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Aktywność ludzkiej rybonukleazy Dicer wspierająca parowanie cząsteczek kwasów nukleinowych: odkrycie, charakterystyka biochemiczna i potencjalne znaczenie biologiczne

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Spis treści

Streszczenie

Rybonukleazy Dicer pełnią ważną funkcję w procesie biogenezy małych regulatorowych RNA wycinając dupleksy mikroRNA (miRNA) z jednoniciowych prekursorów przyjmujących strukturę spinki (pre-miRNA) oraz dupleksy zawierające małe interferujące RNA (siRNA), z długich dwuniciowych RNA. Cząsteczki miRNA lub siRNA, wraz z białkami Argonaute (Ago), tworzą kompleksy biorące udział w zależnym od sekwencji RNA procesie wyciszania ekspresji genów. Pojawia się jednak coraz więcej doniesień opisujących funkcje Dicer niepowiązane z jej aktywnością rybonukleazową i procesem biogenezy małych regulatorowych RNA.

Badania przedstawione w niniejszej pracy doktorskiej dotyczą niepoznanych obszarów aktywności rybonukleazy Dicer człowieka. Ludzka Dicer, podobnie jak większość rybonukleaz tego typu, to białko wielodomenowe. W przypadku wszystkich domen Dicer, z wyjątkiem jednej, zwanej DUF283 (ang. *domain of unknown function 283*), opisano ich udział w rozpoznawaniu, wiązaniu lub cięciu substratów RNA. W przypadku DUF283, jedyną zaproponowaną do tej pory funkcją był udział tej domeny w oddziaływaniu z partnerami białkowymi Dicer. Biorąc pod uwagę przedstawione powyżej fakty, celem badań prowadzonych w ramach pracy doktorskiej było ustalenie, czy domena DUF283 rybonukleazy Dicer człowieka może uczestniczyć w procesie wiązania kwasów nukleinowych. Na początku otrzymano preparat białkowy ludzkiej DUF283. Badania prowadzone z wykorzystaniem metody różnicowej migracji cząsteczek w natywnym żelu poliakrylamidowym (EMSA) wykazały, że DUF283 wiąże jednoniciowe cząsteczki RNA oraz DNA, nie wiąże natomiast dwuniciowych RNA lub DNA. Co ciekawe, dalsze, wnikliwe badania wykazały, że DUF283 może również wspierać proces parowania RNA lub DNA, działając tym samym jak białka typu ang. *nucleic acid annealer.* Zgromadzone dane zrodziły kolejne pytania i cele badawcze zmierzające do ustalania, czy Dicer pełnej długości również może wspierać parowanie cząsteczek kwasów nukleinowych, w jakim stopniu struktura RNA wpływa na badany proces oraz jakie jest potencjalne znaczenie biologiczne nowo odkrytej aktywności. Przeprowadzone badania wykazały, że w warunkach *in vitro* ludzka Dicer wpiera hybrydyzację krótkich RNA do komplementarnych miejsc docelowych w obrębie długich RNA, nawet jeśli krótkie RNA obecne są w dupleksach typu miRNA, a miejsca docelowe znajdują się w obrębie stabilnych struktur przyjmowanych przez dłuższe cząsteczki RNA. Równolegle prowadzone badania ujawniły, że ludzka rybonukleaza Ago2 nie wspiera parowania dwóch cząsteczek RNA jeśli sekwencja komplementarna jednego z substratów występuje w obrębie stabilnych struktur drugorzędowych. Następnie, wykorzystując dane pochodzące z eksperymentów głębokiego sekwencjonowania puli RNA wiązanych *in cellulo* przez Dicer pokazano, że wiele miejsc wiązania Dicer w obrębie transkryptów zawiera także miejsca docelowe dla miRNA. W oparciu o dostępną wiedzę i uzyskane wyniki zaproponowano model bezpośredniego udziału Dicer w potranskrypcyjnej regulacji ekspresji genów. Model ten przedstawia dwa scenariusze, zgodnie z którymi Dicer współzawodniczy, bądź współpracuje, z białkami Ago podczas wiązania do miejsc docelowych dla miRNA/siRNA w obrębie transkryptów, co prowadzi do, odpowiednio, stabilizacji bądź degradacji transkryptu.

Wyniki badań prowadzonych w ramach przedłożonej rozprawy doktorskiej wzbogacają dotychczasową wiedzę dotyczącą rybonukleazy Dicer człowieka i otwierają perspektywy dalszych badań odnoszących się do aktywności Dicer niepowiązanych z procesem biogenezy małych regulatorowych RNA.

Abstract

Dicer ribonucleases play an important role in the biogenesis of small regulatory RNAs by cleaving single-stranded precursors adopting stem-loop structures (pre-miRNAs) as well as double-stranded RNAs (dsRNAs) into short RNA duplexes containing functional microRNAs (miRNAs) or small interfering RNAs (siRNAs), respectively. miRNA or siRNA together with Argonaute (Ago) proteins form RNA-induced silencing complex which mediates post-transcriptional gene silencing. However, there is a growing number of reports describing Dicer's functions that are not related to its ribonuclease activity, and the biogenesis of small regulatory RNAs.

The research presented in this doctoral dissertation relates to unknown areas of activity of human ribonuclease Dicer. Human Dicer, similar to other ribonucleases of this type, is a multi-domain protein. All of Dicer's domains except one, named DUF283 (the domain of unknown function 283), have already been described in relation to their involvement in RNA substrate recognition, binding or cleavage. In the case of DUF283, its interaction with Dicer's protein partners has been the only function proposed so far. Taking these facts into consideration, the aim of the doctoral project was to establish whether the DUF283 domain of the human ribonuclease Dicer is capable of binding nucleic acids. First, a protein preparation of human DUF283 was obtained. By using the electrophoretic mobility shift assay (EMSA), it was demonstrated that DUF283 binds single-stranded RNA and DNA molecules, but not double-stranded RNAs or DNAs. Interestingly, subsequent investigations revealed that DUF283 can support RNA or DNA base pairing, thus acting as a nucleic acid annealer. These results generated further questions and research objectives on whether the full-length Dicer can also facilitate base pairing between nucleic acid molecules, how RNA structure influences this base pairing process, and what are the potential biological implications of the newly discovered annealing activity of human Dicer. The conducted studies demonstrated that *in vitro* human Dicer supports hybridization between short RNA and a complementary sequence of a longer RNA, even when both complementary sequences are trapped within secondary structures. Furthermore, under applied conditions, human Ago2, a core component of RNA-induced silencing complex, presented very limited annealing activity. In-depth analysis of the available data from new-generation sequencing experiments, concerning the RNA pool bound to Dicer *in cellulo*, revealed that multiple Dicer-binding sites within RNA transcripts also contain miRNA targets. Based on the literature data and the collected results, a model of direct Dicer involvement in the posttranscriptional control of gene expression was proposed. This model assumes two scenarios in which Dicer either cooperates or competes with Ago proteins for binding to the same miRNA/siRNA target within a transcript that may result in degradation or stabilization of the transcript, respectively.

The collected results expand the knowledge on the human ribonuclease Dicer, and open prospects for further studies on Dicer's activities that extend beyond the biogenesis of small regulatory RNAs.

Wykaz artykułów zawartych w rozprawie doktorskiej

[1] **The many faces of Dicer: the complexity of the mechanisms regulating Dicer gene expression and enzyme activities**

Kurzynska-Kokorniak A, Koralewska N, **Pokornowska M**, Urbanowicz A, Tworak A, Mickiewicz A, Figlerowicz M

Nucleic Acids Res. 2015 May 19;43(9):4365-80. doi: 10.1093/nar/gkv328

Wartość współczynnika *Impact Factor* w 2015 roku: **9,202**

[2] **Revealing a new activity of the human Dicer DUF283 domain in vitro**

Kurzynska-Kokorniak A, **Pokornowska M**, Koralewska N, Hoffmann W, Bienkowska-Szewczyk K, Figlerowicz M

Sci Rep. 2016 Apr 5;6:23989. doi: 10.1038/srep23989.

Wartość współczynnika *Impact Factor* w 2016 roku: **4,259**

[3] **The RNA-RNA base pairing potential of human Dicer and Ago2 proteins**

Pokornowska M, Milewski MC, Ciechanowska K, Szczepańska A, Wojnicka M, Radogostowicz Z, Figlerowicz M, Kurzyńska-Kokorniak A

Cell Mol Life Sci. 2019 Oct 26. doi: 10.1007/s00018-019-03344-6

Wartość współczynnika *Impact Factor* w 2019 roku: **7,014**

Wykaz skrótów

5LO	(ang. *5-lipooxygenase*) 5-lipooksygenaza
Å	angstrem
ADAR1	ang. *adenosine deaminase acting on RNA 1*
Ago	Argonaute
ARE	ang. *AU-rich element*
BRCA1	ang. *tumor suppressor breast cancer 1*
BSA	(ang. *bovine serum albumin*) surowicza albumina wołowa
CAT-1	ang. *cationic amino acid transporter 1*
CCR4-NOT	ang. *carbon catabolite repressor 4 – negative regulator of transcription*
ceRNA	ang. *competing endogenous RNA*
DCL	(ang. *Dicer-like protein*) białko typu Dicer
DCP	ang. *decapping protein*
DDRNA	ang. *DNA damage response RNA*
DDX6	ang. *DEAD-box helicase 6*
DGCR8	ang. *DiGeorge syndrome critical region gene*
diRNA	ang. DNA *damage-induced small RNA*
DRB4	ang. *dsRNA-binding protein 4*
DSB	(ang. *DNA double-strand break*) dwuniciowe uszkodzenie DNA
dsRBD	(ang. *dsRNA binding domain*) domena wiążąca dsRNA
dsRNA	(ang. *double-stranded RNA*) długi dwuniciowy RNA
DUF283	(ang. *domain of unknown function 283*) domena o nieznanej funkcji 283
egzo-siRNA	egzogenny siRNA
EMSA	(ang. *electrophoretic mobility shift assay*) metoda różnicowej migracji w żelu poliakrylamidowym
endo-siRNA	endogenny siRNA
GiDicer	rekombinowana rybonukleaza Dicer *Giardia intestinalis* (producent: My Biosource)
hAgo2	rekombinowana ludzka rybonukleaza Ago2 (producent: Active Motif)
hDicer	rekombinowana ludzka rybonukleaza Dicer, wyprodukowana we współpracy z zespołem prof. Krystyny Bieńkowskiej-Szewczyk (Zakład Biologii Molekularnej Wirusów, Międzyuczelniany Wydział Biotechnologii Uniwersytetu Gdańskiego i Gdańskiego Uniwersytetu Medycznego)
hDicer-His6	rekombinowana ludzka rybonukleaza Dicer, wyprodukowana przez autorkę ninjeszej rozprawy doktorskiej
HEK293	ang. *human embryonic kidney 293*
HERNA	ang. *helicase with RNase motif*
HF	High Five
hg38	ang. *Genome Reference Consortium Human Build 38*
His6	znacznik histydynowy 6xHis

His6-DUF283	rekombinowane białko domeny DUF283 ludzkiej rybonukleazy Dicer, wyprodukowane przez autorkę niniejszej rozprawy doktorskiej
HMW-RISC	(ang. *high molecular weight RISC*) wysokocząsteczkowy kompleks RISC
HuR	ang. *Hu-Antigen R*
HYL-1	ang. *hyponastic levaes 1*
K_d	stała wiązania
kDa	kilodalton
KSRP	ang. *KH-type splicing regulatory protein*
LINE-1	ang. *long interspersed element-1*
LMW-RISC	(ang. *low molecular weight RISC*) niskocząsteczkowy kompleks RISC
LTR	ang. *long terminal repeat*
MDa	megadalton
mESC	(ang. *mouse embryonic stem cell*) mysia embrionalna komórka macierzysta
miRNA	(ang. *microRNA*) mikroRNA
MT	ang. *mouse transcript*
MVB	(ang. *multivesicular body*) ciałko wielopęcherzykowate
NER	(ang. *nucleotide excision repair*) naprawa przez wycinanie nukleotydu
NMR	(ang. *nuclear magnetic resonanse*) magnetyczny rezonans jądrowy
nt	nukleotyd
OB	ang. *oligonucleotide/oligosaccharide binding*
PABC	ang. *polyadenylate binding protein 1*
PACT	ang. *protein activator of protein kinase R*
PAR-CLIP	ang. *photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation*
PARN2-PARN3	ang. *polyA-specific ribonuclease 2/3*
PAZ	PIWI-Argonaute-Zwille
PB	(ang. *processing body, P-body*) ciałko degradujące
pI	punkt izoelektryczny
PRE	ang. *Pumilio recognition element*
pre-miRNA	(ang. *miRNA precursor*) precursor miRNA
pri-miRNA	(ang. *primary miRNA transcript*) pierwotny transkrypt miRNA
PTGS	(ang. *post-transcriptional gene silencing*) potranskrypcyjne wyciszanie ekspresji genów
pz	para zasad
Q	ang. *quaternary ammonium*
RBP	(ang. *RNA-binding protein*) białko wiążące RNA
RISC	ang. *RNA-induced silencing complex*
RLC	ang. *RISC-loading complex*
RNAi	(ang. *RNA interference*) interferencja RNA
SDS	(ang. *sodium dodecyl sulfate*) siarczan dodecylu sodu
SG	(ang. *stress granule*) granula stresu
shRNA	ang. *short hairpin RNA*

siRNA	(ang. *small interfering RNA*) mały interferujący RNA
snoRNA	(ang. *small nucleolar RNA*) mały jąderkowy RNA
srRNA	(ang. *small regulatory RNA*) mały regulatorowy RNA
ssRNA	(ang. *single-stranded RNA*) jednoniciowy RNA
TGS	ang. *transcriptional gene silencing*) transkrypcyjne wyciszanie genów
TNRC6	ang. *trinucleotide repeat-containing gene 6 protein*
TRAF4	ang. *TNF receptor-associated factor 4*
TRBP	ang. *trans-activation response RNA binding protein*
tRNA	(ang. *transfer RNA*) transportujący RNA
TSS	(ang. *transcription start site)* miejsce rozpoczęcia transkrypcji
TSS-miRNA	ang. *transcriptional start site miRNA*
UTR	(ang. untranslated region) region niepodlegający translacji
XRN1	ang. *5'-3' exoribonuclease 1*

