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Proliferation and Tumorigenesis of a Murine Sarcoma Cell Line in the Absence of DICER1

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SUMMARY

MicroRNAs are a class of short ~22 nucleotide RNAs predicted to regulate nearly half of all protein coding genes, including many involved in basal cellular processes and organismal development. Although a global reduction in miRNAs is commonly observed in various human tumors, complete loss has not been documented, suggesting an essential function for miRNAs in tumorigenesis. Here we present the finding that transformed or immortalized Dicer1 null somatic cells can be isolated readily in vitro, maintain the characteristics of DICER1-expressing controls and remain stably proliferative. Furthermore, Dicer1 null cells from a sarcoma cell line, though depleted of miRNAs, are competent for tumor formation. Hence, miRNA levels in cancer may be maintained in vivo by a complex stabilizing selection in the intratumoral environment.

INTRODUCTION

MicroRNAs (miRNAs) are short ~22 nucleotide RNAs that comprise an essential class of regulators predicted to repress over half of all genes posttranscriptionally (Bartel, 2009; Friedman et al., 2009). Consistent with computational predictions of widespread targeting, they have been implicated experimentally in a variety of fundamental cellular processes such as cell cycle (Wang et al., 2008), apoptosis (Chivukula and Mendell, 2008), and differentiation (Herranz and Cohen, 2010; Stefani and Slack, 2008). Given these broad roles, the relationship between miRNAs and cancer is understandably complex. At the level of individual miRNAs, either gains or losses may promote tumor formation. However, analysis of global miRNA levels in tumors suggests a surprisingly unidirectional relationship, with multiple human tumors showing decreased miRNA content (Gaur et al., 2007; Lu et al., 2005). In some cases, this downregulation may be directly achieved by decreased expression of DICER1 and DROSHA, key processing enzymes of miRNA production (Lin et al., 2010; Martello et al., 2010; Torres et al., 2011) or mutations in their binding partners (Melo et al., 2009).

Despite these trends toward decreased miRNA expression, a number of observations suggest that miRNAs may in fact be important for a variety of tumor types. For instance, although heterozygous somatic mutations in DICER1 can be found in tumor genotyping atlases, homozygous loss has not been reported in these databases (Kumar et al., 2009). Similarly, in rare cases of heterozygous germine DICER1 mutations,

Significance
The nearly global decrease in miRNAs observed across a range of human tumors suggests that restoration of miRNA levels may have a valuable therapeutic role. Here we report that some tumor cells devoid of miRNA activity are viable and form tumors under certain conditions. However, the failure to detect human tumors with complete loss suggests that the opposite approach, namely a further decrease in these levels, may surprisingly also be beneficial. We explore this alternative in a well-characterized sarcoma model, demonstrating that miRNA depletion can indeed inhibit tumor growth rates through reduced proliferation and increased cell death. These findings suggest that the targeted inhibition of miRNA pathway elements, particularly DICER1, may be a potential therapy for the treatment of cancer.
the pleuropulmonary blastomas to which patients are predisposed retain an intact DICER1 allele in tumor tissue (Hill et al., 2009). Somatic point mutations in DICER1 associated with nonepithelial ovarian cancers are hypomorphic, likely resulting in expression of a full-length protein but in loss of some specific miRNAs and retention of others, further suggesting a requirement for DICER1 and miRNA expression in tumors (Heravi-Moussavi et al., 2012). Mouse models of Dicer1 loss in cancer also suggest an advantage of retaining miRNA regulation. In a mouse model of Dicer1 deletion in the liver, tumors emerge several months after deletion following a period of hepatic repopulation by Dicer1-intact “escapers” (Sekine et al., 2009). In Dicer1-conditional mouse models of either soft tissue sarcoma or lung adenocarcinoma, haploinsufficiency of Dicer1 promotes tumor development but homozygous loss of Dicer1 is not observed (Kumar et al., 2009). Similarly, in both an Eμ-myc lymphoma model and a retinoblastoma model, viable tumors could not be identified following homozygous Dicer1 deletion (Arrate et al., 2010; Lambertz et al., 2010). These studies suggest that complete Dicer1 loss and the subsequent misregulation of gene expression are highly deleterious to tumor development.

