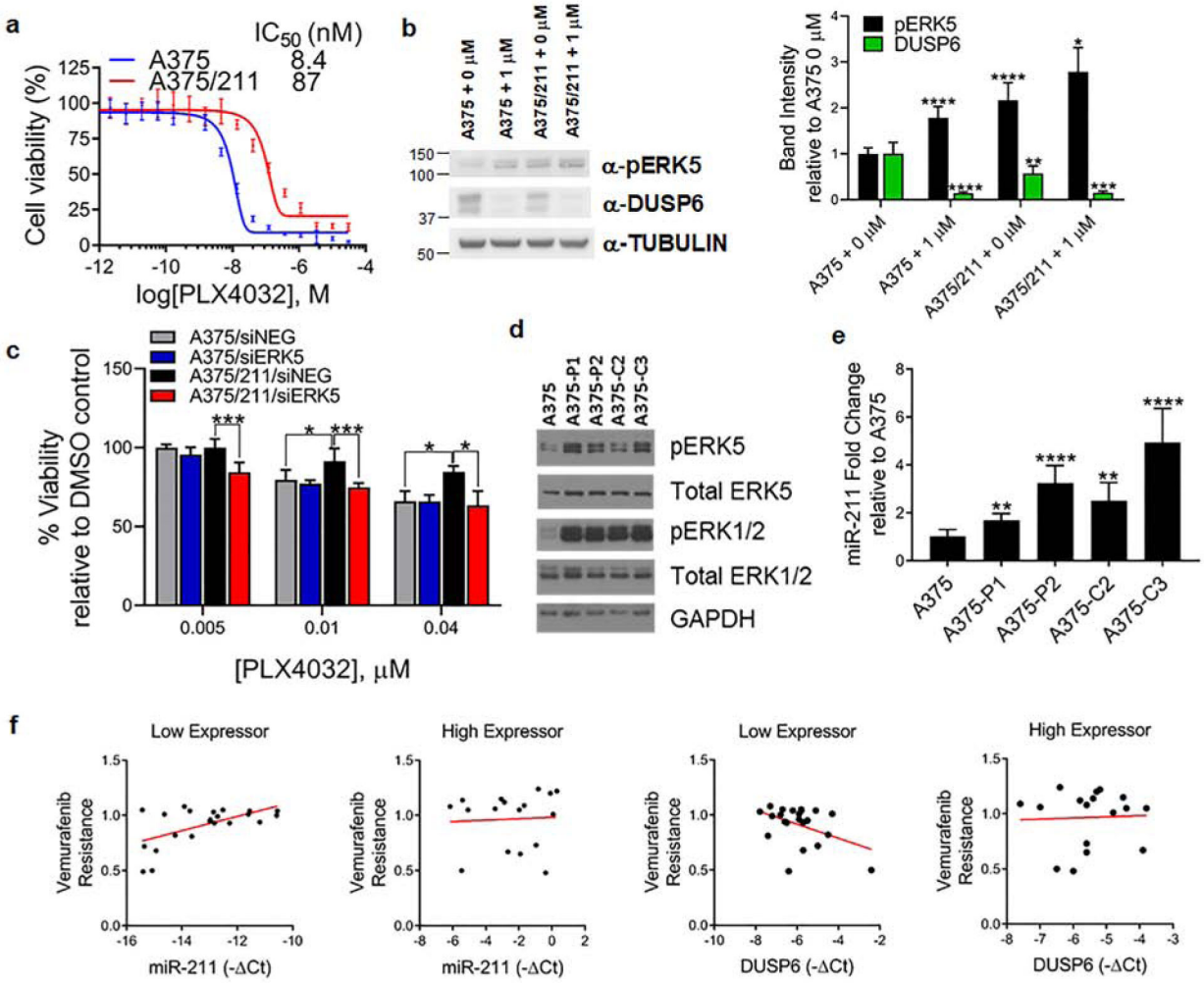

Figure 5. miR-211 contributes to vemurafenib resistance via ERK5.

(**a**) IC_{50} of PLX4032 for A375 and A375/211 cells. The data shown represent the mean ± SD from two independent experiments. (**b**) Western blot showing pERK5 and DUSP6 levels upon vemurafenib treatment. (**c**) Cell viability assay showing ERK5 knockdown in A375/211 cells restored vemurafenib sensitivity. (**d**) The phosphorylation level of ERK1/2 and ERK5 in A375 cells with acquired vemurafenib resistance. (**e**) miR-211 levels in vemurafenib-resistant cell lines compared to A375 cells. (**f**) Correlation of vemurafenib resistance with miR-211 levels and inverse correlation of vemurafenib resistance with DUSP6 levels in melanoma cell lines with low expression level of miR-211. Student t-test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$