1 Wprowadzenie

1.1 Rola rybonukleazy Dicer w procesie biogenezy małych regulatorowych RNA u ssaków

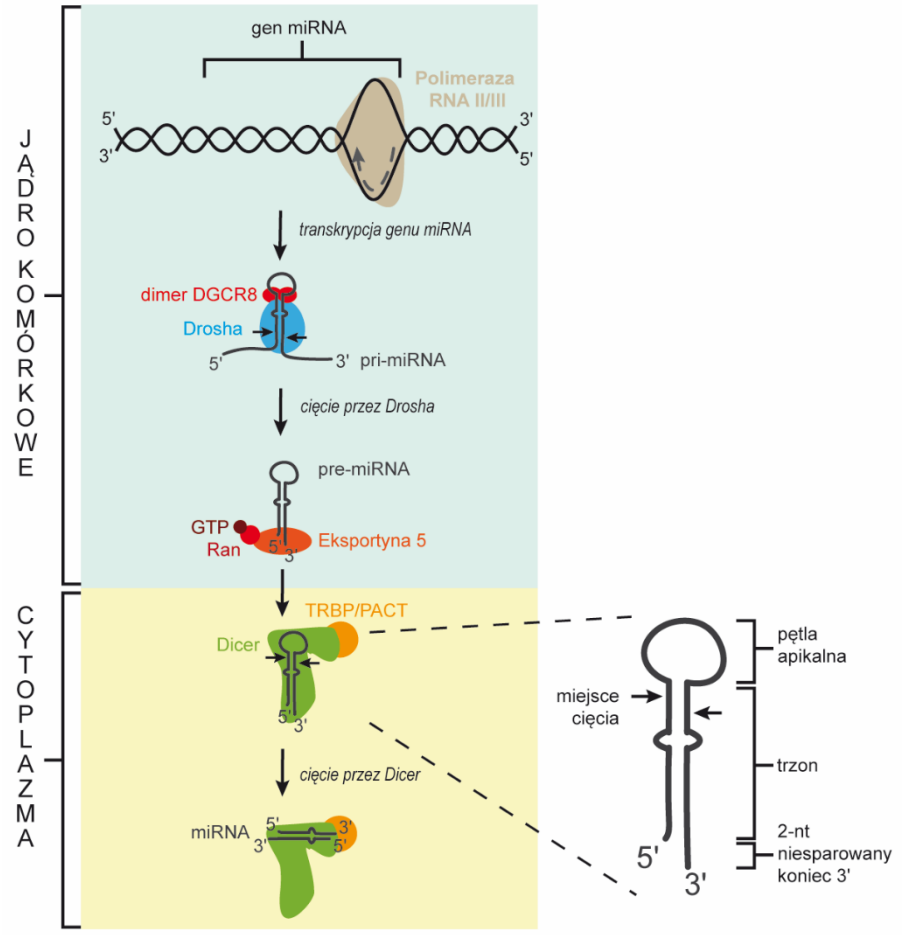
MikroRNA (miRNA, ang. *microRNA*) oraz małe interferujące RNA (siRNA, ang. *small interfering RNA*) reprezentują grupę małych regulatorowych RNA (srRNA, ang. *small regulatory RNA*). Są to cząsteczki o długości od ~20 do ~30 nukleotydów (nt) [4]. Wraz z białkami Argonaute (Ago) współtworzą kompleksy RISC (ang. *RNA-induced silencing complex*) – centralne kompleksy efektorowe uczestniczące w procesie interferencji RNA (RNAi, ang. *RNA interference*), zachowawczym ewolucyjnie mechanizmie wyciszania ekspresji genów, który jest inicjowany przez dwuniciowy RNA (dsRNA, ang. *double-stranded RNA*) [5-7]. W ramach kompleksu RISC, srRNA działają jak specyficzne sondy umożliwiające swoim partnerom białkowym rozpoznawanie komplementarnych transkryptów lub rejonów DNA, które mają podlegać regulacji. W procesie biogenezy srRNA bardzo ważną rolę odgrywa rybonukleaza Dicer, która wycina dupleksy miRNA z jednoniciowych prekursorów miRNA przyjmujących strukturę spinki (pre-miRNA) oraz siRNA z długich dsRNA [8].

W ostatnim czasie pojawiły się doniesienia literaturowe opisujące nową klasę srRNA, które powstają w jądrze komórkowym przy współudziale rybonukleazy Dicer. Te nowo odkryte srRNA zostały nazwane diRNA (ang. DNA *damage-induced small RNA*), ponieważ zaobserwowano, że ich powstawanie związane jest z odpowiedzią na dwuniciowe uszkodzenia DNA (DSB, ang. *double-strand breaks*) [9-12].

1.1.1 Cząsteczki miRNA

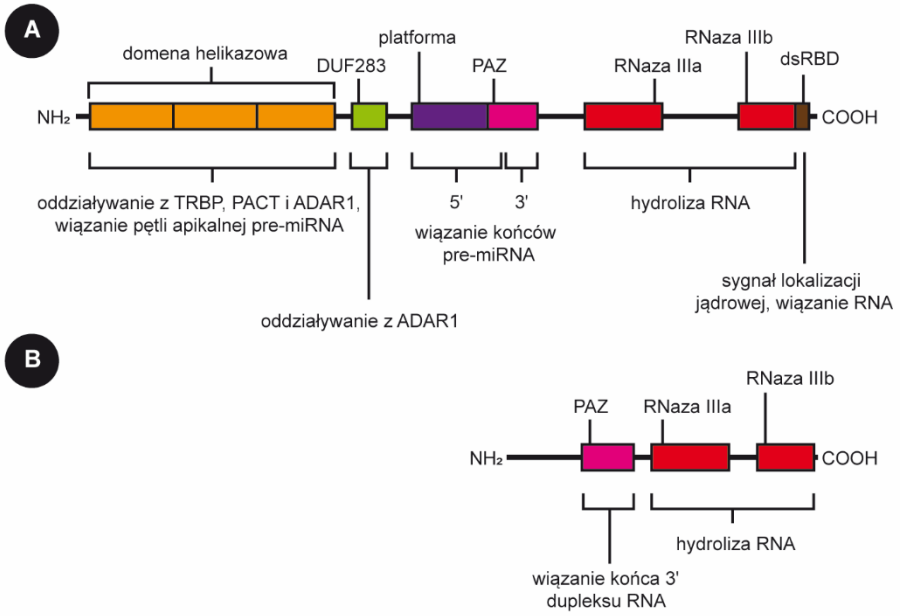
Cząsteczki miRNA stanowią dominującą klasę srRNA u ssaków, w tym człowieka. Szacuje się, że ekspresja ponad 60% ludzkich mRNA kontrolowana jest przez miRNA [13]. Cząsteczki miRNA powstają są z genów miRNA w wyniku transkrypcji do pierwotnych transkryptów miRNA (pri-miRNA, ang. *primary miRNA transcript*), najczęściej przez polimerazę RNA II [14, 15]. Geny miRNA znajdujące się w pobliżu elementów powtórzonych, takich jak Alu, transkrybowane są przez polimerazę RNA III [16]. Wykazano, że około połowa wszystkich miRNA jest transkrybowana z genów kodujących białka; zdecydowaną większość tych sekwencji to introny, rzadko egzony. Pozostałe miRNA są transkrybowane z genów miRNA znajdujących się pod kontrolą niezależnych promotorów transkrypcji [17]. Ponadto, szacuje się, że około połowa genów miRNA występuje w postaci klastrów transkrybowanych do policistronowych transkryptów pri-miRNA [18].

Pri-miRNA zawierają jedną lub kilka struktur drugorzędowych typu spinki oraz jednoniciowe sekwencje oskrzydlające. Struktury typu spinki są rozpoznawane i cięte przez kompleks mikroprocesora, w skład którego wchodzą rybonukleaza Drosha, kofaktor DGCR8 (ang. *DiGeorge syndrome critical region gene*) i kilka białek pomocniczych, w tym helikazy RNA p68 (DDX5) i p72 (DDX17) oraz białko naprawy DNA, BRCA1 (ang. *tumor suppressor breast cancer 1*) [19-22]. Zgodnie z obowiązującym modelem funkcjonowania kompleksu mikroprocesora, Drosha wiąże pri-miRNA w miejscu rozgałęzienia struktury typu spinki do struktur jednoniciowych, od którego mierzy odległość odpowiadającą około 11 parom zasad (pz) RNA w trzonie struktury i dokonuje cięcia obu nici [23]. Homodimer DGCR8 wiąże pri-miRNA w rejonie między pętlą apikalną a dwuniciowym trzonem struktury pri-miRNA [23, 24], oraz oddziałuje bezpośrednio z Drosha, stabilizując cały kompleks i aktywując cięcie RNA [25]. Produktem cięcia kompleksu mikroprocesora jest pre-miRNA o długości ~60-70 nt i strukturze drugorzędowej typu spinki, posiadający grupę fosforanową na końcu 5' i dwa niesparowane nukleotydy na końcu 3' [26, 27]. Pre-miRNA jest następnie eksportowany z jądra komórkowego do cytoplazmy przez eksportynę 5 w kompleksie z kofaktorem Ran-GTP **(rysunek 1)**. Uwolnienie pre-miRNA w cytoplazmie następuje w wyniku hydrolizy GTP [28, 29].



Rysunek 1 Kanoniczna ścieżka biogenezy miRNA u ssaków. Poziomymi strzałkami zaznaczono miejsca cięcia endorybonukleaz Drosha i Dicer. Z prawej strony zamieszczono schemat struktury drugorzędowej pre-miRNA.

Cząsteczki pre-miRNA są rozpoznawane w cytoplazmie przez rybonukleazę Dicer, która wycina z nich dupleksy, zawierające funkcjonalne miRNA. Ludzka Dicer to białko wielodomenowe, na którego końcu aminowym znajduje się domena helikazy RNA, a dalej, w kierunku końca 3', leżą kolejno: domena DUF283 (ang. *domain of unknown function 283*), Platformy, PAZ (PIWI-Argonaute-Zwille), RNazy IIIa, RNazy IIIb oraz domena wiążąca dsRNA (dsRBD, ang. *dsRNA binding domain*) (**rysunek 2A**). Niemniej, u pierwotniaka *Giardia intestinalis* (Ogoniastek jelitowy) Dicer występuje w postaci enzymu o prostej budowie, często nazywanego „minimalną Dicer". Dicer *Giardia intestinalis* składa się z zaledwie trzech domen: domeny PAZ oraz domen RNazy IIIa i RNazy IIIb [27]; za specyficzne wiązanie prekursora miRNA odpowiada domena PAZ, rozpoznająca charakterystyczny, niesparowany koniec 3' OH pre-miRNA lub dsRNA (**rysunek 2B**). Ludzka Dicer funkcjonuje w kompleksach z białkami TRBP (ang. *trans-activation response RNA binding protein*) i PACT (ang. *protein activator of protein kinase R*) (**rysunek 1**), które wpływają na powinowactwo Dicer o substratów [30-33].



Rysunek 2 Schematy przedstawiające domenową budowę ludzkiej rybonukleazy Dicer (A) oraz Dicer *Giardia intestinalis* (B). Poniżej schematów podsumowano funkcje poszczególnych domen (szczegóły w rozdziale 1.3).

