Site-specific DICER and DROSHA RNA products control the DNA-damage response

Sofia Francia1,2, Flavia Michelini1, Alka Saxena3, Dave Tang3, Michiel de Hoon3, Viviana Anelli1†, Marina Mione1†, Piero Carninci3 & Fabrizio d’Adda di Fagagna1,4

Non-coding RNAs (ncRNAs) are involved in an increasingly recognized number of cellular events1. Some ncRNAs are processed by DICER and DROSHA RNases to give rise to small double-stranded RNAs involved in RNA interference (RNAi)2. The DNA-damage response (DDR) is a signalling pathway that originates from a DNA lesion and arrests cell proliferation3. So far, DICER and DROSHA RNA products have not been reported to control DDR activation. Here we show, in human, mouse and zebrafish, that DICER and DROSHA, but not downstream elements of the RNAi pathway, are necessary to activate the DDR upon exogenous DNA damage and oncogene-induced genotoxic stress, as studied by DDR foci formation and by checkpoint assays. DDR foci are sensitive to RNase A treatment, and DICER- and DROSHA-dependent RNA products are required to restore DDR foci in RNase-A-treated cells. Through RNA deep sequencing and the study of DDR activation at a single inducible DNA double-strand break, we demonstrate that DDR foci formation requires site-specific DICER- and DROSHA-dependent small RNAs, named DDRNAs, which act in a MRE11—RAD50—NBS1—complex-dependent manner (MRE11 also known as MRE11A; NBS1 also known as NBN). DDRNAs, either chemically synthesized or in vitro generated by DICER cleavage, are sufficient to restore the DDR in RNase-A-treated cells, also in the absence of other cellular RNAs. Our results describe an unanticipated direct role of a novel class of ncRNAs in the control of DDR activation at sites of DNA damage.

Mammalian genomes are pervasively transcribed, with most transcripts apparently not associated with coding functions4,5. An increasing number of ncRNAs have been shown to have a variety of relevant cellular functions, often with very low estimated expression levels6–8. DICER and DROSHA are two RNAse type III enzymes that process ncRNA hairpin structures to generate small double-stranded RNAs9 (see Supplementary Information).

Detection of a DNA double-strand break (DSB) triggers the kinase activity of ATM, which initiates a signalling cascade by phosphorylating the histone variant H2AX (γH2AX) at the DNA-damage site and recruiting additional DDR factors. This establishes a local self-feeding loop that leads to accumulation of upstream DDR factors in the form of cytologically detectable foci at damaged DNA sites10. The DDR has been considered to be a signalling cascade made up exclusively of proteins, with no direct contributions from RNA species to its activation.

Oncogene-induced senescence (OIS) is a non-proliferative state characterized by a sustained DDR11 and senescence-associated heterochromatoc foci (SAHF)12. Because ncRNAs participate in heterochromatin formation13, we investigated whether they could control SAHF and OIS. We used small interfering RNAs (siRNAs) to knockdown DICER or DROSHA in OIS cells and monitored SAHF and cell-cycle progression. Knockdown of either DICER or DROSHA, as well as ATM as control14, restored DNA replication and entry into mitosis (Supplementary Figs 1 and 2); we did not detect overt SAHF changes, however (Supplementary Fig. 3a, b). Instead, we observed that DICER or DROSHA inactivation significantly reduced the number of cells positive for DDR foci containing 53BP1, the autophosphorylated form of ATM (pATM) and the phosphorylated substrates of ATM and ATR (pS/TQ), but not γH2AX, without decreasing the expression of proteins involved in the DDR (Supplementary Fig. 3a–c). Importantly, the simultaneous inactivation of all three GW182-like proteins, TNRC6A, B and C, essential for the translational inhibition mediated by microRNAs (miRNAs; canonical DICER and DROSHA products involved in RNAi)15, does not affect DDR foci formation (Supplementary Fig. 4).

