

Characterization of a naturally occurring truncated Dicer

Nicola Mosca¹ · Julia Starega-Roslan² · Filomena Castiello¹ · Aniello Russo¹ ·
Włodzimierz J. Krzyzosiak² · Nicoletta Potenza¹

Received: 22 December 2014 / Accepted: 16 April 2015 / Published online: 25 April 2015
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Abstract Dicer is central to small RNA silencing pathways, thus playing an important role in physiological and pathological states. Recently, a number of mutations in dicer gene have been identified in diverse types of cancer, implicating Dicer in oncogenic cooperation. Here we report on the properties of a rare splice variant of the human dicer gene, occurring in neuroblastoma cells, and not detectable in normal tissues. Due to the skipping of one exon, the alternatively spliced transcript encodes a putative truncated protein, t-Dicer, lacking the dsRNA-binding domain and bearing altered one of the two RNase III catalytic centers. The ability of the exon-depleted t-dicer transcript to be translated in vitro was first investigated by the expression of flagged t-Dicer in human cells. We found that t-dicer transcript could be translated in vitro, albeit not as efficiently as full-length dicer transcript. Then, the possible enzymatic activity of t-Dicer was analyzed by an in vitro dicing assay able to distinguish the enzymatic activity of the individual RNase III domains. We showed that t-Dicer preserved partial dicing activity. Overall, the results indicate that t-dicer transcript could produce a protein still able

to bind the substrate and to cleave only one of the two pre-miRNA strands. Given the increasing number of mutations reported for dicer gene in tumours, our experimental approach could be useful to characterize the activity of these mutants, which may dictate changes in selected classes of small RNAs and/or lead to their aberrant maturation.

Keywords Dicer · Neuroblastoma · Dicing assay · miRNA · Cancer

Introduction

Dicer is a ribonuclease playing key roles in microRNA (miRNA) pathway and RNA interference [1]. It is required for biogenesis of miRNA and small interfering RNA (siRNA) during the processing of double-stranded RNA (pre-miRNAs and long dsRNA). Dicer is also involved in the assembly of the RNA-induced silencing complex (RISC) that mediates the effector steps of RNA silencing [2].

Human Dicer, as most metazoan Dicer proteins, contains an N-terminal helicase domain, followed by a domain of unknown function (DUF283), the tightly associated PAZ and Platform domains, two RNase III domains (RIIIa and RIIIb), and a C-terminal dsRNA-binding domain (dsRBD) (Fig. 1). The PAZ/Platform, dsRBD and RNase III domains are involved in dsRNA binding and cleavage. In particular, PAZ and Platform domains enfold binding pockets for the 3'-overhang and 5'-phosphate moieties of the dsRNA substrate, respectively [3]; dsRBD domain binds the dsRNA [4]; RNase IIIa and IIIb domains function as an intramolecular pseudo-dimer forming a single processing centre containing two independent catalytic ‘‘half sites’’, with the RIIIa domain cleaving the 3' pre-miRNA

Nicola Mosca and Julia Starega-Roslan authors are joint First Authors.

✉ Włodzimierz J. Krzyzosiak
wlokrzy@ibch.poznan.pl

✉ Nicoletta Potenza
nicoletta.potenza@unina2.it

¹ Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, Second University of Naples, via Vivaldi 43, 81100 Caserta, Italy

² Department of Molecular Biomedicine, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland

