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**Citation:** Ravi, Arvind, Allan M. Gurtan, Madhu S. Kumar, Arjun Bhutkar, Christine Chin, Victoria Lu, Jacqueline A. Lees, Tyler Jacks, and Phillip A. Sharp. "Proliferation and Tumorigenesis of a Murine Sarcoma Cell Line in the Absence of DICER1." *Cancer Cell* 21, no. 6 (June 2012): 848–855. © 2012 Elsevier Inc.

**As Published:** <http://dx.doi.org/10.1016/j.ccr.2012.04.037>

**Publisher:** Elsevier

**Persistent URL:** <http://hdl.handle.net/1721.1/91549>

**Version:** Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

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

Arvind Ravi,<sup>1,3,5</sup> Allan M. Gurtan,<sup>4,5</sup> Madhu S. Kumar,<sup>1,6</sup> Arjun Bhutkar,<sup>4</sup> Christine Chin,<sup>1</sup> Victoria Lu,<sup>1</sup> Jacqueline A. Lees,<sup>1,4</sup> Tyler Jacks,<sup>1,2,4</sup> and Phillip A. Sharp<sup>1,4,\*</sup>

<sup>1</sup>Department of Biology

<sup>2</sup>Howard Hughes Medical Institute, Department of Biology  
Massachusetts Institute of Technology, Cambridge, MA 02139, USA

<sup>3</sup>Harvard-MIT Health Sciences and Technology Program, Cambridge, MA 02139, USA

<sup>4</sup>David H. Koch Institute for Integrative Cancer Research, Cambridge, MA 02139, USA

<sup>5</sup>These authors contributed equally to this work

<sup>6</sup>Present address: Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

\*Correspondence: sharppa@mit.edu

DOI 10.1016/j.ccr.2012.04.037

## 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

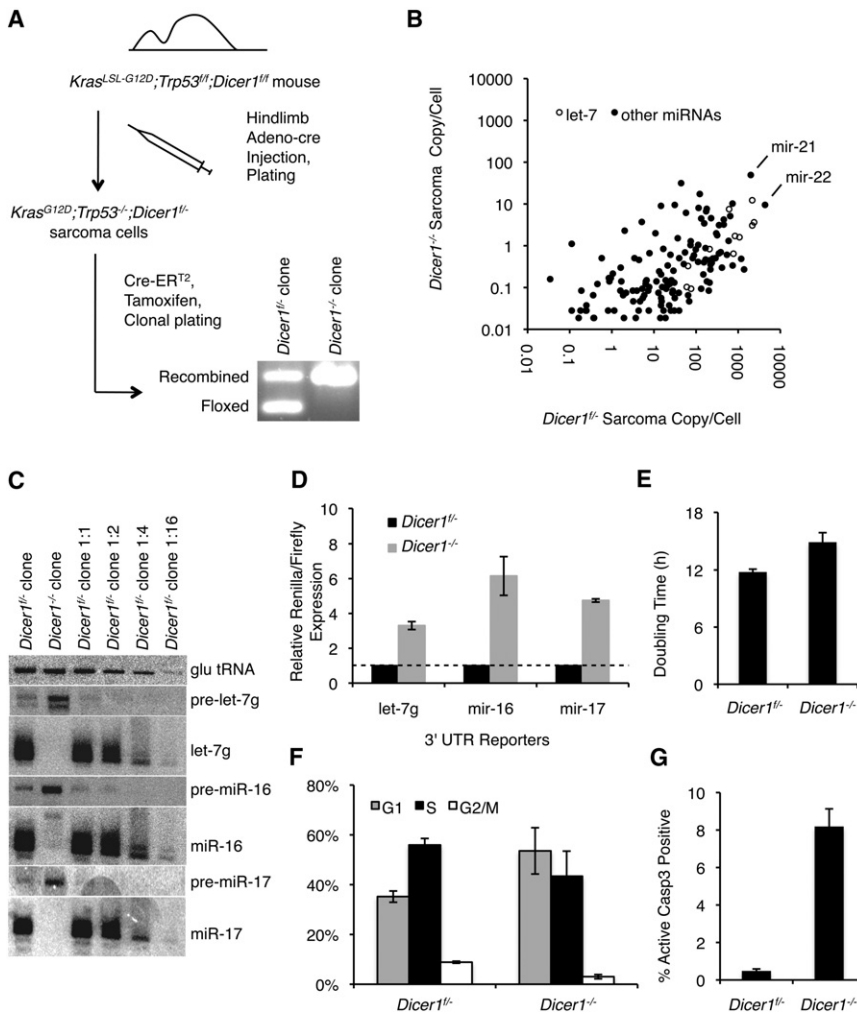
MicroRNAs (miRNAs) are short ~22 nucleotide RNAs that comprise an essential class of regulators predicted to repress over half of all genes posttranscriptionally (Bartel, 2009; Friedman et al., 2009). Consistent with computational predictions of widespread targeting, they have been implicated experimentally 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 germline *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.