We next asked whether DICER or DROSHA inactivation also affects ionizing-radiation-induced DDR activation. We transiently inactivated DICER or DROSHA by siRNA in human normal fibroblasts (HNFs), exposed cells to ionizing radiation, and monitored DDR foci. We observed that a few hours after exposure to ionizing radiation, DICER or DROSHA inactivation impairs the formation of pATM, pS/TQ and MDC1, but not γH2AX, foci without decreasing their protein levels (Fig. 1a, b and Supplementary Fig. 5). Furthermore, at an earlier time point (10 min) after ionizing radiation, 53BP1 foci were significantly reduced (Supplementary Fig. 6a). Using an RNAi-resistant form of DICER in DICER knockdown cells, we observed that re-expression of wild-type DICER, but not of a DICER endonuclease mutant (DICER44ab)16, rescues DDR foci formation (Supplementary Fig. 6b–d). The simultaneous knockdown of TNRC6A, B and C, or DICER has a comparable impact on a reporter system specific for miRNA-dependent translational repression17, but only DICER inactivation reduces DDR foci formation (Supplementary Fig. 7). To confirm further the involvement of DICER in DDR activation, we used a cell line carrying a hypomorphic allele of DICER in DICER knockdown cells, and observed that ATM autophosphorylation is reduced in DICER knockdown cells, but not of a DICER endonuclease mutant (DICER44ab)16, rescues DDR foci formation (Supplementary Fig. 7). The simultaneous inactivation of all three GW182-like proteins, TNRC6A, B and C, essential for the translational inhibition mediated by microRNAs (miRNAs; canonical DICER and DROSHA products involved in RNAi)15, does not affect DDR foci formation (Supplementary Fig. 4).
wild-type DICER re-expression in DICERexon5 cells restores checkpoint functions whereas two independent mutant forms of DICER fail to do so (Supplementary Figs 11–13). Thus, DICER and DROSHA are required for DNA-damage-induced checkpoint enforcement.

To test the role of DICER in DDR activation in a living organism, we inactivated it by morpholino antisense oligonucleotide injection in Danio rerio (zebrafish) larvae19. Such Dicer inactivation results in a marked impairment of pAtm and zebrafish γH2AX accumulation in irradiated larvae as detected both by immunostaining and immunoblotting of untreated or Dicer morpholino-injected larvae and of chimaeric animals (Supplementary Figs 14 and 15).

Previous reports have shown that mammalian cells can withstand transient membrane permeabilization and RNase A treatment, enabling investigation of the contribution of RNA to heterochromatin organization and 53BP1 association to chromatin20,21. We used this approach to address the direct contribution of DICER and DROSHA RNA products in DDR activation. Irradiated HeLa cells were permeabilized and treated with RNase A, leading to degradation of all RNAs, without affecting protein levels (Supplementary Fig. 16a). We observed that 53BP1, pATM, pS/TQ and MDC1 foci become markedly reduced in number and intensity upon RNA degradation whereas, similarly to DICER- or DROSHA-inactivated cells, γH2AX is unaffected (Fig. 2a and Supplementary Fig. 16b). Notably, 53BP1, MDC1 and γH2AX triple staining shows that RNA degradation reduces 53BP1 and MDC1 accumulation at unperturbed γH2AX foci (Supplementary Fig. 16c). When RNase A is inhibited, DDR foci progressively reappear within minutes and α-amanitin prevents this (Supplementary Fig. 17a, b), suggesting that DDR foci stability is RNA polymerase II dependent.

We tested whether DDR foci can reform upon addition of exogenous RNA to RNase-A-treated cells. We observed that DDR foci robustly reform in RNase-A-treated cells following their incubation with total RNA purified from the same cells, but not with transfer RNA (tRNA) control (Fig. 2b–d). Similar conclusions were reached using an inducible form of PpoI and AsiSI site-specific endonucleases22,23 (data not shown).

Next, we attempted to characterize the length of the RNA species involved in DDR foci reformation, which we refer to as DDRNAs. We observed that an RNA fraction enriched by chromatography for species <200 nucleotides was sufficient to restore DDR foci (Supplementary Fig. 17c–e). To attain better size separation, we resolved total RNA on a polyacrylamide gel and recovered RNA fractions of different lengths (Supplementary Fig. 17f, g). Using equal amounts of each fraction, we observed that only the 20–35-nucleotide fraction could restore DDR foci (Fig. 2b), consistent with the size range of DICER and DROSHA RNA products.

