

Characterization of Dicer-deficient murine embryonic stem cells

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Communicated by James D. Watson, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, June 30, 2005 (received for review February 18, 2005)

Dicer is an RNase III-family nuclease that initiates RNA interference (RNAi) and related phenomena by generation of the small RNAs that determine the specificity of these gene silencing pathways. We have previously shown that Dicer is essential for mammalian development, with Dicer-deficient mice dying at embryonic day 7.5 with a lack of detectable multipotent stem cells. To permit a more detailed investigation of the biological roles of Dicer, we have generated embryonic stem cell lines in which their single Dicer gene can be conditionally inactivated. As expected, Dicer loss compromises maturation of microRNAs and leads to a defect in gene silencing triggered by long dsRNAs. However, the absence of Dicer does not affect the ability of small interfering RNAs to repress gene expression. Of interest, Dicer loss does compromise the proliferation of ES cells, possibly rationalizing the phenotype previously observed in Dicer-null animals. Dicer loss also affects the abundance of transcripts from mammalian centromeres but does so without a pronounced effect on histone modification status at pericentric repeats or methylation of centromeric DNA. These studies provide a conditional model of RNAi deficiency in mammals that will permit the dissection of the biological roles of the RNAi machinery in cultured mammalian cells.

centromeres | RNA interference

Dicer is a multidomain ribonuclease with specificity for dsRNA. Dicer's catalytic role in the production of small RNAs is central to dsRNA-mediated gene silencing or RNA interference (RNAi) (1–3). Dicer is essential for the response of many organisms to dsRNAs encountered from exogenous sources, including those generated by viral infection or those that have been experimentally delivered. Additionally, Dicer must process endogenous dsRNAs that trigger silencing responses directed at repetitive elements such as transposons and centromeric sequences. Finally, Dicer must promote the maturation of endogenous noncoding RNAs, the microRNAs (miRNAs) that enter the RNAi pathway to regulate the expression of protein coding genes. In all of these cases, Dicer is directly responsible for producing from a longer precursor the ≈22-nt dsRNAs bearing signature 2-nt 3' overhangs that are a hallmark of RNAi and related pathways.

The Dicer enzyme is organized as a modular structure, with a canonical Dicer containing an N-terminal DEAD-box helicase domain, a domain of unknown function (DUF283), a PAZ domain, a pair of catalytic RNaseIII domains, and a C-terminal dsRNA-binding domain. Structural models of Dicer predict that the RNaseIII domains combine as an intramolecular dimer to produce a single compound catalytic center that measures the position of scissile bonds precisely from an end of its dsRNA substrate (4–7).

Mounting evidence also suggests additional roles for Dicer enzymes. In *Drosophila*, Dcr-2 acts, along with its partner R2D2, in a loading complex responsible for sensing small interfering RNA (siRNA) asymmetry and placing the correct strand of the siRNA into RISC (8–13). Similar activities must also exist for miRNAs because one strand of the precursor miRNA predominantly contributes to the production of a functional RISC (12, 13). In mice and

humans, Dicer is encoded by a single locus, whose protein product must account for all proposed Dicer activities.

Based on genetic studies in other systems, mouse Dicer has a predicted involvement in numerous biological processes. Thus, it is not surprising that Dicer-deficient mice die very early in development, around embryonic day 7.5, with essentially a complete loss of pluripotent stem cells (1). In addition, mouse embryos hypomorphic for Dicer die mid-gestation (14). Dicer-deficient zebrafish progress much further in development, probably owing to the presence of maternally deposited Dicer transcripts (15). Depletion of maternal Dicer achieved by germ-line transplantation in zebrafish revealed that Dicer is required for morphogenesis but not cell fate specification during zebrafish embryogenesis and that the absence of miRNAs is responsible, at least in part, for this phenotype (16).

Recently it has become clear that Dicer is essential for viability and proliferation of some cell types but is dispensable for others. Chicken DT40 cells in which Dicer has been inactivated by homologous recombination become aneuploid and undergo growth arrest upon loss of Dicer (17). Specific deletion of Dicer during T cell development in the mouse thymus showed that survival is compromised in the $\alpha\beta$ but not $\gamma\delta$ lineage, but that transcriptional gene silencing during CD4/8 differentiation is not perturbed (18). In addition, Dicer-deficient mouse ES cells selected for survival *in vitro* are defective in differentiation (19). These phenotypes are consistent with an essential role for Dicer, at least in some cell types, in cellular metabolism and proliferation. However, the precise underlying cause of the requirement for Dicer in these cells remains unclear.

