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Oncogene dependent control of miRNA biogenesis and metastatic progression in a model of undifferentiated pleomorphic sarcoma

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Abstract

Undifferentiated pleomorphic sarcoma (UPS) is one of the most common soft tissue malignancies. Patients with large, high grade sarcomas often develop fatal lung metastases. Understanding the mechanisms underlying sarcoma metastasis are needed to improve treatment of these patients. Micro-RNAs (miRNAs) are a class of small RNAs that post-transcriptionally regulate gene expression. Global alterations in miRNAs are frequently observed in a number of disease states including cancer. The signaling pathways that regulate miRNA biogenesis are beginning to emerge. To test the relevance of specific oncogenic mutations on miRNA biogenesis in sarcoma, we used primary soft tissue sarcomas expressing either $Braf^{V600E}$ or $Kras^{G12D}$. We find that $Braf^{V600E}$ mutant tumors, which have increased MAPK signaling, have higher levels of mature miRNAs and enhanced miRNA processing. To investigate the relevance of oncogene dependent alterations in miRNA biogenesis, we introduce conditional mutations in *Dicer* and show that *Dicer* haploinsufficiency promotes the development of distant metastases in an oncogene dependent manner. These results demonstrate that a specific oncogenic mutation can cooperate with mutation in *Dicer* to promote tumor progression *in vivo*.

Keywords

soft tissue sarcoma; miRNA biogenesis; undifferentiated pleomorphic sarcoma; metastasis; and mouse models

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Statement of author contributions

JM carried out experiments, analyzed data, and conceived the experiments. HM and LD carried out experiments and analyzed data. YM, JC and BB carried out experiments. DD, MM, and DK conceived experiments and analyzed data. All authors contributed to the writing of the manuscript.

Introduction

Undifferentiated Pleomorphic Sarcoma (UPS) is one of the most common soft tissue malignancies of adulthood [1]. Even with surgery and radiation therapy, the development of fatal distant lung metastases still occurs in up to fifty percent of patients with large, high grade tumors [2]. In order to improve the outcomes of these patients, understanding the mechanisms that regulate sarcoma progression may lead to improved prognostic biomarkers and novel therapies for these patients. Because human UPS samples and cell lines are relatively rare compared to common epithelial cancers, utilizing a mouse model of UPS may provide insights into sarcoma biology that lead to new therapies for sarcoma patients [3, 4].

miRNAs are small ~22nt RNAs capable of post-transcriptional gene regulation. Recently, miRNAs have been shown to have a role regulating a diverse set of cellular processes including all events required for metastatic spread of cancer cells [5]. Primary miRNA transcripts (pri-miRNA) are transcribed in a canonical fashion by RNA polymerase II [6]. Pri-miRNAs form extended hairpin structures that are recognized by the microprocessor, which is composed of the ribonuclease Drosha and the double stranded RNA binding protein Dgcr8 [7]. The microprocessor cleaves these pri-miRNAs to shorter ~70nt pre-miRNA within the nucleus [8]. Upon export into the cytoplasm, pre-miRNAs are further processed into their mature form by Dicer and its binding partner Trbp [9]. These ~22nt mature miRNAs are then loaded into a complex with Ago proteins to form the RNA-induced silencing complex, where miRNAs function as post-transcriptional regulators of gene expression through direct base pairing with target mRNAs [10].

Widespread loss of miRNA expression has been found in many cancers when compared to normal tissue [11] and loss of mature miRNA expression has been correlated to tumor progression and the development of distant metastases in renal cell carcinoma and hepatocellular carcinoma [12, 13]. Moreover, some have hypothesized that loss of mature miRNA expression could be caused by defects in miRNA processing [14], but how miRNA biogenesis is regulated in cancer remains to be fully defined.

Mutations in a number of enzymes that process pri-miRNA transcripts into mature miRNAs have been identified in human cancer. For example, *DICER* mutations have been identified in ovarian cancer and soft tissue sarcomas including UPS and rhabdomyosarcoma [15–17]. Moreover, loss of one allele of Dicer is a common feature of many other malignancies [18]. In some human tumors, altered expression of enzymes that perform miRNA processing have been correlated to clinical outcomes in a tumor type dependent manner. For instance, decreased DICER or DROSHA levels are associated with worse outcomes in ovarian cancer and neuroblastoma [19, 20]. Conversely, overexpression of DICER has been linked to worse outcome in colorectal and prostate cancer [21, 22].

