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Supplemental Information

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

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Inventory of Supplemental Information

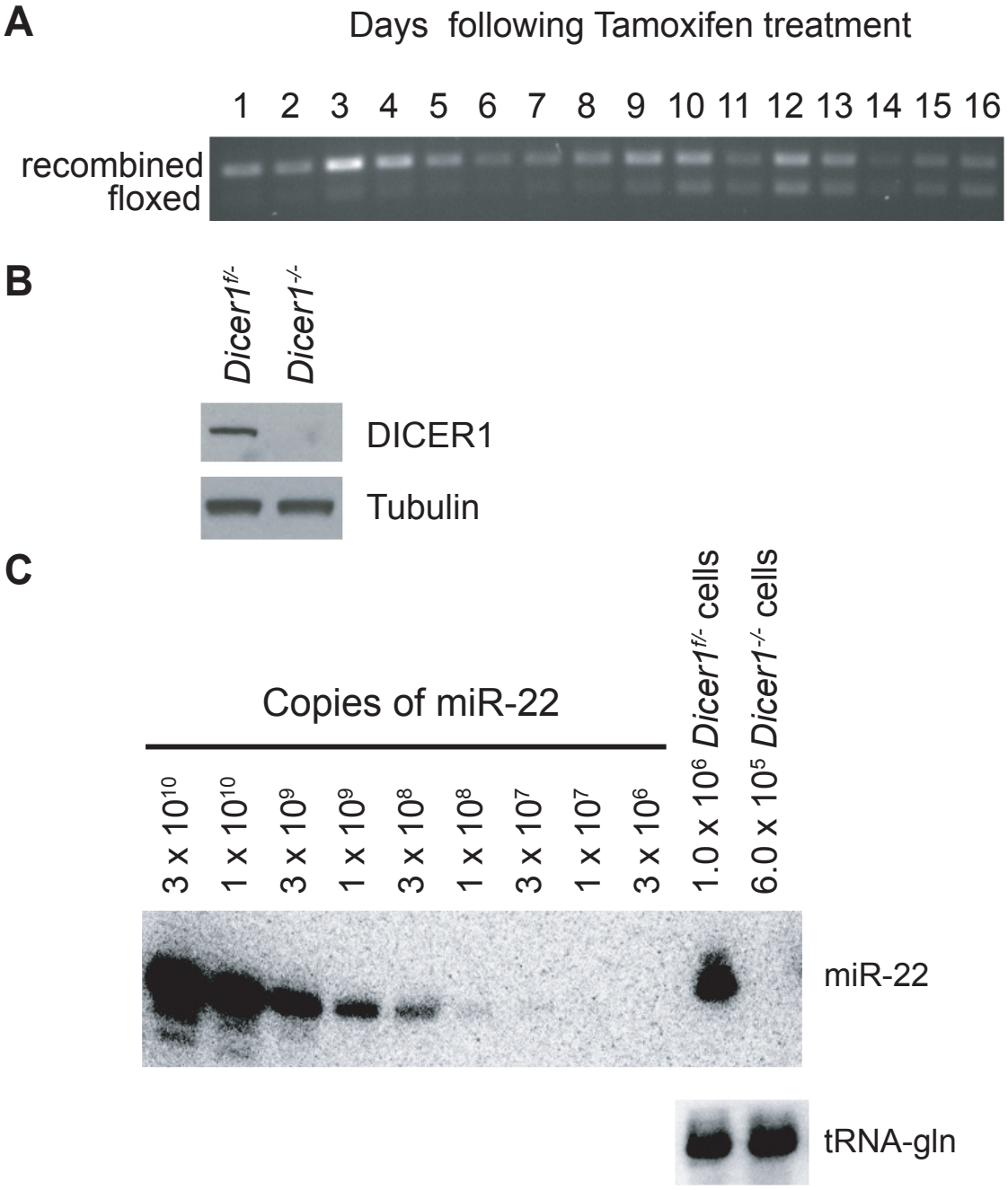

Figure S1. Related to Figure 1. Generation and characterization of *Kras^{G12D};Trp53$^{-/-}$;Dicer1$^{-/-}$* sarcoma cells. A) Time course of acute *Dicer1* deletion in *Dicer1$^{f/-}$* sarcomas. *Dicer1$^{f/-}$* sarcoma cells containing Cre-ER were treated with tamoxifen to induce excision of the remaining *Dicer1* allele, and then genotyped daily for 16 days. B) Western blot of DICER1 protein in *Dicer1$^{f/-}$* and *Dicer1$^{-/-}$* sarcomas. C) Quantitative Northern blot of miR-22. Synthetic