

Dicer is essential for mouse development

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To address the biological function of RNA interference (RNAi)-related pathways in mammals, we disrupted the gene *Dicer1* in mice. Loss of *Dicer1* lead to lethality early in development, with *Dicer1*-null embryos depleted of stem cells. Coupled with our inability to generate viable *Dicer1*-null embryonic stem (ES) cells, this suggests a role for Dicer, and, by implication, the RNAi machinery, in maintaining the stem cell population during early mouse development.

RNAi is an evolutionarily conserved gene-silencing pathway that has been used to determine gene function in a variety of biological models (reviewed in ref. 1). The biochemical mechanisms underlying RNAi have recently emerged (reviewed in ref. 1). Initiation of silencing occurs after processing of double-stranded RNAs (dsRNAs) by Dicer into small interfering RNAs (siRNAs; ref. 2). Dicer also processes numerous endogenous small RNAs, known collectively as microRNAs, which then enter gene-silencing pathways^{3–6}. Both types of processed silencing triggers can enter related effector complexes, known generically as RNA-induced silencing complex (RISC). However, in some cases (*e.g.*, with perfectly complementary siRNAs and some miRNAs), silencing occurs by mRNA cleavage, and in other cases (*e.g.*, with most imperfectly complementary miRNAs) silencing occurs through a block in protein synthesis⁷. In plants and fungi, dsRNA can also initiate silencing at the transcriptional level, and in *Drosophila*, the RNAi machinery has been genetically linked to transcriptional cosuppression (reviewed

in ref. 1). Although several protein components of RISC associate with multiple types of silencing triggers (reviewed in ref. 1), the precise mechanistic relationship between distinct modes of dsRNA-mediated silencing has yet to be elucidated.

So far, genetic strategies have been used to examine the biological functions of the RNAi machinery in *Caenorhabditis elegans*, *Arabidopsis*, *Drosophila* and fungi (reviewed in ref. 1). Now, we begin to assess the biological roles of RNAi in mammals by creating a mouse strain with a chromosomal lesion in *Dicer1*. To prepare a gene-targeting vector for *Dicer1*, we used an *in vivo* recombination strategy⁸ to replace exon 21 of *Dicer1* with a neomycin-resistance (*neo^r*) cassette (Fig. 1a,b). Splice donor and acceptor sites of the exon remained intact, increasing the chance that the entire PGK-*neo^r* cassette would be incorporated into the *Dicer1* transcript, producing stops in all three reading frames.