RESULTS

Dicer1 Null Cells Derived from a Mouse Sarcoma Proliferate Indefinitely In Vitro

Previously, we generated Dicer1-homozygous tumors by injection of Adeno-Cre virus into the hindlimbs of KrasG12D;Trp53−/−;Dicer1−/− mice. The resultant tumors always retained at least one conditional Dicer1 allele (Kumar et al., 2009). From these KrasG12D;Trp53−/−;Dicer1−/− tumors, we established sarcoma cell lines and deleted the remaining allele of Dicer1 by transducing the cells with a retroviral construct encoding MSCV.CreERT2.puro and then activating recombination in vitro with tamoxifen treatment (Figure 1A). A genotyping time course indicated efficient homozygous deletion (Figure S1A available online). After multiple passages, however, genotyping PCR indicated the outgrowth of heterozygous recombinants. To better understand how cancer cells respond to loss of miRNA expression, we characterized the effects of homozygous deletion of Dicer1-conditional alleles on the tumorigenicity of an established line of murine sarcoma cells and on the cellular phenotype of immortalized murine mesenchymal stem cells (MSCs).

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To prevent the preferential outgrowth of DICER1-expressing cells, we isolated monoclonal populations by plating low-density cultures immediately after a 24 hr treatment with tamoxifen. The resulting clones appeared at comparable frequencies in tamoxifen-treated and control cultures, and were also morphologically similar to the parental cell lines. Genotyping PCR indicated that the majority of isolated clones had deleted the second allele of Dicer1 (Figure 1A). We also confirmed recombination of the conditional Dicer1 allele at the protein level by western blot against DICER1 (Figure S1B). Once a KrasG12D;Trp53−/−;Dicer1−/− clonal line was established, we did not observe outgrowth of KrasG12D;Trp53−/−;Dicer1−/− cells, even after several months of continual passage. Hereafter, we will refer to the monoclonal homozygous KrasG12D;Trp53−/−;Dicer1−/− line as Dicer1−/− cells and the parental heterozygous KrasG12D;Trp53−/−;Dicer1−/− cell line as Dicer1−/+. These results suggest that sarcoma cells survive after homozygous Dicer1 deletion but have a growth disadvantage relative to cells retaining Dicer1 expression. To prevent outgrowth of Dicer1−/− sarcoma cells, all subsequent experiments were carried out with monoclonal Dicer1−/− sarcoma cell lines.

To determine whether Dicer1−/− clones lacked miRNAs, we carried out massively parallel sequencing of small RNAs (small RNA-seq) (~15–50 nucleotides in length, from Dicer1−/− and Dicer1−/− sarcoma cells. Both libraries contained comparable sequencing depths at 9.3 and 9.6 million reads, respectively. However, due to miRNA loss, sequence complexity was greater in Dicer1−/− cells, which contained 830,000 unique sequences, relative to 190,000 unique sequences in sarcoma cells. Approximately 48% of mature miRNAs detected in Dicer1−/− cells became undetectable in Dicer1−/− cells, whereas the remainder of miRNAs underwent a median decrease of 111-fold, confirming the global loss of mature miRNAs following Dicer1 deletion. By quantitative northern blot, miR-22 was present at ~4,000 copies per cell in Dicer1−/− sarcoma cells (Figure S1C). Based on the ratio of miRNA reads in the Dicer1−/− to Dicer1−/− small RNA-seq libraries normalized to the copy number of miR-22 in Dicer1−/− sarcoma cells, miR-22 is present at fewer than 10 copies per cell in Dicer1−/− sarcoma cell. Similarly, based on normalization to miR-22, other abundant miRNAs such as individual let-7 family members are also expressed at fewer than ten copies per cell in Dicer1−/− cells, as compared to several thousand in Dicer1−/− cells (Figure 1B). miR-451, a DICER1-independent miRNA processed by Ago2 and expressed abundantly in red blood cells, is not detectable in Dicer1−/− sarcoma cells and is present at extremely low levels (0.4 copies/cell) in Dicer1−/− sarcoma cells (Cheloufi et al., 2010; Cifuentes et al., 2010). These results indicate near-complete loss of miRNAs upon Dicer1 deletion.