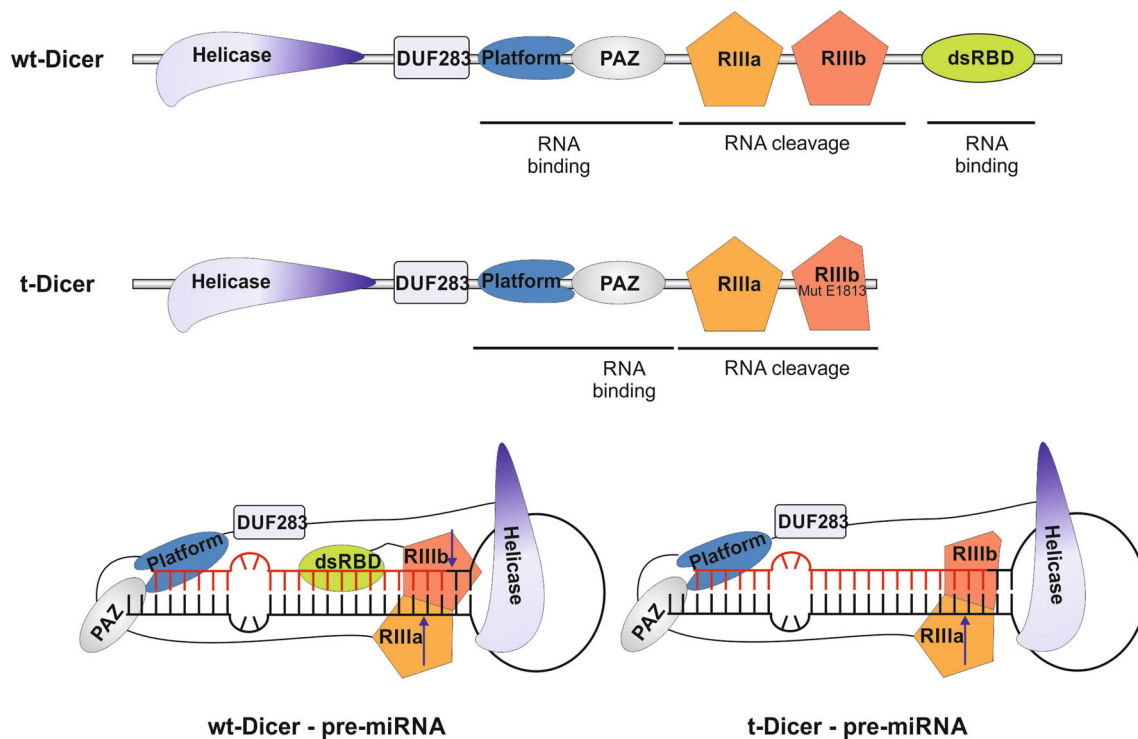


Fig. 1 A schematic overview of composition of wt-Dicer and t-Dicer domains. Below, the model of RNA and wt-Dicer or t-Dicer interaction. Based on electron microscopy data Dicer forms an L-shaped molecule [10]. PAZ/Platform and RNase III domains form the long arm of the L, wherein PAZ and Platform domains bind the 3' and 5' ends of RNA, respectively, and the RIIIa and RIIIb domains cleave the 3'-OH- and 5'-P-bearing pre-miRNA arm, respectively, as

arm, and RIIIb cleaving the 5' arm of pre-miRNA [5]. The helicase domain has a role in the control of enzyme activity [6] and interacts with the two dsRBD-containing cofactors of Dicer in RISC loading complex (RLC), i.e., TRBP and PACT, involved in siRNA-induced RNAi and in miRNA accumulation [7–9]. Recent study based on electron microscopy reported reconstructions of the Dicer architecture as an L-shaped molecule with morphologically discrete regions (Fig. 1) [10].

Since its discovery, the prevalent role of Dicer in development, physiological and pathological states has been progressively revealed [11, 12]. Most recently, a number of mutations in dicer gene have been identified in diverse types of cancer, with the RNase IIIb domain emerging as an hotspot domain for somatic mutations [13, 14].

In the neuroblastoma cells, apart from the prevailing full-length dicer transcript, an alternatively spliced transcript has been observed. In the splice variant, the skipping of one exon removes part of the coding sequence and changes the reading frame, leading to a premature stop codon [15, 16]. The putative encoded protein, called t-Dicer (truncated Dicer), should result 93 aminoacids shorter than wt-Dicer, lacks the C-terminal dsRBD and

indicated by the arrows. The helicase domain is adjacent to the catalytic core and occupies the short arm of the L clamping the pre-miRNA loop. The C-terminal dsRBD lies adjacent to the RNase IIIb domain. Thus, a single continuous channel runs through clamp of the helicase, past the RNase III active site, and ends with the RNA-binding pocket of the PAZ domain

possesses an altered RNase IIIb domain lacking one of the two essential catalytic residues, E1813 [5]. It is noteworthy that the splice variant was not detectable in normal tissues, whereas it occurred in primary neuroblastic tumors [16].

Given the emerging role of Dicer in tumours, likely with not only the dosage but also the activity of the mutated proteins at its very centre [14], we have investigated the ability of t-dicer transcript to be translated in vitro and the possible enzymatic activity of the encoded protein. We used a versatile approach consisting of the expression of flagged dicer in human cells and of an in vitro dicing assay [17, 18].

Materials and methods

Preparation of Dicer expression vectors

Wt-Dicer and t-Dicer coding sequences were cloned in the p3XFLAG-CMV-10 expression vector (Sigma Aldrich) in frame with the plasmid sequence encoding three adjacent FLAG[®] epitopes. The cloning strategy consisted of two steps. In the first step, the 5'-end (315 bp) of Dicer coding sequence, without the ATG start codon, was amplified by

PCR using as template Dicer cDNA [14] and the following primers: FH3-Dicer, 5'-CCCAAGCTTAAAAGCCCTGCTTTGCAAC-3', containing *Hind*III restriction site (underlined); RK-Dicer, 5'-GGGGTACCAGAGTTGACCAA GAACACCGTC-3', containing *Kpn*I restriction site (underlined). The PCR product was digested with *Hind*III and *Kpn*I and cloned into the same sites of p3XFLAG-CMV-10 vector. That amplified Dicer sequence contains a *Pme*I restriction site that has been used for the junction of the remaining parts of wt-Dicer or t-Dicer coding sequence. In fact, in the second step, the remaining parts of wt-Dicer or t-Dicer coding sequences were excised from the plasmids containing the respective cDNAs [16] by cleavage with *Pme*I and *Kpn*I and cloned in the same sites of the construct obtained in the previous step. The resulting recombinant plasmids were fully sequenced and designated FLAG-wt-Dicer and FLAG-t-Dicer.

Cell culture and transfections

HEK293T cells were cultured in DMEM containing 10 % fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin and 100 µg/ml streptomycin. SK-N-BE(2)-C were cultured in RPMI 1640 containing FBS and antibiotics as HEK293T cells. The day before transfection, cells were trypsinized and seeded in medium without antibiotics in 10 cm dishes. Transfections were performed with cells at 80–90 % of confluence with 18 µg of plasmid encoding FLAG-wt-Dicer or FLAG-t-Dicer or parental empty vector by using Lipofectamine2000 (Invitrogen), as described by the manufacturer. After 6 h, transfection mix was replaced with complete medium. The analyses were performed 48 h after transfection.