miR-22 RNA standard was loaded with yeast carrier RNA at known copy numbers, and compared to ~20 μg of total RNA from *Dicer1$^{f/-}$* or *Dicer1$^{-/-}$* cells, corresponding to $1x10^6$ and $6x10^5$ cells, respectively. The image was analyzed with

ImageQuant™ . The experiment was carried out in duplicate. One representative blot is shown.

Table S1. Related to Figure 1. MiRNA cloning frequencies in sarcoma deep sequencing libraries. Provided as an Excel file.

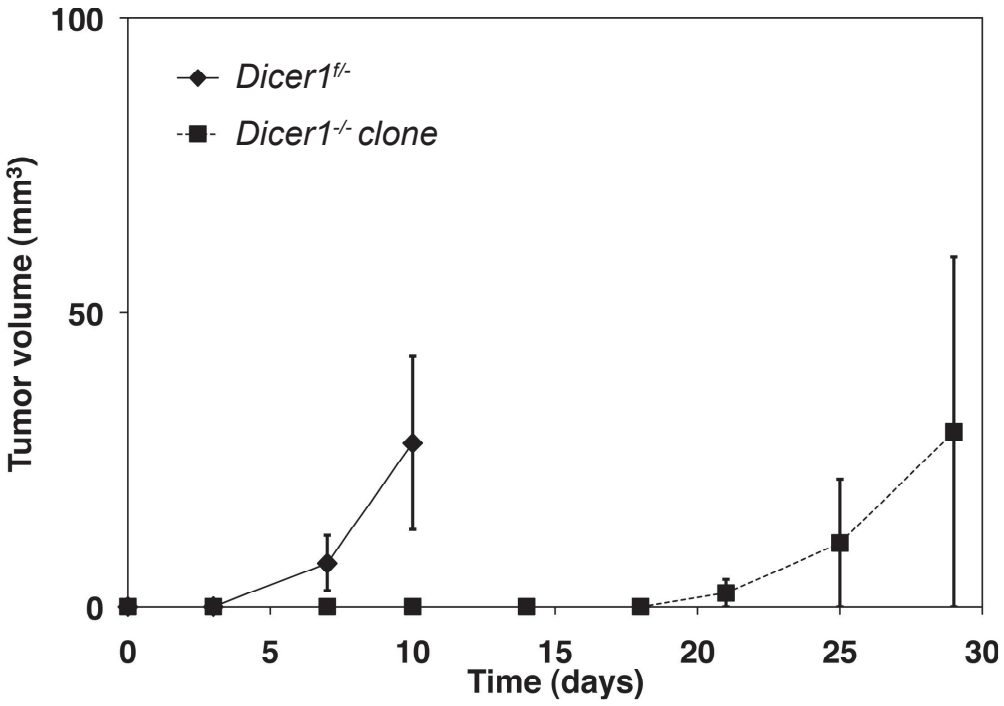

Figure S2. Related to Figure 2. Tumor challenge in immune competent mice. Tumor injection time course for *Dicer1*$^{f/-}$ and *Dicer1*$^{-/-}$ cells in immune competent C57Bl6/SV129 F1 mice. Error bars represent standard error of the mean (SEM).