A series of recent studies has implicated the RNAi machinery in the establishment and maintenance of heterochromatin at centromeres (17, 19–27). In *Schizosaccharomyces pombe*, mutants in the RNAi pathway have a number of mitotic and meiotic defects and display a loss of heterochromatin at the centromeres and a derepression of transcription of centromeric repeats (20, 21, 26–28). Roles for RNAi at heterochromatic mating-type loci are more restricted, with the machinery mainly acting during the initiation phase rather than in the maintenance of heterochromatin (21, 25, 29). Similar roles for RNAi in the husbandry of repetitive elements and formation of heterochromatin have been observed in *Tetrahymena* (22, 30), chicken DT40 cells (17), mouse ES cells (19), and plants (31) but notably not in *Neurospora* (32–34). We set out to examine models of Dicer function in mammalian cells by expanding on our earlier constitutive mutant animals through the creation of conditional Dicer-null ES cells.

ES cells are a transient pluripotent cell population found in the mammalian blastocyst that can be isolated and cultured *in vitro* without a loss of their ability to contribute to all mouse tissues. Mouse ES cells contain Dicer and express a substantial number of miRNAs, including some that are unique to ES cells (35). Here we

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Abbreviations: siRNA, small interfering RNA; RNAi, RNA interference; ES, embryonic stem; RISC, RNA-induced silencing complex; miRNA, microRNA.

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describe the creation and characterization of Dicer conditional ES cell lines that can be induced to inactivate Dicer upon exposure to Cre recombinase. We find that, upon loss of Dicer activity, ES cells have a significant proliferation defect. However, this defect can be overcome with time, probably because of the accumulation of additional mutations. As expected, Dicer-deficient ES cells are unable to process pre-miRNAs or dsRNAs. Interestingly, Dicer-null cells are still able to mount an siRNA-mediated gene silencing response. In addition, ES cells lacking Dicer accumulate transcripts derived from the centromeric major satellite but retain the integrity of centromeric heterochromatin as indicated by the presence of cytosine methylation and histone H3 lysine 9 trimethylation.

Materials and Methods

Gene Targeting and Cell Culture. 4E4 Dicer heterozygous ES cells (1) were electroporated with the linearized conditional targeting construct and selected with puromycin. ES cells were grown on a STO feeder layer or on gelatin-coated plates with media supplemented with leukemia inhibitory factor.

Transfection and FACS. ES cells were transfected with pCMV-Cre-EGFP (a gift from D. Lee and D. W. Threadgill, University of North Carolina, Chapel Hill) by using Lipofectamine 2000 at 5 $\mu\text{g}/\text{ml}$ and pCMV-Cre-EGFP at 1.5 $\mu\text{g}/\text{ml}$. GFP-positive cells were enriched by sorting with a FACSVantage DiVa cell sorter (Becton Dickinson). Cell-cycle analysis was done by using an LSR II FACS analyzer (Becton Dickinson). Cells were fixed and stained with propidium iodide before analysis, and $\approx 25,000$ cells were analyzed per genotype.

Southern and Northern Analyses. Southern blot was probed overnight with either the genotyping probe (flox forward, TTG-GAGCTGTCTAGTTAGTTATGC; flox reverse, GTTGCAA-GATAAACATGGTCACAA) or the minor satellite probe (JPO106, AGTGTATATCAATGAGTTACAATG; JPO107, CATCTAATATGTTCTACAGTGTGG). The major satellite probe and mitochondrial probes were a gift from T. Bestor (Columbia University, New York) (36). Probes used for miRNA Northern blots were as follows: miR292-as, AACTCAAACCTGGCGGCACTT; miR293, AACTACAACTCTGCGGCACT; miR19b, TCAGTTTTGCATGGATTGCACA (35); U6 snRNA, GCTTCACGAATTTGCGTGTCTATCCT.