Protein extraction, immunoprecipitation and Western blotting

Total protein extracts were prepared as described previously [19]. In brief, cells were incubated with lysis buffer D (20 mM HEPES-KOH pH7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 5 % glycerol) supplemented with protease inhibitor (Roche) for 10 min on ice followed by sonication (×8, 5 s, 30 % amplitude) and centrifugation at 12,000 × *g* for 15 min at 4 °C. The supernatants were collected and protein concentration was determined using Bradford reagent with BSA as a standard. The extracts were stored in –80 °C until use in western blotting, immunoprecipitation or Dicer cleavage assay.

For immunoprecipitation of FLAG-wt-Dicer and FLAG-t-Dicer, 5 mg of cell extract was incubated with 80 µl of anti-FLAG antibody conjugated to agarose beads (anti-FLAG M2 affinity gel, Sigma) in buffer D-K'100 (20 mM Tris-pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.2 mM

PMSF) [8] with constant rotation overnight at 4 °C. The beads were washed three times with TBS and the 3XFLAG fusion proteins were eluted with 100 µl of 3XFLAG peptide (Sigma).

Proteins were separated by 5 % Tris acetate SDS-PAGE in XT Tricine buffer (Bio-Rad) and electrotransferred onto a nitrocellulose membrane (Sigma). For Dicer detection, the blots were probed with the rabbit polyclonal primary anti-Dicer antibody (1:1000, Cell Signaling Technology) or with the anti-FLAG primary antibody (1:500, Sigma) and then with HRP-conjugated secondary antibody, anti-rabbit (1:1000, Sigma). The immunoreactions were detected using Westernbright Quantum HRP substrate (Advansta).

Dicing assay and product visualization

Chemically synthesized, phosphorylated pre-miR-549 (IDT) was used as substrate for Dicer cleavage assays. RNA was purified by polyacrylamide gel electrophoresis and stored at –80 °C until use.

The cleavage assays were performed in 12 µl reaction containing either cellular extracts or immunopurified Dicer proteins (amounts depicted in Fig. 4) mixed with 150 ng of pre-miR-549, in the reaction buffer A (32 mM MgCl₂, 5 mM ATP, 200 mM creatine phosphate) [20] and RNaseOUT (Invitrogen). The reaction mixtures were incubated at 37 °C for 90 min followed by phenol/chloroform extraction. RNA was precipitated with ethanol and resuspended in 20 µl of formamide loading buffer with dyes. As a comparison, 150 ng of pre-miR-549 was subjected to cleavage by recombinant Dicer (Genlantis) for 12 min at 37 °C. The reaction was stopped by adding equal volume of urea loading buffer with dyes.

RNA samples from Dicer cleavage reactions were denatured for 1 min at 99 °C, chilled on ice and immediately loaded on 12 % polyacrylamide gels followed by Northern blotting as previously described [17]. The membranes were probed with labeled DNA oligonucleotides complementary to miR-549: 5' arm probe, 5'-ACAGT GACAACATATGGATGAGCT-3'; 3' arm probe, 5'-AGAGCTCATCCATAGTTGTCA-3'.

RNA purification, real-time PCR analyses and luciferase assays

Total RNA was extracted by miRNeasy mini kit (Qiagen) from cell cultures according to the manufacturer's protocol.

MiR-125a-5p was quantified along with RNU6B (reference gene) by RT-qPCR with TaqMan[®] miRNA assays from Applied Biosystems according to the manufacturer's protocol.

Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

All the analyses were performed 48 h after transfections.

Results and discussion

An overview of the experimental approach

To assess whether the exon-depleted t-dicer transcript could be translated in vitro and to analyze the enzymatic activity of the encoded protein, we used an experimental approach based on the overexpression of N-terminal tagged Dicer proteins in human cells and an in vitro enzymatic assay (Fig. 2).

In order to express wt-Dicer or t-Dicer, their coding sequence were cloned into the expression vector p3XFLAG-CMV-10 to obtain FLAG-wt-Dicer and FLAG-t-Dicer plasmids. 3 tandem FLAG epitopes resulted fused to the recombinant proteins, thus increasing their detection and the possible immunopurification. The FLAG peptide is expected to be located on the surface of Dicer and do not obscure other domains or alter the RNase III activity of the C-terminal domains, because of its relatively small (22 amino acids) size and its hydrophilic nature [21]. FLAG-Dicer vectors were then used to transfect human cell lines and the overexpression of recombinant Dicer were verified by Western blot analyses in cellular extracts as well as in immunopurified samples.

Enzymatic activity of recombinant Dicer was analyzed both in the cellular extract and in the immunopurified

samples by an in vitro cleavage assay using unlabeled pre-miRNA as a substrate and northern blotting as a detection method [17]. This procedure does not require special substrate preparation [18] and only uses specific probes to distinguish the enzymatic activity of the individual RNase III domains. We demonstrate the usefulness of this approach by the comparison of the results obtained with wt-Dicer and t-Dicer as well as recombinant Dicer.