In Dicer1−/− cells, the five most abundant miRNAs were miR-22 (13%), let-7i (7%), let-7a (7%), let-7c (7%), and miR-21 (6%) (Table S1). Collapsing miRNAs by seeds, the dominant heptamer seed sequence corresponds to let-7, accounting for 31.6% of all miRNA reads. Let-7 dominance has been observed in other somatic tissues, such as embryonic fibroblasts and neural precursors (Marson et al., 2008). In addition, we observed miRNAs associated with tissue-specific expression and function, such as kidney-specific miR-196a and -196b (1.2% combined) (Landgraf et al., 2007) and miR-96 (2.2%), implicated in progressive hearing loss (Lewis et al., 2009), suggesting broader regulatory roles for these short RNAs. Reads from the miR-290-295 cluster, specific to embryonic stem cells, were negligible in number, distinguishing these cells as somatic. In total, our results establish let-7 as the dominant seed in Dicer1−/− sarcoma cells and confirm the loss of mature miRNAs following Dicer1 deletion.

As a confirmation of these sequencing results, we performed northern analysis for let-7g, miR-16, and miR-17, all detected abundantly in our Dicer1−/− sequencing library. In contrast to Dicer1−/− cells, Dicer1−/− cells showed an absence of mature miRNAs and concomitant accumulation of precursors (Figure 1C). Luciferase reporters containing six bulged sites for let-7g or one perfect site for either miR-16 or miR-17 were also derepressed 3- to 6-fold, consistent with functional loss (Figure 1D). To evaluate proliferative differences, we measured doubling times for each genotype (Figure 1E). Dicer1−/− cells divided more slowly (~15 hr) than the Dicer1−/− controls (~12 hr), but without obvious senescence or onset of crisis. Dicer1−/− sarcoma cells exhibited a delay in G1 phase relative to Dicer1−/− sarcoma cells (Figure 1F). Additionally, the Dicer1−/− sarcoma cells exhibited elevated levels of apoptosis (Figure 1G).

**Dicer1−/− Sarcoma Cells Retain Tumorigenicity In Vivo**

Our findings indicate that genetic ablation of Dicer1 is tolerated in mouse sarcoma cells in vitro. However, in vivo mouse models and human patient data suggest that homozygous deletion of Dicer1 is not tolerated in tumors. To test whether proliferative defects in homozygous Dicer1-deleted tumors, and subsequent loss through competition in vivo by DICER1-expressing cells, account for these differences, we carried out tumor formation assays. Upon subcutaneous injection of 1 × 10⁶ cells into the flanks of immune-compromised mice, Dicer1−/− cells were indeed tumorigenic, forming tumors at 7/18 sites within 24 days, as compared to 4/8 sites by day 14 for the original Dicer1−/− strain. To better evaluate the difference in tumor formation kinetics, we repeated this injection experiment with 2.5 × 10⁴ cells. At this lower cell number, Dicer1−/− sarcoma cells began to develop tumors in ~45 days, as compared to 22 days for the parental Dicer1−/− sarcoma cell line (Figure 2A). Pathologic analysis of either Dicer1−/− or Dicer1−/− tumors identified both as high grade sarcomas with pleomorphic nuclei and abnormal mitoses, consistent with previous reports of KRAS-driven sarcoma models (Figure 2B) (Kirsch et al., 2007; Kumar et al., 2007). Sample genotypes could not be readily distinguished in a blinded analysis.