To test the hypothesis that DDRNAs are DICER and DROSHA products, we tested DDR foci restoration with total RNA extracted from wild-type or DICERexon5 cells. Although RNA extracted from wild-type cells restores pATM, pS/TQ and 53BP1 foci, RNA from DICERexon5 cells does not (Fig. 2c, d). Importantly, RNA from

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**Figure 1** | DICER or DROSHA inactivation impairs DDR foci formation in irradiated cells. a, DICER or DROSHA knockdown WI-38 cells were irradiated (10 Gy) and fixed 7 h later. Original magnification, ×250. b, Histogram shows the percentage of cells positive for pATM, pS/TQ, MDC1 and γH2AX foci. c, Wild-type (WT) and DICERexon5 cells were irradiated (2 Gy) and fixed 2 h later. Histogram shows the percentage of cells positive for pATM, pS/TQ, MDC1 and γH2AX foci. Error bars indicate s.e.m. (n ≥ 3). Differences are statistically significant (*P value < 0.01).
DICER<sup>exon5</sup> cells re-expressing wild-type, but not endonuclease-mutant, DICER allows DDR focus reformation (Supplementary Fig. 18a, b). These results were reproduced using RNA extracted from cells transiently knocked down for DICER or DROSHA (Supplementary Fig. 18c–f).

Ionizing radiation induces DNA lesions that are heterogeneous in nature and random in their genomic location. To reduce this complexity, we studied a single DSB at a defined and traceable genomic locus. We therefore took advantage of NIH2/4 mouse cells carrying an integrated copy of the I-SceI restriction site flanked by arrays of Lac- or Tet-operator repeats at either sites<sup>19</sup>. In this cell line, the expression of the I-SceI restriction enzyme together with the fluorescent protein Cherry-Lac-repressor allows the visualization of a site-specific DDR focus formation, we used a specific MRN inhibitor<sup>26</sup>, mirin, synthesized four potential pairs among the sequences obtained and probed for 53BP1, pATM, pS/TQ, MDC1 and γH2AX focus. Histogram shows the percentage of cells positive for DDR foci (Fig. 3c), indicating that DDRNAs originate from the damaged genomic locus.

The MRE11–RAD50–NBS1 (MRN) complex is necessary for ATM activation<sup>25</sup>, and pATM and MRE11 foci formation is sensitive to RNase A treatment in the NIH2/4 cell system (Supplementary Fig. 19a, b). To probe the molecular mechanisms by which RNA modulates DDR focus formation, we used a specific MRN inhibitor<sup>26</sup>, mirin, which prevents ATM activation also in the NIH2/4 system (Supplementary Fig. 19d). In the presence of mirin, NIH2/4 RNA is unable to restore 53BP1 or pATM focus formation (Fig. 3d, e), indicating that DDRNAs act in a MRN-dependent manner.

To detect potential short RNAs originating from the integrated locus, we deep-sequenced libraries generated from short (<200 nucleotides) nuclear RNAs of cut or uncut NIH2/4 cells, as well as from parental cells expressing I-SceI as negative control. Sequencing revealed short transcripts arising from the exogenous locus (Supplementary Fig. 20a–e), 47 reads in cut cells, 20 reads in uncut cells and none in parental cells, indicating that even an exogenous integrated locus lacking mammalian transcriptional regulatory elements is transcribed and can generate small RNAs.

To test whether the identified locus-specific small RNAs are biologically active and have a causal role in DDR activation, we chemically synthesized four potential pairs among the sequences obtained and used them to attempt to restore the DDR focus in RNase-A-treated focus formation at the I-SceI-induced DSB in RNase-A-treated cells. The formation of the 53BP1 focus was efficiently recovered only by RNA purified from NIH2/4 cells and not from parental cells (Fig. 3c), indicating that DDRNAs originate from the damaged genomic locus.
37–23-nucleotide RNAs arising from the locus significantly

supplementary Fig. 22). Our statistical analyses revealed that the percentage

significantly reduced reads mapping to the known miRNAs (supplementary

Fig. 21). As expected, DICER or DROSHA knockdown

as well as DICER or DROSHA knockdown NIH2/4 cells (supplementary

Fig. 20g, h). Overall, these results indicate that DDRNAs are small RNAs with

activity of RNAs processed by DICER, we

processed the resulting RNAs with recombinant DICER.

transcribed both

in vitro

in vivo

were performed

To investigate the biogenesis of such RNAs in vivo, we performed

deeper sequencing of small nuclear RNAs from cut and uncut wild-type

as well as DICER or DROSHA knockdown NIH2/4 cells (supplementary

Fig. 21). As expected, DICER or DROSHA knockdown

significantly reduced reads mapping to the known miRNAs (supplementary

Fig. 22). Our statistical analyses revealed that the percentage

of 22–23-nucleotide RNAs arising from the locus significantly

increases in the wild-type cut sample compared to the uncut one and

that DICER inactivation significantly reduces it (supplementary

Fig. 23a, b); the detectable decrease in DROSHA-inactivated cells did not

reach statistical significance. Because the fraction of 22–23-nucleotide

RNAs from the locus is significantly higher with respect to

that of non-miRNA genomic loci, the RNAs detected are very unlikely

to be random degradation products (supplementary Fig. 23c). Finally,

22–23-nucleotide RNAs at the locus tend to have an A/U at their 5’

and a G at their 3’ end (supplementary Fig. 23d), a nucleotide bias signifi-

cantly different from the originating locus and from the rest of the genome.