Expression of Dicer proteins

FLAG-Dicer plasmids or the parental vector were used for the transfection of HEK293T cells. 48 h after transfection, cell lysates were analyzed by Western blot with both the anti-Dicer antibody and the anti-FLAG antibody. A band of ~200 kDa, expected for the full-length Dicer, was observed in protein extracts from FLAG-wt-Dicer transfected cells (Fig. 3a). FLAG-t-Dicer was expressed, albeit to a lower degree than full-length Dicer. In particular, FLAG-t-Dicer was estimated to be expressed approximately three-fold less than FLAG-wt-Dicer (Fig. 3b). The same result was observed also by transfecting HeLa (data not shown) and neuroblastoma cells (see later). Intriguingly, the only other reported splice variant encoding a modified Dicer, resulting in a RNase IIIb-defective protein, showed the same degree of expression in comparison to full-length form [22]. The observed difference in FLAG-tagged Dicer proteins was not dependent on a difference in their corresponding transcripts stability (data not shown).

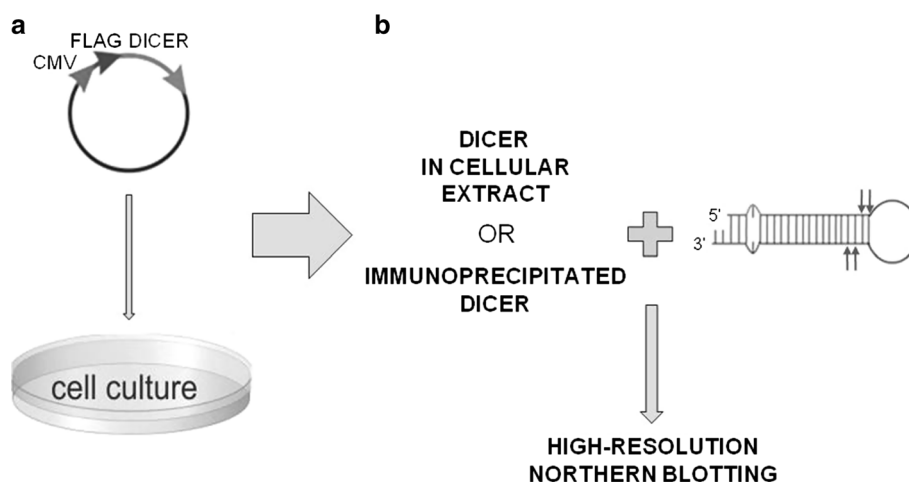


Fig. 2 An overview of the experimental approach. **a** Plasmids encoding FLAG-tagged Dicer proteins were transfected to cell culture. **b** The dicing activity was analyzed in cellular extracts or on anti-FLAG immunoprecipitated samples by an in vitro cleavage assay. The assay is based on the relaxed specificity of Dicer in the

cleavages (indicated by the *arrows*) on its pre-miRNA substrate [18]; the miRNA length heterogeneity of the released products can be visualized by a high-resolution northern blot and hybridization with probes detecting 5'- or 3'-arm, allowing to distinguish between the cleavages generated by the individual RNase III domains

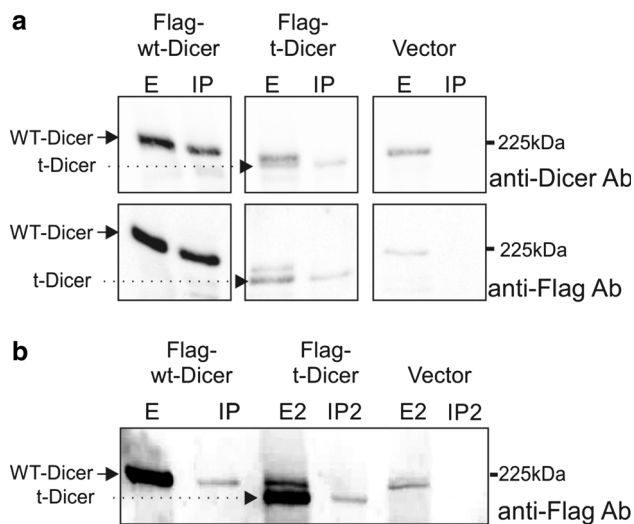


Fig. 3 Western blot analysis of FLAG-tagged Dicer constructs. **a** A total of 50 μ g of cellular extract (E) or 5 μ l of the immunoprecipitated samples (IP) from cells transfected with FLAG-wt-Dicer, FLAG-t-Dicer and parental vector were analyzed by Western blotting with anti-Dicer and anti-FLAG antibodies. Densitometry analysis revealed that signal intensity of the band corresponding to FLAG-wt-Dicer was threefold higher than the signal intensity of the band corresponding to FLAG-t-Dicer. **b** Western blot analysis of cellular extracts and immunopurified samples as in (a) with the exception of using a three times more amount of samples relative to FLAG-t-Dicer and parental vector (E2, IP2)