Antibodies and Western Blots. Whole-cell extract was probed with DICER8 (obtained from the W. Filipowicz laboratory, Friedrich Miescher Institute, Basel) (37) and Dicer 1416 (obtained from the D. Livingston laboratory, Dana Farber Cancer Institute, Boston).

dsRNA and siRNA Silencing Assays. dsRNA was prepared by using a Megascript kit (Ambion) according to the manufacturer's directions. T7-tagged firefly luciferase dsRNA was amplified from pGL3 template (Promega) with the following primer sequences: TAAT-ACGACTCACTATAGGGATAAAGAAAGGCCCGCGCC (firefly luciferase forward) and TAATACGACTCACTATAGG-GACGAACGTGTACATCGACTGAAAT (firefly luciferase reverse). pEGFP-N1 (Clontech) was used as a PCR template for GFP dsRNA, using primers TAATACGACTCACTATAGGGATC-CTGGTTCGAGCTGGAC (T7 GFP forward) and TAATAC-GACTCACTATAGGGTGTCTCAGGTAGTGGTTGT (T7 GFP reverse). DsRNA was transfected into ES cells by using Lipofectamine 2000 (Invitrogen) at 10 $\mu\text{g}/\text{ml}$ with dsRNA at a concentration of 2 $\mu\text{g}/\text{ml}$, along with reporter plasmids pSV40renilla and pGL3 (Promega) at 0.1 $\mu\text{g}/\text{ml}$ and 1 $\mu\text{g}/\text{ml}$ concentrations, respectively. siRNAs were transfected into ES cells at a total concentration of 100 nM, with the total concentration being maintained by mixing variable ratios of target siRNA and control siRNA. Luciferase activity was measured 48 h after transfection by using a Dual Luciferase Detection Kit (Promega).

cDNA Preparation and Quantitation. A total of 0.1 μg of purified RNA was used in cDNA synthesis with 0.1 μg of random nonamer primers in a 20- μl reaction volume. One microliter of this cDNA was used as template for quantitative PCR analysis of actin transcript levels with actin forward and actin reverse primers (actin forward, ACCCACACTGTGCCCATCTAC; actin reverse, AGC-CAAGTCCAGACGCAGG). Relative amplification of duplicate samples for each cDNA was compared with 5-fold serial dilutions of wild-type AB2.2 cDNA, with C_t values plotted against log dilutions. For centromeric transcript analyses, 1 μg of RNA was used for cDNA synthesis with oligo T₁₂, random nonamers, MajF1, or MajR1 (MajF1, GACGACTTGAAAAATGACGAAATC; MajR1, CATATTCCAGGTCCTTCAGTGTGC) (38). One microliter of this cDNA was used for PCR analysis of satellite transcripts. Products were stained with ethidium bromide before photography. PCRs performed with 25 cycles were "spiked" by addition of [α -³²P]dCTP, and products were fractionated on 4% polyacrylamide gels before drying gels and quantifying bands by phosphorimaging.

Chromatin Immunoprecipitations. Chromatin immunoprecipitations were done by using anti-trimethyl H3K9 antibody (1:100, Upstate Biotechnology, Lake Placid, NY) according to Upstate Biotechnology protocol. Immunoprecipitated DNA was used in PCRs with primers specific for the major satellite (38).

Results

Generation of Dicer-Deficient ES Cells. To generate ES cells that conditionally express Dicer, we replaced a wild-type Dicer allele with a floxed allele in ES cells that already contained a null Dicer allele (1) (Fig. 1). We screened puromycin-resistant clones for those in which the intact allele of Dicer had been replaced by the floxed allele. In these clones, we expected that functional Dicer would be expressed from the floxed locus, in which exons 22 and 23, encoding the majority of both catalytic RNaseIII domains, were flanked by loxP sites (Fig. 1).

To examine the phenotypic effects of Dicer loss, we used the Dicer^{flox/null} clones to generate Dicer-deficient cells. Expression of Cre recombinase in Dicer^{flox/null} ES cells was expected to lead to recombination between the loxP sites and excision of exons 22 and 23, resulting in Dicer-deficient ES cells. We transiently expressed Cre by transfection of a Cre-expression plasmid, plated cells onto gelatin-coated plates, and analyzed the plates by Southern blotting for the presence of Dicer-deficient cells. Initially highly enriched, Dicer-deficient cells represented $\approx 30\%$ of the population at 10 days after Cre treatment. However, the Dicer-deficient subpopulation was rapidly depleted and, by 2 weeks after Cre transfection, was no longer detectable by Southern blot analysis (Fig. 2A and B). These results demonstrated that Dicer-deficient ES cells are at a growth disadvantage compared with the Dicer^{flox/null} cells, indicating the absence of Dicer either causes a significant alteration in the growth of ES cells or compromises their survival.