miRNA biogenesis has also been reported to be regulated by the MAPK pathway [23]. Activation of the MAPK pathway is a common feature of a diverse set of human tumors. Mutations in growth factor receptors, Ras, or within the MAPK pathway itself can activate the MAPK pathway leading to cell proliferation [24–26]. MAPK signaling has been reported to regulate miRNA biogenesis in cells *in vitro* through phosphorylation of TRBP, the binding partner of DICER, which can enhance both DICER stability and miRNA biogenesis [23]. Here we show that specific oncogenic mutations can regulate miRNA biogenesis in sarcomas *in vivo*. Using complementary primary mouse models of soft tissue sarcoma, we find that tumors initiated by $Braf^{V600E}$, compared to tumors expressing $Kras^{G12D}$, have increased pERK, miRNA processing, and expression of mature miRNAs. Moreover, we show that sarcomas expressing $Kras^{G12D}$ with Dicer haploinsufficiency have increased metastasis. However, deletion of one allele of *Dicer* in $Braf^{V600E}$ driven tumors does not

increase tumor proliferation or the rate of distant metastases. These results indicate that, in cancer, the consequences of a mutation in a component of the miRNA biogenesis machinery depend on specific oncogenic mutations.

Materials and methods

Animals

All animal work was performed in accordance with Duke University Animal Care and Use Committee approved protocols. Primary soft tissue sarcomas were generated using the following previously described alleles: *LSL-Kras^{G12D}* [27]; *p53^{Fl}* [28], *Braf^{CA}* [29], and *Dicer^{Fl}* [30] in mice with the following genotypes: 1) *Braf$^{CA/+}$; p53$^{Fl/Fl}$*, *Dicer$^{Fl/+}$* (*Dicer-BP*), 2) *Braf$^{CA/+}$; p53$^{Fl/Fl}$* (*BP*), 3) *LSL-Kras$^{G12D/+}$; p53$^{Fl/Fl}$* (*KP*), *or* 4) *LSL-Kras$^{G12D/+}$; p53$^{Fl/Fl}$; Dicer$^{Fl/+}$*(*Dicer-KP*). Tumors were initiated by injection of an Adenovirus expressing Cre-recombinase as described previously [3]. Rearrangement of *Dicer1* was confirmed using DNA from primary sarcomas as described previously [30]. To compare rates of metastasis across genotypes (*KP*, *Dicer-KP*, *BP* and *Dicer-BP*), *Dicer-KP* mice were crossed to *Dicer-BP* mice. The F1 progeny were intercrossed to generate *KP*, *Dicer-KP*, *BP* and *Dicer-BP* mice. Tumors were removed as described previously [31] and animals were monitored daily for signs of developing distant metastases, such as: labored breathing, masses, hunched posture, coat changes, and weigh loss, for 6–12 months.

Immunostaining and Image analysis

Five micron thick tissue sections from formalin fixed paraffin embedded tissue were subjected to standard hematoxylin and eosin staining. Mitoses per high powered field were determined by counting ten 40x fields per sample. Immunohistochemistry was performed with the following antibodies: Dicer1 (Sigma, HPA000694), Ki67 (BD Pharmagen, 556027), pERK1/2 (Cell Signaling, 4370), and pS6 (Cell Signaling, 9234) using the Vectastain Elite ABC Reagent (Vector labs). Ki67 staining was quantified as described previously [4].

Primary-miRNA and mature miRNA Expression

Cells or tumors were harvested with Trizol reagent, per manufacturer's suggestion. Reverse transcription for pri-miRNA transcripts was performed using iScript cDNA synthesis kit (Biorad) with 300ng of total RNA. Reverse transcription for specific mature miRNAs was performed using the Taqman microRNAs Reverse Transcription kit (Applied Biosystems). Q-PCR was carried out with Taqman probes for their respective targets (Applied Biosystems), pri-miRNA and mature miRNA expression were normalized to 18S and SnoRNA202 expression, respectively using the delta-delta-CT method.

Taqman PCR arrays for miRNA expression

Reverse transcription was performed using 1 microgram of total RNA using the Taqman Rodent microRNAs A card v2.0 for 375 mature miRNAs (Applied Biosystems). Samples were normalized to SnoRNA202 expression. Samples were compared in the delta-delta-CT method. Samples were clustered using Pearson correlation and row normalized heatmaps were made using differentially expressed miRNAs based on two-tailed T-test ($p<0.05$) between either sarcomas from: *KP* or *BP* sarcomas (Figure 1E), *Dicer-KP* mice or *BP, KP* and *Dicer-BP* (Figure 2F).

Cell line experiments and western blotting

Cell lines were derived by digestion with trypsin, collagenase IV and dispase (all Invitrogen). Cells were maintained in DMEM (Gibco, 11995-065) supplemented with 10%

FBS (Gibco). Cell proliferation was assessed by plating $5*10^3$ cells per well, in triplicate, in 24-well plates. Plating efficiency was determined at 6 hours, and used to normalize cell counts performed at 48, 72 and 96 hours. Cells were washed with PBS, trypsinized and counted using a coulter counter (Beckman Coulter).