In summary, we demonstrate that different sources of DNA damage,
including oncogenic stress, ionizing radiation and site-specific endonucleases,
activate the DDR in a manner dependent on DDRNAs, which are DICER- and
DROSHA-dependent RNA products with the sequence of the damaged site. DDRNAs control DDR

foci formation and maintenance, checkpoint enforcement and cellular

senescence in cultured human and mouse cells and in different cell
types in living zebrafish larvae. They act differently from canonical

miRNAs, as inferred by their demonstrated biological activity independent of other RNAs and of GW182-like proteins.
Figure 4 | Chemically synthesized small RNAs and in vitro-generated DICER RNA products are sufficient to restore DDR focus formation in RNase-A-treated cells in a sequence-specific manner. a, Chemically synthesized RNA oligonucleotides were annealed and tested to restore DDR focus formation in RNase-A-treated cut NIH2/4 cells. Mixed with a constant amount (800 ng) of parental cell RNA, a concentration range (1 ng·μl⁻¹ to 1 fg·μl⁻¹, tenfold dilution steps) of locus-specific or GFP RNAs was used. Locus-specific synthetic RNAs (down to 100 fg·μl⁻¹) allow site-specific DDR activation. b, Small double-stranded RNAs generated by recombinant DICER were tested to restore DDR focus formation in RNase-A-treated cut NIH2/4 cells. 1 ng·μl⁻¹ RNA was tested mixed with 800 ng of parental cell RNA. Locus-specific DICER RNAs, but not control RNAs, allow site-specific DDR activation. Histograms show the percentage of cells positive for DDR focus. Error bars indicate s.e.m. (*P < 0.05).

METHODS SUMMARY
Details of cell cultures, plasmids, sRNAs and antibodies used, as well as descriptions of methods for immunofluorescence, immunoblotting, checkpoint assays, real-time quantitative polymerase chain reaction (PCR), zebrafish injection and transplantation, RNase A treatment, small RNA extraction and purification from gel, RNA sequencing and statistical analyses are provided in Methods.

Full Methods and any associated references are available in the online version of the paper.
METHODS

Cultured cells. Early-passage WI-38 cells (ATCC) were grown under standard tissue culture conditions (37 °C, 5% CO2) in MEM supplemented with 10% fetal bovine serum, 1% glutamine, 1% non-essential aminoacids, 1% Na pyruvate. HeLa, Phoenix e pocrophic and HEK293T cell lines were grown under standard tissue culture conditions (37 °C, 5% CO2) in DMEM, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin.

NIH2/4 cells were grown in DMEM, supplemented with 10% fetal bovine serum, 1% glutamine, gentamicine (40 μg/ml), and hygromycin (400 μg/ml).

H-RasV12-overexpressing senescent BJ cells were generated as described previously. BrdU incorporation assays were carried out at least 1 week after cultures had fully entered the senescent state, as determined by ceased proliferation, DDR activation and SAHF formation. Ionizing radiation was induced by a high-voltage X-ray generator tube (Faxitron X-Ray Corporation). In general, WI-38 cells were exposed to 5 Gy and transformed cells, 10 Gy for the G1/S checkpoint assays. H.2Ras V12-overexpressing senescent BJ cells were generated as described previously. Short hairpin sequence for p53 was: AGTAGATTACACATCTTCAAGACTTAACT. Short hairpin sequence for DICER is: CCGGCCACACATCTTCAAGACTTAACT

Antibodies. Mouse anti-β-tubulin (clone AA2) and anti-Flag M2 monoclonal antibody (Sigma) were used as control antibodies. Rabbit polyclonal to anti-pH3 (Upstate Biotechnology); anti-pS/TQ (Cell Signaling Technology); anti-H2AX, anti-Lamin A/C (Santa Cruz); anti-vinculin (clone hVIN-1), anti-β-tubulin (clone AA2) and anti-Flag M2 monoclonal antibodies (Sigma).

