

RESEARCH ARTICLE

Open Access

# Two-step cleavage of hairpin RNA with 5' overhangs by human DICER

Yoshinari Ando<sup>1</sup>, Yoshiko Maida<sup>2</sup>, Ayako Morinaga<sup>1</sup>, Alexander M Burroughs<sup>1</sup>, Ryuichiro Kimura<sup>3</sup>, Joe Chiba<sup>3</sup>, Harukazu Suzuki<sup>1</sup>, Kenkichi Masutomi<sup>2,4</sup>, Yoshihide Hayashizaki<sup>1\*</sup>

## Abstract

**Background:** DICER is an RNase III family endoribonuclease that processes precursor microRNAs (pre-miRNAs) and long double-stranded RNAs, generating microRNA (miRNA) duplexes and short interfering RNA duplexes with 20~23 nucleotides (nts) in length. The typical form of pre-miRNA processed by the Drosha protein is a hairpin RNA with 2-nt 3' overhangs. On the other hand, production of mature miRNA from an endogenous hairpin RNA with 5' overhangs has also been reported, although the mechanism for this process is unknown.

**Results:** In this study, we show that human recombinant DICER protein (rDICER) processes a hairpin RNA with 5' overhangs *in vitro* and generates an intermediate duplex with a 29 nt-5' strand and a 23 nt-3' strand, which was eventually cleaved into a canonical miRNA duplex via a two-step cleavage. The previously identified endogenous pre-miRNA with 5' overhangs, pre-mmu-mir-1982 RNA, is also determined to be a substrate of rDICER through the same two-step cleavage.

**Conclusions:** The two-step cleavage of a hairpin RNA with 5' overhangs shows that DICER releases double-stranded RNAs after the first cleavage and binds them again in the inverse direction for a second cleavage. These findings have implications for how DICER may be able to interact with or process differing precursor structures.

## Background

DICER plays a key role in RNA interference pathways through the biogenesis of microRNA (miRNA) and small interfering RNA (siRNA) [1-3]. Most miRNA genes are transcribed as long primary transcripts (pri-miRNAs) where stem-loop structures with mature miRNA sequences embedded in the arm of a stem are cleaved by the Drosha nuclear microprocessor complex releasing a precursor miRNA (pre-miRNA) hairpin [4,5]. The cleavage site is determined mainly by the distance (~11 bp) from the stem-single stranded RNA junction of pri-miRNA and most pre-miRNAs have 2 nt-3' overhangs [6]. Pre-miRNAs, exported into the cytoplasm by Exportin-5 and Ran-GTP [7], are processed by the RISC loading complex (RLC) into 20~23 nt duplexes where the RNase III enzyme DICER plays a central role together with the double stranded (ds) RNA-binding proteins TRBP and PACT and the

miRNA-associated RNA-induced silencing complex (miRISC) core component Argonaute-2 (AGO2) [8-10]. miRNA duplexes processed by RLC are finally loaded to miRISC as a double stranded-structure [11] and separated into the functional guide strand, which is complementary to the target, and the passenger strand, which is subsequently degraded [12,13]. Strand selection of the functional guide strand by AGO2 depends on the thermodynamic stabilities of the base pairs at the 5' ends of the two strands [12,14,15]. Duplexes of siRNA or miRNA produced by DICER can be loaded in either direction to Argonaute [16-18]. Indeed, the mature miRNA either in the 5' or 3' strands can be harboured from pre-miRNA [19-21]. On the other hand, endogenous human AGO2 can bind directly to pre-miRNAs in DICER-knockout cells [22]. Recently, it was reported that human DICER is not essential for loading dsRNAs to AGO2 but functions in pre-selection of effective siRNAs for handoff to AGO2 [23].

Human DICER is a ~220 kDa protein consisting of several domains; an N-terminal DExH-box RNA helicase-like domain, a DUF283 domain, a PAZ domain,

\* Correspondence: yoshihide@gsc.riken.jp

<sup>1</sup>RIKEN Omics Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan

Full list of author information is available at the end of the article

two RNase III domains (RIIIa and RIIIb), and a dsRNA binding motif domain (DARM) [24]. The two RNase III domains of DICER form a single dsRNA processing center via intramolecular dimerization which together cleave the opposite strands of the dsRNA, generating dinucleotide-long 3' overhangs on both ends [25]. The crystal structure of Dicer from *Giardia intestinalis* showed that the hydrophobic pocket of the PAZ domain was responsible for the binding of the 3' dinucleotide overhangs of the substrate and the connector helix between the PAZ domain and RNase III domain functioned as a molecular ruler measuring the distance from the 3' end of pre-miRNA to the cleavage site [26,27]. However, 3'-dinucleotide dsRNA overhangs are not essential for binding with DICER [28]. When the 3' overhang is removed, DICER can still cleave dsRNA through interaction with the remaining 5' overhang [28]. This is consistent with MacRae *et al.* who found that the recombinant Dicer protein of *Giardia intestinalis* could cleave the dsRNA with 5' overhangs [27]. However, they used perfectly matched dsRNAs with no gap, which might resemble an endogenous siRNA precursor. An additional study by Flores-Jasso *et al.* showed that human recombinant DICER protein could nick either strand of a mononucleotide-5' overhanged pre-miRNA with some strand preferences [29]. Despite this, the detailed step mechanism for pre-miRNA cleavage, especially for the pre-miRNA with 5' overhangs, is not yet elucidated.