For western blots, cells were grown in DMEM with 1% FBS for 48 hours. Cells were harvested in RIPA buffer with protease and phosphatase inhibitors (all Sigma). Western blots were performed using the following antibodies: ERK1/2 (Cell Signaling, 4695), Phospho-ERK1/2 (Cell Signaling, 4370), and Actin (BD, 612656). For pERK1/2 and total ERK1/2, the same blot was stripped and reprobed for total ERK1/2.

Results

Oncogene Dependent Alterations in miRNA Biogenesis *in vivo*

To test the functional significance of specific oncogenic mutations on tumor progression *in vivo*, we generated primary mouse soft tissue sarcomas initiated by oncogenic $Braf^{V600E}$ and loss of *p53* or oncogenic $Kras^{G12D}$ and loss of *p53*. Because mutations in upstream receptor tyrosine kinases and $Kras^{G12D}$, but not $Braf^{V600E}$, induce Sprouty and other suppressors of MAPK signaling [32], we hypothesized that $Braf^{V600E}$ mutant sarcomas would have increased MAPK signaling compared to $Kras^{G12D}$ mutant sarcomas. Intramuscular injection of an adenovirus expressing Cre recombinase into $Braf^{CA/+}; p53^{Fl/Fl}$ mice (referred to here as *BP* mice) [29], results in Cre dependent activation of the $Braf^{V600E}$ allele and deletion of *p53,* causing soft tissue sarcomas. Similar to recently published results in a mouse model of lung cancer [33], sarcomas in *BP* mice developed in approximately 55 days (median time to tumor), which is approximately 20 days faster than sarcomas in *KP* mice (Figure 1A, $p<0.03$). Sarcomas in *BP* mice have similar histology to sarcomas from *KP* mice (Figure 1B). However, in contrast to the sarcomas in *KP* mice, which we have described previously [3], sarcomas in *BP* mice have significantly higher levels of steady state MAPK signaling (Figure 1C) compared to their Kras driven counterparts. Similarly, compared to cell lines derived from sarcomas from *KP* mice, cell lines derived from sarcomas from *BP* mice showed significantly higher levels of pERK1/2 by western blot (Figure S1A). Conversely, downstream Ras targets of the PI3K pathway were not consistently different (Figure 1D).

Consistent with a model where MAPK signaling regulates miRNA biogenesis *in vivo*, miRNA profiling of soft tissue sarcomas from *BP* and *KP* mice demonstrates that sarcomas with increased MAPK signaling, such as those in *BP* mice, also have increased expression of mature miRNAs (Figure 1E, and Table S1). It should be noted that, four sarcomas from *KP* mice had higher mature miRNA expression and clustered with sarcomas from *BP* mice. Tissue from three of these four tumors was available for pERK1/2 staining. All three of the tumors examined had elevated levels of pERK1/2 similar to sarcomas from *BP* mice (Figure S1B). In order to determine if processing of miRNAs is more efficient in sarcomas in *BP* mice, we examined 8 miRNAs implicated in tumor progression to measure levels of pri-miRNA and mature miRNA in the primary sarcomas from *BP* and *KP* mice. We found an increased ratio of mature:pri-miRNA in the $Braf^{V600E}$ mutant sarcomas in miRNAs upregulated, or downregulated, in sarcomas from *KP* mice (Figure 1F). This suggests that sarcomas in *BP* mice have more efficient processing of miRNAs compared to sarcomas in *KP* mice in a manner independent of pri-miRNA expression. Together these data suggest that miRNA biogenesis in primary sarcomas is regulated by MAPK signaling in a oncogene dependent manner.

Dicer Haploinsufficiency Enhances Tumor Proliferation in an Oncogene Dependent Manner

