

***Dicer1* functions as a haploinsufficient tumor suppressor**

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While the global down-regulation of microRNAs (miRNAs) is a common feature of human tumors, its genetic basis is largely undefined. To explore this question, we analyzed the consequences of conditional *Dicer1* mutation (*Dicer1* “floxed” or *Dicer1*^{f/f}) on several mouse models of cancer. Here we show *Dicer1* functions as a haploinsufficient tumor suppressor gene. Deletion of a single copy of *Dicer1* in tumors from *Dicer1*^{f/+} animals led to reduced survival compared with controls. These tumors exhibited impaired miRNA processing but failed to lose the wild-type *Dicer1* allele. Moreover, tumors from *Dicer1*^{f/f} animals always maintained one functional *Dicer1* allele. Consistent with selection against full loss of *Dicer1* expression, enforced *Dicer1* deletion caused inhibition of tumorigenesis. Analysis of human cancer genome copy number data reveals frequent deletion of *DICER1*. Importantly, however, the gene has not been reported to undergo homozygous deletion, suggesting that *DICER1* is haploinsufficient in human cancer. These findings suggest *Dicer1* may be an important haploinsufficient tumor suppressor gene and, furthermore, that other factors controlling miRNA biogenesis may also function in this manner.

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MicroRNAs (miRNAs) are short, noncoding RNAs that function to suppress post-transcriptionally the expression

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of target mRNAs, predominately via inhibition of translation. Such translational inhibition relies on imperfect base-pairing between the miRNA and the target transcript, with the interaction at nucleotides 2–8 (or the “seed” region) of the miRNA being required for translational repression. Computational prediction of miRNA targets based on seed regions and sequence conservation has revealed a widespread potential for miRNA-mediated transcript regulation, with hundreds of putative mRNA targets for an individual miRNA (Bartel 2004).

In line with their broad-based effects, miRNAs have been proposed to function as oncogenes or tumor suppressor genes based on their inhibition of a variety of tumor-suppressive and oncogenic mRNAs, respectively (Plasterk 2006; Ventura and Jacks 2009). In particular, three distinct mechanisms have been posited. First, oncogenic miRNAs can undergo gain of function in tumors. This has been most clearly demonstrated for the miR-17~92 cluster, whose amplification in B-cell lymphomas promotes their development, potentially through its control of B-cell differentiation (He et al. 2005; Koralov et al. 2008; Ventura et al. 2008). Furthermore, tumor-suppressive miRNAs could undergo loss of function in tumors. This has been shown for several miRNAs, including the let-7 family, whose expression can limit lung tumorigenesis through inhibition of oncogenes like the Ras family and HMGA2 (Esquela-Kerscher et al. 2008; Kumar et al. 2008). In particular, let-7 family members are in sites of frequent deletion in human tumors, and their processing is inhibited by the oncogenic Lin-28 proteins (Heo et al. 2008; Newman et al. 2008; Viswanathan et al. 2008; Chang et al. 2009). Finally, oncogenes can acquire mutations to remove miRNA-binding sites in tumors. This has been described for HMGA2, whose translocation promotes lipoma development by releasing the transcript from let-7-mediated tumor suppression (Mayr et al. 2007).

We reported a global down-regulation of miRNAs in several types of human and murine cancer (Lu et al. 2005). From this initial study, it was unclear whether this widespread loss of miRNAs was merely a consequence of tumor development or was functionally related to the disease process. We demonstrated previously that this global loss of miRNAs was functionally relevant to oncogenesis, as impairment of miRNA maturation enhanced transformation in both cancer cells and a K-Ras-driven model of lung cancer (Kumar et al. 2007). While these studies provide a framework to explain inhibition of miRNA biogenesis in cancer, the genetic basis of impaired miRNA processing in human cancer has been largely undefined. For a subset of miRNAs, widespread silencing occurs at the transcriptional level via the c-Myc oncogene (Chang et al. 2008). However, it has also been shown that such broad reductions in miRNAs can occur post-transcriptionally, since changes in miRNA levels frequently occur without changes in the levels of the primary miRNA transcript (Thomson et al. 2006). Recently, it was shown that mutations in the miRNA processing component *TARBP2* occur frequently in mismatch repair-deficient colon cancer, and that these mutations promote tumorigenesis by impaired processing of miRNAs (Melo et al. 2009). While interesting, these limited cases do not resolve the common global reduction