An alternative nuclear pathway of pre-miRNA biogenesis was described where a short intron with a hairpin can be spliced and debranched into pre-miRNA hairpin mimics (mirtrons) [30-32]. This processing pathway uses intron splicing machinery instead of the Drosha endonuclease; miRNA precursors generated from intronic sequences (debranched mirtrons) are believed to be incorporated into the canonical miRNA pathway as a substrate of DICER. Interestingly, mouse pre-mir-1982 is a mirtron with an 11 nt tail at the 5' end [33], although most mammalian mirtron are hairpin structures with single nucleotide overhangs at both ends [32-34]. Mature mouse miR-1982\* miRNA emerges without 11 nt-5' overhangs from deep sequencing data of murine cells [33,35] while the elimination mechanism of this 11 nt-5' tail is still unknown.

In this paper, we investigated the detailed processing pattern of hairpin RNAs containing 5' overhangs by human recombinant DICER. We show here that human recombinant DICER is able to process hairpin RNA with 5' overhangs and two-step cleavage by DICER forms the mature miRNA duplex from the hairpin RNAs. Additionally, pre-mmu-mir-1982 RNA, which is a natural hairpin RNA with 5' overhangs, is also

processed by a two-step cleavage mediated by human recombinant DICER protein *in vitro*.

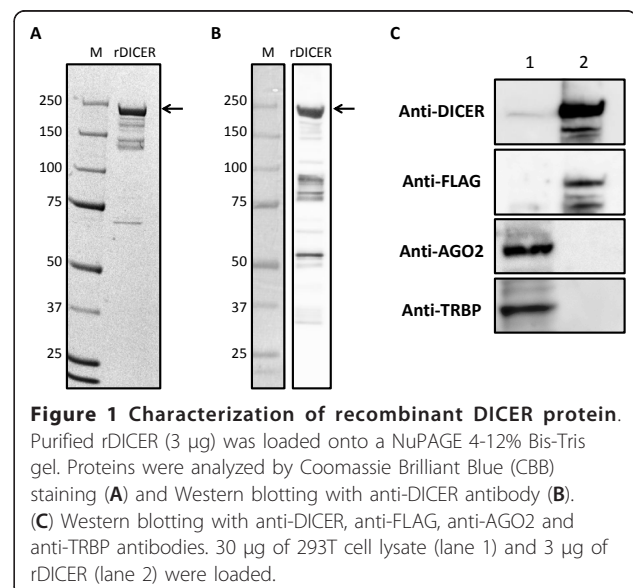
## Results and Discussion

### Processing of the pre-miRNA by recombinant DICER protein

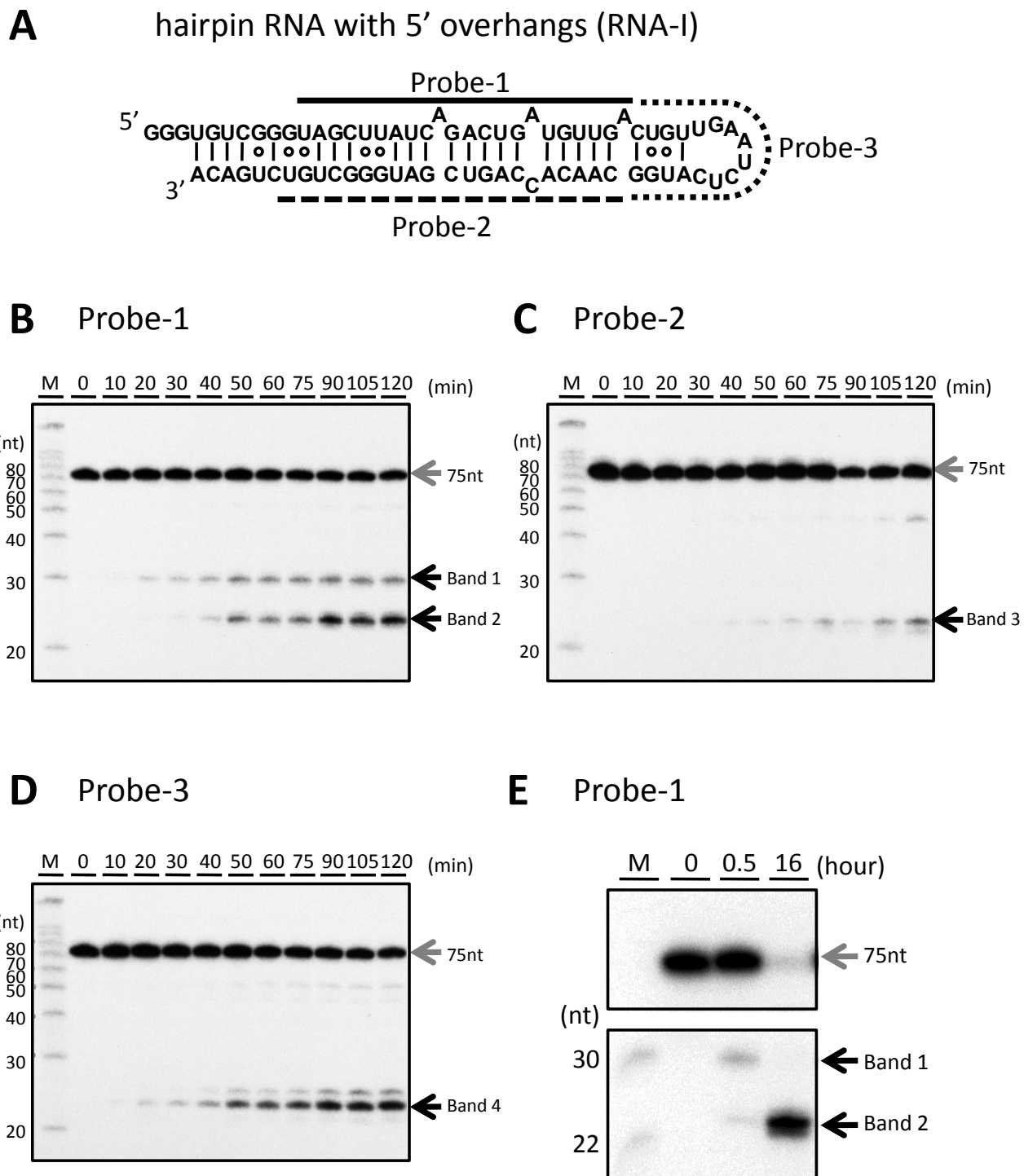
We prepared purified recombinant DICER1 (rDICER) protein containing a FLAG-tag at the N-terminus (see Figures 1A and 1B). This rDICER does not contain known DICER-binding partners, AGO2 and TRBP (see Figure 1C). In order to confirm activity, we attempted to cleave pre-miRNA hairpin RNA using the rDICER. Forty-five pmol of pre-mir-21 RNA (see Figure 2A) was incubated with 2 pmol of the purified rDICER at the indicated times followed by purification. The reacted RNA substrates were subjected to Northern blotting using probe-1, corresponding to the antisense sequence of bases 2-22 of pre-mir-21 (see Figure 2A). A single band, 23 nucleotides in length, appeared after 20 min incubation and gradually increased. Thus, the purified rDICER possessed reasonable pre-miRNA processing activity to produce ~23 nt mature miRNA *in vitro* (see Figure 2B).