Having demonstrated that sarcomas in *BP* mice have increased MAPK signaling and elevated miRNA processing, we next tested the functional significance of these findings in cancer by generating primary sarcomas with Dicer haploinsufficiency. Dicer haploinsufficiency has been observed in a number of human cancers, and specific mutations in Dicer have been identified in several subtypes of soft tissue sarcoma [15–17]. In *KP* mice, Dicer acts as a haploinsufficient tumor suppressor [18]. Therefore, we crossed $Dicer^{Fl}$ mice to *KP* and *BP* mice to generate: $LSL\text{-}Kras^{G12D/+}; p53^{Fl/Fl}; Dicer^{Fl/+}$ and $Braf^{CA/+}; p53^{Fl/Fl}; Dicer^{Fl/+}$ mice (referred to here as *Dicer-KP* and *Dicer-BP* mice, respectively). These mice developed soft tissue sarcomas at the site of Adeno-Cre injection with similar kinetics to mice with wildtype Dicer (Figure 2A–B). Western blot analysis of sarcoma cell lines derived from *Dicer-KP* and *Dicer-BP* mice showed decreased Dicer protein levels compared to sarcomas from *KP* and *BP* mice (Figure 2C). Immunostaining of these sarcomas further demonstrated decreased Dicer protein levels in sarcomas from *Dicer-KP* and *Dicer-BP* mice (Figure 2D). PCR analysis of genomic DNA from primary tumors demonstrated that recombination of the $Dicer^{Fl}$ allele occurred in all sarcomas examined (Figure 2E). Moreover, we isolated RNA from primary tumors and profiled the expression of mature miRNAs from *Dicer-KP, KP, Dicer-BP* and *BP* mice. miRNA profiling revealed loss of miRNA expression in *Dicer-KP* mice compared to the other genotypes (Figure 2F and Table S2). Taken together, these results suggest that oncogene dependent alterations in MAPK signaling, such as differences in pERK, resulting from specific mutations in *Kras* or *Braf*, can regulate miRNA biogenesis *in vivo*.

Previous studies have suggested that decreased miRNA expression from knockdown of Dicer or other components of the miRNA biogenesis machinery enhances tumor cell proliferation both *in vitro* and *in vivo* [34]. Therefore, we examined hematoxylin and eosin stained sections of sarcomas from *KP*, *Dicer-KP*, *BP* and *Dicer-BP* mice and scored the frequency of mitotic figures. We found a significant increase in mitoses per field in *Dicer-KP* mutant sarcomas compared to *KP* littermate controls (Figure 3A–B). In contrast, no increase in mitotic rate was observed in sarcomas from *Dicer-BP* mice compared to *BP* littermate controls. In an independent assay to assess proliferation in these sarcomas, we performed immunohistochemistry for the proliferation marker Ki67, and quantified the data through automated counting. Similar to the results from scoring mitoses, sarcomas from *Dicer-KP* mice had a significantly higher Ki67 index compared to sarcomas from *KP*, *BP* or *Dicer-BP* mice (Figure 3C–D). Moreover, sarcoma cell lines derived from *Dicer-KP* mice showed an increased proliferation phenotype *in vitro* compared to sarcoma cell lines derived from *Dicer-BP* mice (Figure 3E).

Dicer Haploinsufficiency Enhances Metastasis in an Oncogene Dependent Manner

Increased tumor proliferation has been linked to worse outcomes in a number of human tumor types including soft tissue sarcomas [35, 36]. Thus, we hypothesized that sarcomas in *Dicer-KP* mice may have increased rates of distant metastases, which are the most common cause of death in sarcoma patients. To test this hypothesis, a cohort of *Dicer-KP* and *Dicer-BP* mice and their littermate *KP* and *BP* controls were used to generate primary tumors in the distal hind limb. When sarcomas reached approximately $500mm^3$ in volume, they were removed by transfemoral amputation. These mice were then followed for a minimum of 6 months for the development of distant metastases, which occurred most frequently in the lungs (Figure 4A), but also to the body wall and soft tissues (Figure 4B, Table S3). Cellular morphology was maintained between primary (Figure 4C) and metastatic lesions (Figure 4D).

Sarcomas in *Dicer-KP* mice had a significant increase in the rate of metastatic progression compared to their littermate *KP* controls (Figure 4E, $p < 0.03$). In contrast, the rate of distant metastasis in *Dicer-BP* mice was similar to the rate of metastasis in *BP* mice (Figure 4F). Therefore, Dicer haploinsufficiency enhances metastasis in an oncogene dependent manner.

Discussion

miRNA expression is frequently altered in a number of disease states, including cancer [37]. Understanding the regulatory framework for miRNA expression may have important implications for therapeutic interventions that target specific signaling pathways that are perturbed in an oncogenic state. The mechanisms underlying the regulation of proteins that control miRNA processing are slowly being unraveled. Our results in a primary mouse model of soft tissue sarcoma show that mutations in components of the miRNA biogenesis machinery can alter the natural history of tumors by increasing cell proliferation and the development of distant metastases in an oncogene dependent manner. In human cancers, decreased levels of mature miRNAs have been linked to tumorigenesis [11] as well as the development of metastases in renal cell carcinoma and hepatocellular carcinoma [12, 13].