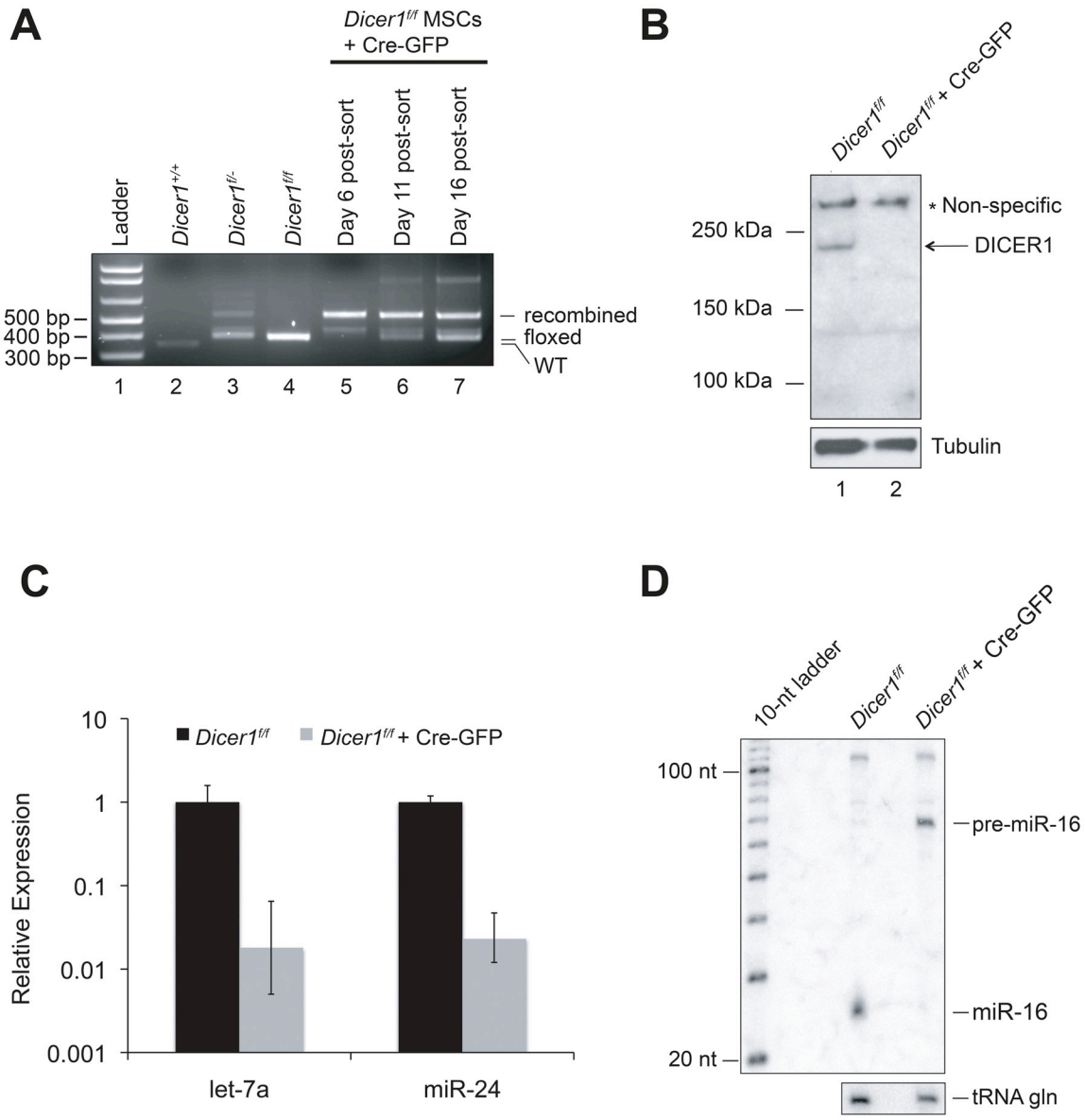

Figure S3. Related to Figure 3. Generation of *Dicer1*$^{-/-}$ murine mesenchymal stem cells. A) PCR genotyping of Adeno-Cre-GFP-infected MSCs after FACS for GFP. B) DICER1 Western blot. C) miRNA qPCR analysis. A representative experiment is shown. Error bars indicate standard deviation. D) Northern blot. For all experiments shown here, MSCs were analyzed 7 days post-sort.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Complete protocols are available upon request.