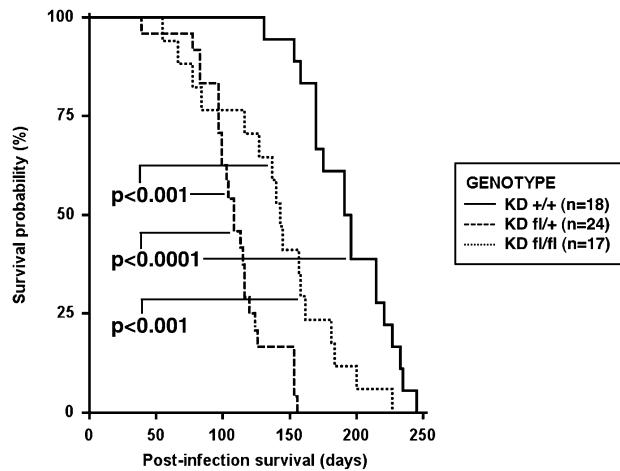


Figure 1. *Dicer1* mutation reduces post-infection survival in a genetically engineered mouse model of K-Ras-driven lung cancer. *Kras^{LSL-G12D}* mice, either wild type or heterozygous or homozygous conditional for *Dicer1* (KD^{+/+}, KD^{fl/+}, and KD^{fl/fl}, respectively), were intranasally infected with Ad-Cre, and survival was assessed. Median survival was 194 d for KD^{+/+} mice, 108 d for KD^{fl/+} mice, and 143 d for KD^{fl/fl} mice. Statistical significance was assessed by the log-rank test.

of miRNAs in human cancers. Moreover, the precise genetics of such changes in tumors is poorly defined, especially as no components of the miRNA processing pathway have been reported to be completely deleted in human tumors. This is not surprising, since it has been shown that germline deletion of miRNA processing components *Dicer1* and *Dgcr8* in mice fails to produce viable progeny (Bernstein et al. 2003; Wang et al. 2007). Thus, conditional deletion of miRNA processing components provides a powerful means of examining the role of miRNAs in tumorigenesis.

Results and Discussion

We previously reported results using the *Kras^{LSL-G12D}* strain, which was either heterozygous or homozygous for a conditional allele of *Dicer1* (hereafter referred to as KD^{fl/+} and KD^{fl/fl}) (Jackson et al. 2001; Harfe et al. 2005). After intranasal infection with adenovirus expressing Cre (Ad-Cre), these compound mutant animals developed significantly more lung tumors compared with *Kras^{LSL-G12D}* mice that were wild type for *Dicer1* (hereafter referred to as KD^{+/+}); moreover, *Dicer1* mutant mice had a modest shift in histological grade, further enforcing its role as a tumor suppressor (Kumar et al. 2007). To extend these observations of *Dicer1*-mediated tumor suppression, we characterized survival of KD^{+/+}, KD^{fl/+}, and KD^{fl/fl} animals after intranasal Ad-Cre infection. Both KD^{fl/+} and KD^{fl/fl} animals had significantly reduced survival relative to KD^{+/+} mice (Fig. 1). Surprisingly, we found that the survival of the KD^{fl/+} cohort was less than that of KD^{fl/fl} animals. To extend these findings to additional cancer types, we used a mouse model of soft-tissue sarcoma generated through intramuscular infection with Ad-Cre of *Kras^{LSL-G12D}*; *Trp53^{fl/fl}* mice (Kirsch et al. 2007). When we compared survival of *Kras^{LSL-G12D}*; *Trp53^{fl/fl}* mice either wild type or heterozygous or homozygous for the conditional allele of *Dicer1* (hereafter referred to as KPD^{+/+}, KPD^{fl/+}, and KPD^{fl/fl}) intramuscularly infected with Ad-Cre, we observed a reduction in survival in only the

KPD^{fl/+} cohort (Supplemental Fig. 1). Overall, these results indicate that induction of heterozygosity for a *Dicer1* mutation in tumors can enhance tumor development.