Previous reports have suggested that Dicer haploinsufficiency can promote tumorigenesis [18, 34]. In this study, we show that sarcoma initiation is not altered by Dicer haploinsufficiency, but that tumor progression is significantly enhanced in Dicer haploinsufficient tumors in an oncogene dependent manner. Moreover, our data suggest that mutations that alter miRNA processing can be partially suppressed by specific oncogenic mutations. For instance, increased MAPK signaling can be provided by oncogenic mutations that strongly activate the MAPK cascade such as mutations in *Braf*, which are less susceptible to feedback inhibition from downstream Sprouty and other proteins [32]. Conversely, tumors with relatively lower levels of MAPK signaling, such as those with *Kras* mutation, have lower basal miRNA processing activity as well as decreased steady state levels of mature miRNAs. Although we did identify a few *Kras* mutant sarcomas with higher levels of mature miRNAs, these tumors also had elevated pERK. It is possible that additional molecular alterations, such as downregulation or mutation of Sprouty or Spred proteins [38, 39], may lead to increased MAPK signaling and higher levels of mature miRNAs within these tumors. Conversely, mutations that decrease MAPK signaling within Braf tumors may lead to the opposite phenotype. Therefore, as we have observed in primary sarcomas in mice, *Kras* mutant tumors, may be more susceptible to mutations in other components of the miRNA biogenesis pathway, such as Dicer, resulting in decreased miRNA levels that promote tumor proliferation and metastasis. It is likely that as additional mechanisms that regulate miRNA biogenesis are uncovered, more mutations in pathways commonly perturbed in cancer will be identified that alter regulation of miRNA processing. For instance, other groups have shown that the tumor suppressor p53 can enhance the processing of a number of growth-suppressive miRNAs in response to DNA damage [40]. Conversely, in this same study, they demonstrated that common point mutations in p53 prevent miRNA maturation. These results suggest that specific p53 mutations such as point-mutations versus deletion, may lead to alterations in miRNA biogenesis and sensitize cells to loss of function mutations in Dicer or other components of the miRNA biogenesis machinery.

The ability of mutations in Dicer to increase cell proliferation and metastasis is likely to be due to the combined downregulation of a number of miRNAs rather than loss of a specific miRNA. Indeed, our data show that several miRNAs are downregulated by Dicer haploinsufficiency in sarcomas from *Dicer-KP* mice (Table S2). Moreover, the downregulated miRNAs that are most important for increasing proliferation and promoting metastasis may vary between tumors depending on the specific gene mutations that are

driving cancer growth. Nevertheless, single miRNAs can significantly alter metastatic progression. Therefore, to fully test the hypothesis that Dicer haploinsufficiency promotes metastasis through multiple miRNAs, it would be necessary to reintroduce specific miRNAs into the Dicer haploinsufficient background at physiologic levels to see if they can rescue the Dicer haploinsufficient phenotype. Alternatively, miRNAs could be deleted from tumors individually or in combination to identify those miRNAs whose downregulation promotes metastasis.