Zasadniczo, ludzkie miRNA mają długość ~22 nt [34], jednakże miRNA generowane z różnych genów miRNA mogą różnić się pod względem długości [35]. Natomiast heterogenność cięcia pre-miRNA pochodzącego z jednego genu miRNA, skutkuje powstawaniem izomiRów (ang. *iso-miRs*) i jest możliwa dzięki elastyczności struktury Dicer [36]. Jak wcześniej wspomniano, cząsteczki pre-miRNA przyjmują struktury typu spinki, jednak struktury te, w obrębie różnych prekursorów, mogą różnić się od siebie. Obecnie wiadomo, że stabilność termodynamiczna końca trzonu pre-miRNA wpływa na wybór miejsca cięcia danego pre-miRNA przez rybonukleazę Dicer [37], podobnie jak obecność symetrycznych i asymetrycznych motywów strukturalnych, takich jak niesparowania, wybrzuszenia i pętle

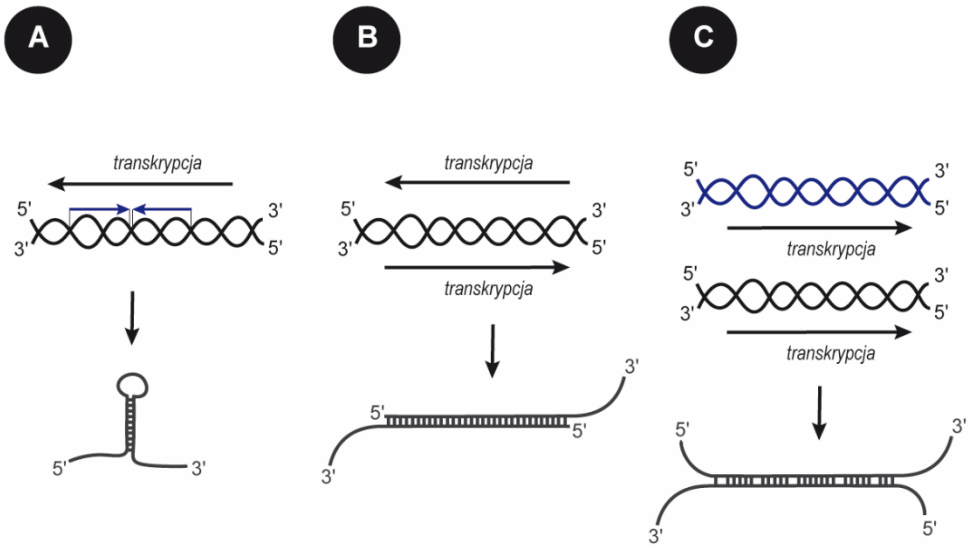
wewnętrzne zlokalizowane w obrębie trzonu spinki [36], czy sekwencja nukleotydowa w pobliżu miejsca cięcia [38]. Ponadto, sekwencja nukleotydowa niesparowanego końca 3' cząsteczek pre-miRNA ma znaczący wpływ na wydajność cięcia prekursora [39]. Poza tym zaobserwowano, że cząsteczki pre-miRNA o całkowicie sparowanym trzonie [40, 41] oraz z małą pętlą apikalną [40] charakteryzuje niska efektywność cięcia przez Dicer. Sekwencja pętli apikalnej może również pośrednio wpływać na wydajność cięcia pre-miRNA; znane są białka specyficznie oddziałujące z rejonami pętli pre-miRNA o określonej sekwencji nukleotydowej (podsumowane w [1]), np. KSRP (ang. *KH-type splicing regulatory protein*), wiążąc pętle bogate w guanozyny niektórych pre-miRNA, zwiększa wydajność cięcia tych prekursorów, lecz mechanizm takiego działania nie został dotąd poznany [42].

Niekanoniczne ścieżki biogenezy miRNA u zwierząt przebiegają z wyłączeniem niektórych spośród wyżej wymienionych białek. Część miRNA powstaje niezależnie od kompleksu mikroprocesora, ale zależnie od Dicer. Przykładem prekursorów takich miRNA są miRtrony, czyli introny, których struktura lassa ulega reorganizacji do struktury spinki, przez co mogą ulec wiązaniu i cięciu przez Dicer [43, 44]. Inne miRNA wywodzą się z niekodujących RNA, np. z transportujących RNA (tRNA, ang. *transfer RNA*) [45-49], lub z małych jąderkowych RNA (snoRNA, ang. *small nucleolar RNA*) [50-52], jednakże ich funkcjonalność pozostaje wciąż słabo zbadana. Warunkiem powstawania miRNA z tRNA jest przyjęcie przez tRNA alternatywnej struktury drugorzędowej w postaci spinki [48, 49]. Kolejnym przykładem prekursorów miRNA są produkty przedwczesnej terminacji transkrypcji. Jeśli takie krótkie transkrypty przyjmują strukturę spinki, Dicer rozpoznaje je i tnie do dupleksów miRNA. Jednakże, ze względu na obecność czapeczki 7-metyloguanozynowej na końcu 5' miRNA powstałego z ramienia 5' prekursora, tylko miRNA generowany z ramienia 3' jest efektywnie wiązany przez Ago [53]. Również bez udziału kompleksu mikroprocesora powstają niedawno odkryte TSS-miRNA (ang. *transcriptional start site miRNA*), czyli niekanoniczne miRNA, których prekursory są transkrybowane z rejonów promotorowych genów kodujących białka [54]. Cząsteczki miRNA mogą także powstawać z udziałem kompleksu mikroprocesora, lecz bez udziału Dicer, czego przykładem jest miR-451. Prekursor miR-451 jest zbyt krótki by był cięty przez Dicer. Jest on natomiast bezpośrednio wiązany i cięty przez białko Ago2, w wyniku czego dojrzały miR-451 występuje w postaci jednoniciowej, a nie w dupleksie, jak inne miRNA [55, 56].

1.1.2 Cząsteczki siRNA

Cząsteczki siRNA produkowane są w organizmach roślinnych [57, 58], niektórych zwierząt, w tym bezkręgowców [59], a także u niższych eukariontów, jak niektóre grzyby [60]. Endogenne siRNA (endo-siRNA) znaleziono również w ssaczych oocytach [61, 62] i embrionalnych komórkach macierzystych [63], lecz brak ich w komórkach somatycznych [64]. Ssacze endo-siRNA, podobnie jak owadzie, mają długość 21-nt i powstają z udziałem

rybonukleazy Dicer, ale niezależnie od kompleksu mikroprocesora. Prekursorami endo-siRNA w ssaczych oocytach są transkrypty zawierające odwrócone powtórzenia, przyjmujące struktury typu spinki o wydłużonym trzonie **(rysunek 3, A)** lub dsRNA powstałe z komplementarnych sensowych i antysensowych transkryptów w układach cis lub trans. W układach cis dochodzi do transkrypcji dwukierunkowej tego samego *loci* i hybrydyzacji powstałych komplementarnych transkryptów **(rysunek 3, B)**, a w układach trans, do hybrydyzacji transkryptów powstałych z różnych *loci* **(rysunek 3, C)** [65, 66]. Na przykład, u człowieka produkty transkrypcji dwukierunkowej rejonu kodującego 5' UTR (ang. *untranslated region*) transpozonu LINE-1 (ang. *long interspersed element-1*) są źródłem siRNA hamujących proces retrotranspozycji [67]. Również transkrypty pseudogenów, hybrydyzując do transkryptów swoich funkcjonalnych odpowiedników, stanowią substrat dla Dicer do generowania siRNA. Sugeruje się, że za pomocą takich siRNA pseudogeny mogą wyciszać ekspresję genów rodzicielskich [61, 62].



Rysunek 3 Prekursory endo-siRNA u ssaków i ich pochodzenie. (A) Transkrypty o strukturze drugorzędowej typu spinki z długimi jednoniciowymi rejonami oskrzydlającymi powstają w wyniku transkrypcji sekwencji zawierających odwrócone powtórzenia (zaznaczone niebieskimi strzałkami). (B) Transkrypcja dwukierunkowa jest źródłem dsRNA. (C) W wyniku hybrydyzacji częściowo komplementarnych transkryptów wygenerowanych w układzie trans, powstają nie w pełni sparowane dsRNA.

Dobrze zbadanym procesem jest produkcja w mysich oocytach skróconej o domenę helikazową izoformy Dicer, która, w porównaniu do pełnej długości enzymu, wydajnie przycina długie dsRNA do siRNA [68]. Podobnie, badania *in vitro* wykazały, że obecność domeny helikazowej obniża aktywność RNazową ludzkiej Dicer wobec długich dsRNA, a jednocześnie nie wpływa na aktywność enzymu, gdy substratami są pre-miRNA [69]. Źródłem produkcji skróconej izoformy Dicer w mysich oocytach jest alternatywny transkrypt powstający w wyniku insercji retrotranspozonu MT (ang. *mouse transcript*) z rodziny LTR (ang. *long terminal repeat*) w obrębie intronu szóstego genu *DICER1* [68]. Retrotranspozon MT jest specyficzny dla

rodziny myszowatych i funkcjonuje w oocytach jako alternatywny promotor transkrypcji przyległych genów [70]. Dotychczas nie znaleziono tej izoformy Dicer w oocytach innych ssaków, co więcej, jej obecność nie wydaje się być konieczna do produkcji endo-siRNA. Endo-siRNA można bowiem znaleźć w krowich, świńskich czy owczych oocytach pozbawionych takiej skróconej Dicer [71-73].

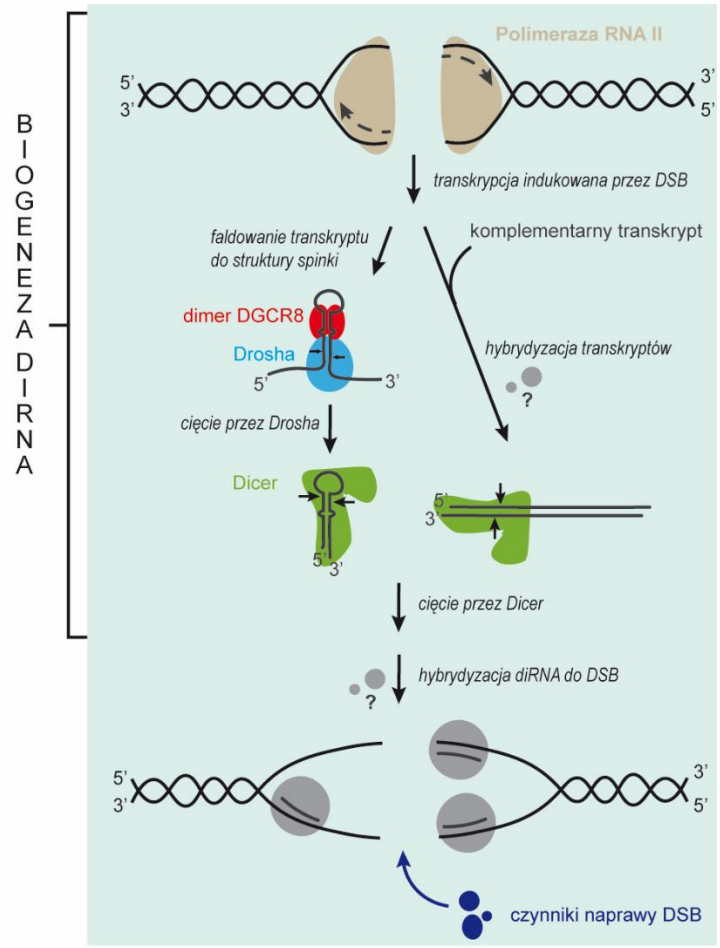
Obecnie wiadomo, że rybonukleazy typu Dicer stanowią ważny element odpowiedzi antywirusowej u roślin czy owadów [59, 74]. Przykładem rybonukleaz biorących udział w cięciu wirusowych RNA do siRNA są np. owadzie białka Dicer-2 czy roślinne białka typu Dicer (ang. *Dicer-like protein*, DCL), DCL2-4 [75]. Wirusowe siRNA, ze względu na źródło ich pochodzenia, nazywane są egzogennymi siRNA (egzo-siRNA). Analizy krótkich RNA pochodzących z ssaczych komórek somatycznych zainfekowanych wirusami wskazują jednak na brak produkcji takich egzo-siRNA przez ssacze Dicer [76-79]. Co ciekawe, mysie i ludzkie komórki pozbawione Dicer, tym samym niezdolne do produkcji kanonicznych miRNA i siRNA, nie różnią się od komórek dzikiego typu pod względem zachodzących w nich procesów replikacji wirusów DNA i RNA [77, 78]. Należy w tym miejscu wspomnieć, że w królestwach roślin, zwierząt i grzybów doszło do, najprawdopodobniej, niezależnych duplikacji genów kodujących Dicer [80]. W królestwie zwierząt wykształciły się dwa homologi Dicer: homolog Dicer-1, odpowiedzialny za produkcję miRNA, oraz homolog Dicer-2, odpowiedzialny za produkcję siRNA [81]. Gen kodujący Dicer-2 został utracony w przypadku zwierząt, które wykształciły alternatywne ścieżki odpowiedzi antywirusowej, np. w przypadku kręgowców posiadających układ odpornościowy [80]. Ponadto, przypuszcza się, że geny kodujące Dicer zostały utracone u niektórych grzybów, takich jak *Saccharomyces cerevisiae*, i pasożytniczych pierwotniaków, takich jak *Leishmania major* oraz *Trypanosoma cruzi* [82, 83]. U roślin wykształciły się cztery homologi białek typu Dicer. Białko DCL1, jako jedyny homolog DCL, zaangażowane jest w produkcję miRNA, natomiast białka DCL2-4 są odpowiedzialne za produkcję różnych siRNA. DCL3 produkuje 24-nt siRNA w odpowiedzi na zakażenie wirusami DNA, a białka DCL2 i DCL4 generują, odpowiednio, 22-nt i 21-nt siRNA z wirusowych transkryptów [84].