These findings stand in opposition to traditional tumor suppressor genes, in which a homozygous mutation provides a direct advantage to tumor cells while a heterozygous mutation promotes tumorigenesis only after subsequent mutation or inactivation of the remaining wild-type allele. In particular, the survival disadvantage of KD^{fl/+} tumors to KD^{fl/fl} suggests active selection against complete loss of *Dicer1* in these tumors. To characterize precisely the mutation status of *Dicer1* conditionally mutant tumors, we generated lung cancer cell lines from KPD^{fl/+} animals and examined the *Dicer1* locus for further deletion. As shown in Figure 2, we observed recombination of the conditional allele of *Dicer1* and retention of the wild-type allele, generating KPD^{+/-} lung cancer cells (Fig. 2A). Similar maintenance of the wild-type allele was found in sarcoma cell lines from KPD^{fl/+} mice (Supplemental Fig. 2A).

We further examined *Dicer1* recombination in lung cancer cell lines from KPD^{fl/fl} animals. In these cell lines, we again found evidence of Cre-Lox recombination. However, the recombination was incomplete, generating KPD^{fl/-} lung cancer cells (Fig. 2B); this incomplete loss of *Dicer1* was also observed in sarcoma cell lines from KPD^{fl/fl} mice (Supplemental Fig. 2A). Thus, it is likely that KD^{fl/fl} lung tumors undergo selection against complete *Dicer1* recombination during tumor progression. To determine if complete loss of *Dicer1* product occurs, *Dicer1* protein levels were examined in lung cancer cells and sarcomas either wild type or mutant for *Dicer1*. Although we observed a general reduction in *Dicer1* protein in *Dicer1* mutant cancer cell lines, all retained some expression of *Dicer1* (Supplemental Fig. 2B). In addition, resequencing of the *Dicer1* ORF in lung cancer and sarcoma cell lines from KPD^{fl/+} and KPD^{fl/fl} mice revealed no further mutations of the retained *Dicer1* allele (data not shown), suggesting that complete loss of *Dicer1* function is not achieved through second site mutation. In sum, these data indicate that only partial loss of *Dicer1* occurs in these genetically engineered mouse models during tumorigenesis.

Based on the protumorigenic effect of hemizygous mutation of *Dicer1* in these tumor models, we went on to assess

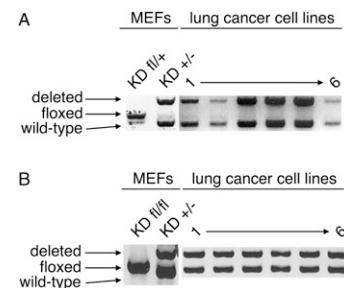


Figure 2. *Dicer1* undergoes hemizygous loss in lung tumors. (A) DNA was prepared from *Kras^{LSL-G12D}* mouse embryonic fibroblasts (MEFs) either heterozygous conditional or mutant for *Dicer1* (KD^{fl/+} and KD^{+/-}) and lung cancer cell lines from KPD^{fl/+} mice, and the *Dicer1* locus was examined by PCR. (B) DNA was prepared from *Kras^{LSL-G12D}* mouse embryonic fibroblasts either homozygous conditional or heterozygous mutant for *Dicer1* (KD^{fl/fl} and KD^{+/-}) and lung cancer cell lines from KPD^{fl/fl} mice, and the *Dicer1* locus was examined by PCR.