### Processing of the hairpin RNA with 5' overhangs, RNA-I, by recombinant DICER protein

Using this rDICER, we performed a cleavage assay on a designed pre-miRNA mimic of hairpin RNA with trinucleotide-5' overhangs (RNA-I, see Figure 3A) to analyze whether DICER could process a hairpin RNA with 5' overhangs. The cleavage products were detected by Northern blotting using three different probes, probe-1, probe-2 and probe-3, corresponding to antisense



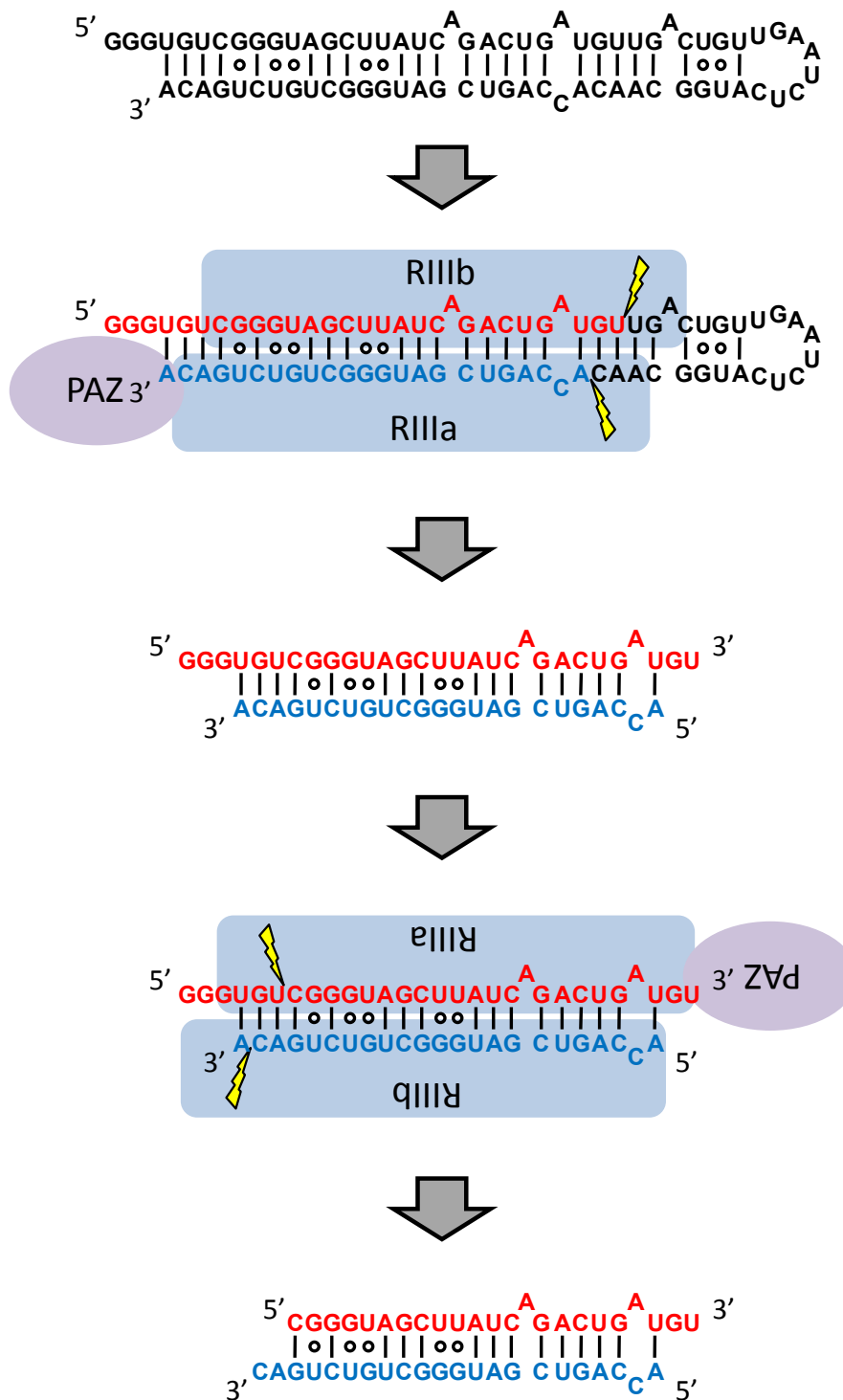




**Figure 3 Processing of the hairpin RNA with 5' overhangs by recombinant DICER protein.** (A) Hairpin RNA with 5' overhangs (RNA-I) and probes used in this study. RNA-I was a hairpin RNA with 5' overhangs based on the "pre-mir-21 RNA" sequence. The secondary structure was predicted using the CentroidFold program [49]. The solid line shows the position of probe-1, the dashed line shows the position of probe-2 and the dotted line shows the position of probe-3. (B-D) Time-course analysis of the processing of RNA-I by the rDICER protein. RNA-I RNAs were incubated with rDICER *in vitro* for the indicated time points (0, 10, 20, 30, 40, 50, 60, 75, 90, 105 and 120 min). The RNAs processed by rDICER were detected using probe-1, probe-2 