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Regular paper

How short RNAs impact the human ribonuclease Dicer activity: putative regulatory feedback-loops and other RNA-mediated mechanisms controlling microRNA processing

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Ribonuclease Dicer plays a pivotal role in RNA interference pathways by processing long double-stranded RNAs and single-stranded hairpin RNA precursors into small interfering RNAs (siRNAs) and microRNAs (miRNAs), respectively. While details of Dicer regulation by a variety of proteins are being elucidated, less is known about non-protein factors, e.g. RNA molecules, that may influence this enzyme's activity. Therefore, we decided to investigate the question of whether the RNA molecules can function not only as Dicer substrates but also as its regulators. Our previous *in vitro* studies indicated that the activity of human Dicer can be influenced by short RNA molecules that either bind to Dicer or interact with its substrates, or both. Those studies were carried out with commercial Dicer preparations. Nevertheless, such preparations are usually not homogeneous enough to carry out more detailed RNA-binding studies. Therefore, we have established our own system for the production of human Dicer in insect cells. In this manuscript, we characterize the RNA-binding and RNA-cleavage properties of the obtained preparation. We demonstrate that Dicer can efficiently bind single-stranded RNAs that are longer than ~20-nucleotides. Consequently, we revisit possible scenarios of Dicer regulation by single-stranded RNA species ranging from ~10- to ~60-nucleotides, in the context of their binding to this enzyme. Finally, we show that siRNA/miRNA-sized RNAs may affect miRNA production either by binding to Dicer or by participating in regulatory feedback-loops. Altogether, our studies suggest a broad regulatory role of short RNAs in Dicer functioning.

Key words: ribonuclease Dicer; miRNA processing; regulatory RNAs; regulation of Dicer activity; regulatory feedback-loops

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Abbreviations: dsRNA, double-stranded RNA; hDicer, recombinant human Dicer protein; miRNA, microRNA; miR-Reg, an oligonucleotide with a sequence identical to a specific miRNA; pre-miRNA, pre-microRNA (microRNA precursor); RNAi, RNA interference; siRNA, small interfering RNA; ssRNA, single-stranded RNA

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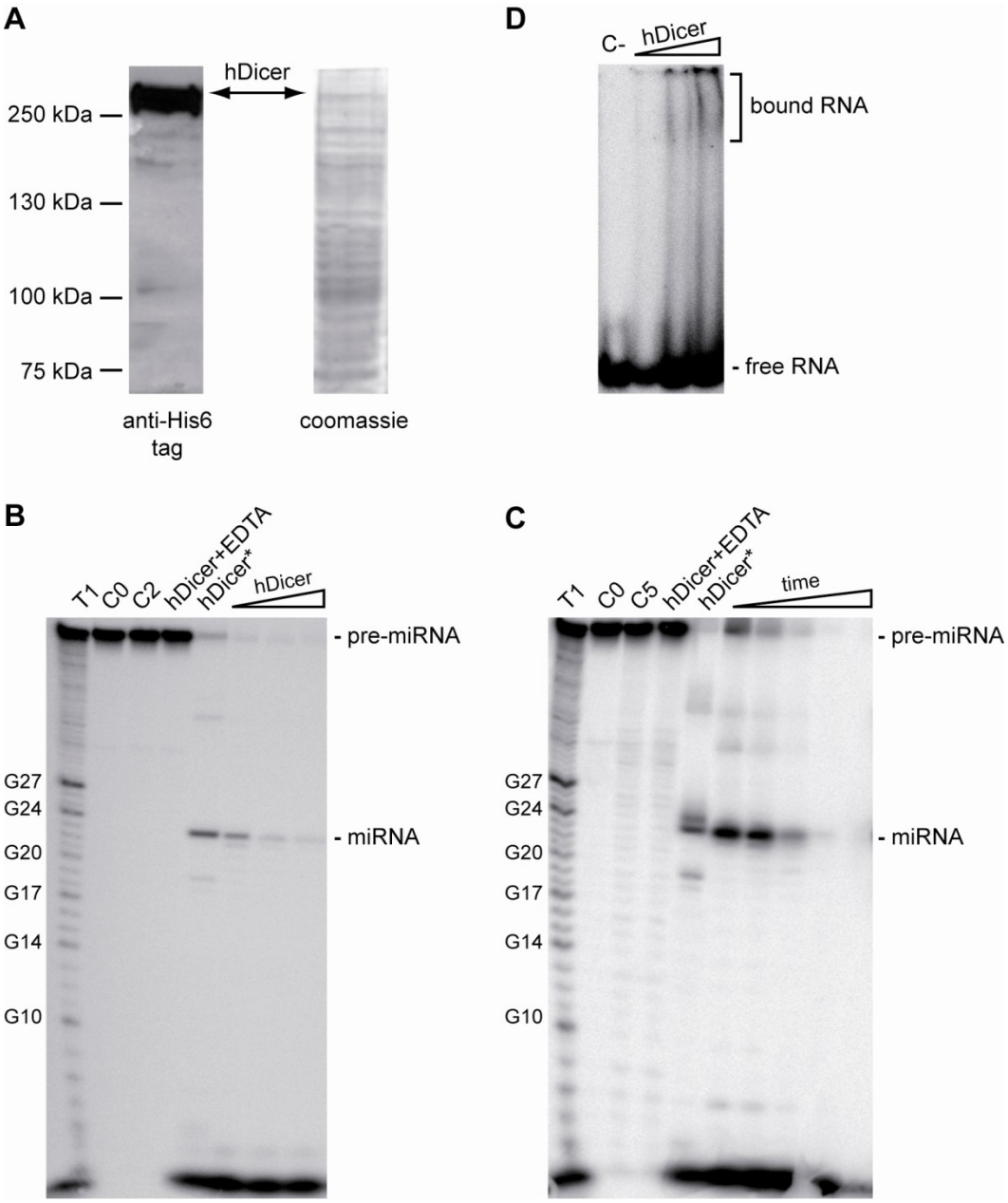