To distinguish between these possibilities, we plated Dicer^{flox/null} populations enriched for Cre-GFP transfection at clonogenic densities. Single clones were picked 6 days after Cre transfection and analyzed by PCR for recombination between loxP sites. Among different experiments, between 50% and 90% of clones analyzed had undergone Cre-mediated excision events. Clones were then transferred to individual wells of a 96-well plate and cultured in isolation either on gelatin or on an irradiated feeder layer. Strikingly, within 1 week of culture in individual wells, all clones that had not undergone Cre-mediated excision events had achieved confluence, whereas no Dicer-deficient clones had reached a similar density. After several weeks, a small number of these Dicer-deficient clones had proliferation rates sufficient to establish continuous Dicer-deficient cell lines. In this study we primarily analyzed two representative Dicer-deficient cell lines isolated from populations derived on gelatin. For comparison,

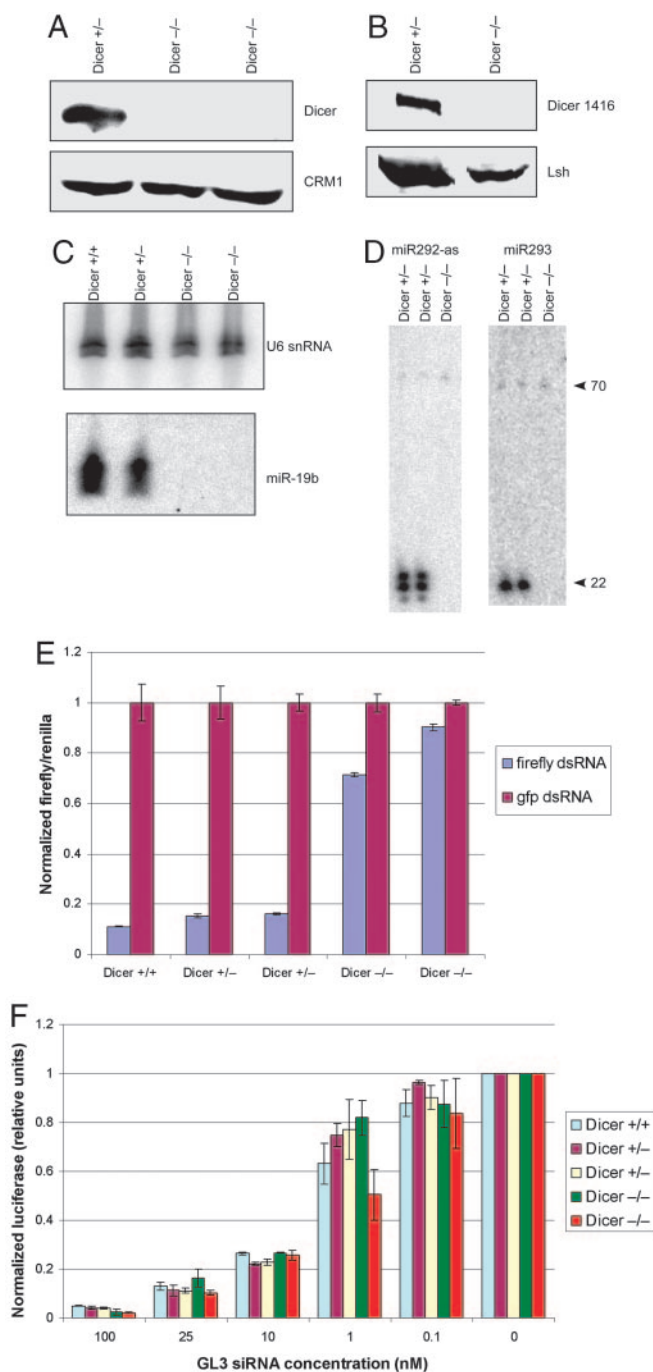


Fig. 3. Dicer-null ES cells do not process dsRNA or pre-miRNAs but are able to respond to siRNAs. No full-length Dicer protein can be detected in whole-cell lysates of Dicer-deficient cells. (A) Western blotting using an antibody recognizing the RNaseIII domains of Dicer. (B) Western blotting using an antibody recognizing the PAZ domain of Dicer. Dicer-null ES cells fail to process pre-miRNAs into mature miRNAs. Total RNA extracted from floxed/null and floxed excised/null ES cells was probed with ^{32}P -labeled oligonucleotides complementary to miR-19b (with equal loading indicated by blotting for U6 snRNA) (C) or miR-293 and miR-292-as (D). (E) Dicer-null ES cells fail to initiate gene silencing in response to dsRNA. dsRNAs, 500 nt in length, corresponding to either firefly luciferase or GFP were introduced into ES cells along with firefly and *Renilla* luciferase reporters. (F) Standard dual luciferase assays were performed on ES cells transfected with siRNAs.