Culturing and Genotyping of Cells

Mesenchymal stem cells

Primary MSC cultures were prepared similarly as previously described (Mukherjee et al., 2008) but with some differences as noted here. A one year-old *Dicer1*$^{f/f}$ mouse (Harfe et al., 2005) was sacrificed by CO_2 asphyxiation. Tibia and femurs were isolated, cleaned of excess soft tissue, and crushed with mortar and pestle in PBS supplemented with 0.5% FBS (PBS/FBS). The suspension was then filtered at 70-μm and centrifuged at 1000 rpm for 5 minutes. After resuspending the pellet in ACK lysis buffer for 4 minutes, PBS/FBS was added to neutralize the lysis buffer. The sample was centrifuged again at 1000 rpm, washed once more with PBS/FBS, and centrifuged again. The pellet was resuspended into 2 ml of alpha-MEM supplemented with pen/strep and 15-20% serum (Alpha-MEM Primary). The cells were plated into two T75 flasks in 10 ml each of Alpha-MEM Primary. The media was replaced 24-48 hours after plating.

Approximately 2 weeks after plating, primary MSCs were infected with a retroviral construct encoding SV40 large T-antigen. Multiple colonies grew out following infection and were passaged together as a polyclonal population in alpha-MEM supplemented with pen/strep and 10% FBS (Alpha-MEM Complete). Individual clones were then isolated from this polyclonal population of cells, infected with Adeno-Cre-GFP, and sorted by FACS to isolate GFP-positive cells. Sorted cells were then plated at low

density and individual clones were grown out, passaged, and genotyped to determine *Dicer1* status.

Luciferase Reporter Assays

Reporters for each microRNA were generated via ligation of a target site containing oligo downstream of a pRL-CMV Renilla luciferase reporter as described previously (Doench et al., 2004). Firefly luciferase (pGL3) was used as a transfection control. Cells were transfected using Lipofectamine 2000 (Invitrogen) and assayed 24 hours later with the Dual Luciferase reporter assay system (Promega) according to manufacturer's instructions. Data shown are summaries of three or more independent trials.

Western Blot

Cells were lysed with 2X sample buffer (Bio-Rad) containing 5% β-mercaptoethanol, boiled for 5 minutes, and subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to PVDF membranes using a submerged transfer apparatus (BioRad, Hercules, CA). After blocking with 5% nonfat dried milk in TBS-T (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20), the membrane was incubated with the primary antibody diluted in TBS-T (1:250 dilution for DICER1, 1:1000 for tubulin), washed extensively, and incubated with the appropriate horseradish peroxidase–linked secondary antibody (Amersham, Piscataway, NJ). Chemiluminescence was used for detection. Rabbit anti-DICER1 and mouse anti-Tubulin (Sigma) antibodies were used.

Northern Blot

RNA was prepared from 10^6 cells using Qiazol Reagent according to the manufacturer's protocol (Qiagen). 20 μg RNA was mixed with an equal volume of formamide loading buffer, denatured at 95º C for 5 minutes, and run for 1 hour at 35W on a 15% denaturing polyacrylamide gel (Sequagel, National Diagnostics) after 30 minutes of pre-running. A semi-dry transfer apparatus set to 18V was used to transfer the RNA to a Hybond-N+ nylon membrane (GE Healthcare Life Sciences) for 1.5 hours at 4°C. RNA was then UV crosslinked at 1.2 E6 uJoules in a Crosslinker 2400 (Stratagene) on top of Whatman paper. The membrane was prehybridized with Ultrahyb oligo (Ambion) for 1 hour, and then probed overnight at 37°C with a 5' end labeled locked nucleic acid (LNA) probe for let-7g. The membrane was washed twice for 30 minutes in 2xSSC/0.1% SDS buffer and then imaged on a Storm scanner (Molecular Dynamics) for 24 hours. The membrane was then stripped and exposed to confirm probe removal prior to reprobing with LNAs for let-7g, mir-16, or mir-17. Loading was confirmed with a DNA oligo probe for glutamine tRNA with 1 hour exposure.