**Figure 1. Characterization of *Kras*<sup>G12D</sup>; *Trp53*<sup>-/-</sup>; *Dicer1*<sup>-/-</sup> Sarcoma Cells**

(A) Derivation scheme for *Dicer1*<sup>-/-</sup> sarcoma cells. Hindlimb injection of Adeno-Cre generates *Kras*<sup>G12D</sup>; *Trp53*<sup>-/-</sup>; *Dicer1*<sup>fl/fl</sup> tumors. Clones isolated following Cre-ER integration and tamoxifen treatment were genotyped by PCR to identify *Dicer1*<sup>-/-</sup> clones.

(B) miRNA expression (copies per cell). Per cell calculations are based on relative representation of each miRNA in *Dicer1*<sup>fl/fl</sup> and *Dicer1*<sup>-/-</sup> small RNA-seq libraries, normalized to quantitative northern blot of miR-22 in *Dicer1*<sup>fl/fl</sup> cells (shown in Figure S1C). miR-21, miR-22, and let-7 family members are indicated.

(C) Northern analysis for precursor and mature miRNAs. Glutamine tRNA was used to control for loading, and a dilution series of *Dicer1*<sup>fl/fl</sup> RNA (1:1 to 1:16) is provided for quantitation.

(D) Luciferase reporter assays for abundant miRNAs. The Renilla luciferase reporter contains six bulged sites for the let-7 family, and two perfect sites for miR-16 and miR-17. Targeted Renilla luciferase reporters were normalized to non-targeted firefly luciferase reporters. Renilla/firefly luciferase expression was normalized to expression in the *Dicer1*<sup>fl/fl</sup> sarcoma cell line.

(E) Proliferation assay.

(F) Cell cycle distribution determined by BrdU labeling.

(G) Apoptosis determined by caspase-3 cleavage assay.

All error bars represent the SEM (D–G). See also Figure S1 and Table S1.

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.

labeled line of murine sarcoma cells and on the cellular phenotype of immortalized murine mesenchymal stem cells (MSCs).

**RESULTS**

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

Previously, we generated *Dicer1*-heterozygous tumors by injection of Adeno-Cre virus into the hindlimbs of *Kras*<sup>LSL-G12D</sup>; *Trp53*<sup>fl/fl</sup>; *Dicer1*<sup>fl/fl</sup> mice. The resultant tumors always retained at least one conditional *Dicer1* allele (Kumar et al., 2009). From these *Kras*<sup>G12D</sup>; *Trp53*<sup>-/-</sup>; *Dicer1*<sup>fl/fl</sup> tumors, we established sarcoma cell lines and deleted the remaining allele of *Dicer1* by transducing the cells with a retroviral construct encoding MSCV.CreER<sup>T2</sup>.puro and then activating recombination in vitro with tamoxifen treatment (Figure 1A). A genotyping time course indicated efficient homozygous recombination (Figure S1A available online). After multiple passages, however, genotyping PCR indicated the outgrowth of heterozygous cells, consistent with previous findings in both this sarcoma model and an *Eμ-myc*/*Dicer1* lymphoma model (Arrate et al., 2010; Kumar et al., 2009).