We also performed syngeneic injections into immunocompetent C57Bl6/SV129 F1 mice. As before, the rate was slower than the parental Dicer1−/− line, with the first tumors appearing 7 days after injection of the Dicer1−/− cells and 21 days after injection of the Dicer1−/− cells (Figure S2). Thus, the absence of DICER1 impairs but does not preclude tumor formation, even in an immunocompetent background.

Although the sarcoma cells at the time of the injection were Dicer1−/−, it is possible that in vivo selection resulted in outgrowth of contaminating Dicer1−/− cells. Therefore, we...
genotyped DNA prepared from primary tumor tissue and confirmed a significant recombinant band corresponding to the injected Dicer1−/− cells with an accompanying background wild-type band contributed by contaminating host tissue (Figure 2C). Northern analysis of primary tumors revealed accumulation of precursors as well as significant but incomplete depletion of mature miRNAs (Figure 2D).

To test if the residual miRNAs were a result of contaminating wild-type tissue, we generated cell lines from these tumors. PCR genotyping confirmed a depletion of the wild-type tissue during this process (Figure 2E). By northern blotting, the level of mature miRNAs was lower than the detection limit of the blot, and this was again accompanied by enrichment in the pre-miRNA (Figure 2F). The residual mature miRNA observed is likely due to host tissue contamination, as evidenced by the greatest miRNA signal in the tumor sample showing the greatest wild-type contaminant band by PCR (Figure 2E) and northern (F) analysis following one round of in vitro passage of secondary tumors. In (C–F), each sample ID contains a prefix identifying the injected sarcoma cell clone followed by a suffix identifying the tumor replicate (e.g., sample 1-3 corresponds to clone 1 and tumor replicate 3).

See also Figure S2.

Mesenchymal Stem Cells Were Generated as an Alternative Model of Somatic Dicer1 Deletion

The viability of Dicer1 null sarcoma cells, which lack TRP53 and express oncogenic KRAS, may be a function of the strong oncogenic background required for rapid in vivo growth or may require additional genetic alterations that occur during tumor formation. Therefore, we tested whether Dicer1 loss could be tolerated in a defined immortalized cell model. Because sarcomas are thought to be mesenchymal in origin (Clark et al., 2005), we turned to mesenchymal stem cells (MSCs), a multipotent population of cells that can differentiate into osteoblasts, chondrocytes, adipocytes, or myocytes (Pittenger et al., 1999).

From a 1-year-old adult Dicer1f/f mouse, we prepared a primary culture of MSCs that was then immortalized with a retroviral vector encoding SV40 large T-antigen (Figure 3A). Individual clones were isolated and analyzed by flow cytometry to confirm the expression of CD49e and CD106 (Figure 3B, left panels), surface markers associated with MSCs (Pittenger, 2008), and the absence of CD31, specific to endothelial cells, and CD45, a marker of hematopoietic stem cells (data not shown).

To delete Dicer1, we carried out Adeno-Cre-GFP infection and sorted the infected cells by GFP. This protocol enriched for Dicer1−/− cells, as seen by the predominance of the deletion-specific PCR product 6 days after sorting (Figure 3A). This signal was accompanied by loss of Dicer1 protein (Figure S3B), as well as a decrease in mature miRNA levels by qPCR (Figure S3C) and northern blot at day 7 (Figure S3D). However, as observed for the sarcoma cells, additional passage
Figure 3. Derivation and Characterization of Dicer1−/− Mesenchymal Stem Cells

(A) Schematic of MSC preparation. Primary MSC cultures were prepared from the tibia, femur, and pelvic bones of a 1-year-old Dicer1f/f mouse. The primary cells were then infected with retrovirus encoding SV40 large T-antigen. Monoclonal cultures were then isolated, infected with Adeno-Cre-GFP, sorted by FACS for GFP-positive cells, and plated at low density to isolate Dicer1-recombined clones.