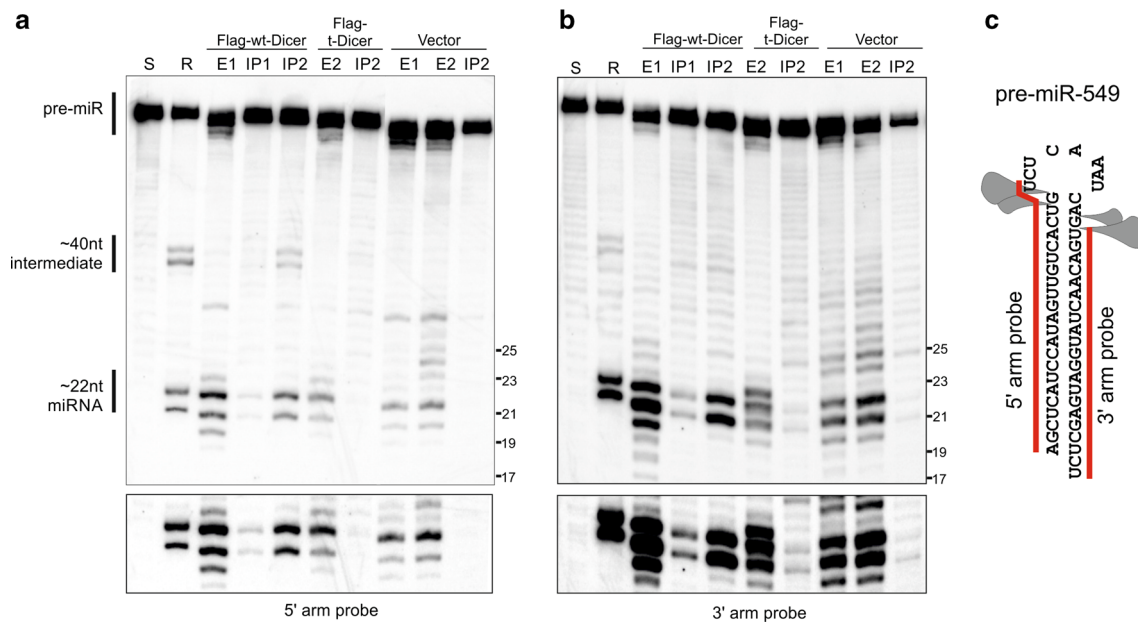


Fig. 4 Northern blot analysis of the pre-miR-549 dicing assay. **a** and **b** Representative experiment showing the analysis of products generated by recombinant Dicer (R), Dicer present in the extracts (E) or immunopurified samples (IP) from cells transfected with FLAG-wt-Dicer, FLAG-t-Dicer or parental vector. S, uncleaved pre-miR-549 substrate; E1, analysis of enzymatic assay performed with 80 μ g of cellular extract; E2, analysis of enzymatic assay performed with 240 μ g of cellular extract; IP1, analysis of enzymatic assay performed with 100 μ g of immunopurified sample; IP2, analysis of

The overexpressed Dicer proteins were then successfully immunopurified (Fig. 3) by anti-FLAG antibody for the subsequent analyses.

Dicing activity of the recombinant proteins

Different approaches have been used to investigate how Dicer cleaves its pre-miRNA substrates. Recombinant Dicer, alone or within in vitro reconstituted complex with its molecular partners [5, 18, 23], or present in cellular extracts [24, 25] or from immunoprecipitates [8, 26], was used in cleavage assays with labelled pre-miRNA and the reaction products revealed by PAGE and autoradiography. In particular, the most used assay employs end-labelled pre-miRNA as substrate, which assume a special substrate preparation, especially, when both 5' and 3' end labelled RNA are to be used as a homologous substrates for Dicer (ligation of pCp to 1nt shorter substrate to obtain 3' end labeled RNA, homologous to the one labeled at 5' end) [18, 27].

Here we used an unlabelled precursor as substrate for Dicer cleavage followed by the separation of products according to their size and their visualization by a northern blotting analysis able to detect miRNAs with single-nucleotide resolution (Fig. 4) [17]. This approach does not

enzymatic assay performed with 250 μ g of immunopurified samples. The migration of 17-, 19-, 21-, 23-, 25-nt oligonucleotide marker are indicated on the right. Below, the same gels having longer exposure time to visualize products \sim 22nt long. **c** The structure of synthetic pre-miR-549 used as a substrate in cleavage assays with marked positions of recombinant Dicer cleavage. In red, the span of 5' and 3' arm probe, detecting the cleavage products generated by RIIIb and RIIIa, respectively. (Color figure online)

require special substrate preparation, and only uses specific-labeled probes detecting 5' or 3' arm products. In particular, we used the pre-miR-549 as synthetic substrate, having 2nt 3' overhangs, representing a product of Drosha cleavage (Fig. 4). pre-miR-549 is poorly expressed in HEK293T cells (our data not published), thus allowing a null or low background in our extract. Recombinant Dicer enzyme was used as a positive control for the assay, showing that the cleavage products generated by the RNase IIIa or IIIb domain are clearly visualized and distinguishable using the two different probes detecting miRNA 3'- or 5'-arm, respectively (Fig. 4, lane R).

The activity of exogenous wt-Dicer in the cell extracts was also clearly detectable (Fig. 4, lane E1); the signal intensities of the cleavage products are higher in the lanes where the extracts from FLAG-wt-Dicer transfected cells were analyzed in comparison to those observed for the empty vector transfected cells, where only the endogenous Dicer contributed to the enzymatic activity (Fig. 4, lanes E1, E2). The dicing assay performed on immunopurified samples confirmed the activity of FLAG-wt-Dicer, because the cleavage products were detectable in the immunoprecipitates obtained from FLAG-Dicer vector transfected cells (Fig. 4, lanes IP1, IP2 relative to FLAG-wt-Dicer) and no from immunoprecipitates obtained from empty vector transfected cells (Fig. 4, lane IP2 relative to Vector). The profile of the cleavages of immunopurified wt-Dicer resemble those of recombinant Dicer (Fig. 4, lane R): both ~22nt miRNA fraction and ~40nt intermediate fraction were observed. The occurrence of ~40nt intermediate (Fig. 4a, lane IP2 relative to FLAG-wt-Dicer) results from cleavage of only RIIIa domain in 3' pre-miRNA arm. This results confirm previous observations showing that sole Dicer cleaves pre-miRNA in less synchronised manner compared to Dicer cooperating with its protein partners in cells [28]. The heterogeneity of the miRNA fraction is somewhat different in extract where Dicer exists within RLC with Dicer protein partner compared to recombinant Dicer and immunopurified Dicer, where sole Dicer binds and cleaves pre-miRNA. This observation confirm previous reports showing that TRBP, a Dicer protein partner, may influence the precision of Dicer cleavage [9, 29].