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 *Kras*<sup>G12D</sup>; *Trp53*<sup>-/-</sup>; *Dicer1*<sup>-/-</sup> clonal line was established, we did not observe outgrowth of *Kras*<sup>G12D</sup>; *Trp53*<sup>-/-</sup>; *Dicer1*<sup>fl/fl</sup> cells, even after several months of continual passage. Hereafter, we will refer to the monoclonal homozygous *Kras*<sup>G12D</sup>; *Trp53*<sup>-/-</sup>; *Dicer1*<sup>-/-</sup> line as *Dicer1*<sup>-/-</sup> cells and the parental heterozygous *Kras*<sup>G12D</sup>; *Trp53*<sup>-/-</sup>; *Dicer1*<sup>fl/fl</sup> cell line as *Dicer1*<sup>fl/fl</sup> cells. 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*<sup>fl/fl</sup> sarcoma cells, all subsequent experiments were carried out with monoclonal *Dicer1*<sup>-/-</sup> sarcoma cell lines.

To determine whether *Dicer1*<sup>-/-</sup> clones lacked miRNAs, we carried out massively parallel sequencing of small RNAs (small RNA-seq), ~15–50 nucleotides in length, from *Dicer1*<sup>fl/fl</sup> and *Dicer1*<sup>-/-</sup> 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*<sup>-/-</sup> cells, which contained 830,000 unique sequences, relative to 190,000 unique sequences in *Dicer1*<sup>fl/fl</sup> cells. Of all reads mapping to the genome with 0 or 1 mismatch, 58% correspond to mature miRNAs in *Dicer1*<sup>fl/fl</sup> cells in comparison to 0.8% in *Dicer1*<sup>-/-</sup> cells. Approximately 48% of mature miRNAs detected in *Dicer1*<sup>fl/fl</sup> cells became undetectable in *Dicer1*<sup>-/-</sup> cells, whereas the remainder of miRNAs underwent a median decrease of 111-fold, confirming the global loss of mature miRNAs with homozygous *Dicer1* loss. By quantitative northern blot, miR-22 was present at ~4,000 copies per cell in *Dicer1*<sup>fl/fl</sup> sarcoma cells (Figure S1C). Based on the ratio of miRNA reads in the *Dicer1*<sup>fl/fl</sup> to *Dicer1*<sup>-/-</sup> small RNA-seq libraries normalized to the copy number of miR-22 in *Dicer1*<sup>fl/fl</sup> sarcoma cells, miR-22 is present at fewer than 10 copies per *Dicer1*<sup>-/-</sup> 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*<sup>-/-</sup> cells, as compared to several thousand in *Dicer1*<sup>fl/fl</sup> cells (Figure 1B). miR-451, a DICER1-independent miRNA processed by Ago2 and expressed abundantly in red blood cells, is not detectable in *Dicer1*<sup>fl/fl</sup> sarcoma cells and is present at extremely low levels (0.4 copies/cell) in *Dicer1*<sup>-/-</sup> sarcoma cells (Cheloufi et al., 2010; Cifuentes et al., 2010). These results indicate near-complete loss of miRNAs upon *Dicer1* deletion.

In *Dicer1*<sup>fl/fl</sup> cells, the five most abundant miRNAs were miR-22 (13%), let-7f (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 func-

tion, 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*<sup>fl/fl</sup> sarcoma cells and confirm the loss of mature miRNAs following *Dicer1* deletion.