absence of mature miRNAs in Dicer-deficient cells and, in some cases, a slight accumulation of Dicer substrates, the pre-miRNAs (Fig. 3 C and D).

ES cells have previously been shown to lack prominent nonspecific (e.g., PKR) responses to long dsRNAs and instead to mount a sequence-specific silencing response to these triggers (37, 39, 40). To confirm that Dicer is essential for these sequence-directed responses, we transfected 500-bp dsRNAs corresponding in sequence to either firefly luciferase or GFP into our Dicer-deficient cells and their Dicer-expressing siblings. Wild-type and heterozygous ES cell lines were greatly reduced in firefly luciferase activity when transfected with firefly dsRNA, as compared with cells transfected with GFP dsRNA (Fig. 3E). However, Dicer-deficient cells failed to silence firefly luciferase to a significant extent after firefly dsRNA treatment (Fig. 3E). Considered together, these studies confirm that a single mouse Dicer enzyme is required for both miRNA and dsRNA processing pathways.

Responses to siRNAs. Synthetic siRNAs mimic Dicer cleavage products and presumably act downstream of Dicer. Therefore, there is not an *a priori* expectation that these RNAi triggers would require Dicer for their action. However, previous studies concluded that Dicer is required for siRNAs to trigger silencing in mammalian cells (41). Therefore, we asked whether siRNAs are functional in the absence of Dicer in mouse ES cells.

We transfected a mixture of a firefly luciferase siRNA and a second siRNA that does not target our reporter into cells, varying the concentration of the relevant siRNA but keeping the overall siRNA concentration constant. When the dual luciferase activity was analyzed 48 h after transfection, there was a clear concentration-dependent reduction in firefly luciferase activity. Interestingly, the effect was comparable in wild-type ES cells (AB2.2), Dicer^{flox/null} cells, or cells that are null for Dicer (Fig. 3F). These studies suggested that siRNAs can indeed enter RISC and function in the absence of an active Dicer protein in mammals. It has been reported elsewhere that Dicer-deficient mammalian cells are competent for siRNA-mediated silencing (19). Our data extend these findings by showing that lack of Dicer does not change the IC₅₀ for inhibition by a small RNA.

Accumulation of Centromeric Transcripts in Dicer-Deficient ES Cells. In a number of organisms, RNAi has been implicated in the regulation of heterochromatin, especially at centromeres (17, 20, 22, 23, 25–27). It has been suggested that transcripts derived from heterochromatic repeats are recognized and processed by Dicer, producing siRNAs that enter complexes involved in nucleating or maintaining centromeric heterochromatin (20, 24, 42). Mouse centromeres are composed of highly repetitive heterochromatic loci, divided into two classes of repeats known as the major satellite and the minor satellite. In accord with the situation previously characterized in *S. pombe*, transcripts derived from both strands of the satellite repeats have been detected in mouse cells and in mouse embryos (18, 19, 38, 43, 44). Therefore, these studies are consistent with a possible role of Dicer in maintaining the integrity of mammalian centromeres.

To investigate the role of Dicer at mammalian centromeres, we used RT-PCR to quantify transcript accumulation from the major satellite repeats. Using actin as a standardization control, we found an accumulation of major satellite transcripts in cDNA primed with random primers, oligo(dT), and major satellite-specific primers (see Fig. 6, which is published as supporting information on the PNAS web site). This effect was quantified, and Dicer-deficient cells showed a consistently greater than 2-fold increase in major satellite transcript levels, as compared with heterozygous and wild-type cells (AB2.2) (Fig. 4A).

There are several possible interpretations of these findings. In one model, loss of Dicer would lead to deconstruction of centromeric heterochromatin, similar to what is seen in *S. pombe*. The status of heterochromatin at centromeres can be monitored in part by examining patterns of cytosine methylation, one important marker of constitutive heterochromatin in mammals. We used

normally involved in degrading transcripts that are generated from these repeated loci. These studies do not rule out the possibility that Dicer might be involved in initiating DNA methylation at centromeric regions, e.g., during neocentromere formation.