Finally, our data may have important prognostic and therapeutic implications for cancer. We have observed that loss of miRNA expression through Dicer haploinsufficiency in primary sarcomas is linked to the subsequent development of distant metastases in the setting of a specific oncogenic mutation. If this finding is replicated in human patient samples, then downregulation of mature miRNAs in primary tumors may act as a biomarker to help identify sarcoma patients at high risk for developing distant metastases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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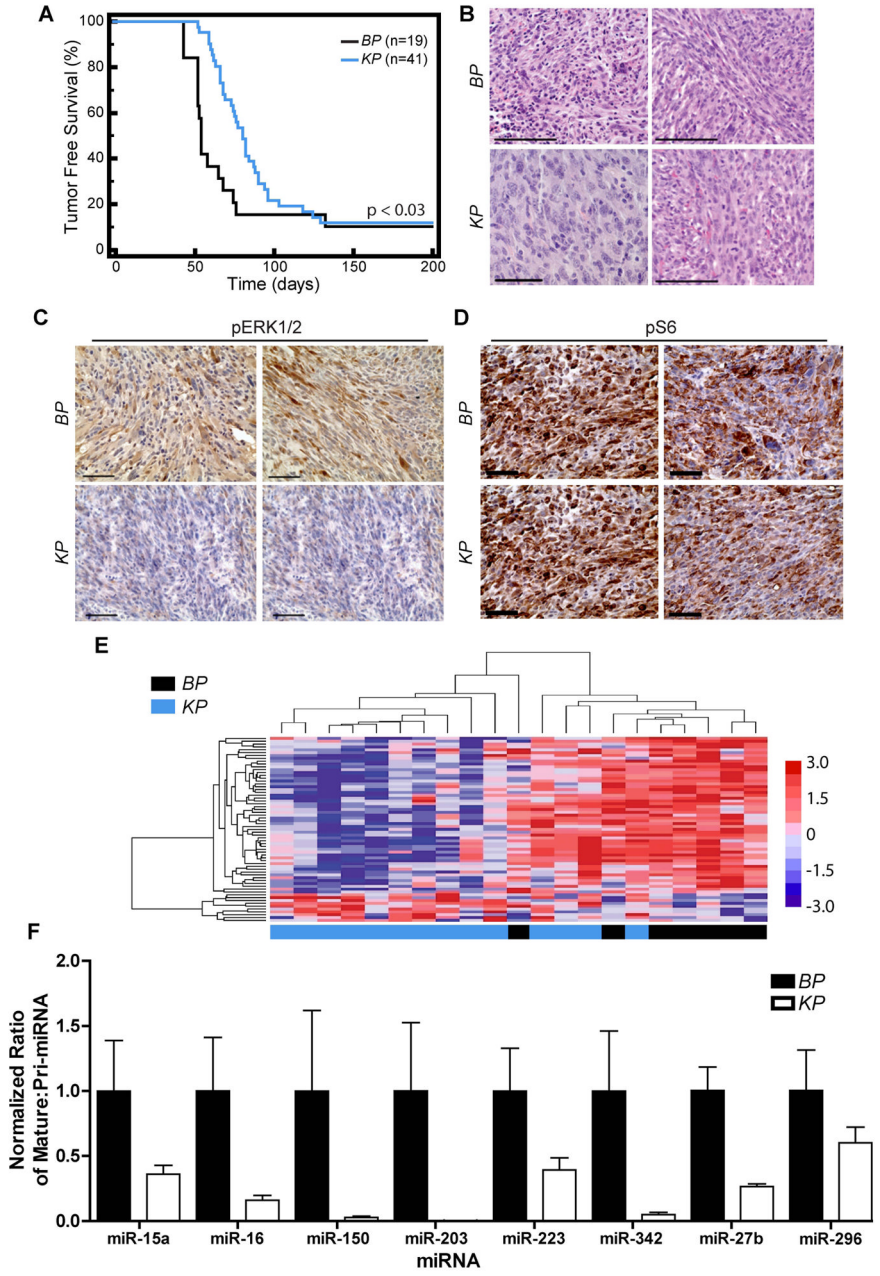

Figure 1.

Oncogenic *Braf*^{V600E} and deletion of *p53* can initiate high grade soft tissue sarcomas with elevated MAPK signaling and miRNA processing. (A) *Braf*^{V600E} sarcomas develop in *BP* mice with accelerated kinetics compared to *Kras*^{G12D} sarcomas in *KP* mice ($p<0.03$). (*B*) *Braf*^{V600E} initiated soft tissue sarcomas share similar histologic appearance to *Kras* initiated sarcomas. Higher levels of (C) pERK1/2, but not (D) pS6, were consistently detected by IHC in *Braf* mutant sarcomas (n=8) compared to *Kras* driven sarcomas (n=8). (E) *Braf*^{V600E} mutant soft tissue sarcomas have increased mature miRNAs, measured by Taqman PCR arrays. (F) *Braf*^{V600E} sarcomas have increased efficiency of miRNA processing compared to *Kras*^{G12D} mutant sarcomas as measured by the ratio of mature to pri-miRNA levels. Scale bars represent 100 microns, error bars represent SEM.