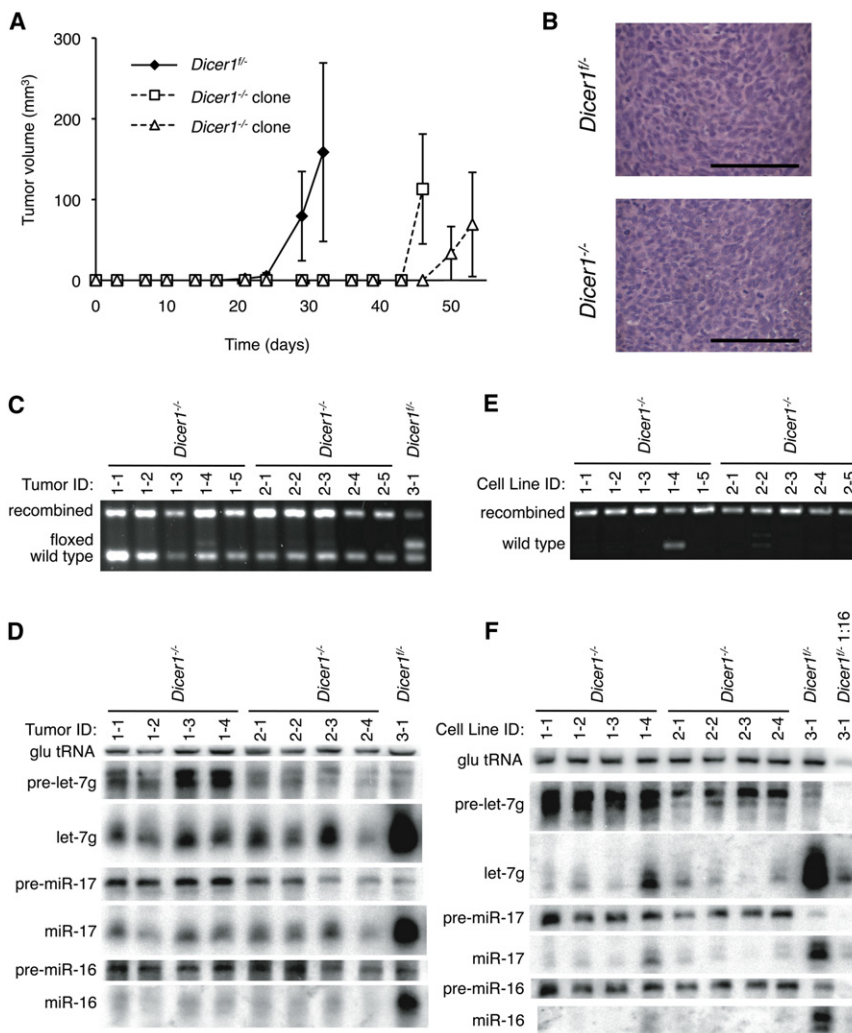
As a confirmation of these sequencing results, we performed northern analysis for let-7g, miR-16, and miR-17, all detected abundantly in our *Dicer1*<sup>fl/fl</sup> sequencing library. In contrast to *Dicer1*<sup>fl/fl</sup> cells, *Dicer1*<sup>-/-</sup> cells showed an absence of mature miRNAs and 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*<sup>-/-</sup> cells divided more slowly (~15 hr) than the *Dicer1*<sup>fl/fl</sup> controls (~12 hr), but without obvious senescence or onset of crisis. *Dicer1*<sup>-/-</sup> sarcoma cells exhibited a delay in G1 phase relative to *Dicer1*<sup>fl/fl</sup> sarcoma cells (Figure 1F). Additionally, the *Dicer1*<sup>-/-</sup> sarcoma cells exhibited elevated levels of apoptosis (Figure 1G).

### ***Dicer1*<sup>-/-</sup> 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  $1 \times 10^6$  cells into the flanks of immune-compromised mice, *Dicer1*<sup>-/-</sup> 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*<sup>fl/fl</sup> strain. To better evaluate the difference in tumor formation kinetics, we repeated this injection experiment with  $2.5 \times 10^4$  cells. At this lower cell number, *Dicer1*<sup>-/-</sup> sarcoma cells began to develop tumors in ~45 days, as compared to 22 days for the parental *Dicer1*<sup>fl/fl</sup> sarcoma cell line (Figure 2A). Pathologic analysis of either *Dicer1*<sup>-/-</sup> or *Dicer1*<sup>fl/fl</sup> 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*<sup>fl/fl</sup> line, with the first tumors appearing 7 days after injection of the *Dicer1*<sup>fl/fl</sup> cells and 21 days after injection of the *Dicer1*<sup>-/-</sup> 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*<sup>-/-</sup>, it is possible that in vivo selection resulted in outgrowth of contaminating *Dicer1*<sup>fl/fl</sup> cells. Therefore, we