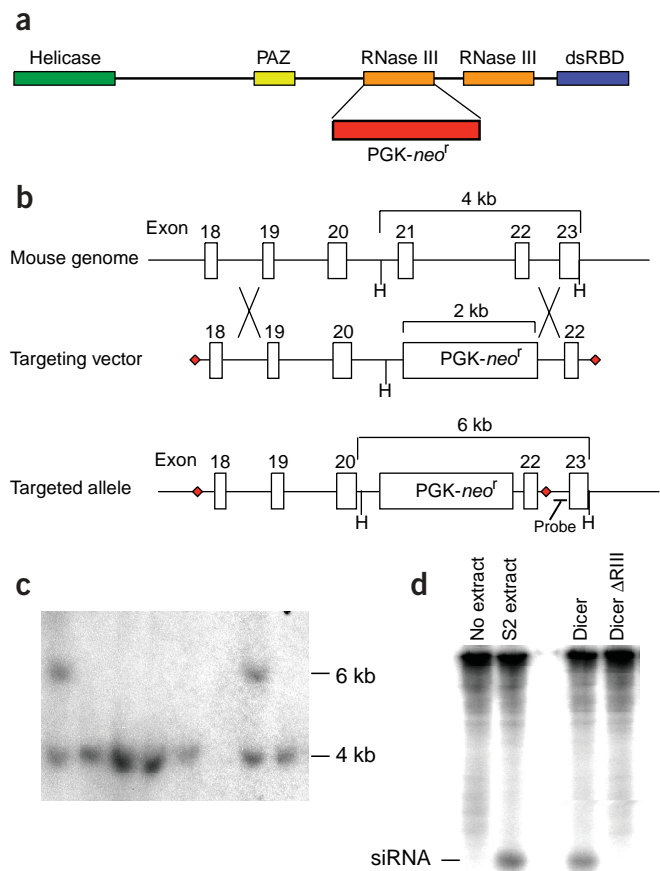


Figure 1 Disruption of the mouse gene *Dicer1*. (a) The targeting strategy for the *Dicer1* disruption replaces the first RNase III domain with a PGK-*neo^r* expression cassette. This domain is encoded in exon 21. (b) Details of the targeting strategy. Red diamonds indicate the limits of the homology region in the targeting vector. ES cell lines containing homologous recombinants (designated by arrows) were identified by Southern blotting (c) with a probe derived from regions outside the targeting vector (indicated in b). Dicer activity of a mutated *DICER1* cDNA comprising essentially the deletion introduced into the mouse gene was tested using immunoaffinity-purified protein (d). Comparison of wild-type (Dicer) and mutant (Dicer Δ R111) proteins shows lack of siRNA production by the mutant. For comparison, processing reactions lacking extract and in *Drosophila* S2 cell extracts are shown.

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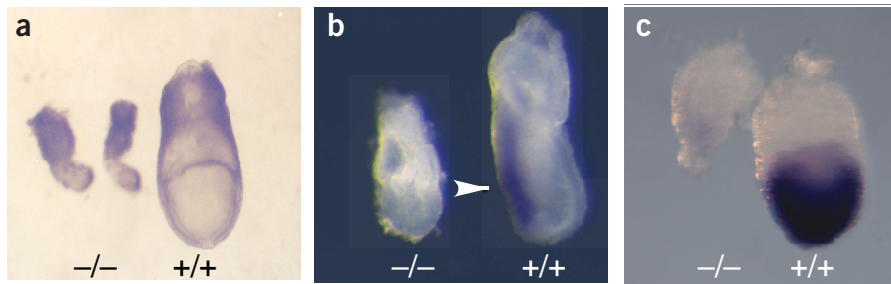


Figure 2 Characterization of *Dicer1* mutant embryos. (a) Typical E7.5 mutant ($-/-$) and wild-type ($+/+$) embryos are shown for morphological comparison. (b) Mutant ($-/-$) and wild-type ($+/+$) embryos were stained by *in situ* hybridization with a probe for *T* (brachyury), a primitive streak marker. (c) Mutant ($-/-$) and wild-type ($+/+$) embryos were stained with a probe for Oct4, a marker for stem cell populations in the early embryo.

Our choice of mutation was informed by previous structural and biochemical studies of *Aquifex* RNase III (ref. 9). Dicer has two catalytic RNase III domains per molecule². The first RNase III domain, encoded in the mouse by exon 21, has canonical active sites, but crucial catalytic residues in the second RNase III domain are not conserved in Dicer enzymes. Evidence that the second RNase III domain is defective comes from studies in *Escherichia coli*. Introduction of mutations corresponding to the naturally occurring changes in the catalytic site of the second RNase III domain of Dicer render the *E. coli* protein inactive⁹.

Even if alterations in the *Dicer1* splicing pattern produced a variant mRNA, we predicted that the loss of coding potential in the targeted allele would render Dicer incapable of producing siRNAs from dsRNA. To test this prediction explicitly, we generated a deletion in the human *DICER1* cDNA that removed residues 1,686–1,728. These residues substantially overlap with those sequences that are encoded by mouse exon 21. The catalytic core of the first RNase III domain (QRLEFLGDAILDYLITKHLIYE) is missing from both mutants. We tested the ability of the mutant Dicer to produce siRNAs by expressing T7-tagged versions of the wild-type and mutant Dicer proteins in 293 cells². Dicer activity was measured using immunoaffinity purified proteins. Notably, wild-type Dicer produced siRNAs, but the mutant protein produced no detectable siRNAs (Fig. 1d). Thus, by functional criteria, the *Dicer1* allele created in our genetic mutant can be considered null with respect to this activity.

Approximately 4% of ES cell lines obtained after gene targeting contained the disrupted *Dicer1* allele (Fig. 1c). Independent ES cell clones were used to create chimeric mice, two of which transmitted the mutated *Dicer1* allele through the germ line. Of 62 mice born from heterozygous intercrosses, none were homozygous mutants, suggesting that the *Dicer1*-deficient mice are not viable. Consistent with a normal mendelian ratio, viable progeny comprised 26 wild types and 36 heterozygotes.