1.1.3 Cząsteczki diRNA

Od momentu wykrycia Dicer w jądrze komórkowym [85] coraz więcej badań koncentruje się na potencjalnych funkcjach jądrowych tego białka [85-92]. Odkryto, że naprawa uszkodzeń DNA jest ściśle powiązana z obecnością Dicer [93, 94]. Dicer lokalizuje się w obrębie uszkodzeń DNA powstających w wyniku naświetlania komórek promieniowaniem nadfioletowym, aby wziąć udział w rozluźnieniu struktury chromatyny poprzedzającej proces naprawczy, nazywany naprawą przez wycinanie nukleotydu (ang. *nucleotide excision repair*, NER) [95]. Wykazano również, że w obrębie DSB, w sposób zależny od Dicer, powstają małe niekodujące RNA, tzw. diRNA lub DDRNA (ang. *DNA damage response RNA*) [10, 12].

Powstawanie diRNA jest charakterystyczne nie tylko dla ssaczych komórek, obecność tych małych cząsteczek RNA zaobserwowano również u niektórych grzybów [9], roślin [10], bezkręgowców [11] i niższych kręgowców [12]. Rolą diRNA jest rekrutacja czynników odpowiedzialnych za indukcję procesów naprawczych, na przykład, białka MDC1 [96], 53BP1 [96, 97], Rad51 [98, 99], BRCA1 [98] czy metylotransferaz i acetylotransferaz histonowych odpowiedzialnych za rozluźnienie struktury chromatyny [98] w miejscu uszkodzenia.

Mechanizm biogenezy diRNA nie został w pełni poznany. Sugeruje się, że w miejscu dwuniciowego pęknięcia DNA, w sposób zależny od polimerazy RNA II, inicjowana jest produkcja transkryptów (**rysunek 4**). Transkrypty powstałe w odpowiedzi na uszkodzenie DNA ulegają zwijaniu w struktury spinkowe i cięciu przez białka Drosha i Dicer, podobnie do pri-miRNA. Natomiast, gdy dwa procesy transkrypcji zachodzą zbieżnie (tj. jedna nić DNA transkrybowana jest konstytutywnie, a druga w wyniku indukcji związanej z uszkodzeniem) powstające sensowe i antysensowe transkrypty hybrydyzują ze sobą i stają się substratami dla Dicer [100].



Rysunek 4 Proponowany udział Dicer w biogenezie diRNA. „?" – nieznane czynniki białkowe. Schemat wykonano na podstawie Hawley i wsp., 2017 [100].

Obecnie wiadomo, że uszkodzenie DNA indukuje fosforylację seryny w pozycji 1016 ludzkiej Dicer (S1016), dzięki czemu białko migruje z cytoplazmy do jądra komórkowego.

Zjawisko to jest zachowawcze ewolucyjnie u ssaków. Przypuszcza się, że dodatkowa fosforylacja końca karboksylowego Dicer w obrębie dwóch reszt serynowych (S1728, S1852) jest niezbędna dla procesu cięcia dwuniciowych RNA, które pojawiają się w odpowiedzi na dwuniciowe pęknięcia DNA [90].

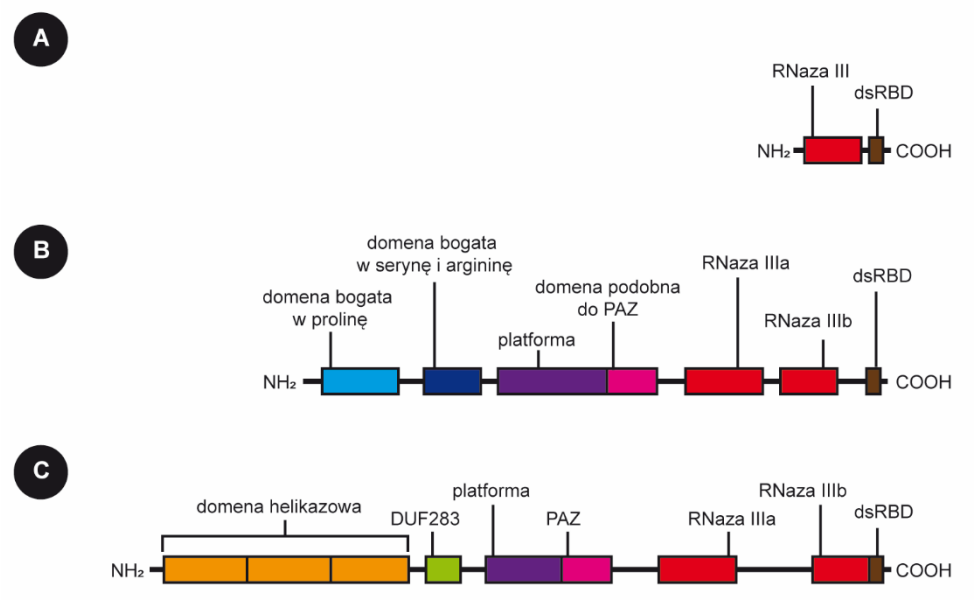
1.2 Budowa i model działania ludzkiej rybonukleazy Dicer

Ludzka rybonukleaza Dicer to białko o masie 220 kDa (kilodaltonów), złożone ze 1922 aminokwasów. Jest kodowane przez pojedynczy gen *DICER1* (ENSG0000010069), zlokalizowany na długim ramieniu chromosomu 14 w rejonie subtelomerowym 14q32.13. Gen *DICER1* zawiera 26 egzonów kodujących białko i jest uznawany za gen metabolizmu podstawowego (ang. *housekeeping gene*), jednakże, w porównaniu z innymi genami tego typu, charakteryzuje się długim rejonem 3' UTR (powyżej 4000 pz) [101].

Pierwsze literaturowe doniesienie o ludzkiej Dicer pochodzi z pracy Provost i wsp. z 1999 roku [102], w której Dicer opisywana jest jako białko oddziałujące z 5-lipooksygenazą (5LO, ang. *5-lipooxygenase*), enzymem odpowiedzialnym za syntezę przeciwzapalnych związków, leukotrienów. Zidentyfikowane białko wykazywało homologię względem helikazy K12H4.8 z *Caenorhabditis elegans*, jak również względem bakteryjnych RNaz z rodziny RNaz III [102]. Następnie, w roku 2000 opublikowano szczegółowy opis genu *DICER1*, ówcześnie pod nazwą *HERNA* (ang. *helicase with RNase motif*) [103]. Wykazano, że profil ekspresji *HERNA* jest szeroki i tkankowo specyficzny, a białkowy produkt ekspresji tego genu posiada m.in. zachowawcze ewolucyjnie motywy charakterystyczne dla ATP-zależnej helikazy RNA: motyw wiążący ATP i kasetę DEXD/H (Asp-Glu-X-Asp/His) oraz motywy wiążące RNA [103]. Rok później Dicer została przypisana do rodziny Rybonukleaz III (RNaz III) [8].

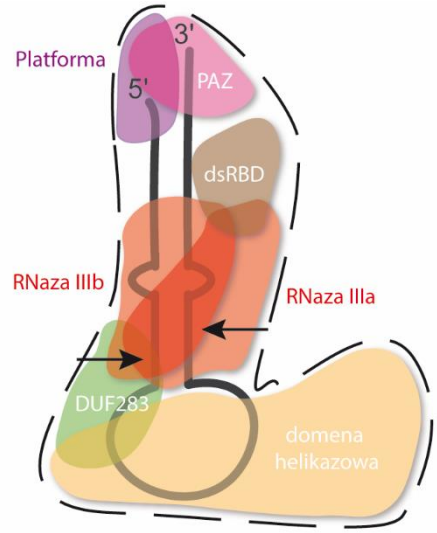
RNazy III występują u bakterii, fagów i eukariontów, lecz tylko sporadycznie pojawiają się u archeonów. Istnieje hipoteza, zgodnie z którą enzym pojawił się w organizmach eukariotycznych w wyniku endosymbiozy pierwotnych komórek eukariotycznych z bakteriami [104]. Rodzinę RNaz III dzieli się na trzy klasy. Klasę 1 reprezentuje RNaza III z *Escherichia coli* (Pałeczka okrężnicy), która składa się z jednej domeny RNazowej i jednej domeny dsRBD o zachowawczym motywie α-β-β-β-α **(rysunek 5, A)** [105, 106]. RNazy III klasy 1 funkcjonują w postaci homodimeru, który wiąże dsRNA i przeprowadza po jednym cięciu wiązania fosfodiestrowego w obrębie każdej z nici dupleksu [104, 107]. Klasę 2 i 3 RNaz III reprezentują, odpowiednio, ludzkie białka Drosha i Dicer, które posiadają po dwie domeny RNazowe, tj. RNazę IIIa i RNazę IIIb, tworzące wewnątrzcząsteczkowy dimer katalityczny **(rysunek 5, B i C)**. W wyniku trawienia dsRNA przez RNazy III powstają specyficzne produkty posiadające grupę fosforanową na końcu 5' i dwa niesparowane nukleotydy na końcu 3' [104]. RNazy III klasy 2, w przeciwieństwie do RNaz III klasy 3, nie posiadają domen specyficznie wiążących dsRNA. Za specyficzne wiązanie substratów odpowiadają ich partnerzy białkowi. Przykładem

jest kompleks mikroprocesora, w którym kofaktor DGCR8 odpowiada za rozpoznawanie charakterystycznych elementów strukturalnych w pri-miRNA, tj. w substracie Drosha [108].



Rysunek 5 Schemat przedstawiający domenową budowę poszczególnych klas RNaz III: klasy 1 – reprezentowanej przez bakteryjną rybonukleazę III (A), klasy 2 - reprezentowanej przez ludzką rybonukleazę Drosha (B) i klasy III - reprezentowanej przez ludzką rybonukleazę Dicer (C).

Badania z użyciem mikroskopii elektronowej wykazały, że ludzka Dicer przyjmuje strukturę przypominającą kształtem literę L; krótsze ramię zajmuje domena helikazowa, a dłuższe ramię zajmują pozostałe domeny. Domena PAZ wraz z domeną Platformy znajdują się na szczycie struktury. Domeny RNazy IIIa i RNazy IIIb, które formują wewnątrzcząsteczkowy dimer katalityczny, znajdują się w centrum struktury trzeciorzędowej cząsteczki. Domeny dsRBD i DUF283 oskrzydlają dimer katalityczny, przy czym domena DUF283 jest zlokalizowana w pobliżu miejsca rozgałęzienia ramion struktury litery L **(rysunek 6)** [34, 109, 110].



Rysunek 6 Schemat struktury trzeciorzędowej ludzkiej Dicer w kompleksie z pre-miRNA, przygotowany na podstawie Liu i wsp. [110]. Strzałkami zaznaczono miejsca cięcia pre-miRNA przez domeny RNazowe.

1.2.1 Domeny RNazy IIIa, RNazy IIIb i dsRBD

Dwie domeny RNazowe Dicer działają jako pojedynczy moduł katalityczny [26]. Domena RNazy IIIa odpowiada za cięcie w obrębie ramienia 3', natomiast domena RNazy IIIb odpowiada za cięcie w obrębie ramienia 5' pre-miRNA [26]. Białka Dicer, podobnie jak inne RNazy III, wymagają do swojej aktywności hydrolitycznej związania dwuwartościowego jonu metalu w centrum katalitycznym, a preferowanym jonem jest jon magnezu, Mg^{2+} [111-113]. Struktury krystaliczne Dicer *Giardia intestinalis* i domeny RNazy IIIb ludzkiej Dicer ujawniły obecność dwóch dwuwartościowych jonów metalu w miejscu aktywnym [27, 114]. Pierwszy z jonów jest wiązany przez cztery zachowawcze ewolucyjnie reszty aminokwasowe (dwie reszty kwasu asparaginowego i dwie reszty kwasu glutaminowego) i jest bezpośrednio zaangażowany w katalizę. Rola drugiego jonu jest niejasna, a miejsce wiązania jonu różni się w strukturze Dicer *G. intestinalis* i RNazy IIIb ludzkiej Dicer [114]. Takeshita i wsp. zaproponowali mechanizm działania domen RNazowych ludzkiej Dicer, w którym jeden jon metalu zaangażowany jest w katalizę, a drugi w wiązanie dsRNA [114].

Domena dsRBD obecna jest na końcu karboksylowym ludzkiej Dicer i charakteryzuje się wysokim powinowactwem do prekursorów miRNA i siRNA, przez co znacząco przyczynia się do wiązania kanonicznych substratów Dicer [115].