Discussion

We have constructed floxed Dicer mouse ES cell lines that can be induced by Cre recombinase to excise the catalytic RNaseIII domain-encoding exons of Dicer. This system has allowed us to examine the acute effects of Dicer loss on ES cells in culture as well as to establish Dicer-deficient cell lines.

Studies of Dicer-deficient animals have revealed that Dicer plays critical roles during development (1, 14, 15). However, in part owing to the extremely early lethality in Dicer-deficient organisms, the causes underlying the phenotypes have remained unclear. Using our floxed Dicer ES cell line, we have shown that loss of Dicer in ES cells leads to a pronounced proliferation defect, an effect that can be eventually partially overcome, perhaps by accumulation of secondary mutations. This phenotype could result from an absence of one or more mature miRNAs, perhaps one required to repress a cell-cycle inhibitor that would otherwise be expressed in ES cells. Alternatively, a more global effect on genome organization and structure could trigger checkpoint responses that arrest proliferation.

We show here that removal of Dicer from ES cells also results in accumulation of transcripts derived from the major satellite of the mouse centromere. Additionally, the transcripts seem to be dynamically regulated during mouse development (18, 43). Dicer-deficient ES cells similarly have a clearly increased level of major satellite transcripts, but we cannot distinguish between the possibilities that this is due to transcript stabilization or a secondary effect of loss of Dicer on the expression of a gene that normally controls expression from centromeres. Interestingly, we detect no effect of Dicer loss on cytosine methylation or histone H3 lysine 9 trimethylation status at the centromeres. Thus, our results suggest that Dicer is dispensable for the maintenance of pericentric heterochromatin in mouse.

A recent study has shown that Dicer is required by mouse ES cells for differentiation *in vitro* and *in vivo* and that loss of Dicer can lead to transcriptional derepression of major and minor satellite repeats accompanied by loss of cytosine methylation at these regions (19). In our current study, however, we were unable

to detect DNA methylation defects in ES cells deficient in Dicer. There are several explanations for these apparently contradictory results. It is possible that small amounts of catalytically inert, truncated Dicer protein are produced from either or both the allele described here or that described by C. Kanellopoulou *et al.* (19) and that this is sufficient to retain or perhaps inhibit cytosine methylation at the centromeres. Alternatively, perhaps selective pressure accompanied by prolonged cell culture has led to two distinct outcomes with respect to DNA methylation at the major and minor satellite repeats. Interestingly, loss of Dicer in developing mouse thymocytes leads to no apparent loss of constitutive heterochromatin, suggesting that there may be cell-type-specific regulation of heterochromatin.

The observations indicate that Dicer-deficient ES cells retain their ability to incorporate siRNAs into RISC. Thus, RISC loading in mammals must differ in some way from the process that has been studied in *Drosophila*. In mice, loading of siRNAs may depend more heavily on an as yet unidentified ortholog of *Drosophila* R2D2, with the role played by Dicer in this process being either diminished or completely eliminated. Of course, we cannot absolutely eliminate the possibility that small amounts of a truncated, catalytically inert Dicer protein are produced in our Dicer-null cells that might be selectively able to participate in RISC assembly.

The conditional Dicer^{fllox/null} ES cell that we have generated will be useful in dissecting in a mammalian setting the many roles of Dicer and, by implication, the RNAi machinery, in gene regulation, genomic organization, and genome defense.

We thank members of the G.J.H. laboratory for helpful discussions. C. Marsden, P. Moody, and J. Morris provided critical technical assistance. We are grateful to W. Filipowicz, C. Kanellopoulou (Dana Farber Cancer Institute), D. Livingston, D. Lee, D. Threadgill, T. Jenuwein (Research Institute of Molecular Pathology, Vienna), and T. Bestor for their generous gifts of materials. E.P.M. is an Elisabeth Sloan Livingstone Fellow and is supported by a fellowship from the Department of Defense Breast Cancer Research Program (X81XWH-05-1-0256). This work was supported by National Cancer Institute Cancer Center Support Grant Developmental Funds 2 P30 CA 21765-25, -26 (to J.F.P.) and the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital. G.J.H. is supported by funds from the Department of Defense Breast Cancer Research program and the National Institutes of Health.

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