and probe-3 (B-D, respectively) by Northern blotting. The gray arrow shows the band of unprocessed RNA-I and the black arrow shows the bands of small RNA processed from the 5' strand, 3' strand and loop region of RNA-I respectively. M: decade marker. (E) The processing of RNA-I by the rDICER protein at a longer incubation time. RNA-I RNAs were incubated with rDICER for 30 min and 16 hours. The RNAs processed from the 5' strand of RNA-I were detected using probe-1 by Northern blotting.



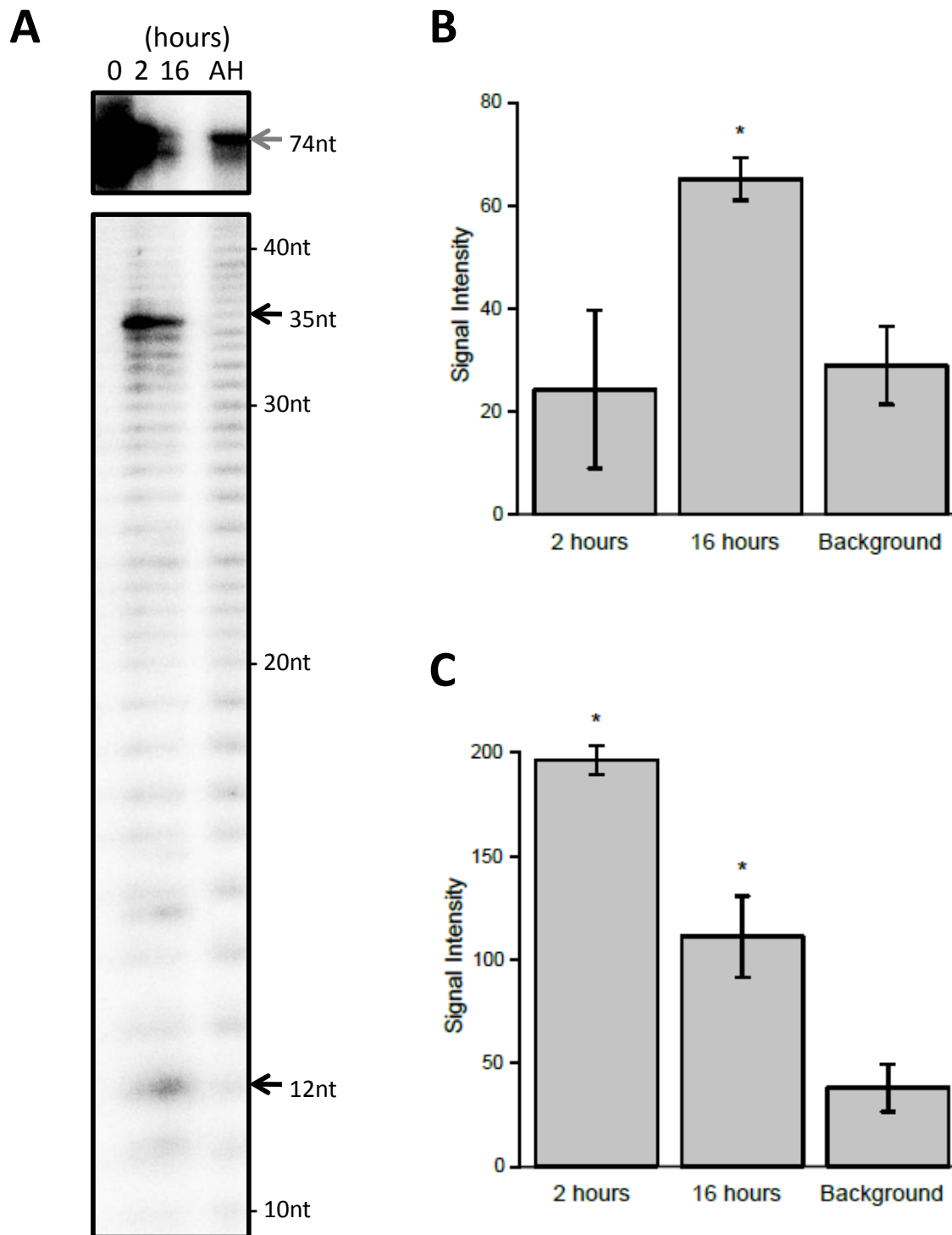




**Figure 5 Model of the two-step processing of hairpin RNA with 5' overhangs (RNA-I) by DICER protein.** rDICER processes hairpin RNA with 5' overhangs (RNA-I) to dsRNA with 29 nt-5' strand and 23 nt-3' strand after the first cleavage reaction and releases once from the binding site. Then, the dsRNA is bound in the inverse direction with the same or different rDICER molecule and is measured after the anchoring 3' end of the 29-nt strand to generate dsRNA with 23 nt cleaved from the 29-nt strand and 22 nt cleaved from the 23-nt strand. "PAZ" domain of rDICER colored purple; "RIIIa" and "RIIIb" domain of rDICER colored blue. Lightning marks indicate the cleavage sites in the RNA.