To calculate miRNA copies per cell, a quantitative Northern blot was carried out, in duplicate, against total RNA from known cell numbers of *Dicer1*$^{f/-}$ sarcoma cells alongside a titration curve of synthetic murine miR-22 RNA standard (IDT) loaded with 20 μg of yeast carrier RNA (Ambion, 8546G). miR-22 was probed overnight at 37-42 °C with a 5' end labeled complementary DNA oligo probe (IDT). Based on signal intensities of the standard and total RNA samples as measured with ImageQuant™ , copy numbers of miR-22 per cell were calculated for *Dicer1*$^{f/-}$ sarcoma cells. To calculate copy numbers of all other miRNAs, small RNA-seq read counts for each miRNA were normalized relative to total mapped reads in the *Dicer1*$^{f/-}$ and *Dicer1*$^{-/-}$ libraries, and then

each read count was subsequently converted to copies per cell by normalization to miR-22 read counts, determined by quantitative Northern as described above, in the *Dicer1*$^{f/-}$ and *Dicer1*$^{-/-}$ small RNA-seq libraries.

miScript qPCR

MiRNA quantitation was performed using the miScript PCR reagents (Qiagen) per manufacturer's protocol. Total RNA isolated from MSCs was assayed for representative miRNAs let-7a, miR-24, miR-26, and miR-31.

Cell Cycle and Apoptosis assays

For cell cycle assays, sarcoma cells were plated at 50% confluency in 6-well plates. Cells were harvested 24 hours later following a 30-45 minute BrdU pulse. Wells were washed twice with PBS prior to trypsinization, fixation, and DNAse treatment per manufacturer's protocol (BD Pharmingen, FITC BrdU Flow Kit). The final samples were resuspended in 20 ul of 7-AAD solution prior to resuspension in 200 ul wash buffer and analysis on FACSCalibur. MSC cell cycle analysis was performed similarly to sarcoma cells but at a plating density of 80,000 cells per well of a 6-well plate.

For apoptosis assays, sarcoma cells or MSCs were seeded at 50,000 and 25,000 cells per well, respectively, into 24-well plates. Cells were harvested for analysis approximately 3 days later. For sarcomas, the cell culture medium was aspirated, while for MSCs, the cell culture medium was retained. Wells were washed once with PBS, which was retained and pooled to collect floating cells. Wells were then trypsinized, and collected with cell culture medium, and then spun at 2,000 rcf at 4 minutes on a table top centrifuge. The cells were resuspended in cell fixation/permeabilization buffer (BD Biosciences, Active Casp3-FITC kit) on ice for 15 minutes. Each sample was then

diluted in 200 ul of 1X wash buffer ((BD Biosciences, Active Casp3-FITC kit) and spun at full speed for ~2 seconds. Two more washes were carried out. The wash buffer was then aspirated completely and the samples were resuspended in 25 ul of undiluted anti-Active Caspase-3 antibody (BD Biosciences, Active Casp3-FITC kit) for 30 minutes at room temperature. The samples were then washed twice, resuspended in 400 ul of wash buffer, filtered into polystyrene tubes, and analyzed by FACSCalibur.

Flow Cytometry and Fluorescence-Activated Cell Sorting

Mesenchymal stem cells were washed with PBS, incubated with biotin-conjugated primary antibodies against CD49e or CD106 (BD Biosciences), washed, stained with PerCP-Cy5.5-streptavidin (BD Biosciences), and sorted on a BD FACSCalibur flow cytometer (Swanson Biotechnology Center). As a negative control, primary antibody was omitted and cells were incubated only with PerCP-Cy5.5-streptavidin. GFP positive cell sorting was similarly performed on live cells following Adeno-Cre-GFP infection to enrich for infected cells.

Analysis of Small RNA Cloning Reads

Raw sequence reads (after linker stripping) were mapped to the mouse genome (build mm9) using the Bowtie short read alignment tool (Langmead et al., 2009), allowing for a single base pair mismatch per unique alignment (U01) to accommodate sequencing errors. All such unique matches to the genome were first processed to identify hits to microRNA annotations (Chiang et al., 2010). Reads mapping to full-length mature miRNA or miRNA* annotations with exact ends were categorized as mature miRNAs. These were subsequently used to determine the level of mature miRNAs in various datasets under study.

SUPPLEMENTAL REFERENCES

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