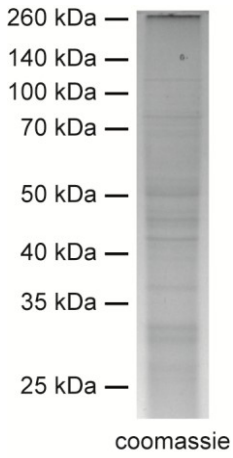
Supplementary Figure S1. Characterization of the one-step purified hDicer.

(A) PAGE analysis of the hDicer preparation. The polyhistidine-tagged hDicer protein was expressed in the Baculovirus Expression System, partially purified by Ni^{2+} affinity chromatography, and analyzed by SDS–PAGE followed by Western blotting with anti-His6 tag antibody (*left*), or Coomassie Blue staining (*right*). The band corresponding to hDicer is indicated.

(B) The effect of an increasing hDicer amount on miRNA formation. The ^{32}P-labeled pre-mir-21 was incubated with increasing amounts of the tested hDicer preparation (1, 2.5 and 5 µl; represented by *a triangle*) or a commercially available hDicer (*hDicer**, 1U), and analyzed by denaturing PAGE. *C0* and *C2* – controls incubated without hDicer for 0h and 2h, respectively; *hDicer+EDTA* – a reaction carried out with hDicer in a buffer supplemented with 50 mM EDTA; *T1* – G-ladder generated with RNase T1.

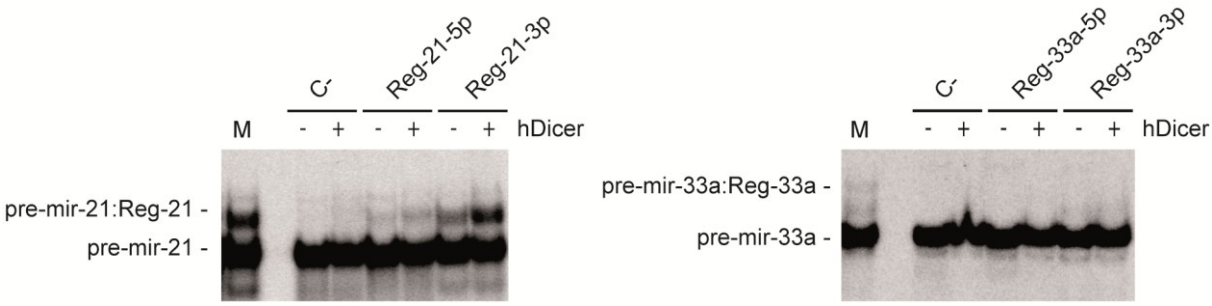
(C) The time-course assay of the hDicer cleavage activity. The ^{32}P-labeled pre-mir-21 was incubated with the tested hDicer preparation (5 µl) for 10 min, 30 min, 1.5h, 3h and 5h (represented by *a triangle*) or with a commercially available hDicer (*hDicer**, 1U) for 5h, and analyzed by denaturing PAGE. *C0* and *C5* – controls incubated without hDicer for 0h and 5h, respectively. Other captions as in Fig. 1B.