In the case of FLAG-t-Dicer, an amount ~3-fold higher of cell extract or immunoprecipitate samples were used for the dicing assays, to compensate its lower expression (Fig. 3). The northern blotting showed that the cleavage products generated by the defective RNase IIIb domain are not detectable (Fig. 4a, lane IP2). This observation agrees with published data of point mutations of RIIIb domains of Dicer [5]. However, the products released by the RNase IIIa domain are detectable (Fig. 4b, lane IP2), albeit to a lower degree, likely because of a minor binding of the substrate due to the truncation of its C-terminal domain

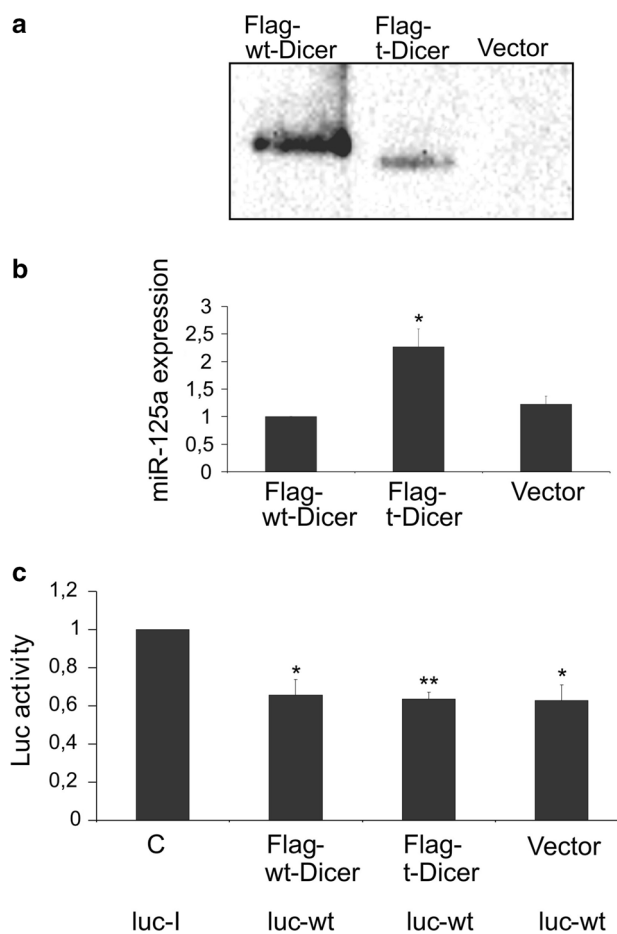


Fig. 5 Possible association of t-Dicer overexpression with the loss of the -5p strand cleavage of pre-miRNA-125a and/or an impaired functioning in the silencing of the target. **a** Western blot analysis by anti-FLAG antibody of extracts from SK-N-BE(2)-C cells transfected with FLAG-wt-Dicer, FLAG-t-Dicer constructs or parental vector (Vector). **b** miR-125a-5p expression was determined in the same samples as above by q-PCR. The expression level of miR-125a-5p was normalized by the $2^{-\Delta\Delta Ct}$ method and reported as fold-change [31]. **c** The efficiency of the silencing activity performed by miR-125a-5p was evaluated by co-transfecting the FLAG-wt-Dicer, FLAG-t-Dicer constructs or parental vector along with luciferase-based reporter plasmid psiCheck-2 containing a validated target sequence (luc-WT) of miR-125a-5p or a control inverted sequence (luc-I) [30]. Luciferase activities (Luc) registered with luc-WT construct were always lower than that observed with the luc-I construct, indicating that the overexpression of FLAG-wt-Dicer or FLAG-t-DICER do not influence the performance of miR-125a-5p at silencing its target. C, control, i.e. the maximum of luciferase activity registered from cells transfected with FLAG-wt-Dicer, or FLAG-t-Dicer or parental vector along with the luc-I construct. * $p < 0.05$; ** $p < 0.01$ at Student's *t* test referred to "FLAG-wt-Dicer" (b) and to "C" (c)

and/or some conformational changes resulting in a suboptimal Dicer structure. In particular, densitometry analysis revealed that signal intensities of the product bands generated by FLAG-t-Dicer was threefold lower than those generated by FLAG-wt-Dicer (Fig. 4b, compare lanes IP1

relative to FLAG-t-Dicer and IP2 relative to FLAG-wt-Dicer). To this regard, previous reports have shown that human Dicer deleted for dsRBD is active in RNA processing, however its cleavage efficiency was reduced 2–3 fold [5, 6].