(B) Cell surface marker expression in Dicer1f/f (left) and Dicer1f/−/− (right) MSCs. Cells were analyzed by flow cytometry with antibodies against CD49e and CD106.

(C) PCR genotyping of clonally isolated Dicer1f/f or Dicer1f/−/− MSCs. Clones 6.8 and 6.9 (lanes 3, 4) were derived from parental clone 6 (lane 2), and clones 12.2 and 12.4 (lanes 6, 7) were derived from parental clone 12 (lane 5). PCR genotyping of a Dicer1f/f sarcoma cell line was used as a heterozygous control (lane 1).

(D) Expression of miRNAs in Dicer1f/f and Dicer1f/−/− MSCs. Total RNA was analyzed with a QIAGEN miScript qPCR assay for let-7a, miR-24, -26, and -31. A representative qPCR experiment is shown. Error bars represent standard deviation.

(E) Luciferase reporter assay for let-7g. The reporter contains six bulged sites. Targeted Renilla luciferase reporters were normalized to nontargeted firefly luciferase reporters. Renilla/firefly luciferase expression was normalized to expression in the Dicer1f/f MSC line.

(F) Proliferation assay.

(G) Cell cycle distribution determined by BrdU labeling.

(H) Apoptosis determined by caspase-3 cleavage assay. Error bars represent SEM (E–H). See also Figure S3.
led to a decrease in the deletion-specific PCR band (Figure S3A), suggesting outgrowth of DICER1-expressing cells.

To prevent competition by cells retaining Dicer1, we repeated the strategy used for the sarcoma cell lines and isolated clones. MSCs were infected with Adeno-Cre-GFP, sorted for GFP 2 days later, then plated at low-density after an additional 10 days. Clones were then isolated, expanded, and PCR genotyped to confirm recombination (Figure 3C). Of these clones, a majority (55%) had undergone homozygous deletion of Dicer1, indicating that immortalized MSCs can readily tolerate loss of Dicer1. Dicer1−/− clones were stably proliferative and remained Dicer1 null after multiple passages (>3 months) as determined by PCR genotyping (data not shown). All subsequent experiments were carried out with a monoclonal Dicer1−/− MSC line. Following loss of Dicer1, we observed an ~100-fold reduction of miRNA expression by qPCR analysis of abundant miRNAs (let-7a, miR-24, -26, and -31) (Figure 3D). We also observed ~5-fold derepression of a let-7g luciferase reporter in Dicer1−/− cells (Figure 3E). Dicer1−/− MSCs exhibited a proliferative lag, with a doubling time of ~20 hr relative to ~14 hr for Dicer1+/+ cells (Figure 3F). Dicer1−/− MSCs also exhibited a G1 delay in cell cycle (Figure 3G) and elevated levels of basal apoptosis (Figure 3H). Notably, Dicer1−/− MSCs remained positive for CD49e and CD106 (Figure 3B, right panels) and negative for CD31 and CD45 (data not shown), suggesting a retention of cell identity in the absence of miRNAs.

**DISCUSSION**

We have carried out homozygous Dicer1 deletion in a Dicer1-null sarcoma cell line and observed resultant loss of miRNA expression by small RNA sequencing. Relative to Dicer1+/+ sarcoma cells, these miRNA-depleted cells proliferate more slowly, exhibit a cell cycle delay in G1 phase, and have a higher level of basal apoptosis. Additionally, we have generated a second in vitro model of homozygous Dicer1-deletion in murine mesenchymal stem cells, related in cell type to sarcomas, established from an adult Dicer1+/− mouse and immortalized in vitro. Similar to the sarcoma model, MSCs that had undergone homozygous Dicer1 deletion were readily isolatable but exhibited a reduction in proliferation, a delay in G1, and an increase in basal apoptosis.