To determine at which developmental stage Dicer is required, we carried out timed heterozygous matings. In embryos collected at embryonic day (E) 11.5, we found numerous empty and necrotic decidua. We obtained similar results from studies at E10.5 and E9.5. At E8.5, we observed a subset of small necrotic embryos in an otherwise relatively normal yolk sac (data not shown). All the morphologically normal E8.5 embryos were either heterozygous or wild-type with respect to *Dicer1*, whereas all of the abnormal embryos were *Dicer1* mutants. At E7.5, a subset of embryos also appeared small and morphologically abnormal, although distinctions between the embryonic and extraembryonic regions were recognizable (Fig. 2a). In contrast to wild types and heterozygotes, E7.5 *Dicer1*-deficient embryos did not express *T* (brachyury), a primitive streak marker (Fig. 2b). This suggests that development of *Dicer1* mutants is arrested before the body plan is configured during gastrulation. At E7.5, the number of *Dicer1*-null embryos was ~50% lower than expected from mendelian ratios, probably because a fraction of the embryos died at an earlier stage of development.

The RNAi machinery has been implicated in maintaining stem cell character in both plants and animals. The Argonaute proteins are key components of RISC complexes and are represented by multiprotein families in most multicellular eukaryotes. Lesions in these genes affect stem cells in a variety of tissues in diverse organisms, indicating that the RNAi pathway may contribute in part to fundamental mechanisms influencing stem cell fate. In *Drosophila*, *piwi* is required for the self-renewing asymmetric division of both male and female germline stem cells (reviewed in ref. 10). In *C. elegans*, RNAi-mediated knockdown of the family members closest to *piwi*, *prg-1* and *prg-2*, produces markedly similar germline defects. In *Arabidopsis*, the shoot apical meristems of *zwille* and some *ago1* mutants lose their stem cell character and terminally differentiate, often forming a single central organ in place of the meristem (reviewed in ref. 10).

These examples highlight the role of the RNAi machinery in various biological contexts that share the theme of stem cells and the control of differentiation. Additionally, our own efforts to produce homozygous *Dicer1*-null ES cells either by sequentially targeting both alleles or by isolating cell lines from blastocysts of heterozygous intercrosses failed (0 of 15, with wild types and heterozygotes present at normal mendelian ratios). We therefore considered the possibility that loss of Dicer might affect stem cell pools in the early embryo.

Oct4 expression in the early mammalian embryo defines a pool of pluripotent stem cells whose maintenance is essential for proliferation of cells in the inner cell mass of the blastocyst and later the epiblast. Oct4 is a key regulator of ES cell maintenance and proliferation. Loss of Oct4 in blastocysts leads to terminal differentiation of the inner cell mass into trophoblast lineages¹¹, and precise Oct4 dosage is required for cell fate decisions¹². Phenotypes associated with Oct4 downregulation in the epiblast are lethal, probably owing to premature terminal differentiation of stem cells (see ref. 13 for example).

In situ hybridization of wild-type and heterozygous embryos with probes specific for Oct4 results in strong staining of the epiblast that precisely mirrors the expression patterns indicated from analysis of an allele encoding an Oct4-LacZ fusion construct¹⁴. Oct4 staining was much reduced in the *Dicer1* mutant embryos (Fig. 2c). This indicates a loss of stem cells from the *Dicer1* mutant embryos and provides a potential explanation for the lethality that we observed. We cannot yet determine whether Oct4 is directly or indirectly regulated by Dicer-controlled networks or whether loss of Oct4 serves simply as an indicator of stem cell loss.

Overall, our studies indicate that Dicer, and, by extension, the RNAi machinery, are required for vertebrate development. Previous studies in plants and worms have also pointed to a developmental role for the RNAi machinery¹. In *Arabidopsis*, *DICER-LIKE 1* is maternally contributed to the seed and is required for patterning of the early embryo. Null alleles of *DICER-LIKE 1* result in arrest at the