**Figure 7 Two-step processing of the 5'-end labelled pre-mmu-mir-1982 RNA by recombinant DICER protein.** (A) *In vitro* processing of the pre-mmu-mir-1982 RNA by the rDICER protein at a longer incubation time. 5' labelled pre-mmu-mir-1982 RNAs were incubated with rDICER for 0, 2 and 16 hours. The gray arrow shows the band of unprocessed RNA and the black arrow shows the bands of small RNA processed from pre-mmu-mir-1982 RNA. AH: the alkaline hydrolysis ladder of pre-mmu-mir-1982 RNA. The size of each band was determined by the AH ladder. (B-C) The signal intensities were quantified from the 12 nt (B) and 35 nt (C) bands in Figure 7A. These plots show average values bracketed by s. e.m. error bars; calculated from two independent experiments. Background refers to the signal intensity of the same sized band in the AH lane. The p-value was calculated using a simple t-test for each time point (2 hrs and 16 hrs) relative to the background. Significant differences ( $p < 0.05$ ) in signal intensities are denoted with an asterisk. The significant calculated p-values are as follows: the 12-nt band at 16 hours,  $p = 0.017$ ; the 35-nt band at 2 hours,  $p = 0.0073$ ; and the 35-nt band at 16 hours,  $p = 0.024$ .

## Conclusions

We show human rDICER recognizes and processes a hairpin RNA bearing a trinucleotide-5' overhang, and the two-step cleavage by rDICER forms canonical miRNA duplexes from the hairpin RNAs. It indicates that human rDICER functions as a molecular ruler by anchoring the 3' end of both the hairpin RNA with 5' overhangs and the 5' strand in the intermediate duplex. Moreover, an endogenously-expressed pre-miRNA with 5' overhangs, pre-mmu-mir-1982, also can be utilized as a substrate of rDICER and processed into a canonical miRNA duplex by the two-step cleavage reaction. While pre-mmu-mir-1982 RNA is a naturally expressed pre-miRNA [33,35], this 5'-overhanged structure is not a suitable substrate for nuclear export by Exportin-5 [43] and, assuming the absence of possible alternative export pathways, may not be presented to cytoplasmic DICER in the cells. However, it is worth noting a recent report, that mammalian DICER might be located in the nucleus and associate with ribosomal DNA chromatin [44]. We have also observed human DICER localized in both cytoplasm and nucleus (unpublished data, Ando *et al.*). These findings raise the intriguing possibility that nuclear DICER could process hairpin RNA with 5'-overhangs, like pre-mmu-mir-1982 RNA.

The two-step cleavage of a hairpin RNA with 5' overhangs shows that rDICER can release dsRNAs after the first cleavage and binds them again in the inverse direction for a second cleavage. The DICER protein's ability to release and bind dsRNA again indicates DICER could be capable of binding and processing dsRNA multiple times during short RNA maturation. DICER has recently been linked to the processing of diverse non-coding RNA precursors with as-yet undetermined structures. The experiments performed above suggest DICER has considerable flexibility in processing precursors, contributing to an ability to generate various short RNA products for incorporation into functional RISCs.

## Methods

### Preparation of hairpin RNA substrates

Pre-hsa-mir-21 RNA (pre-mir-21), pre-miRNA mimic hairpin RNA (RNA-I) and pre-mmu-mir-1982 RNA used in this study were generated by *in vitro* transcription using the Ampliscribe T7 High Yield Transcription kits (Epicentre) according to manufacturer's instructions. We made double-stranded DNA templates with T7 RNA polymerase promoter sequence by overlap-PCR using the following oligonucleotide pair; pre-mir-21-sense 5'-taatacagactcactatagAGCTTATCAGACTGATGTTGACTG-3' and pre-mir-21-antisense 5'-ACAGCCATCGACTGGTGTGCCATGAGATTCAACAGTCAACATC-3', RNAI-sense 5'-taatacagactcactatagg

TGTCGGGTAGCTTATCAGACTGATGTTGA-3' and RNAI-antisense 5'-TGTCAGACAGCCCATCGACTGTGTTGCCATGAGATTCAACAGTCAACA-3', pre-mmu-mir-1982-sense 5'-taatacagactcactataGTTGGTATTGCTTGGGAGGGTCTGGGGAGGGGAGTT-3' and pre-mmu-mir-1982-antisense 5'-CTGTGGGAGAACATAGGGTGAGAGGTTGGGGTGCCAGAACTCCCTCCCCA-3'. The overlapped sequences are underlined and the lower-case characters show the sequence of the T7 RNA polymerase promoter. *In vitro* transcription reactions were performed at 37°C overnight. Transcripts were run on 10% denaturing polyacrylamide gels in 0.5x TBE (45 mM Tris-borate, 1 mM EDTA), gel-excised, eluted from the gel in 1 M NaCl at 4°C overnight, and precipitated with ethanol. The pellet was resuspended in an appropriate volume of water and stored into the freezer at -30°C. Before use, RNA substrates were heated to 70°C for 5 min and then slowly cooled to room temperature.