Strikingly, Dicer1−/− sarcoma cells retain the ability, upon transplant, to form tumors in both immunocompromised and immunocompetent recipient mice, albeit at slower rates relative to Dicer1+/+ sarcoma cells. We present three compelling lines of evidence excluding the possibility that these tumors arose from contaminating Dicer1+/+ cells:

1. Clonal Dicer1−/− sarcoma cells were used for the tumor injection studies to preclude the possibility of Dicer1+/+ outgrowth.
2. Tumors derived from Dicer1−/− injections exhibit strong PCR genotyping bands for (1) recombinant Dicer1, and (2) wild-type Dicer1, derived from host wild-type tissue associated with the tumors. In contrast, the control tumor derived from Dicer1+/+ cells exhibits a third PCR band, representing unrecombined floxed Dicer1. This stark difference in genotype demonstrates that the tumors that arose from Dicer1−/− injections are composed predominantly of Dicer1−/− cells.
3. In vitro passage of cells derived from Dicer1−/− tumors resulted in depletion of wild-type primary host tissue and outgrowth of Dicer1−/− cells, as determined by PCR genotyping, and concomitant depletion in miRNA signal by northern blot. Furthermore, in most tumor samples, miRNAs were present at levels less than one-sixteenth of the Dicer1+/− control, likely an overestimate given the low level of contaminating host-derived cells.

Our results stand in stark contrast to many published reports. The failure to observe Dicer1 null tumors from Dicer1−/−-transplanted cells in vivo mouse models of cancer have been interpreted to suggest that DICER1 and miRNAs are essential for tumor formation and, furthermore, may be required for tumor cell survival. Contrary to these observations, our results indicate that miRNAs are not essential for in vitro survival or proliferation. In vivo, Dicer1 null sarcoma cells, though depleted of miRNAs, are competent for tumor development. Furthermore, our observation of tumor growth in an immunocompetent background demonstrates that miRNAs are not essential for escape from immune surveillance. In addition, the histological resemblance of Dicer1−/− sarcomas to Dicer1+/− sarcomas, as well as the retention of cell type-specific cell surface markers in Dicer1−/− MSCs, suggest that cellular identity is largely retained despite loss of miRNAs. Given the high frequency with which Dicer1 null cells can be isolated in both the sarcoma and MSC models, secondary mutational or other low frequency events beyond the initial immortalization are likely not necessary to tolerate DICER1 loss.

Based on the outgrowth of DICER1-expressing cells in vitro relative to Dicer1−/− cells, and the proliferative delay observed in monoclonal Dicer1−/− cells, we conclude that the absence of Dicer1 null cells in previously characterized mouse models of cancer is due in part to the preferential outgrowth, in vivo, of cells expressing DICER1. Notably, tumor genotyping analyses in published studies have typically been carried out in whole tumor samples and, as such, do not exclude the possibility that Dicer1 null cells comprise a subpopulation of the samples.

Furthermore, factors additional to proliferative capacity may contribute to the preferential outgrowth of DICER1-expressing cells in vivo. The viability of Dicer1 null transformed cells in our study indicates that total miRNA loss itself, and resultant genetic misregulation, is not intrinsically catastrophic, but rather triggers secondary signals that initiate changes in proliferation or cell death. Numerous studies have reported that miRNAs mediate stress responses (Hermeking, 2007; Leung and Sharp, 2010; Leung et al., 2011a) and loss of DICER1 in embryonic stem cells results in increased sensitivity to stress (Zheng et al., 2011). Similarly, we have observed that sarcoma cells and MSCs that lack DICER1 exhibit increased apoptosis. Given the intrinsically stressful nature of the in vivo tumor environment, the retention of a miRNA-mediated stress response may provide an additional growth advantage to tumor cells that retain at least one copy of Dicer1.