It should also be noted that the truncated protein does not seem to elicit dominant-negative effects on the endogenous counterpart (compare lanes E2 relative to FLAG-t-Dicer and Vector). However, we inquired whether the defective RIIIb domain could be indeed associated to a loss of the –5p strand cleavage of the pre-miRNA and/or an impaired functioning in the silencing of its targets. Therefore, FLAG-wt-Dicer and FLAG-t-Dicer were overexpressed in the SK-N-BE(2)-C (the source of t-Dicer transcript) (Fig. 5a) and the level of the –5p strand of a specific pre-miRNA, miR-125a-5p, was quantified by Q-PCR. The data showed that no differences in the levels of miR-125a-5p were detectable when the amount of t-Dicer was increased (endogenous t-Dicer plus FLAG-t-DICER), whereas the overexpression of FLAG-Dicer increases the accumulation of mature –5p miRNA (Fig. 5b). Finally, no differences were detectable in the ability of miR-125a-5p to silence one of its validated target sequence (Fig. 5c) [30].

Overall, these results indicate that t-Dicer is still able to bind the substrate (Fig. 1) and to cleave only one of the two pre-miRNA strands with the active RIIIa domain, without the activity of RIIIb due to the absence of the catalytic E1813. Furthermore, the presence of t-Dicer, even if overexpressed, seems to have no consequences on the level and functioning of the –5p strand of a pre-miRNA.

Conclusion

Previous analyses using Dicer proteins having either deleted dsRBD or point mutated residues located in the active site were focused on finding the role of these protein fragments or residues on Dicer cleavage. Here, we focused on the in vitro expression and the activity of a rare splice variant of Dicer, t-Dicer, lacking the dsRBD and having altered RIIIb domain. We found that t-dicer transcript could be translated in vitro, albeit not as efficiently as wt-Dicer, and the encoded truncated protein is still capable of binding pre-miRNA and preserves partial activity of cleavage by RIIIa domain. Moreover, the overexpression of t-Dicer does not seem to elicit a dominant negative effects on the endogenous Dicer, since a loss of –5p strand cleavage of the pre-miRNA and a possible reduction of its silencing activity were not detectable. At this stage, it still remains elusive whether t-Dicer plays a role in the tumorigenesis or it is simply a sign of neuroblastoma. In the

last case, it could be interesting to inquire whether t-Dicer may be a new possible marker predictive for pathological behaviour of neuroblastoma in clinical setting, as already demonstrated for other transcripts [32–34].

Given the increasing number of mutations reported for dicer gene in diverse types of cancer, future investigations will be necessary to characterize the activity of these mutants, which may dictate specific changes in selected classes of small RNAs [14, 35]. The experiments and protocols reported here show the usefulness of the approach for the study of Dicer and its mutants, allowing the analysis of the RNase activity discriminating between the enzymatic activities attributable to each RNase III domain.

Acknowledgments This work was supported by Regione Campania (L5/2007 and 2008) and National Science Centre [2011/03/B/NZ1/03259 to W.J.K]. Financial support for young investigators from the Polish Ministry of Science and Higher Education (statutory funds) is gratefully acknowledged (J.S-R).

References

- Hammond SM (2005) Dicing and slicing: the core machinery of the RNA interference pathway. *FEBS Lett* 579:5822–5829
- Jaskiewicz L, Filipowicz W (2008) Role of Dicer in posttranscriptional RNA silencing. *Curr Top Microbiol Immunol* 320:77–97
- Tian Y, Simanshu DK, Ma JB, Park JE, Heo I, Kim VN, Patel DJ (2014) A phosphate-binding pocket within the platform-PAZ-connector helix cassette of human Dicer. *Mol Cell* 53(4):606–616
- Wostenberg C, Lary JW, Sahu D, Acevedo R, Quarles KA, Cole JL, Showalter SA (2012) The role of human Dicer-dsRBD in processing small regulatory RNAs. *PLoS ONE* 7(12):e51829
- Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W (2004) Single processing center models for human dicer and bacterial Rnase III. *Cell* 118:57–68
- Ma E, MacRae IJ, Kirsch JF, Doudna JA (2008) Autoinhibition of human dicer by its internal helicase domain. *J Mol Biol* 380:237–243
- Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* 7051:740–744
- Lee Y, Hur Y, Park SY, Kim YK, Suh MR, Kim VN (2006) The role of PACT in the RNA silencing pathway. *EMBO J* 25:522–523
- Lee HY, Zhou K, Smith AM, Noland CL, Doudna JA (2013) Differential roles of human Dicer-binding proteins TRBP and PACT in small RNA processing. *Nucleic Acids Res* 41(13):6568–6576
- Lau PW, Guiley KZ, De N, Potter CS, Carragher B, MacRae IJ (2012) The molecular architecture of human Dicer. *Nat Struct Mol Biol* 19(4):436–440
- Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409(6818):363–366
- Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ (2003) Dicer is essential for mouse development. *Nat Genet* 35:215–217
- Slade I, Bacchelli C, Davies H, Murray A, Abbaszadeh F, Hanks S, Barfoot R, Burke A, Chisholm J, Hewitt M, Jenkinson H, King