(D) The RNA-binding capacity of hDicer. The ^{32}P-labeled pre-mir-21 was incubated in the absence (*C-*) or presence of increasing amounts of hDicer (1, 2.5 and 5 µl; represented by *a triangle*). Reaction mixtures were analyzed by EMSA.



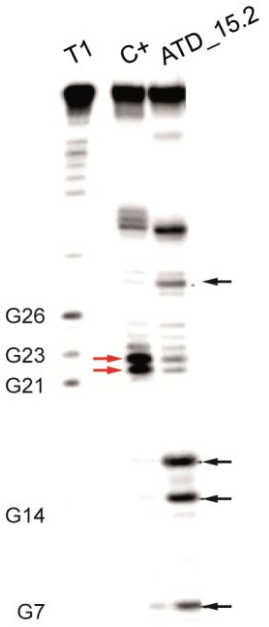
Supplementary Figure S2. Characterization of a commercially available hDicer.

PAGE analysis of a commercial hDicer preparation. 1U of a commercial hDicer was analyzed by SDS–PAGE followed by Coomassie Blue staining. Molecular-weight size standards are marked on the left.



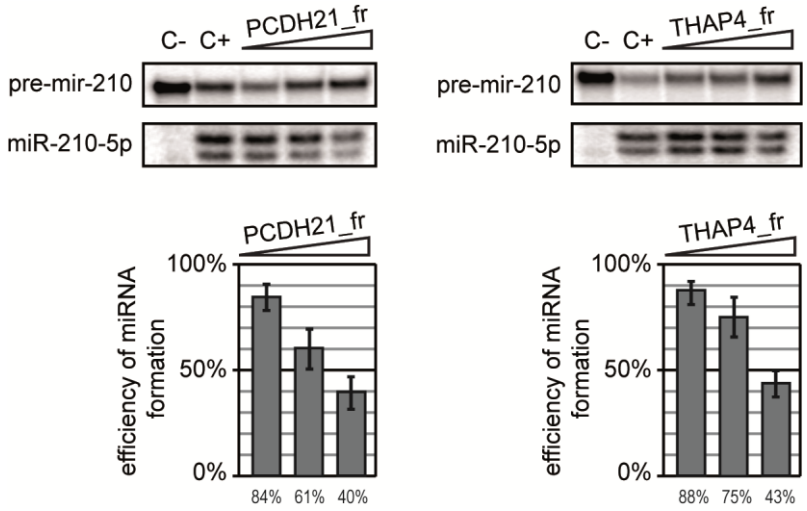
Supplementary Figure S3. Characterization of the interactions between pre-miRNAs and the corresponding miR-regulators.

The interactions between pre-miRNAs (pre-mir-21 or pre-mir-33a) and their corresponding miR-regulators (Reg-21 and Reg-33a, respectively) were tested by EMSA. The ^{32}P-labeled pre-miRNA was incubated without (*C*-) or with the miR-regulator (as indicated), in the absence (-) or presence (+) of hDicer. *M* – molecular mass ladder.



Supplementary Figure S4. Changes in the pre-mir-210 cleavage pattern upon addition of the complementary oligonucleotide.

The ^{32}P-labeled pre-mir-210 was incubated with hDicer in the absence (*C+*) or in the presence of ATD_15.2 (having a complementary sequence to pre-mir-210), and analyzed by denaturing PAGE. Red arrows indicate miR-210, black arrows indicate products generated upon addition of ATD_15.2. *T1* – G-ladder generated with RNase T1.



Supplementary Figure S5. Influence of ~30-nt single-stranded RNAs, identical with the mRNA fragments containing sequences complementary to pre-mir-210, on the precursor processing by hDicer.

The ^{32}P-labeled pre-mir-210 was incubated with hDicer in the presence of either 30-nt PCDH21_fr or 35-nt THAP4_fr, and analyzed by denaturing PAGE. Control reactions lacked the enzyme and oligonucleotide (*C*-) or oligonucleotide only (*C*+). *Triangles* represent increasing amounts of the oligonucleotide (pre-miRNA and oligonucleotide molar ratios of 1:1, 1:10, and 1:100). Diagrams show an average efficiency of miRNA production in comparison to (*C+)*; error bars represent standard deviation of three independent experiments.