Indirect immunofluorescence. Cells were grown on poly-l-lysinated coverslips (poly-l-lysine was used at 50 μg/ml) and plated (15-20 x 10^4 cells per cover) 1 day before staining. BrdU and BrdU staining was performed as described previously4. Cells were fixed in 4% paraformaldehyde or methanol:acetone 1:1. NIH2/4 mouse cells were fixed by 4% paraformaldehyde for 10 minutes. HeLa, Phoenix ecotrophic and HEK293T cell lines were grown under standard conditions. Cells were grown on poly-D-lysinated coverslips and plated (15–20 x 10^4 cells per cover) 1 day before staining. Confocal sections were obtained with a Leica TCS SP2 or AOBS confocal laser microscope by sequential scanning. Confocal sections were screened for each antigen. Cells with more than two DDR foci were scored positive. Cells were screened for each antigen. Cells with more than two DDR foci were scored positive. Immunofluorescence was performed in parallel with identical acquisition parameters; at least 100 cells were analysed for each experiment.

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Primers for real-time quantitative PCR. RPS19 expression levels (assay numbers 000397, 001094 and 001003). 18S or RNU19 expression levels (assay numbers 001759, 003755, 001984, 001197, 000741 and 000591). 18S or RNU19 expression levels (assay numbers 001759, 003755, 001984, 001197, 000741 and 000591). Primers were designed using the Roche LightCycler 480 Sequence Detection System. The reactions were preheated using SYBR Green reaction mix from Roche. Ribosomal protein P0 (RP0) was used as a human and mouse control gene for normalization.

Plasmids. DICER-Flag, DICER44ab-Flag and DICER110ab-Flag were a gift from R. Shiekhattar. DICER110ab-Flag and DICER44ab-Flag double mutants carry two amino acid substitutions in the RNase III domains of DICER (Asp 1320 Ala and Asp 1709 Ala for 44ab; and Glu 1652 Alda and Glu 1813 Ald for 110ab mutant; both mutants were reported to be deficient in endonuclease activity6,7). PKL.01shDICER-expressing vector was a gift from W. C. Hahn. Short hairpin sequence for DICER is: CGCGCCGACAACATCTCCTGGAAGCTTATATCGTGAAGTTGGATGTTGATTGTTGTTTTTG. pRETROSUPER shps3 was as described previously8. Short hairpin sequence for p53 was as described previously. Short hairpin sequence for p53 was as described previously. Short hairpin sequence for p53 was as described previously.

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AAGU-3’, oligonucleotide 8: 5’-CUUCCACUUCUCUCUACACUAGAGGAGUG-3’. GFP 1: 5’-GUUACGCUGUCCCGCGGAGUU-3’, GFP 2: 5’-CUCCGGCCGACCGCGAACC-3’. RNAs were resuspended in 60 mM KCl, 6 mM HEPES, pH 7.5, 0.2 mM MgCl2, at the stock concentration of 12 μM, denatured at 95 °C for 5 min and annealed for 10 min at room temperature.

Dicer RNA products were generated as follows. A 350-bp DNA fragment carrying the central portion of the genomic locus studied (three Lac repeats, the I-SceI site and two Tet repeats) was flanked by T7 promoters at both ends and was used as a template for in vitro transcription with the TurboScript T7 transcription kit (Ambion). The 500-nucleotide-long RNAs were purified and incubated with human recombinant Dicer enzyme (Ambion) to generate 22–23-nucleotide RNAs. RNA products were purified, quantified and checked on gel. As a control, the same procedure was followed with a 700-bp construct containing the RFP DNA sequence. Equal amounts of Dicer RNA products generated in this way were used in a complementation experiment in NIH/2A cells following Rnase A treatment.

**Small RNA preparation**. Total RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. To generate small RNA-enriched fraction and small RNA-devoid fraction we used the mirVana microRNA Isolation Kit (Ambion) according to the manufacturer’s instructions. The mirVana microRNA isolation kit uses an organic extraction followed by immobilization of RNA on glass-fibre (silica-fibres) filters to purify either total RNA, or RNA enriched for small species. For total RNA extraction ethanol is added to samples, and they are passed through a filter cartridge containing a glass-fibre filter, which immobilizes the RNA. The filter is then washed a few times and the RNA is eluted with a low ionic-strength solution. To isolate RNA that is highly enriched for small RNA species, ethanol is added to bring the samples to 25% ethanol. When this lysate/ethanol mixture is passed through a glass-fibre filter, large RNAs are immobilized, and the small RNA species are collected in the filtrate. The ethanol concentration of the filtrate is then increased to 55%, and it is passed through a second glass-fibre filter where the small RNAs become immobilized. This RNA is washed a few times, and eluted in a low ionic strength solution. Using this approach consisting of two sequential filtrations with different ethanol concentrations, an RNA fraction highly enriched in RNA species <200 nucleotides can be obtained15–17.