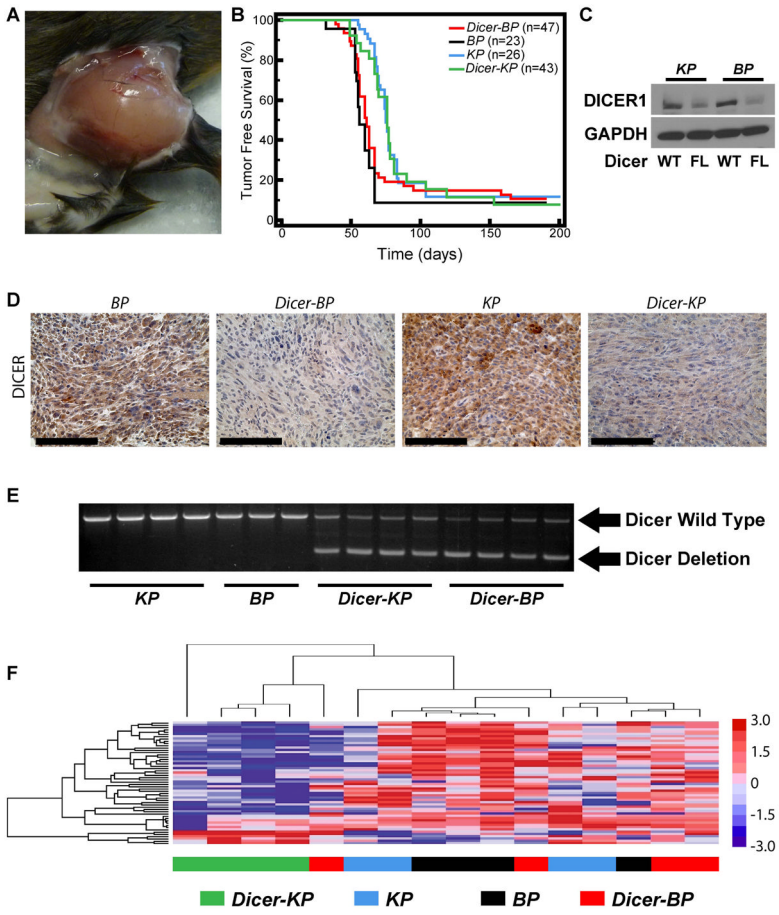

Figure 2.

Deletion of one allele of Dicer alters miRNA expression in an oncogene dependent manner. *Dicer-KP* and *Dicer-BP* mice develop (A) sarcomas at the site of injection with (B) similar kinetics to *KP* and *BP* littermate controls. (C) Decreased Dicer protein levels by western blot in sarcoma cells derived from primary tumors from $Dicer^{Fl/+}$ mice. (D) Decreased Dicer protein levels by IHC in primary tumors from $Dicer^{Fl/+}$ mice. (E) PCR analysis of genomic DNA from primary sarcomas in *Dicer-KP* and *Dicer-BP* mice shows efficient recombination of the $Dicer^{Fl}$ allele. (F) miRNA profiling of mature miRNAs by taqman PCR array demonstrates decreased miRNA expression in sarcomas from *Dicer-KP* mice.

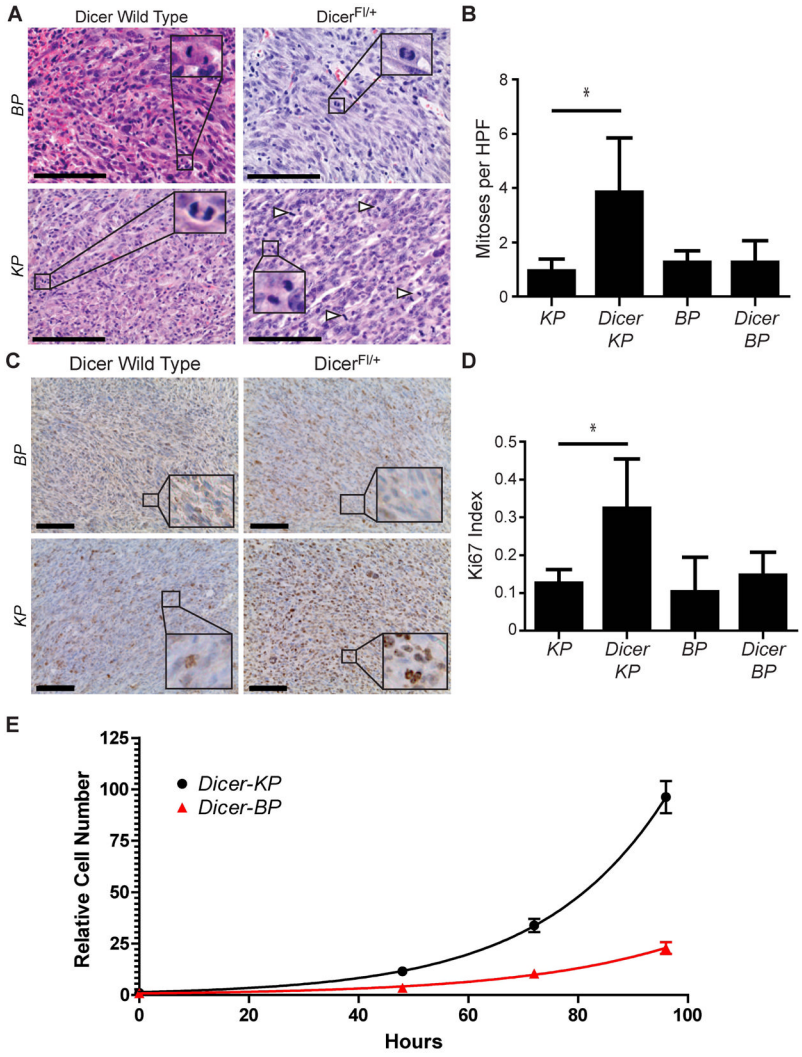

Figure 3.