1.2.2 Domeny Platformy i PAZ

Domena PAZ wiąże dwa niesparowane nukleotydy obecne na końcu 3' substratów pre-miRNA oraz dsRNA. Motyw wiążący RNA, obecny w domenach typu PAZ, to motyw OB (ang. *OB fold*, *oligonucleotide/oligosaccharide binding fold*), formujący tzw. kieszeń 3' (ang. *3' binding pocket*) [27, 116-118]. Ludzka Dicer posiada również tzw. kieszeń 5' (ang. *5' binding pocket*), która wiążę grupę fosforanową obecną na końcu 5' prekursorów miRNA i siRNA. Reszty aminokwasowe tworzące kieszeń 5' występują zarówno w obrębie domeny Platformy, jak i PAZ [37, 119]. Ponadto, w obrębie domeny PAZ w toku ewolucji wykształcił się segment, który w przypadku ludzkiej Dicer posiada strukturę helisy α oddzielającej kieszenie 3' i 5' i prawdopodobnie odgrywa rolę na etapie przekazywania produktu cięcia (tj. dupleksu miRNA lub siRNA) do Ago [37].

Dicer, ze względu na zdolność generowania dupleksu o określonej dla danego homologu długości, nazywana jest często „linijką molekularną" (ang. *molecular ruler*). W związku z występowaniem dwóch kieszeni wiążących RNA, opisano dwie reguły wyznaczania miejsca cięcia substratu przez rybonukleazy typu Dicer. Pierwsza z nich, opisana dla ludzkiej Dicer i Dicer *Giardia intestinalis*, polega na odmierzaniu odległości odpowiadającej ~22 nt (dla ludzkiej Dicer) lub ~25 nt (dla Dicer *G. intestinalis*) od miejsca związania substratu w obrębie kieszeni 3' do miejsca jego cięcia w obrębie centrum katalitycznego RNazy IIIa [26, 27, 39, 120, 121]. Na podstawie struktury krystalicznej Dicer *G. intestinalis* obliczono, że

odległość między domeną PAZ a dimerem katalitycznym domen RNazowych wynosi 65 Å (angstremów) i równa się odległości, na jaką rozciąga się helisa RNA typu A o długości 25 pz [27]. Druga reguła, opisana dla owadziej Dicer-1 i Dicer-2 oraz ludzkiej Dicer, polega na wykorzystywaniu kieszeni 5' celem wyznaczania miejsca cięcia substratu [119, 122]. Wiązanie grupy fosforanowej obecnej na końcu 5' w obrębie kieszeni 5' ludzkiej Dicer jest możliwe w przypadku substratów o niestabilnych termodynamicznie końcach, na przykład takich jak pre-miRNA [37, 119]. W związku z tym postuluje się, że kieszeń 5' wykształciła się u tych homologów Dicer, które wyspecjalizowały się w produkcji miRNA, ale nie siRNA [37]. Ponadto, z uwagi na fakt, że końce 5' pre-miRNA są bardziej homogenne od końców 3', przypuszcza się, że wykorzystanie kieszeni 5' umożliwia generowanie homogennej puli miRNA, w przeciwieństwie do bardziej heterogennej puli miRNA uwalnianych z ramienia 3' pre-miRNA [119]. Wiadomo bowiem, że końce 3' pre-miRNA ulegają licznym modyfikacjom przez egzonukleazy i nukleotydylotransferazy [123-127]. Niemniej, owadzia Dicer-2 również wykształciła kieszeń 5', której obecność jest konieczna dla zachowania precyzji cięcia dsRNA do 21-nt siRNA [122], przy czym kieszenie 5' owadziej Dicer-1 i ludzkiej Dicer znacząco różnią się składem reszt aminokwasowych od kieszeni 5' owadziej Dicer-2 [119, 122].

1.2.3 Domena helikazy

Helikazy RNA wspierają proces rozplatania dwuniciowych struktur RNA. Białka te hydrolizują wysokoenergetyczne wiązania fosfodiestrowe obecne w trójfosforanach nukleozydów, a uwolnioną energię wykorzystują do rozplatania dupleksu [128]. W przypadku rybonukleaz typu Dicer funkcjonalność domeny helikazowej najczęściej oceniana jest poprzez badanie wpływu usunięcia całej domeny helikazowej, lub jej wybranych fragmentów, na aktywność wiązania i cięcia pre-miRNA oraz dsRNA przez uzyskiwane warianty Dicer. Między innymi, badania z użyciem takich wariantów delecyjnych wykazały, że obecność domeny helikazowej nie jest wymagana do zachowania aktywności RNazowej ludzkiej Dicer [32], a wręcz ją maskuje [69]. Niemniej, funkcjonalna domena helikazowa jest niezbędna dla zachowania aktywności cięcia pre-miRNA o niestabilnych termodynamicznie strukturach spinkowych, posiadających krótkie trzony [129].

Klasyfikacji helikaz dokonuje się w oparciu o obecność charakterystycznych motywów sekwencyjnych, które odpowiadają za aktywności ATPazowe i helikazowe oraz za wiązanie RNA. Zgodnie z powyższym, domena helikazowa ludzkiej Dicer należy do rodziny helikaz posiadających charakterystyczny motyw DEXD/H (ang. *DEXD/H-box helicase*), należącej do nadrodziny helikaz SF2 [129]. Motyw DEXD/H jest zaangażowany w wiązanie i hydrolizę ATP [128]. Liczne publikacje traktują o przyporządkowaniu domeny helikazowej ludzkiej Dicer do podrodziny helikaz typu RIG-I (ang. *RIG-I-like helicase*) [34, 130-133]. Helikaza RIG-I to receptor wrodzonego układu immunologicznego u kręgowców, który wchodzi w skład czynników tworzących pierwszą linię obrony przed wirusami RNA. Ten cytoplazmatyczny

sensor wirusowego RNA aktywuje ścieżki sygnałowe czynników transkrypcyjnych, w wyniku czego następuje produkcja interferonu β i aktywacja ekspresji wielu genów, których produkty zaangażowane są w mobilizację układu immunologicznego do walki z infekcją [132].

Domena helikazowa w ludzkiej Dicer służy za platformę do wiązania białka TRBP [134], które zwiększa powinowactwo Dicer do dsRNA i wydajność cięcia substratów [33, 135], a także wpływa na wybór miejsca cięcia pre-miRNA [136]. Co więcej, TRBP stanowi pomost między Dicer a białkiem Ago podczas przekazywania miRNA i wpływa na wybór nici efektorowej [134, 137, 138]. Wiadomo również, że domena helikazowa ludzkiej Dicer oddziałuje z pętlą apikalną prekursorów miRNA [129, 139], dzięki czemu enzym pozostaje w konformacji „otwartej", umożliwiającej wydajne cięcie pre-miRNA [40, 41], podobnie jak ma to miejsce podczas oddziaływania z partnerami białkowymi, TRBP i PACT [109].

Nie opublikowano dotychczas badań, które potwierdziłyby lub zaprzeczyły aktywności ATPazowej, czy rozplatania dsRNA domeny helikazowej ludzkiej Dicer . Wiadomo jednak, że domena helikazowa owadziej Dicer-2, która również posiada motyw DEXD/H, wykorzystuje energię pochodzącą z hydrolizy ATP do przemieszczania się wzdłuż dsRNA i procesywnego cięcia substratu [140-142]. Tymczasem ludzki enzym generuje siRNA z długich dsRNA niezależnie od ATP [120, 143], jednakże wydajność cięcia dsRNA jest znacznie niższa w porównaniu do cięcia pre-miRNA [33, 69].

1.2.4 Domena DUF283

Badania profesor Doudny i jej zespołu stanowią bogate źródło informacji na temat domeny DUF283. Grupa badaczy wykazała, że dwa fragmenty ludzkiej Dicer, uzyskane niezależnie w bakteryjnym systemie produkcji białek, mogą odtwarzać funkcjonalną rybonukleazę Dicer i generować produkty o charakterystycznych długościach ~22 pz. Jeden fragment obejmował domeny: RNazę IIIa, RNazę IIIb i dsRBD (tzw. fragment C), a drugi fragment obejmował domeny: DUF283 i PAZ (tzw. fragment DP). Fragment C przeprowadzał cięcie dsRNA do produktu o długości 15 pz, podobnie do RNazy III *E. coli*, która cięła ten sam substrat do produktu o długości 12 pz. Dodanie fragmentu DP do fragmentu C spowodowało, że dsRNA było cięte do produktu o długości 22 pz, jak ma to miejsce w przypadku pełnej długości ludzkiej Dicer [139]. Co więcej, we wcześniejszej pracy zespół wykazał, że rekombinowana Dicer pozbawiona domeny DUF283 (wariant ΔDUF283) posiada znacznie obniżoną aktywność RNazową wobec prekursorów siRNA względem białka typu dzikiego. Ponadto, zauważono różnice w powinowactwie Dicer dzikiego typu i wariantu ΔDUF283 do prekursorów miRNA i prekursorów siRNA [69].

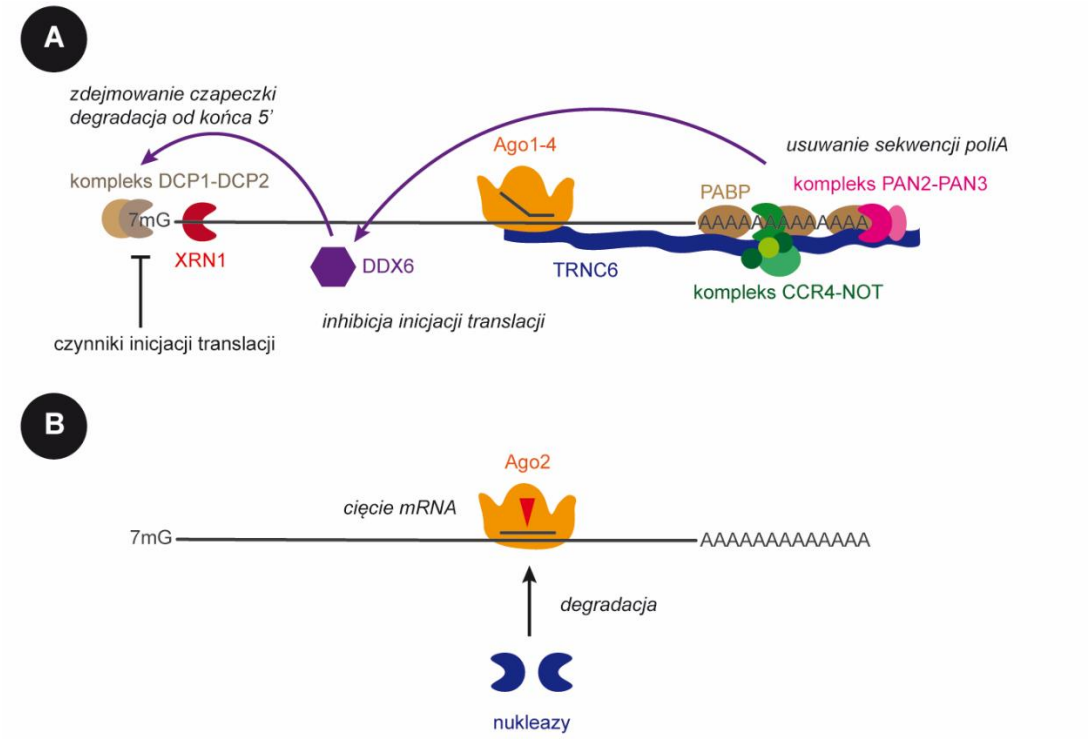
Struktura domeny DUF283 ludzkiej Dicer nie jest znana, zaproponowano natomiast jej model, który przedstawia kanoniczny motyw dsRBD [144]. Jedyna jak dotąd poznana struktura domeny DUF283 to struktura DUF283 roślinnego białka typu Dicer, DCL4, która również

zawiera motyw dsRBD. Nie wykazano jednak, by domena DUF283 DCL4 oddziaływała z dsRNA [145]. Wykazano natomiast, że domeny DUF283 DCL1 i DCL4 *Arabidopsis thaliana* (Rzodkiewnik pospolity) odpowiadają za oddziaływanie z, odpowiednio, HYL1 (ang. *hyponastic levaes 1*) i DRB4 (ang. *dsRNA-binding protein 4*), białkami partnerującymi DCL1 i DCL4 [145]. Podobnie, domena DUF283 ludzkiej Dicer odpowiada za wiązanie białka ADAR1 (ang. *adenosine deaminase acting on RNA 1*), które pozytywnie wpływa na, zarówno, wydajność cięcia pre-miRNA przez Dicer, jak i wydajność przekazywania miRNA do Ago [146]. Zgodnie z powyższym, domenę DUF283 nazywana jest często „domeną dimeryzacji".