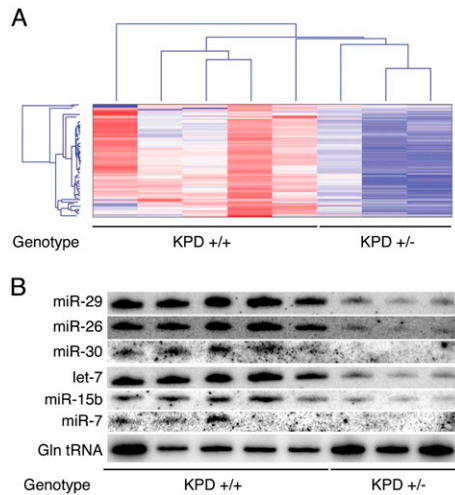


Figure 3. Hemizygous deletion of *Dicer1* causes a global reduction in steady-state miRNA levels. (A) miRNA profiling and hierarchical clustering were performed on lung cancer cell lines either wild type (KPD^{+/+}) or heterozygous for *Dicer1* (KPD^{+/-} and KPD^{fl/-}). (B) Small RNA Northern blotting analysis of miRNAs and glutamine tRNA was performed on lung cancer cell lines wild type or heterozygous for *Dicer1* as above.

the consequence of partial loss of *Dicer1* on global miRNA expression. Comparing miRNA profiles in *Dicer1* wild-type and heterozygous mutant lung cancer cells, there was a global decrease in steady-state miRNA levels in KPD^{+/-} cells (Fig. 3A; Supplemental Table 1). A small RNA Northern blot analysis verified these reductions in miRNA levels (Fig. 3B). Thus, a *Dicer1* mutation is capable of significantly altering miRNA levels, even when incomplete.

While the genetic analysis of the *Dicer1* locus in our mouse cancer models suggested that *Dicer1* was functioning as a haploinsufficient tumor suppression gene, it was still formally possible that *Dicer1* mutant tumors eventually underwent complete inactivation of *Dicer1* via an alternative mechanism like epigenetic silencing. Thus, we assessed the consequences of complete loss of *Dicer1* in the genetically engineered mouse models. First, to promote complete loss of *Dicer1* in the lung, we infected KD^{fl/+} and KD^{fl/fl} mice with a lentivirus expressing Cre (Lenti-Cre). In contrast to the recombinant adenoviruses, which do not integrate in the genome of the infected cells and are eventually lost, lentiviral integration is expected to allow for stable, longer-term expression of Cre and, more likely, complete recombination at the *Dicer1* locus. Infection of mice with Lenti-Cre led to a striking decrease in tumor burden in KD^{fl/fl} compared with KD^{fl/+} mice (Supplemental Fig. 3A–C). This result contrasts sharply with previous studies in which KD^{fl/+} and KD^{fl/fl} mice created a similar tumor burden after Ad-Cre infection. Notably, when we isolated DNA from the tumors that did arise in KD^{fl/fl} mice and assessed *Dicer1* loss, all tumors were once again found to have undergone incomplete recombination, suggesting that there is selection against total *Dicer1* loss in lung tumors (Supplemental Fig. 3D).

To further examine the effects of complete *Dicer1* loss, we transduced a set of sarcoma cell lines with CreER^{T2} to permit tamoxifen-dependent Cre activity. Treatment with 4-hydroxytamoxifen (4-OHT) led to efficient deletion of

Dicer1 in vitro (Fig. 4A). To assess the effect of complete *Dicer1* loss on tumorigenesis, we transplanted untreated KPD^{fl/-} sarcoma cells expressing CreER^{T2} into immune-competent hosts, and recombination was induced by systemic tamoxifen administration. Compared with control treatments, tamoxifen administration substantially slowed tumor growth in KPD^{fl/-} sarcomas (Fig. 4B). Of note, this system allowed for Cre-mediated deletion of *Dicer1*, as the tumors that developed in tamoxifen-treated animals had extensive *Dicer1* recombination (Fig. 4C). Importantly, tumor suppression was not merely an effect of tamoxifen- or Cre-mediated toxicity, as tamoxifen treatment of animals injected with KPD^{+/+} sarcoma cells expressing CreER^{T2} did not impair tumor growth (Supplemental Fig. 4). Taken together, these studies demonstrate that complete deletion of *Dicer1* is deleterious to tumor development, strongly suggesting that *Dicer1* is a haploinsufficient tumor suppressor. Of note, in a separate study using these cancer cell lines, A Ravi, MS Kumar, C Chin, T Jacks, and PS Sharp (in prep.) have shown that full loss of *Dicer1* function can be tolerated, but that *Dicer1*-null cells have impaired proliferative capacity.