- D, Morland B, Pizer B, Prescott K, Saggar A, Side L, Traunecker H, Vaidya S, Ward P, Futreal PA, Vujanic G, Nicholson AG, Sebire N, Turnbull C, Priest JR, Pritchard-Jones K, Houlston R, Stiller C, Stratton MR, Douglas J, Rahman N (2011) DICER1 syndrome: clarifying the diagnosis, clinical features and management implications of a pleiotropic tumour predisposition syndrome. *J Med Genet* 48(4):273–278
14. Foulkes WD, Priest JR, Duchaine TF (2014) DICER1: mutations, microRNAs and mechanisms. *Nat Rev Cancer* 14(10):662–672
15. Potenza N, Papa U, Russo A (2009) Differential expression of Dicer and Argonaute genes during the differentiation of human neuroblastoma cells. *Cell Biol Int* 33:734–738
16. Potenza N, Papa U, Scaruffi P, Mosca N, Tonini GP, Russo A (2010) A novel splice variant of the human dicer gene is expressed in neuroblastoma cells. *FEBS Lett* 584(15):3452–3457
17. Koscianska E, Starega-Roslan J, Czubala K, Krzyzosiak WJ (2011) High-resolution northern blot for a reliable analysis of microRNAs and their precursors. *Sci World J* 11:102–117
18. Starega-Roslan J, Krol J, Koscianska E, Kozlowski P, Szlachcic WJ, Sobczak K, Krzyzosiak WJ (2011) Structural basis of microRNA length variety. *Nucleic Acids Res* 1:257–268
19. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Rådmark O, Kim S, Kim VN (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425(6956):415–419
20. Lee Y, Jeon K, Lee JT, Kim S, Kim VN (2002) MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J* 21(17):4663–4670
21. Hernan R, Heuermann K, Brizzard B (2000) Multiple epitope tagging of expressed proteins for enhanced detection. *Biotechniques* 28(4):789–793
22. Wu MK, Sabbaghian N, Xu B, Addidou-Kalucki S, Bernard C, Zou D, Reeve AE, Eccles MR, Cole C, Choong CS, Charles A, Tan TY, Iglesias DM, Goodyer PR, Foulkes WD (2013) Biallelic DICER1 mutations occur in Wilms tumours. *J Pathol* 230(2):154–164
23. MacRae IJ, Ma E, Zhou M, Robinson CV, Doudna JA (2008) In vitro reconstitution of the human RISC-loading complex. *Proc Natl Acad Sci USA* 105(2):512–517
24. Flores-Jasso CF, Arenas-Huertero C, Reyes JL, Contreras-Cubas C, Covarrubias A, Vaca L (2009) First step in pre-miRNAs processing by human Dicer. *Acta Pharmacol Sin* 30(8):1177–1185
25. Leuschner PJ, Martinez J (2007) In vitro analysis of microRNA processing using recombinant Dicer and cytoplasmic extracts of HeLa cells. *Methods* 43(2):105–109
26. Park JE, Heo I, Tian Y, Simanshu DK, Chang H, Jee D, Patel DJ, Kim VN (2011) Dicer recognizes the 5' end of RNA for efficient and accurate processing. *Nature* 475(7355):201–205
27. Kolb FA, Zhang H, Jaroczyk K, Tahbaz N, Hobman TC, Filipowicz W (2005) Human dicer: purification, properties, and interaction with PAZ PIWI domain proteins. *Methods Enzymol* 392:316–336
28. Koscianska E, Starega-Roslan J, Krzyzosiak WJ (2011) The role of Dicer protein partners in the processing of microRNA precursors. *PLoS ONE* 6(12):e28548
29. Fukunaga R, Han BW, Hung JH, Xu J, Weng Z, Zamore PD (2012) Dicer partner proteins tune the length of mature miRNAs in flies and mammals. *Cell* 151(3):533–546
30. Potenza N, Papa U, Mosca N, Zerbini F, Nobile V, Russo A (2011) Human microRNA hsa-miR-125a-5p interferes with expression of hepatitis B virus surface antigen. *Nucleic Acids Res* 39(12):5157–5163
31. Mosca N, Castiello F, Coppola N, Trotta MC, Sagnelli C, Pisaturo M, Sagnelli E, Russo A, Potenza N (2014) Functional interplay between hepatitis B virus X protein and human miR-125a in HBV infection. *Biochem Biophys Res Commun* 449(1):141–145
32. D'Angelo V, Pecoraro G, Indolfi P, Iannotta A, Donofrio V, Errico ME, Indolfi C, Ramaglia M, Lombardi A, Di Martino M, Gigantino V, Baldi A, Caraglia M, De Luca A, Casale F (2014) Expression and localization of serine protease Htra1 in neuroblastoma: correlation with cellular differentiation grade. *J Neurooncol* 117(2):287–294
33. Iannaccone M, Giuberti G, De Vivo G, Caraglia M, Gentile V (2013) Identification of a FXIIIa variant in human neuroblastoma cell lines. *Int J Biochem Mol Biol* 4(2):102–107
34. Nakaguro M, Kiyonari S, Kishida S, Cao D, Murakami-Tonami Y, Ichikawa H, Takeuchi I, Nakamura S, Kadomatsu K (2015) The nucleolar protein PES1 is a marker of neuroblastoma outcome and is associated with neuroblastoma differentiation. *Cancer Sci* 106:237–243
35. Anglesio MS, Wang Y, Yang W, Senz J, Wan A, Heravi-Moussavi A, Salamanca C, Maines-Bandiera S, Huntsman DG, Morin GB (2013) Cancer-associated somatic DICER1 hotspot mutations cause defective miRNA processing and reverse-strand expression bias to predominantly mature 3p strands through loss of 5p strand cleavage. *J Pathol* 229(3):400–409