Notably, inactivation of TRP53 is a common feature in both the sarcoma and MSC models presented here and may facilitate or
be required for viability in the absence of DICER1. This possibility is consistent with the observation that TRPS3 loss allows primary MEFs to bypass an immediate senescence phenotype induced by DICER1 loss (Mudhasani et al., 2008). However, TRPS3 loss alone only prolongs proliferative capacity of Dicer1−/− MEFs for a few additional passages (S.N. Jones, personal communication), suggesting that other events, such as activation of oncogenes or inactivation of additional tumor suppressor genes, are required.

In addition to expanding our understanding of DICER1 in tumorigenesis, the cell lines reported here may complement existing models such as the hypomorphic DICER1 HCT116 line, a human colorectal cancer line containing homozygous deletion of exon 5 in the DICER1 gene (Cummins et al., 2006). The availability of Dicer1−/− cancer cells will permit further characterization of somatic miRNA families through miRNA transfection and “add-back” cell culture experiments. Finally, the observations that DICER1 loss leads to a negative selective pressure in vitro and in vivo suggests that DICER1 activity could be a therapeutic target.

EXPERIMENTAL PROCEDURES

Animal Work
All animal studies were performed with protocols approved by the NIH and the Massachusetts Institute of Technology Committee for Animal Care, and were consistent with the Guide for Care and Use of Laboratory Animals, National Research Council, 1996 (Institutional Animal Welfare Assurance No. A-3125-01).

Cell Culture
Sercoma cells were generated from hindlimb injection of Adeno-Cre into KrasG12D/Trp53R172H/Dicer1−/− mice as described previously (Kirsch et al., 2007). Sarcoma cells infected with MSCV.CreER T2.puro were treated with 250 nM 4-hydroxy tamoxifen for 24 hr to recombine the floxed Dicer1 allele and plated at clonal density. Primary MSC cultures were prepared from an adult Dicer1−/− mouse using a previously described protocol (Mukherjee et al., 2008), immortalized with SV40 large T-antigen, and infected with Adeno-Cre-GFP to recombine the floxed alleles. Cells were genotyped as described previously (Calabrese et al., 2007).

Histology
Tumor-bearing animals were sacrificed with CO2 asphyxiation. Tumors were embedded in paraffin. Tumors were then sectioned and stained with hematoxylin and eosin.

Tumor Injection
A total of 2.5 × 104, 105, or 106 KrasG12D/Trp53−/− sarcoma cells of Dicer1−/− or Dicer1−/− genotype were suspended in PBS and subcutaneously injected into the flanks of Rag2−/− or C57/B6/SV129 F1 mice. Tumors were measured by time calipers and volumes were assessed as described previously (Sage et al., 2003). DNA, RNA, and histological sections were prepared from tumors. Some tumors were trypsinized and replated for the development of secondary cell lines as described above.

Small RNA Northern and Cloning
Small RNA northern blots were performed using 20 μg total RNA on a 15% denaturing polyacrylamide gel. Following semi-dry transfer to a Hybond-N+ membrane, a DNA oligo probe for glutamine tRNA or LNA probes for let-7, mir-16, and mir-17 were used for visualization. Small RNA sequencing from sarcoma lines was carried out as described previously (Leung et al., 2011b). The sequencing data are available under Gene Expression Omnibus accession GSE34825.

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A.R., A.M.G., and M.S.K. designed and performed the experiments. A.B. performed the informatics for the sequencing data. C.C. and V.L. performed experiments. A.R., A.M.G., and P.A.S. wrote the paper, J.A.L., and P.A.S. provided supervision and assisted with manuscript preparation. All authors reviewed and approved the manuscript.

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SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2012.04.037.

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A.R., A.M.G., and M.S.K. designed and performed the experiments. A.B. performed the informatics for the sequencing data. C.C. and V.L. performed experiments. A.R., A.M.G., and P.A.S. wrote the paper, J.A.L., and P.A.S. provided supervision and assisted with manuscript preparation. All authors reviewed and approved the manuscript.

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