### Affinity purification of recombinant FLAG-DICER fusion proteins

We assembled a full-length cDNA of human DICER1 protein from HeLa total RNA. This cDNA sequence was identical to the coding sequence cited in the Swiss-Prot Protein Database (Swiss-Prot) [Swiss-Prot: Q9UPY3]. N-terminally FLAG-tagged human DICER1 protein was purified from 293T cells transfected with the plasmid pCA-FLAG-DICER1. This vector contained the full-length human DICER1 protein FLAG-tagged at the amino terminus in a pCA-FLAG-DEST vector [45]. We purified the recombinant FLAG-DICER1 fusion protein (rDICER) using ANTI-FLAG M2-Agarose Affinity Gel (Sigma) and eluted by 0.1 M Glycine-HCl (pH3.5). Then, the eluate was neutralized by Tris-HCl (pH8.0). The average yield was 50-100 µg of the active form of rDICER protein from 1 × 10<sup>8</sup> culture cell. Purified rDICER protein was detected by Coomassie Brilliant Blue (CBB) staining and Western blotting using anti-DICER (H212, Santa Cruz) antibody to check for successful homogenous purification (see Figures 1A and 1B). The contamination of known DICER-binding proteins in rDICER samples was checked by Western blotting using anti-FLAG (M2, Sigma), anti-AGO2 (07-590, Upstate) and anti-TRBP (ab42018, Abcam) antibody, respectively (see Figure 1C).

### Processing of RNA substrates using recombinant DICER enzyme

The affinity-purified rDICER protein (2 pmol) was incubated with 45 pmol of RNA substrates (pre-mir-21 RNA, RNA-I or pre-mmu-mir-1982 RNA) in 1x reaction buffer (300 mM NaCl, 50 mM Tris-HCl, 20 mM

HEPES, 5 mM MgCl<sub>2</sub>, pH 9.0) and 40 units of RNase-OUT (Invitrogen). These mixtures were incubated at 37°C for the indicated times. The reactions were purified by phenol-chloroform extraction followed by sodium acetate-ethanol precipitation at -20°C. The RNA pellet was resuspended in water at a final concentration of approximately 1 pmol/μl.

#### Northern blotting

rDICER-processed RNAs (1 pmol) were separated on 7 M urea-denaturing 20% polyacrylamide gels, then blotted onto Hybond-N+ membranes (GE Healthcare) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Hybridization was performed in Church buffer (0.5 M NaHPO<sub>4</sub>, pH 7.2, 1 mM EDTA and 7% SDS) containing 10<sup>6</sup> c.p.m./ml of each <sup>32</sup>P-labelled probe for 14 h. The membranes were washed in 2x SSC, and the signals were detected by autoradiography. All experiments were repeated and replicated consistently.

The probe sequences in this study were as follows: probe-1 (5'-TCAACATCAGTCTGATAAGCTA-3'), probe-2 (5'-ACAGCCCATCGACTGGTGTG-3'), probe-3 (5'-CCATGAGATTC AACAG-3'), probe-4 (5'-CCTCCCCAGGACCCTCCCA-3') and probe-5 (5'-CTGTGGGAGAACATAGGGTGAGA-3'). The probes were 5'-end labelled using T4 polynucleotide kinase (TaKaRa Bio) with [ $\gamma$ -<sup>32</sup>P] ATP (6000Ci/mmol) at 37°C for 4 h.

#### Cloning of cleavage products

rDICER-processed RNAs (1 pmol) were separated on 7 M urea-denaturing 15% polyacrylamide gels, then the gel was stained by SYBR Gold (Invitrogen). The band around 23 nt was excised from the gel and purified as described above. The purified RNA was cloned by the Small RNA cloning kit (TaKaRa Bio) and sequenced by capillary sequencing.

#### 5'-end labelling of the transcript

For the 5'-end labelling, RNA (45 pmol) was dephosphorylated with CIP at 37°C for 60 min. The reaction was inactivated by phenol-chloroform extraction and precipitated by sodium acetate-ethanol at -20°C. The pellet was resuspended in an appropriate volume of water. The dephosphorylated transcript was 5' end-labelled using T4 polynucleotide kinase (TaKaRa Bio) with [ $\gamma$ -<sup>32</sup>P] ATP (3000Ci/mmol) at 37°C for 4 h. The 5'-end labeled transcript was PAGE-purified as described above and the RNA pellet was resuspended in water at a final concentration of approximately 0.5 pmol/μl. One microliter of this was used for the processing reaction by rDICER. These processed samples were run on 7.5 M urea-denaturing 20% polyacrylamide gels in 1x TBE buffer with RNA molecular

marker or the products of alkaline hydrolysis of the same RNA molecule. The alkaline hydrolysis ladder was generated by incubating the labelled RNA in alkaline hydrolysis buffer (Ambion) at 100°C for 10 min. The signals were detected by autoradiography and quantified using ImageJ software (National Institutes of Health; <http://rsb.info.nih.gov/ij/>). The signal intensities were calculated as the mean of pixel value of selected area.

#### Additional material

Additional file 1: Supplementary information.