1.3 Potranskrypcyjne wyciszanie ekspresji genów u ssaków

Dojrzałe cząsteczki miRNA i siRNA stają się ważnym składnikiem kompleksów, odpowiednio miRISC i siRISC, pełniąc rolę przewodnika dla białka Ago w szukaniu komplementarnych sekwencji docelowych obecnych w transkryptach. Białka Ago odpowiadają natomiast za rozpoczęcie kaskady sygnałów zmierzających do wyciszenia ekspresji genów na drodze represji translacji, deadenylacji oraz degradacji mRNA. Zjawisko to nosi nazwę potranskrypcyjnego wyciszana ekspresji genów (ang. *post-transcriptional gene silencing*, PTGS) [147-149].

Generalnie, u zwierząt brak jest pełnego zakresu komplementarności pomiędzy cząsteczkami miRNA a sekwencją docelową transkryptu [149]. Po związaniu kompleksu miRISC do miejsca docelowego proces translacji zostaje zahamowany, lecz transkrypt jest nadal stabilny. W kolejnym kroku następuje deadenylacja transkryptu, która przyczynia się do jego destabilizacji [150, 151]. Proces ten rozpoczyna białko Ago rekrutując TNRC6 (ang. *trinucleotide repeat-containing gene 6 protein*), białko z rodziny GW z wielokrotnymi powtórzeniami motywu glicyna-tryptofan, które stanowi platformę dla wiązania kolejnych białek o funkcjach efektorowych [152] (**rysunek 7, A**). Następnie TRNC6 rekrutuje czynnik PABC (ang. *polyadenylate binding protein*) [153], białko wiążące sekwencję poliadenylową na końcu 3' transkryptu, tzw. „ogon poli(A)". Deadenylację ogona poli(A) przeprowadzają kompleksy CCR4-NOT (ang. *carbon catabolite repressor 4 – negative regulator of transcription*) i PARN2-PARN3 (ang. *polyA-specific ribonuclease 2/3*). Nukleaza PARN2 inicjuje proces deadenylacji, a nukleaza CCR4 jest odpowiedzialna za dalsze jego etapy [154]. Skrócenie sekwencji poli(A) uruchamia proces zdejmowania czapeczki 7-metyloguanozynowej na końcu 5' transkryptu przez kompleks DCP1-DCP2 (ang. *decapping protein 1/2*). Helikaza DDX6 (u ludzi, RCK lub p54) koordynuje wyżej wymienione procesy [149, 155]. Ponadto, DDX6 wiąże białko 4E-T - transporter czynnika EIF4E inicjującego translację, uniemożliwiając interakcję czynników EIF4E i EIF4G podczas składania kompleksu inicjującego translację (EIF4F) [156, 157]. Ostatecznie transkrypt jest degradowany od końca 5' przez egzorybonukleazę XRN1 (ang. *5'-3' exoribonuclease 1*) [149, 158].



Rysunek 7 Schemat przedstawiający mechanizmy potranskrypcyjnego wyciszania ekspresji genów w przypadku częściowego (A) lub pełnego (B) zakresu komplementarności między srRNA i mRNA

Zdarza się jednak, że cała cząsteczka srRNA hybrydyzuje do sekwencji docelowej. W przypadku takiego scenariusza transkrypt ulega przecięciu w miejscu związania kompleksu RISC dzięki aktywności endonukleolitycznej Ago2, po czym jest degradowany przez nukleazy komórkowe **(rysunek 7, B)** [147, 159]. Taka sytuacja ma miejsce, na przykład, podczas wykorzystywania narzędzi stosowanych w technologii RNAi, dokładniej zastosowania syntetycznych siRNA lub shRNA (ang. *short hairpin RNA*) w celu wyciszenia ekspresji wybranych genów (ang. *gene knockdown*) [160, 161].

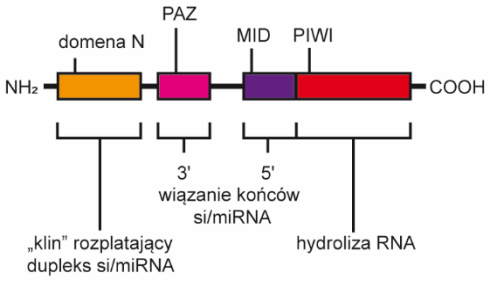
Gdy w potranskrypcyjnym wyciszaniu ekspresji genów udział biorą cząsteczki miRNA, mówi się o tzw. ścieżce miRNA, a gdy siRNA, mowa o tzw. ścieżce siRNA. U ssaków, w przeciwieństwie do *Drosophila*, miRNA i siRNA oddziałują z tymi samymi homologami białek Ago, dlatego ścieżka miRNA i ścieżka siRNA łączą się już na etapie cięcia prekursora srRNA przez Dicer [65]. Co ciekawe, jeśli nukleotydy w pozycji 2-11 miRNA, licząc od końca 5' cząsteczki, hybrydyzują do sekwencji docelowej, wówczas Ago2 może przeprowadzić cięcie nici transkryptu docelowego [162-164]..

1.3.1 Białka Argonaute

Ludzki genom koduje cztery białka Ago (Ago 1-4), które wykazują podobieństwo sekwencji aminokwasowej na poziomie 80% [165]. Ago2 jest jedynym paralogiem wykazującym aktywność endonukleolityczną (ang. *slicer activity*) *in vivo* [166]. Ekspresja paralogów Ago u ssaków jest swoista tkankowo [167], niemniej, wykazano, że w kompleksach RISC najczęściej występuje Ago2 [168, 169]. Ustalono, że w procesie identyfikacji miejsc

docelowych przez aktywny kompleks RISC, kluczową rolę odgrywa parowanie się zasad obecnych w pozycjach 2-4 (ewentualnie 2-5) cząsteczki miRNA (licząc od końca 5' miRNA) z komplementarnymi zasadami występującymi w mRNA [170, 171]. Następnie dochodzi do zmian konformacyjnych w obrębie Ago2, które wspierają dalsze parowane zasad pomiędzy miRNA a transkryptem, co skutkuje powstaniem stabilnego oddziaływania, angażującego nukleotydy 2-8 od końca 5' miRNA; rejon obejmujący 2-8 nt miRNA nazywany jest rejonem *seed* (ang. *seed region*) [172, 173].

Białka Ago posiadają szereg domen umożliwiających specyficzną interakcję z RNA (**rysunek 8**). Domena PAZ wiąże 2-nt niesparowane końce 3' dupleksu [174], natomiast domena MID wiąże grupy fosforanowe na końcach 5' [175]. Domena N, zlokalizowana na końcu aminowym Ago, przyczynia się do inicjacji procesu rozplatania dupleksów siRNA i miRNA [176]. Domena PIWI przypomina budową RNazę H, a jej aktywność nukleolityczna jest zależna od motywu DEDH (Asp-Glu-Asp-His) [166]. Funkcjonalny motyw katalityczny DEDH obecny jest w białkach Ago2 i Ago3 [177, 178], jednakże struktura kanału wiążącego RNA w białku Ago3 ogranicza aktywność nukleazową tego paralogu [179].



Rysunek 8 Schemat przedstawiający domenową budowę ludzkich białek Ago. Poniżej zamieszczono funkcje poszczególnych domen w kontekście oddziaływań z RNA.

1.3.2 Kompleksy RLC i RISC

Proces kształtowania się aktywnego kompleksu RISC można podzielić na dwa etapy: etap formowania się kompleksu „ładującego", RLC (ang. *RISC-loading complex*) oraz etap dojrzewania kompleksu „wyciszającego", RISC. W pierwszym etapie białka Dicer, TRBP i Ago tworzą kompleks RCL, którego zadaniem jest przekazanie dupleksu srRNA wygenerowanego przez Dicer do Ago [30, 180, 181]. Proces ten wspierany jest przez zależne od ATP białka opiekuńcze Hsp70 i Hsp90, które pomagają białku Ago w przyjęciu „otwartej" konformacji [182, 183]. Już na etapie formowania się kompleksu RLC następuje wybór nici efektorowej dupleksu srRNA. Wykazano, że Ago2 wykazuje preferencję wyboru nici, na której końcu 5' znajduje się urydyna lub adenozyna oraz wiązania tego końca dupleksu, który jest mniej stabilny termodynamicznie. Asymetria dupleksu rozpoznawana jest przez domenę MID Ago2 [184]. Niemniej, zbadano, że to Dicer spełnia rolę pierwszego „sensora" asymetrii srRNA [137]. Dicer wiąże substraty i produkty cięcia wzdłuż dwóch różnych osi. Jak wcześniej wspomniano, długie

dsRNA lub pre-miRNA wiązane są wzdłuż domeny PAZ i domen RNazowych, dzięki czemu generowane dupleksy srRNA mają długość ~22 pz. Dupleksy srRNA zostają uwolnione z centrum aktywnego Dicer, a następnie ponownie związane, najprawdopodobniej wzdłuż domeny helikazowej. Taki sposób zakotwiczenia dupleksów srRNA umożliwia ich jednoczesne związanie przez białko partnerujące TRBP lub PACT [137, 185]. Dicer i TRBP wiążą dupleks srRNA w określonej orientacji: TRBP wiąże bardziej stabilny termodynamicznie koniec dupleksu, podczas gdy Dicer koniec mniej stabilny [134, 137, 186]. Dalsze badania skupiające się na sekwencji, strukturze i stabilności termodynamicznej dupleksów srRNA potwierdziły, że wybór nici wiodącej srRNA przez Ago2 ściśle zależy od obecności białek Dicer, TRBP i PACT [138]. Istnieją jednakże doniesienia zaprzeczające wyżej opisanym wynikom badań, między innymi, Betancur i Tomari wykazali, że asymetryczny sposób wiązania srRNA przez Dicer nie wpływa na wybór nici efektorowej przez Ago [187]. Dojrzewanie kompleksu RISC polega na usunięciu nici pasażerskiej srRNA z kompleksu. Podczas dojrzewania kompleksu siRISC zawierającego Ago2, nić pasażerska siRNA jest nacinana przez Ago2 i usuwana z kompleksu [188, 189]. Również Ago1, pomimo braku kompletnej tetrady katalitycznej DEDH w domenie PIWI, posiada aktywność cięcia nici pasażerskiej siRNA *in vitro* [190]. Natomiast w przypadku dojrzewania kompleksu miRISC, wykazano, że białka Ago1 i Ago2 rozplatają nici dupleksu miRNA, a proces ten jest niezależny od ATP [176, 190].

Minimalny kompleks RISC składa się z białka Ago i nici wiodącej (efektorowej) miRNA lub siRNA, jednakże zidentyfikowano wiele białek, które mogą współtworzyć RISC, na przykład MOV10, helikaza RNA A oraz Dicer [191-194]. Yoda i wsp. wykazali, że w układach izolowanych aktywność minimalnego kompleksu siRISC jest znacznie słabsza od aktywności minimalnego kompleks miRISC [183]. Efekt ten można wytłumaczyć mniejszą stabilnością termodynamiczną dupleksów miRNA, w porównaniu do dupleksów siRNA; dupleksy miRNA w warunkach fizjologicznych mogą być częściowo rozplecione, co jest korzystne dla procesu dojrzewania kompleksu RISC [195]. O różnicach dotyczących składu kompleksów miRISC i siRISC pisano we wcześniejszych doniesieniach [30, 180, 196, 197]. Przypuszczalnie, po załadowaniu Ago dupleksem siRNA, pozostałe białka kompleksu RLC, tj. TRBP i Dicer, pozostają związane z siRISC i stymulują jego aktywność [30, 196], podczas gdy kompleks RLC ma tendencję do dysocjowania po przekazaniu dupleksu miRNA do Ago2 [180, 197]. Niemniej, w mysich embrionalnych komórkach macierzystych nie zaobserwowano, by brak Dicer negatywnie wpływał na funkcjonowanie siRISC [198, 199].

Kompleksy RISC występują zarówno w postaci nieaktywnych (niezwiązanych z docelowym mRNA) niskocząsteczkowych kompleksów (ang. *low molecular weight RISC,* LMW-RISC) o masie około ~100 kDa, w których skład wchodzi jedynie białko Ago i nić efektorowa miRNA, jak i wysokocząsteczkowych kompleksów (ang. *high molecular weight RISC,* HMW-RISC) o masie przekraczającej 2 MDa (megadaltony) [191, 200, 201].