Deletion of one allele of Dicer alters tumor proliferation in an oncogene dependent manner. (A,B) Proliferation of sarcomas as measured by mitoses per high powered field (A, arrowheads denote additional mitotic figures in a field) was increased in sarcomas from *Dicer-KP* mice, but proliferation was not increased in sarcomas from *Dicer-BP* mice. (C,D) Similarly, staining for the proliferation marker Ki67 showed increased proliferation only in sarcomas from *Dicer-KP* mice (n=6, each genotype). (E) Dicer-KP sarcoma cell lines proliferate at increased rates compared to Dicer-BP sarcoma cell lines. Representative data shown, all cell proliferation experiments were repeated three times and verified in a second independent cell line of each genotype. Scale bars represent 100 microns, error bars represent (B,D) standard error or (E) standard deviation from the mean, * represent $p \leq 0.01$ by two-tailed T-test.

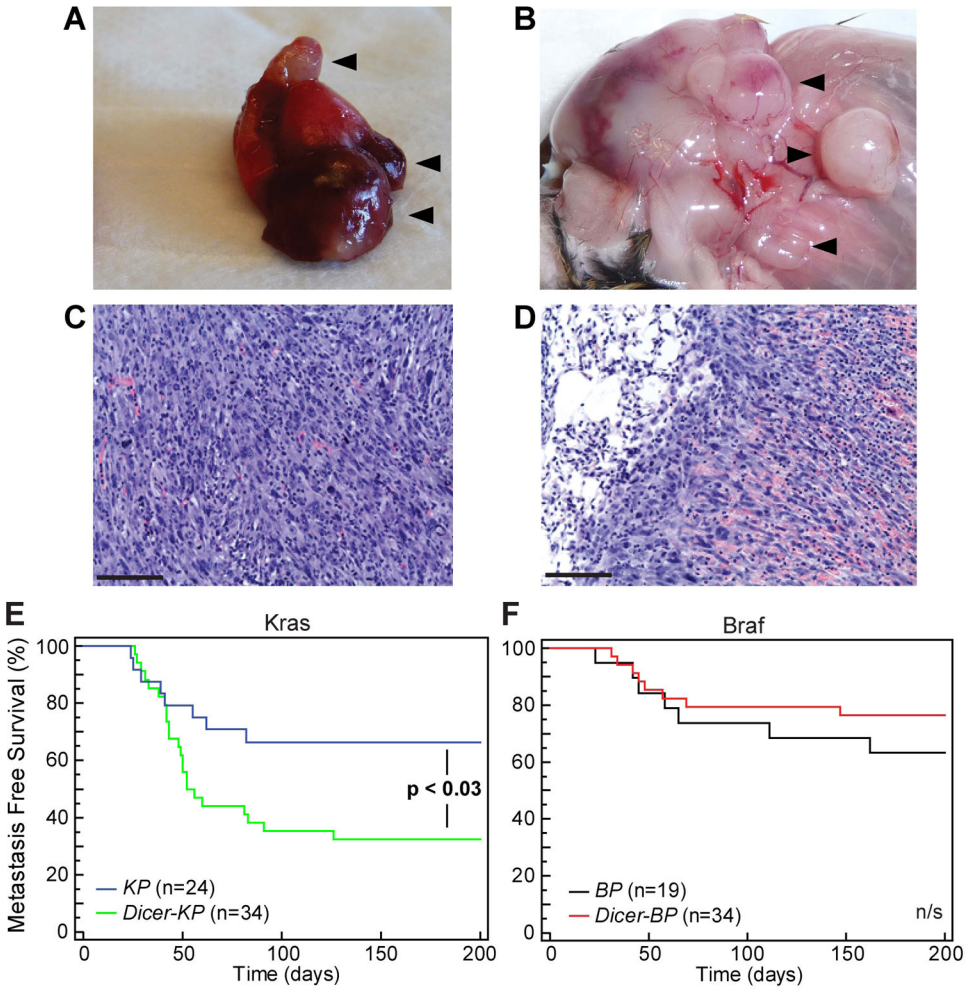

Figure 4.

Dicer haploinsufficiency promotes metastasis in an oncogene dependent manner. Sarcomas from *Dicer-KP* and *Dicer-BP* mice develop distant metastases to both the (A) lung and (B) body wall (metastases noted by arrow head), while the morphology of (C) primary sarcomas is maintained in (D) distant metastases. (E) Dicer haploinsufficiency enhances metastatic spread of $Kras^{G12D}$ mutant tumors with low MAPK signaling, while (F) $Braf^{V600E}$ mutant tumors with high MAPK signaling were protected from Dicer haploinsufficiency promoting metastasis. Significance determined by Kaplan-Meier analysis.