#### Acknowledgements

We thank Drs. Yasuhiro Tomaru, Timo Lassmann and Masanori Suzuki for their helpful discussion. We also thank Dr. Junichi Yano (Nippon Shinyaku Co. Ltd., Kyoto) for the gift of materials. We acknowledge to RIKEN GenAS for their support of our sequencing data production. This work was supported by a Research Grant for the RIKEN Omics Science Center from the Ministry of Education, Culture, Sports, Science and Technology of Japan to YH.

#### Author details

<sup>1</sup>RIKEN Omics Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan. <sup>2</sup>Cancer Stem Cell Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. <sup>3</sup>Department of Biological Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan. <sup>4</sup>PREST, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan.

#### Authors' contributions

YA conceived the study, designed and performed experiments and drafted the manuscript. YM and AM participated in the experimental design and performed experiments. RK and JC participated in the experimental design and purified recombinant FLAG-DICER fusion proteins. AMB, HS and KM participated in the design of the study and revised the manuscript. YH designed the research project, provided funding, supervised the study and critically reviewed the manuscript. All authors read and approved the final manuscript.

Received: 21 June 2010 Accepted: 9 February 2011

Published: 9 February 2011

#### References

1. Sontheimer EJ: Assembly and function of RNA silencing complexes. *Nat Rev Mol Cell Biol* 2005, **6**(2):127-138.
2. Kim VN, Han J, Siomi MC: Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* 2009, **10**(2):126-139.
3. Jinek M, Doudna JA: A three-dimensional view of the molecular machinery of RNA interference. *Nature* 2009, **457**(7228):405-412.
4. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ: Processing of primary microRNAs by the Microprocessor complex. *Nature* 2004, **432**(7014):231-235.
5. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R: The Microprocessor complex mediates the genesis of microRNAs. *Nature* 2004, **432**(7014):235-240.
6. Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, Sohn SY, Cho Y, Zhang BT, Kim VN: Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* 2006, **125**(5):887-901.
7. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U: Nuclear export of microRNA precursors. *Science* 2004, **303**(5654):95-98.
8. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R: Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 2005, **123**(4):631-640.