**RNA extraction from gel**. Total RNA samples (15 ng) were heat denatured, loaded and resolved on a 15% denaturing acrylamide gel (1× TBE, 7 M urea, 15% acrylamide (29:1 acryl-bis-acryl)). Gel was run for 1 h at 180 V and stained in GelRed solution. Gel slices were excised according to the RNA molecular weight marker, moved to a 2 ml clean tube, washed and RNA was eluted in 2 ml of ammonium acetate 0.5 M, EDTA 0.1 M in Rnase-free water, rocking overnight at 4 °C. Tubes were then centrifuged 5 min at top speed, the aqueous phase was recovered and RNA was precipitated and resuspended in Rnase free water.

**G1/S Checkpoint assay.** WI-38 cells were irradiated with 10 Gy and 1 h afterwards incubated with BrdU (10 μg/ml) for 7 h; HCT116 cells were irradiated at 2 Gy and incubated with BrdU for 2 h. Cells were fixed with 4% paraformaldehyde and probed for BrdU immunostaining. At least 100 cells per condition were analysed. G2/M checkpoint was analyzed on HEK 293 cells cultured on coverslip in 3% paraformaldehyde for 2 h at room temperature. After equilibration in 10 and 15% sucrose in PBS, larvae were frozen in OCT compound on coverslips on dry ice. Sections were cut with a cryostat at a nominal thickness of 14 μm and collected on Superfrost slides (BDH). Antisera used were zebrafish anti-rH2AX (gift from J. Amatruda19) and pATM (Rockland). GFP fluorescence in transplanted embryos was still easily visible in fixed embryos. Images were acquired with a confocal (Leica SP2) microscope and ×63 oil immersion lens.

**RNA sequencing.** Nuclear RNA shorter than 200 nucleotides was purified using mirVana microRNA Isolation Kit. RNA quality was checked on a small RNA chip (Agilent) before library preparation. For Illumina hi Seq Version3 sequencing, spike RNA was added to each RNA sample in the RNA: spike ratio of 10,000:1 before library preparation and libraries for Illumina GA IIX were prepared without spike. An improved small RNA library preparation protocol was used to prepare libraries20. In brief, adenylated 3′ adapters were ligated to 3′ ends of 3′-OH small RNAs using a truncated RNA ligase enzyme followed by 5′ adaption ligation to 5′-monophosphate ends using RNA ligase enzyme, ensuring specific ligation of non-degraded small RNAs. cDNA was prepared using a primer specific to the 3′ adaptor in the presence of dimer eliminator and amplified for 12–15 PCR cycles. The amplified cDNA library was run on a 6% polyacrylamide gel and the 100 bp band containing cDNAs up to 33 nucleotides long was extracted using standard extraction protocols. Libraries were sequenced after quality check on a DNA high sensitivity chip (Agilent). Multiplexed barcode sequencing was performed on Illumina GA-IIX (35 bp single end reads) and Illumina Hi seq version3 (51 bp single end reads).

**Statistical analyses.** Results are shown as means ± s.e.m. P value was calculated by Chi-squared test. Quantitative PCR with reverse transcription results are shown as means ± s.d. and P value was calculated by Student’s t-test. A value of P < 0.05 was considered significant in independent biological experiments.

**Statistical analysis of small RNA sequencing data.** Statistical significance of downregulation of normalized miRNAs in DICER and DROSHA knockdown samples was calculated using the Wilcoxon signed-rank test.

The differences in the fraction of 22–23 nucleotides versus total small RNAs at the locus between the wild-type, DICER knockdown and DROSHA knockdown before and after cut were calculated by fitting a negative binomial model to the small RNAs count data and performing a likelihood ratio test, keeping the fraction of 22–23 nucleotides over total small RNAs at the locus fixed across conditions under the null hypothesis and allowing it to vary between conditions under the alternative hypothesis.

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