W przypadku komórek proliferujących większość kompleksów RISC występuje w postaci wysokocząsteczkowej, natomiast wiele dojrzałych tkanek zawiera niskocząsteczkowe kompleksy RISC [201, 202]. Generalnie, ludzkie komórki cechuje nadmiar miRNA w stosunku do białek Ago [203, 204], aczkolwiek niska ekspresja Ago nie musi stanowić czynnika ograniczającego funkcjonalność cząsteczek miRNA. Prawdopodobnie Ago działa w sposób cykliczny, tj. asystuje w hybrydyzacji miRNA do miejsca docelowego w obrębie mRNA, a następnie oddysocjowuje z kompleksu RISC i natychmiast wiąże kolejny dupleks miRNA [148]. Poza tym, w zależności od typu komórek, częstość występowania cząsteczek miRNA w kompleksach RISC może różnić się znacząco, nawet 100-krotnie. Jednakże, w ramach jednego typu komórek, miRNA o podobnej sekwencji asocjują z Ago w RISC z podobnym powinowactwem [203]. Jednocześnie zauważono, że poziomy Ago i miRNA są od siebie zależne. Ago niezaładowane miRNA ulegają degradacji [205, 206], z kolei, Ago stabilizują związane miRNA [207]. Również proporcja między miRNA a docelowym mRNA jest indywidualną cechą komórek. Przyglądając się miRNA o najwyższym poziomie ekspresji i ich docelowym mRNA, co ma miejsce na przykład w mysich embrionalnych komórkach macierzystych (mESC, ang. *mouse embryonic stem cell*), pula sekwencji docelowych przewyższa liczbowo pulę miRNA. Jednakże w przypadku tych szybko dzielących się komórek całkowita pula mRNA jest 10-krotnie niższa niż na przykład w ludzkich hepatocytach [208]. Niemniej, średni okres półtrwania miRNA w przeciętnej komórce ssaczej to około 5 dni, natomiast średni okres półtrwania mRNA to 10 godzin [209], co oznacza, że, średnio, miRNA są ponad 10-krotnie stabilniejsze od mRNA [210]. W określonych typach komórek, np. w neuronach, rozpad miRNA może następować szybciej, do kilku godzin [211]. Dodatkowo, proporcja między funkcjonalnymi miRNA i docelowymi mRNA może ulegać regulacji poprzez ceRNA (ang. *competing endogenous RNA*), czyli transkrypty wiązane z dużym powinowactwem przez te same miRNA [212].

Liczebność danego miRNA względem miejsc docelowych ma duży wpływ na wydajność inhibicji translacji docelowego mRNA, aczkolwiek sama sekwencja miejsca docelowego ma również duże znaczenie we wspomnianym procesie [213]. Im większy stopień komplementarności między miRNA a sekwencją docelową, tym silniejszy efekt represji genu [213]. Kolejnym ważnym aspektem dotyczącym wydajności inhibicji translacji docelowego mRNA jest dostępność sekwencji docelowych (miejsca wiązania miRNA) dla kompleksu RISC. Badania Ameres i wsp. wykazały, że jeśli sekwencje docelowe dla miRNA zlokalizowane są w obrębie stabilnych struktur dwuniciowych, wówczas kompleks RISC ma ograniczony dostęp do takich miejsc [214]. Wiadomo również, że liczne białka wiążące mRNA mogą współpracować, bądź współzawodniczyć z kompleksem RISC o wiązanie do miejsca docelowego dla określonego miRNA. Przykładem białka współpracującego z RISC jest Pumilio-1, które wiąże zakonserwowane ewolucyjnie motywy PRE (ang. *Pumilio recognition element*), występujące, na przykład, w pobliżu miejsc docelowych dla miR-221 i miR-333,

w obrębie 3' UTR transkryptu białka p72. Po związaniu Pumilio-1, dochodzi do lokalnych zmian konformacyjnych w obrębie transkryptu, dzięki czemu miR-221 i miR-222 mogą związać się do swoich miejsc docelowych, indukując tym samym inhibicję translacji p27 [215]. Innym białkiem wiążącym mRNA jest HuR (ang. *Hu-Antigen R*). HuR wiążąc sekwencje bogate w AU (ARE, ang. *AU-rich elements*) [216], blokuje dostęp miR-122 do 3' UTR mRNA białka CAT-1 (ang. *cationic amino acid transporter 1*) [217].

Ago, mRNA i miRNA występują w cytoplazmie komórek, między innymi, w granulach stresu (ang. *stress granules, SG*), ciałkach degradujących (ang. *processing bodies, P-bodies, PB*), czy polisomach, w obrębie siateczki śródplazmatycznej i cytoszkieletu (podsumowane w [17]). Polisomy są miejscem inicjacji procesu PTGS, który kończy się w endosomach i ciałkach wielopęcherzykowatych (ang. *multivesicular bodies, MVB*) degradacją mRNA [218, 219]. Z kolei, SG stanowią przedziały, gdzie mRNA, małe podjednostki rybosomu oraz białka inicjacji translacji agregują w postaci kompleksów preinicjacyjnych, w odpowiedzi na stres komórkowy [220]. Wykazano, że w SG powstają nieaktywne, niskocząsteczkowe kompleksy RISC [221]. Z kolei PB, tj. przedziały komórkowe pozbawione rybosomów lecz zawierające białka zaangażowane w degradację mRNA [222, 223], uważa się za miejsca występowania aktywnych kompleksów miRISC [224, 225]. Przypuszcza się, że SG i PB mogą współpracować i wymieniać się zawartością [220, 226].

2 Cel pracy

W momencie podjęcia problemu badawczego nieznana była rola domeny DUF283 rybonukleaz typu Dicer w procesie rozpoznawania, wiązania lub cięcia substratów RNA. Z uwagi na zgłębianą problematykę badawczą dotyczącą rybonukleazy Dicer człowieka, celem badań prowadzonych w ramach pracy doktorskiej było ustalenie, czy domena DUF283 ludzkiej rybonukleazy Dicer może wiązać kwasy nukleinowe.

Osiągnięcie tego celu wymagało realizacji następujących zadań:

i. dokonania przeglądu danych literaturowych dotyczących rybonukleaz Dicer, w szczególności danych odnoszących się do domeny DUF283 Dicer;
ii. stworzenia konstruktu genetycznego kodującego DUF283 Dicer człowieka;
iii. otrzymania preparatu DUF283 Dicer człowieka;
iv. charakterystyki biochemicznej otrzymanego preparatu DUF283.

Wyniki przeprowadzonych badań ujawniły, iż DUF283 pochodząca z rybonukleazy Dicer człowieka wiąże jednoniciowe kwasy nukleinowe, a także, co ciekawe, wspiera proces parowania RNA oraz DNA. Zgromadzone dane zrodziły kolejne pytania i cele badawcze zmierzające do ustalania, czy rybonukleaza Dicer człowieka również wspiera parowanie cząsteczek kwasów nukleinowych, a w przypadku uzyskania pozytywnej odpowiedzi, w jakim stopniu struktura RNA wpływa na badany proces oraz jakie jest potencjalne znaczenie nowo odkrytej aktywności dla funkcjonowania komórek.

Osiągnięcie tych celów wymagało realizacji kolejnych zadań badawczych:

v. otrzymania preparatu ludzkiej Dicer typu dzikiego;
vi. zbadania potencjału ludzkiej Dicer do wspierania parowania cząsteczek kwasów nukleinowych;
vii. zbadania wpływu struktury RNA na aktywność wspierającą parowanie RNA ludzkiej Dicer;
viii. analizy danych pochodzących z głębokiego sekwencjonowania puli RNA wiązanej *in cellulo* przez ludzką Dicer;
ix. zaproponowania, w oparciu o uzyskane wyniki, komórkowej roli i schematu funkcjonowania Dicer w odniesieniu do nowo odkrytej aktywności.

3 Streszczenie prac

3.1 Kurzynska-Kokorniak i wsp., The many faces of Dicer: the complexity of the mechanisms regulating Dicer gene expression and enzyme activities, Nucleic Acids Res. 2015

Ze względu na szybko rosnącą liczbę badań i publikacji poświęconych rybonukleazom typu Dicer, stan wiedzy w tym zakresie wymaga ciągłej systematyzacji. Praca przeglądowa, której jestem współautorką [1], opisuje zarówno rolę rybonukleaz Dicer w procesie biogenezy srRNA, jak i nowo poznane aktywności Dicer, niepowiązane ze ścieżkami miRNA oraz siRNA. Zagadnienia przedstawione w pracy dotyczą w większości ludzkiej Dicer. W artykule przedstawione zostały różne mechanizmy regulacji ekspresji genu kodującego białko Dicer u człowieka (*DICER1*). Szczególna uwaga została poświęcona analizie regulacji aktywności białka Dicer przez różnorodne czynniki, w tym białka i RNA. W pracy omawiana jest również problematyka Dicer jako onkogenu i supresora procesu nowotworzenia.

W niniejszym rozdziale, ze względu na problem badawczy ujęty w rozprawie doktorskiej, krótko podsumowane zostaną informacje dotyczące oddziaływań Dicer z różnego typu cząsteczkami RNA. Jak wspomniano wcześniej (rozdziały 1.1.1 i 1.1.2), kanoniczną funkcją Dicer jest produkcja dupleksów miRNA i siRNA. Dicer, poza rozpoznawaniem i cięciem prekursorów miRNA i siRNA, oddziałuje również z produktami przeprowadzanej hydrolizy. Grupa badawcza profesor J. Doudny wykazała w badaniach *in vitro*, że dupleks siRNA, generowany przez Dicer, jest uwalniany i ponownie wiązany przez tę rybonukleazę. W przeciwieństwie do substratów siRNA, wiązanie produktu, tj. siRNA, nie angażuje domen RNazowych Dicer, lecz domenę helikazową. Jak wspomniano wcześniej, domena helikazowa Dicer wiąże mniej stabilny koniec dupleksu siRNA, podczas gdy białko partnerujące TRBP, współtworzące z Dicer kompleks RLC, wiąże bardziej stabilny koniec dupleksu. Kooperacja tych dwóch białek determinuje sposób wiązania dupleksu srRNA przez Ago w kompleksie RISC, co w konsekwencji przekłada się na wybór nici efektorowej biorącej czynny udział w potranskrypcyjnym wyciszaniu ekspresji genów [137].

Jądrowa Dicer, poprzez produkcję siRNA, przyczynia się do obniżenia poziomu akumulacji toksycznych dsRNA. Wykazano, że w komórkach HEK293 Dicer wiąże się do chromatyny, między innymi w miejscach rozpoczęcia transkrypcji (ang. *transcription start sites*, TSS). Jest to możliwe dzięki oddziaływaniu tej rybonukleazy z polimerazą RNA II i długim dsRNA, który powstaje w wyniku transkrypcji dwukierunkowej. Długi dsRNA jest cięty („neutralizowany") przez Dicer, co w konsekwencji przeciwdziała apoptozie związanej z pojawieniem się odpowiedzi interferonowej aktywowanej dsRNA. Wykazano również, że wiązane przez Dicer *loci* są poddawane procesowi transkrypcyjnego wyciszania genów

(ang. *transcriptional gene silencing*, TGS), gdzie w sposób zależny od kompleksów Ago1:siRNA formowana jest nieaktywna transkrypcyjnie forma chromatyny - heterochromatyna [89]. Dicer oddziałuje również z transkryptami, których syntezę inicjują dwuniciowe pęknięcia w obrębie chromosomalnego DNA, o czym pisano w rozdziale 1.1.3. Transkrypty te są cięte przez Dicer do diRNA, których rolą jest rekrutacja białek biorących udział w naprawie uszkodzeń DNA. Ze względu na długość diRNA można przypuszczać, że sposób, w jaki odbywa się produkcja diRNA przez Dicer, jest podobny do produkcji miRNA i siRNA (**rysunek 4**).

Dicer potrafi wiązać transkrypty także w tzw. sposób „pasywny" (ang. *„passive binding"*); dokładniej, enzym rozpoznaje lokalne struktury drugorzędowe typu spinki obecne w transkryptach, wiąże je, lecz ich nie przecina. Takie miejsca wiązania Dicer nazwano „miejscami pasywnymi" (ang. *„passive sites"*), w odróżnieniu od „miejsc aktywnych" (ang. *„active sites"*), które ulegają cięciu przez Dicer, a produkty cięcia są włączane do kompleksów RISC. Przypuszczalnie, za brak cięcia „miejsc pasywnych" odpowiada brak możliwości dokowania transkryptu w obrębie kieszeni 5' i 3' Dicer. Dane pochodzące z eksperymentów PAR-CLIP (ang. *photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation*) wykazały, że sekwencje „miejsc pasywnych" często odpowiadają sekwencjom pętli spinek zidentyfikowanych w drugorzędowej strukturze transkryptów [227]. Zważywszy na fakt, że za wiązanie pętli apikalnej pre-miRNA odpowiada domena helikazowa Dicer [109, 139], można przypuszczać, że w wiązaniu „miejsc pasywnych" udział bierze również domena helikazowa. Wykazano, że w ludzkich komórkach HEK293 (ang. *human embryonic kidney 293*) „pasywne" wiązanie transkryptów przez Dicer powodowało ich stabilizację [227].

Co ciekawe, w sposób „pasywny" mogą być także wiązane krótsze cząsteczki RNA [228]. Zaobserwowano, iż niektóre ~60 nt RNA wiązane przez Dicer mogą inhibować proces cięcia pre-miRNA. Spośród oligomerów RNA wiązanych przez Dicer autorzy wyszczególnili takie, które były przez Dicer cięte (inhibitory kompetycyjne) oraz takie, które nie ulegały cięciu (inhibitory allosteryczne) [228]. Wspomniane badania stanowią kolejny przykład wiązania cząsteczek RNA poza doliną katalityczną Dicer. Zebrane wiadomości zostały podsumowane w **Tabeli 1**.