While these findings indicate that partial loss of *Dicer1* promotes tumor development, it was not clear whether a comparable situation occurs in human cancer. To explore this question, we assessed *DICER1* copy number data from the Cancer Genome Project at the Sanger Institute (Forbes et al. 2008). In these data sets, there was frequent loss of one allele of *DICER1* (via hemizygous deletion) in several different tumor types (Table 1); similar results were obtained from analysis of copy number data sets of glioblastomas and ovarian cancers from The Cancer Genome Atlas (TCGA) (data not shown). Although the deletions of *DICER1* seen in human cancer were generally broad (>1 Mb), there were

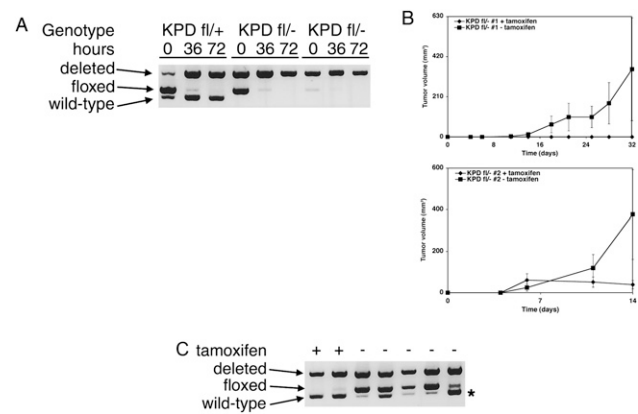


Figure 4. Complete deletion of *Dicer1* inhibits sarcoma development. (A) KPD^{fl/+} and KPD^{fl/-} sarcoma cell lines were infected with MSCV.CreER^{T2}.puro. Cre-mediated recombination was induced by treatment with 4-OHT for defined time points. DNA was prepared, and recombination of the *Dicer1* locus was assessed by PCR. (B) Two independent KPD^{fl/-} sarcoma cell lines infected with MSCV.CreER^{T2}.puro were injected subcutaneously into C57Bl6/129SV F1 animals. Animals were treated with or without tamoxifen by intraperitoneal injection, and tumor growth was measured over time. Values are mean \pm SEM ($n = 8$ each). (C) Tumors were isolated from KPD^{fl/-} sarcoma cell line transplants treated with or without tamoxifen. DNA was prepared, and recombination of the *Dicer1* locus was assessed by PCR. Asterisk corresponds to the wild-type *Dicer1* locus from the C57Bl6/129SV F1 host animals.

Table 1. DICER1 is frequently deleted in various human cancers

Tumor type	Fraction of tumors with DICER1 hemizygous loss
Breast	17/45
Kidney	13/21
Large intestine	14/39
Liver	3/9
Lung	37/149
Ovary	6/22
Pancreas	6/16
Stomach	9/21

never high-level amplifications of the *DICER1* locus in these tumor sets, suggesting these losses were not due to random genome instability. More importantly, homozygous deletion of *DICER1* was never reported, in line with our findings of *Dicer1* functioning as a haploinsufficient tumor suppressor (Hill et al. 2009; Melo et al. 2009).