**Figure 2. Tumorigenesis of *Kras*<sup>G12D</sup>; *Trp53*<sup>-/-</sup>; *Dicer1*<sup>-/-</sup> Sarcoma Cells in Transplant Assays**

(A) Injection of  $2.5 \times 10^4$  *Dicer1*<sup>fl/fl</sup> and *Dicer1*<sup>-/-</sup> sarcoma cells into the flanks of nude mice. Error bars represent the SEM.

(B) Hematoxylin and eosin section of *Dicer1*<sup>fl/fl</sup> and *Dicer1*<sup>-/-</sup> tumors. In (C–F) each lane represents an independent tumor derived from one injection of the indicated *Dicer1*<sup>-/-</sup> sarcoma cell line. Scale bar represents 100  $\mu$ m.

(C) PCR genotyping of *Dicer1*<sup>-/-</sup> tumors. Recombined and floxed bands are derived from the injected tumor cells, whereas wild-type bands derive from host tissue.

(D) Northern analysis of tumor tissue derived from sarcoma injections.

(E and F) PCR (E) 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

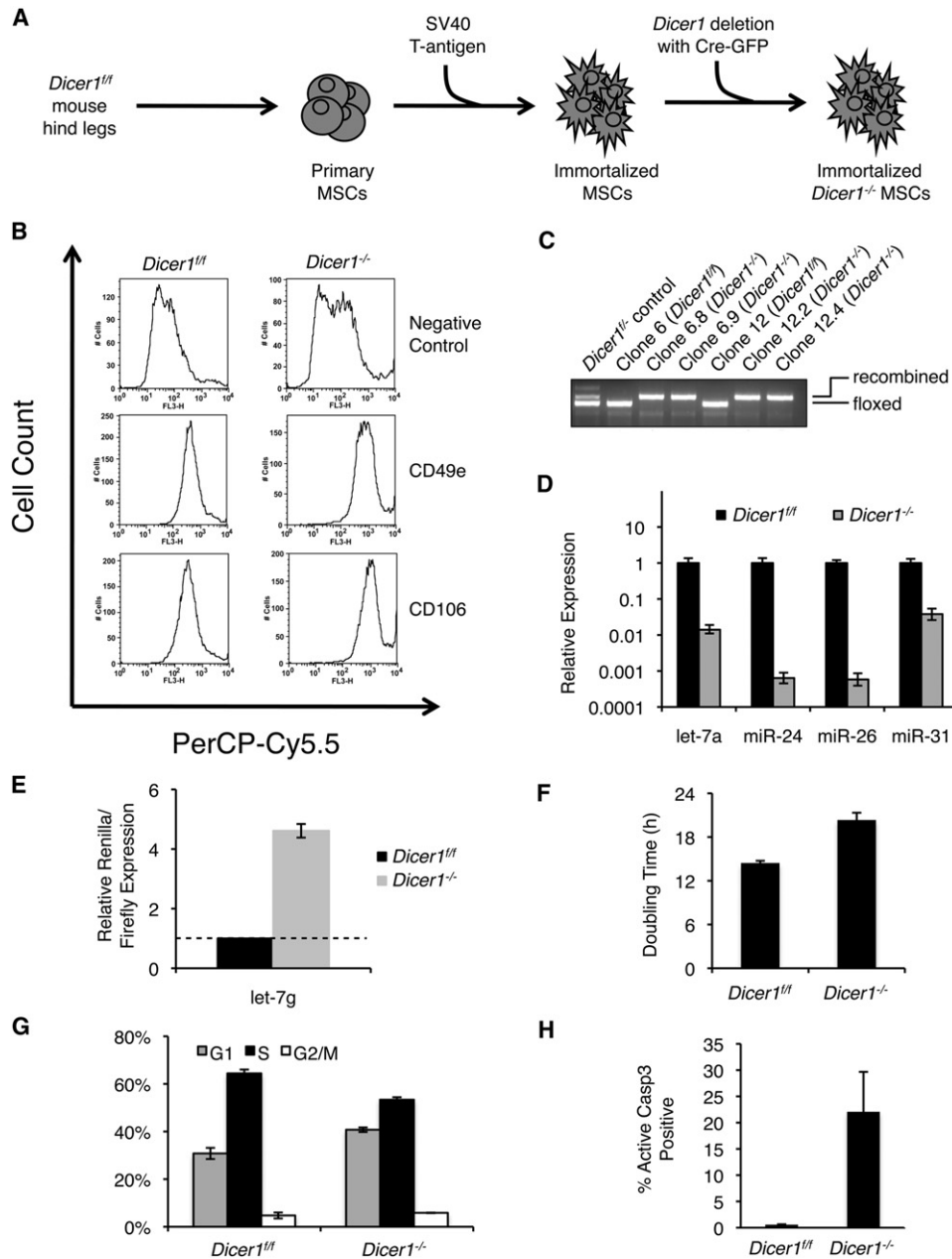
genotyped DNA prepared from primary tumor tissue and confirmed a significant recombined band corresponding to the injected *Dicer1*<sup>-/-</sup> cells with an accompanying background wild-type band contributed by contaminating host tissue (Figure 2C). Northern analysis of primary tumors revealed accumulation 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 (Figures 2E and 2F). Thus, injected *Dicer1*<sup>-/-</sup> cells survived and proliferated in vivo without recovery of miRNA processing. The earlier in vitro results extend to an in vivo setting, with sarcoma cells retaining the capacity to form phenotypically similar tumors, albeit more slowly, in the absence of DICER1 and miRNAs.