heart stage of embryogenesis (reviewed in ref. 15), and hypomorphic alleles lead to a variety of developmental defects involving meristem fate determination, regulation of meristem proliferation and control of flowering time (reviewed in ref. 15). Double mutant analyses have shown that *DICER-LIKE 1* is partially redundant with the genes *CLAVATA* and *SUPERMAN* involved in stem cell maintenance and floral identity (reviewed in ref. 15). In *C. elegans*, *Dicer* (*dcr-1*) mutants develop to adulthood with heterochronic defects, which probably arise from an inability to process endogenous miRNAs^{3–6}. The absence of a more severe effect of *Dicer1* loss in worms may derive from the presence of maternally contributed protein and mRNA, which are sufficient to rescue RNAi in the soma. Similarly, *Dicer1* is maternally contributed in mice (P. Svoboda, P. Stein and R. Schultz, personal communication), and this may affect the timing of the lethality that we observed in the *Dicer1* mutant embryos. Phenotypic defects in *Dicer1*-deficient mice may result in part from a failure to process endogenous miRNAs, some of which might regulate developmental events, as they do in *C. elegans*. We attempted by various means to examine pre-miRNA processing in E6.5–7.5 embryos but were unable to obtain sufficient amounts of RNA for analysis. Considering the roles of components of the RNAi machinery in the regulation of heterochromatin formation (reviewed in ref. 1), more general defects in epigenetic programming could also lead to the phenotypes that we observe. Definitive resolution of such issues will await the development of conditional systems from which sufficient material for detailed mechanistic studies can be derived.

The microRNA-producing enzyme Dicer1 is essential for zebrafish development

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MicroRNAs (miRNAs) are produced by the Dicer1 enzyme; the role of Dicer1 in vertebrate development is unknown. Here we report target-selected inactivation of the *dicer1* gene in zebrafish. We observed an initial build-up of miRNA levels, produced by maternal Dicer1, in homozygous *dicer1* mutants, but miRNA accumulation stopped after a few days. This resulted in developmental arrest around day 10. These results indicate that miRNA-producing Dicer1 is essential for vertebrate development.

MicroRNAs have essential roles in the development of plants¹, nematodes² and flies³. These miRNAs are produced by the Dicer1 enzyme^{4–7}, which is conserved from fungi to vertebrates. No genetic analysis of Dicer1 has been done in vertebrates, however, and its role in vertebrate development is not known. We cloned the zebrafish *dicer1* ortholog (Fig. 1) and applied a method for target-selected gene inactivation that we recently developed⁸. We identified three different premature stop alleles induced by N-ethyl-N-nitrosourea, all of

ACKNOWLEDGMENTS

We thank L. Bianco and J. Coblenz for assistance with animals; B. Holdener, M.J. Garcia-Garcia and the Stony Brook University Histology Facility for help with initial embryo dissections; and M. Zhang and Z. Xuan for bioinformatics. E.M. is a Elisabeth Sloane Livingston Fellow of the Watson School of Biological Sciences. M.C. is supported by a US Army Breast Cancer Research Program Predoctoral Fellowship. This work was supported in part by grants from the US National Institutes of Health (G.J.H., S.J.E. and K.V.A.) and the Howard Hughes Medical Institute (S.J.E.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 17 August; accepted 19 September 2003

Published online at <http://www.nature.com/naturegenetics/>

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which probably cause loss of function (Fig. 1). The alleles had identical recessive phenotypes (developmental arrest), did not complement each other and cosegregated with the phenotype. Of 49 fish with a mutant phenotype, 41 were *dicer1*^{-/-} (wild-type larvae occasionally arrest in development). Of 112 fish with normal phenotype, none were *dicer1*^{-/-}. These data show that the phenotype is caused by disruption of *dicer1*.

Homozygous and trans-heterozygous (having two different null alleles) *dicer1*^{-/-} embryos appeared normal during the first week but at 8 d post fertilization (d.p.f.) had lethargic behavior and developmental growth arrest. Most *dicer1*^{-/-} embryos died after 14–15 d (Fig. 2a), and no *dicer1*^{-/-} fish were alive after 3 weeks of development ($n = 112$). Microscopic analysis indicated no obvious defect in one specific organ but rather a general arrest of growth (Fig. 2a).

Morpholino knockdown experiments resulted in an earlier arrest, indicating that maternal *dicer1* mRNA is necessary for embryonic development (Fig. 2c and Supplementary Fig. 1 and Supplementary Note online). Strong expression of maternal *dicer1* mRNA and ubiquitous expression up to 2 d.p.f. has been detected by whole-mount *in situ* hybridizations (data not shown). Morpholinos target only mRNA; thus, we cannot rule out the possibility that maternal Dicer1 protein is essential for the earliest stages of embryogenesis.

Dicer1 may be essential in vertebrate development because it is required to make miRNAs. Hundreds of miRNAs are expected to be present in the fish^{9,10}, and many of them may act in different organs and stages of development. There was a swift build-up of miRNA levels

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Published online 5 October 2003; doi:10.1038/ng1251