Our results suggest a major cause for the global loss of miRNAs in human cancer (Lu et al. 2005), via partial loss of function of the miRNA processing machinery in human tumors. Although the frequent *DICER1* single-copy deletion noted in human cancers provides a relevant mechanism of impairing miRNA biogenesis, additional mechanisms may occur. In particular, in light of the frequent mutation of *TARBP2* in mismatch repair-deficient colon cancer (Hill et al. 2009) and heterozygous germline point mutations in *DICER1* in patients with pleuropulmonary blastoma (Melo et al. 2009), it is possible that point mutations of *DICER1* or other components of the miRNA processing machinery can occur in different types of human cancer. Indeed, this has been described for *DICER1* and *DROSHA* in a small number of ovarian cancer cell lines (Merritt et al. 2008). Importantly, while this study describes *Dicer1* as a haploinsufficient tumor suppressor in the context of K-Ras-driven cancer, the analysis of miRNA processing machinery mutations has found changes in cancers of a variety of genotypes, suggesting a broad role for *Dicer1* as a tumor suppressor.

Beyond such mutational analyses, these results represent an expansion of the list of haploinsufficient tumor suppressors to include components of the miRNA processing machinery. The breadth of tumor suppressor genes that function via haploinsufficiency is only beginning to be appreciated (Santarosa and Ashworth 2004). In fact, the traditional reliance on complete loss of tumor suppressor genes by heterozygous mutation and subsequent loss of heterozygosity (LOH) is likely to overlook factors whose effects do not require complete loss, such as p27^{Kip1} and Dmp1 (Inoue et al. 2001; Muraoka et al. 2002). Moreover, such analyses are certain to ignore genes like *Dicer1*, for which partial loss is advantageous to tumors while complete loss is disadvantageous. As genome-wide studies begin to explore the functional role of chromosomal deletions in human cancer, it will be important to consider haploinsufficiency of deleted genes in such contexts.

Materials and methods

Mice

Kras^{LSL-G12D}; Dicer1^{+/+}, fl^{+/+}, fl/fl animals were generated as described previously (Kumar et al. (2007)). *Kras^{LSL-G12D}; Trp53^{fl/fl}* animals were bred

with *Kras^{LSL-G12D}; Dicer1^{fl/fl}* animals to produce *Kras^{LSL-G12D}; Trp53^{fl/+}; Dicer1^{fl/+}* animals. These animals were backcrossed to *Kras^{LSL-G12D}; Trp53^{fl/fl}* animals to produce *Kras^{LSL-G12D}; Trp53^{fl/fl}; Dicer1^{fl/+}* animals, which were interbred to produce the experimental cohort.

Intranasal, intratracheal, and intramuscular infection

Mice were infected intranasally, intratracheally, and intramuscularly with Ad-Cre and Lenti-Cre as described (Jackson et al. 2001; Kirsch et al. 2007; Kumar et al. 2008). Lung and tumor areas were determined using Bioquant Image Analysis software as described previously (Kumar et al. 2007).

Cell line injection and tamoxifen treatment

C57Bl6/129SV F1 animals were subcutaneously injected with 2.5×10^4 sarcoma cells. Mice were subsequently treated with corn oil with and without tamoxifen as described previously (Ventura et al. 2007). Tumors were measured as described previously (Kumar et al. 2007).

Cell culture

Primary sarcoma and lung cancer cell lines were generated by dissection of tumors from mice infected with Ad-Cre, digestion with trypsin, and plating and propagation in serum-containing media. *Kras^{LSL-G12D}; Dicer1^{fl/+; fl/fl}* mouse embryonic fibroblasts were generated as described previously (Tuveson et al. 2004). Sarcoma cell lines were infected with MSCV.CreER^{T2}.puro and treated with 4-OHT for defined time points as described previously (Ventura et al. 2007). Cells were maintained using standard conditions.

Recombination analysis

DNA was prepared from tumors and cancer cell lines, and *Dicer1* and *Kras* recombination was assessed by PCR using previously described primers (Harfe et al. 2005). *Kras* primer information is provided in the Supplemental Material.

Northern blotting

Small RNA Northern blotting was performed as described previously (Ventura et al. 2004). Probe information is provided in the Supplemental Material.

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