9. Lee Y, Hur I, Park SY, Kim YK, Suh MR, Kim VN: **The role of PACT in the RNA silencing pathway.** *EMBO J* 2006, **25**(3):522-532.
10. MacRae IJ, Ma E, Zhou M, Robinson CV, Doudna JA: **In vitro reconstitution of the human RISC-loading complex.** *Proc Natl Acad Sci USA* 2008, **105**(2):512-517.
11. Tahbaz N, Kolb FA, Zhang H, Jaronczyk K, Filipowicz W, Hobman TC: **Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer.** *EMBO Rep* 2004, **5**(2):189-194.
12. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD: **Asymmetry in the assembly of the RNAi enzyme complex.** *Cell* 2003, **115**(2):199-208.
13. Winter J, Jung S, Keller S, Gregory RI, Diederichs S: **Many roads to maturity: microRNA biogenesis pathways and their regulation.** *Nat Cell Biol* 2009, **11**(3):228-234.
14. Khvorovova A, Reynolds A, Jayasena SD: **Functional siRNAs and miRNAs exhibit strand bias.** *Cell* 2003, **115**(2):209-216.
15. Yoda M, Kawamata T, Paroo Z, Ye X, Iwasaki S, Liu Q, Tomari Y: **ATP-dependent human RISC assembly pathways.** *Nat Struct Mol Biol* 2010, **17**(1):17-23.
16. Tomari Y, Matranga C, Haley B, Martinez N, Zamore PD: **A protein sensor for siRNA asymmetry.** *Science* 2004, **306**(5700):1377-1380.
17. Tomari Y, Du T, Zamore PD: **Sorting of Drosophila small silencing RNAs.** *Cell* 2007, **130**(2):299-308.
18. Wang HW, Noland C, Siridechadilok B, Taylor DW, Ma E, Felderer K, Doudna JA, Nogales E: **Structural insights into RNA processing by the human RISC-loading complex.** *Nat Struct Mol Biol* 2009, **16**(11):1148-1153.
19. Tomari Y, Zamore PD: **Perspective: machines for RNAi.** *Genes Dev* 2005, **19**(5):517-529.
20. Hu HY, Yan Z, Xu Y, Hu H, Menzel C, Zhou YH, Chen W, Khaitovich P: **Sequence features associated with microRNA strand selection in humans and flies.** *BMC Genomics* 2009, **10**:413.
21. Burroughs AM, Ando Y, de Hoon MJ, Tomaru Y, Nishibu T, Ukekawa R, Funakoshi T, Kurokawa T, Suzuki H, Hayashizaki Y, et al: **A comprehensive survey of 3' animal miRNA modification events and a possible role for 3' adenylation in modulating miRNA targeting effectiveness.** *Genome Res* 2010, **20**(10):1398-1410.
22. Tan GS, Garchow BG, Liu X, Yeung J, Morris JPt, Cuellar TL, McManus MT, Kiriakidou M: **Expanded RNA-binding activities of mammalian Argonaute 2.** *Nucleic Acids Res* 2009, **37**(22):7533-7545.
23. Sakurai K, Amarzguioui M, Kim DH, Alluin J, Heale B, Song MS, Gatignol A, Behlke MA, Rossi JJ: **A role for human Dicer in pre-RISC loading of siRNAs.** *Nucleic Acids Res* 2010.
24. MacRae IJ, Doudna JA: **Ribonuclease revisited: structural insights into ribonuclease III family enzymes.** *Curr Opin Struct Biol* 2007, **17**(1):138-145.
25. Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W: **Single processing center models for human Dicer and bacterial RNase III.** *Cell* 2004, **118**(1):57-68.
26. Macrae IJ, Zhou K, Li F, Repic A, Brooks AN, Cande WZ, Adams PD, Doudna JA: **Structural basis for double-stranded RNA processing by Dicer.** *Science* 2006, **311**(5758):195-198.
27. MacRae IJ, Zhou K, Doudna JA: **Structural determinants of RNA recognition and cleavage by Dicer.** *Nat Struct Mol Biol* 2007, **14**(10):934-940.
28. Zhang H, Kolb FA, Brondani V, Billy E, Filipowicz W: **Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP.** *EMBO J* 2002, **21**(21):5875-5885.
29. Flores-Jasso CF, Arenas-Huertero C, Reyes JL, Contreras-Cubas C, Covarrubias A, Vaca L: **First step in pre-miRNAs processing by human Dicer.** *Acta Pharmacol Sin* 2009, **30**(8):1177-1185.
30. Ruby JG, Jan CH, Bartel DP: **Intronic microRNA precursors that bypass Drosha processing.** *Nature* 2007, **448**(7149):83-86.
31. Okamura K, Hagen JW, Duan H, Tyler DM, Lai EC: **The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila.** *Cell* 2007, **130**(1):89-100.
32. Berezikov E, Chung WJ, Willis J, Cuppen E, Lai EC: **Mammalian mirtron genes.** *Mol Cell* 2007, **28**(2):328-336.
33. Babiarz JE, Ruby JG, Wang Y, Bartel DP, Blelloch R: **Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs.** *Genes Dev* 2008, **22**(20):2773-2785.
34. Glazov EA, Kongsuwan K, Assavalapsakul W, Horwood PF, Mitter N, Mahony TJ: **Repertoire of bovine miRNA and miRNA-like small regulatory RNAs expressed upon viral infection.** *PLoS One* 2009, **4**(7):e6349.
35. Chiang HR, Schoenfeld LW, Ruby JG, Auyeung VC, Spies N, Baek D, Johnston WK, Russ C, Luo S, Babiarz JE, et al: **Mammalian microRNAs: experimental evaluation of novel and previously annotated genes.** *Genes Dev* 2010, **24**(10):992-1009.
36. Starega-Roslan J, Krol J, Koscianska E, Kozlowski P, Szlachcic WJ, Sobczak K, Krzyzosiak WJ: **Structural basis of microRNA length variety.** *Nucleic Acids Res* 2011, **39**(1):257-268.
37. Tam OH, Aravin AA, Stein P, Girard A, Murchison EP, Cheloufi S, Hodges E, Anger M, Sachidanandam R, Schultz RM, et al: **Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes.** *Nature* 2008, **453**(7194):534-538.
38. Watanabe T, Totoki Y, Toyoda A, Kaneda M, Kuramochi-Miyagawa S, Obata Y, Chiba H, Kohara Y, Kono T, Nakano T, et al: **Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes.** *Nature* 2008, **453**(7194):539-543.
39. Maida Y, Yasukawa M, Furuuchi M, Lassmann T, Possemato R, Okamoto N, Kasim V, Hayashizaki Y, Hahn WC, Masutomi K: **An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA.** *Nature* 2009, **461**(7261):230-235.
40. Burroughs AM, Ando Y, Hoon ML, Tomaru Y, Suzuki H, Hayashizaki Y, Daub CO: **Deep-sequencing of human Argonaute-associated small RNAs provides insight into miRNA sorting and reveals Argonaute association with RNA fragments of diverse origin.** *RNA Biol* 2011.
41. Azuma-Mukai A, Oguri H, Mituyama T, Qian ZR, Asai K, Siomi H, Siomi MC: **Characterization of endogenous human Argonautes and their miRNA partners in RNA silencing.** *Proc Natl Acad Sci USA* 2008, **105**(23):7964-7969.
42. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T: **Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate.** *EMBO J* 2001, **20**(23):6877-6888.
43. Okada C, Yamashita E, Lee SJ, Shibata S, Katahira J, Nakagawa A, Yoneda Y, Tsukihara T: **A high-resolution structure of the pre-microRNA nuclear export machinery.** *Science* 2009, **326**(5957):1275-1279.
44. Sinkkonen L, Hugenschmidt T, Filipowicz W, Svoboda P: **Dicer is associated with ribosomal DNA chromatin in mammalian cells.** *PLoS One* 2010, **5**(8): e12175.
45. Kimura R, Yoda A, Hayashizaki Y, Chiba J: **Novel ELISA using intracellularly biotinylated antigen for detection of antibody following DNA immunization.** *Jpn J Infect Dis* 2010, **63**(1):41-48.
46. Griffiths-Jones S: **The microRNA Registry.** *Nucleic Acids Res* 2004, **32** Database: D109-111.
47. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ: **miRBase: microRNA sequences, targets and gene nomenclature.** *Nucleic Acids Res* 2006, **34** Database: D140-144.
48. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ: **miRBase: tools for microRNA genomics.** *Nucleic Acids Res* 2008, **36** Database: D154-158.
49. Sato K, Hamada M, Asai K, Mituyama T: **CENTROIDFOLD: a web server for RNA secondary structure prediction.** *Nucleic Acids Res* 2009, **37** Web Server: W277-280.

doi:10.1186/1471-2199-12-6

Cite this article as: Ando et al.: Two-step cleavage of hairpin RNA with 5' overhangs by human DICER. *BMC Molecular Biology* 2011 **12**:6.

**Submit your next manuscript to BioMed Central and take full advantage of:**

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at  
www.biomedcentral.com/submit