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 *Dicer1*<sup>fl/fl</sup> 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*<sup>-/-</sup> cells, as seen by the predominance of the deletion-specific PCR product 6 days after sorting (Figure S3A). 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*<sup>-/-</sup> 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 *Dicer1*<sup>fl/fl</sup> 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 *Dicer1*<sup>fl/fl</sup> (left) and *Dicer1*<sup>-/-</sup> (right) MSCs. Cells were analyzed by flow cytometry with antibodies against CD49e and CD106.

(C) PCR genotyping of clonally isolated *Dicer1*<sup>fl/fl</sup> or *Dicer1*<sup>-/-</sup> 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 *Dicer1*<sup>fl/fl</sup> sarcoma cell line was used as a heterozygous control (lane 1).

(D) Expression of miRNAs in *Dicer1*<sup>fl/fl</sup> and *Dicer1*<sup>-/-</sup> 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 *Dicer1*<sup>fl/fl</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> cells (Figure 3E). *Dicer1*<sup>-/-</sup> MSCs exhibited a proliferative lag, with a doubling time of ~20 hr relative to ~14 hr for *Dicer1*<sup>+/+</sup> cells (Figure 3F). *Dicer1*<sup>-/-</sup> MSCs also exhibited a G1 delay in cell cycle (Figure 3G) and elevated levels of basal apoptosis (Figure 3H). Notably, *Dicer1*<sup>-/-</sup> 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*-conditional KRAS-activated, *Trp53* null sarcoma cell line and observed resultant loss of miRNA expression by small RNA sequencing. Relative to *Dicer1*<sup>+/+</sup> 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*<sup>+/+</sup> 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*<sup>-/-</sup> sarcoma cells retain the ability, upon transplant, to form tumors in both immunocompromised and immunocompetent recipient mice, albeit at slower rates relative to *Dicer1*<sup>+/+</sup> sarcoma cells. We present three compelling lines of evidence excluding the possibility that these tumors arose from contaminating *Dicer1*<sup>+/+</sup> cells:

- (1) Clonal *Dicer1*<sup>-/-</sup> sarcoma cells were used for the tumor injection studies to preclude the possibility of *Dicer1*<sup>+/+</sup> outgrowth.
- (2) Tumors derived from *Dicer1*<sup>-/-</sup> injections exhibit strong PCR genotyping bands for (1) recombined *Dicer1*, and (2) wild-type *Dicer1*, derived from host wild-type tissue associated with the tumors. In contrast, the control tumor derived from *Dicer1*<sup>+/+</sup> cells exhibits a third PCR band, representing unrecombined floxed *Dicer1*. This stark

difference in genotype demonstrates that the tumors that arose from *Dicer1*<sup>-/-</sup> injections are composed predominantly of *Dicer1*<sup>-/-</sup> cells.

- (3) In vitro passage of cells derived from *Dicer1*<sup>-/-</sup> tumors resulted in depletion of wild-type primary host tissue and outgrowth of *Dicer1*<sup>-/-</sup> 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*<sup>+/+</sup> 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*-conditional 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*<sup>-/-</sup> sarcomas to *Dicer1*<sup>+/+</sup> sarcomas, as well as the retention of cell type-specific cell surface markers in *Dicer1*<sup>-/-</sup> 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*<sup>-/-</sup> cells, and the proliferative delay observed in monoclonal *Dicer1*<sup>-/-</sup> 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 TRP53 loss allows primary MEFs to bypass an immediate senescence phenotype induced by DICER1 loss (Mudhasani et al., 2008). However, TRP53 loss alone only prolongs proliferative capacity of *Dicer1*<sup>-/-</sup> 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*<sup>-/-</sup> 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

Sarcoma cells were generated from hindlimb injection of Adeno-Cre into *Kras*<sup>LSL-G12D</sup>;*Trp53*<sup>fl</sup>;*Dicer1*<sup>fl/fl</sup> mice as described previously (Kirsch et al., 2007). Sarcoma cells infected with MSCV.CreER<sup>T2</sup>.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*<sup>fl/fl</sup> 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 CO<sub>2</sub> asphyxiation. Tumors were isolated, fixed in 4% paraformaldehyde, transferred to 70% ethanol, and embedded in paraffin. Tumors were then sectioned and stained with hematoxylin and eosin.

### Tumor Injection

A total of 2.5 × 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> *Kras*<sup>G12D</sup>;*Trp53*<sup>-/-</sup> sarcoma cells of *Dicer1*<sup>fl/-</sup> or *Dicer1*<sup>-/-</sup> genotype were suspended in PBS and subcutaneously injected into the flanks of *Rag2*<sup>-/-</sup> or C57Bl6/SV129 F1 mice. Tumors were measured over time by calipers and volumes were assessed as described previously (Sage et al., 2000). 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 Blots 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.

## 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.

## ACKNOWLEDGMENTS

We thank members of the Sharp, Jacks, and Lees Labs for many helpful discussions. We are also specifically grateful to Margaret Ebert, Amy Seila, and Eliezer Calo for their generous contribution of reagents. We thank Allison Landman for providing assistance with mesenchymal stem cell isolation and cultures. We thank Roderick Bronson for analysis of histology slides. Massively parallel Illumina sequencing was carried out by the Biopolymers & Proteomics Core Facility in the Swanson Biotechnology Center at the David H. Koch Institute for Integrative Cancer Research at M.I.T. Bioinformatic analysis was carried out in the Bioinformatics & Computing division of the Swanson Biotechnology Center. This work was supported by a Fannie and John Hertz Foundation Fellowship (A.R.), a Leukemia and Lymphoma Society grant 5198-09 (A.M.G.), an NIH grant RO1-CA133404 (P.A.S.), an NCI grant PO1-CA42063 (P.A.S., T.J., J.A.L.), and partially by the NCI Cancer Center Support (core) grant P30-CA14051.

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., T.J., and P.A.S. provided supervision and assisted with manuscript preparation. All authors reviewed and approved the manuscript.

Received: December 25, 2011

Revised: March 7, 2012

Accepted: April 24, 2012

Published: June 11, 2012

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