Tabela 1 Funkcje Dicer powiązane z jej oddziaływaniami z RNA

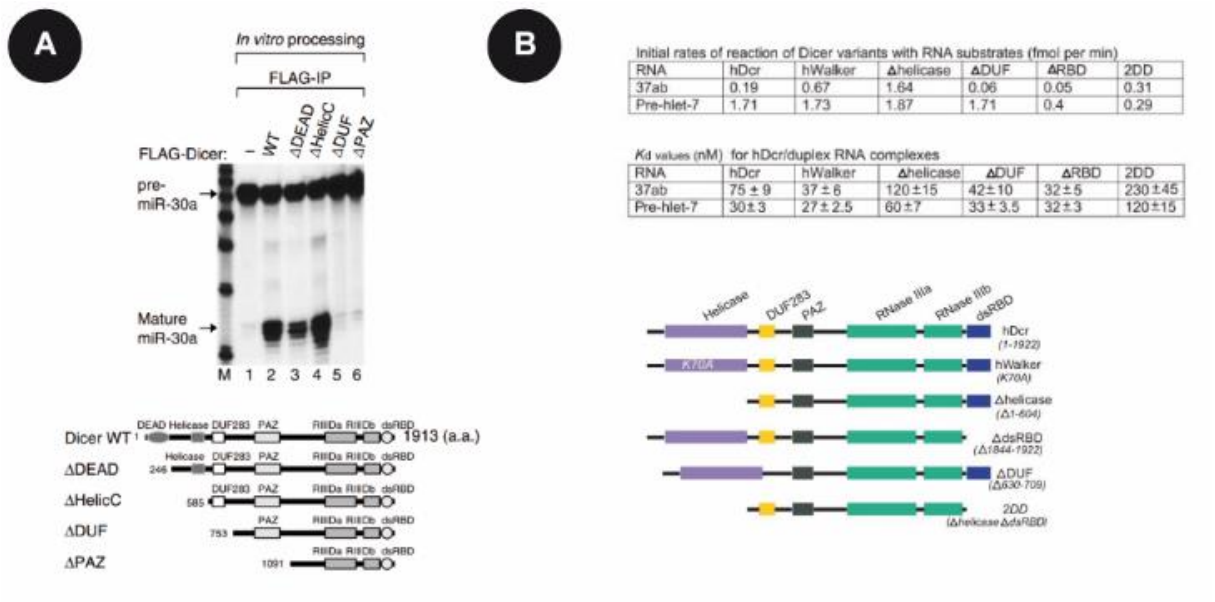
Funkcja Dicer	Dane na temat oddziaływań Dicer z RNA	Aktywność RNazowa Dicer	Literatura
Produkcja miRNA i siRNA w procesie biogenezy srRNA	Prekursor miRNA lub siRNA jest wiązany przez domenę PAZ i wzdłuż domen RNazowych, co umożliwia hydrolizę substratu. Domena helikazowa oddziałuje z pętlą apikalną prekursora miRNA.	Tak	[129, 139]
Transfer dupleksu miRNA lub siRNA w ramach RLC	Dupleks miRNA lub siRNA generowany przez Dicer jest uwalniany z domen RNazowych, a następnie wiązany w obrębie domeny helikazowej.	Nie	[137]

Funkcja Dicer	Dane na temat oddziaływań Dicer z RNA	Aktywność RNazowa Dicer	Literatura
Degradacja toksycznych dla komórki transkryptów, zapobieganie apoptozie	Produkty transkrypcji dwukierunkowej hybrydyzują ze sobą, a następnie są wiązane w formie dsRNA i cięte przez Dicer.	Tak	[89]
Produkcja diRNA	Prawdopodobnie: wiązanie transkryptów generowanych w miejscu uszkodzenia DNA i cięcie ich do diRNA. Prekursory diRNA przyjmują strukturę spinki lub hybrydyzują do komplementarnych mRNA, formując długie dsRNA.	Tak	[10-12]
Stabilizacja transkryptów	Wiązanie transkryptów w rejonie tzw. „miejsc pasywnych" przyjmujących strukturę spinki; w wiązaniu prawdopodobnie bierze udział domena helikazowa Dicer.	Nie	[227]

W pracy przeglądowej omówiono również rolę poszczególnych domen ludzkiej Dicer w rozpoznawaniu, wiązaniu lub cięciu RNA (podsumowane w rozdziale 1.2). Jedyną domeną, która nie została scharakteryzowana pod kątem oddziaływań z RNA była domena DUF283. Wiadomo było jedynie, że DUF283 bierze udział w wiązaniu określonych partnerów białkowych Dicer w komórkach roślinnych [145] i ludzkich [146], dlatego domena ta często nazywana jest domeną dimeryzacji.

3.2 Kurzynska-Kokorniak i wsp., Revealing a new activity of the human Dicer DUF283 domain in vitro, Sci Rep. 2016

W momencie rozpoczęcia badań, w 2012 roku, znaleźliśmy zaledwie cztery publikacje poświęcone domenie DUF283 [32, 69, 144, 145]. W 2006 roku zespół profesor V. Narry Kim opublikował wyniki badań przeprowadzonych z udziałem wariantów delecyjnych Dicer. Z badań tych wynikało, że brak domen helikazowej oraz DUF283 w ludzkiej Dicer powoduje całkowite zahamowanie cięcia pre-miRNA, natomiast delecja samej domeny helikazowej nie prowadzi do obniżenia wydajności cięcia pre-miRNA względem białka typu dzikiego (**rysunek 9, A**) [32]. W badaniach zespołu profesor J. Doudny, opublikowanych w 2008 roku, użyto wariantów delecyjnych ludzkiej Dicer do zbadania roli poszczególnych domen w wiązaniu i cięciu prekursorów siRNA i miRNA [69]. Brak domeny DUF283 spowodował, że enzym silniej wiązał prekursor siRNA, ale ciął go z niższą wydajnością. Jednocześnie, ten sam wariant delecyjny wiązał i ciął pre-miRNA z podobną wydajnością co Dicer typu dzikiego (**rysunek 9, B**) [69].



Rysunek 9 Wyniki eksperymentów cięcia i wiązania prekursorów miRNA oraz siRNA przez warianty delecyjne ludzkiej Dicer. Zdjęcia pochodzą z publikacji (A) Lee i wsp., 2006 [32] oraz (B) Ma i wsp. ,2008 [69]. (A) Górny panel: Rekombinowane białka Dicer izolowane były z komórek HEK293T metodą immunoprecypitacji, następnie przeprowadzano inkubację tak izolowanej Dicer z wyznakowanym radioizotopowo na końcu 5' pre-miR-30a. Produkty reakcji rozdzielano w żelu poliakrylamidowym w warunkach denaturujących. Dolny panel: schemat budowy wariantów delecyjnych ludzkiej Dicer, wykorzystanych w badaniach Lee i wsp. (B) Rekombinowane ludzkie białka Dicer produkowane były w systemie bakulowirusowym, a następnie oczyszczane z wykorzystaniem chromatografii powinowactwa i chromatografii żelowej. Górny panel: tabela przedstawiająca wartości prędkości początkowych reakcji cięcia prekursora siRNA (37ab) i miRNA (pre-hlet-7) dla rekombinowanych białek Dicer oraz, poniżej, tabela przedstawiająca wartości stałych wiązania dla kompleksów RNA:Dicer (zgodnie z opisem). Eksperymenty wiązania RNA przeprowadzane były metodą wiązania kompleksów białko:RNA do membrany nitrocelulozowej (ang. *dot blot*). Dolny panel: schemat budowy wariantów delecyjnych ludzkiej Dicer, wykorzystanych w badaniach Ma i wsp.

Kolejne dwie publikacje traktowały o strukturze DUF283 ludzkiej Dicer i roślinnych białek typu Dicer oraz o związanej z tą strukturą przypuszczalnej funkcji domeny DUF283. W pracy Qin i wsp. z 2010 roku opublikowano strukturę domeny DUF283 DCL-4 *Arabidopsis thaliana*, zbadaną za pomocą spektroskopii magnetycznego rezonansu jądrowego (ang. *nuclear magnetic resonanse*, *NMR*). Okazało się, że DUF283 posiada motyw strukturalny α-β-β-β-α charakterystyczny dla domen wiążących dsRNA, aczkolwiek badania biochemiczne nie potwierdziły, by DUF283 DCL-4 *A. thaliana* mogła oddziaływać z dsRNA [145]. Podobny motyw wiążący dsRNA przewidziano metodami bioinformatycznymi dla domeny DUF283 ludzkiej Dicer [144], jednakże żadnej grupie badawczej nie udało się uzyskać preparat domeny DUF283 ludzkiej Dicer [2]. Biorąc pod uwagę ówczesny stan wiedzy dotyczący rybonukleaz typu Dicer, a także zgłębianą problematykę badawczą podjęłam się próby otrzymania preparatu białkowego domeny DUF283 ludzkiej Dicer.

Domenę DUF283 ludzkiej rybonukleazy Dicer otrzymano w bakteryjnym systemie produkcji białek, w postaci białka fuzyjnego His6-DUF283 zawierającego znacznik

histydynowy (His6) na końcu aminowym oraz fragment odpowiadający domenie DUF283 ludzkiej Dicer (aminokwasy 625-752, NM_030621). Konstrukt genetyczny kodujący His6-DUF283 przygotowany został w oparciu o dane literaturowe opisujące warianty delecyjne ludzkiej Dicer [32, 69], jak również dane dotyczące przewidzianej struktury drugorzędowej domeny DUF283 ludzkiej Dicer [144].

Produkcja białka His6-DUF283 odbywała się w komórkach *E. coli* BL21 Star (Thermo Fisher Scientific). Białko oczyszczane było dwuetapowo. Pierwszym z etapów była chromatografia powinowactwa wykorzystująca powinowactwo znacznika histydynowego do złoża zawierającego kationy niklu (Ni^{2+}). Po pierwszym etapie oczyszczania preparat cechowała 80% homogenność. W drugim etapie zastosowano chromatografię jonowymienną. Przewidywany punkt izoelektryczny (pI) His6-DUF283, w zależności od użytego programu, wynosi od 6,2 do 7,0. Do oczyszczania białka użyto złoża anionitowego typu Q (ang. *quaternary ammonium*). Złoże to stanowi żywica, na powierzchni której znajduje się czwartorzędowa amina, która silnie wiąże ujemnie naładowane reszty aminokwasowe białka. Zgodnie z zasadą oczyszczania białek na złożach anionitowych, wartość pH buforu użytego do oczyszczania białka była wyższa o 1 jednostkę pH od wartości pI białka i wynosiła 8,0 (skład buforu: 0,05 M Tris (pH 8,0), 0,1 M NaCl, 0,1% Triton X-100). W celu elucji białek ze złoża zastosowano gradient NaCl w przedziale 0,1-1,0 M. Białko His6-DUF283 ulegało elucji w buforze o stężeniu NaCl wynoszącym 0,25 M. Uzyskany w ten sposób preparat charakteryzował się wysoką homogennością (>95%) (rysunek: Supplementary Fig. S1 [2]). W toku prowadzonych badań zauważono, iż białko 6His-DUF283 wykazywało tendencję do agregacji i wytrącania z buforu o niskim stężeniu NaCl, dlatego stężenie NaCl w buforze podczas przechowywania preparatu nie było niższe niż 0,3 M. W celu ograniczenia agregacji, zastosowano również 5% glicerol [229] i 0,1% niejonowy detergent Triton X-100 [230].

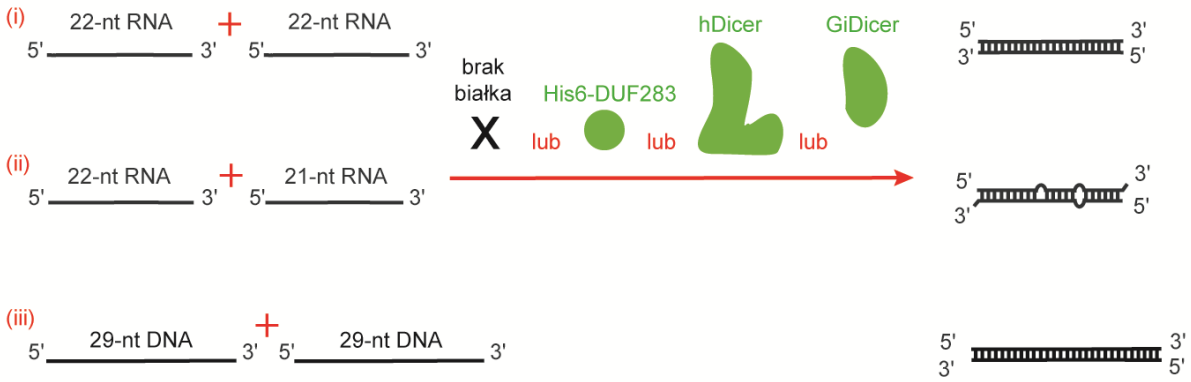
Jak wspomniano wcześniej, domena DUF283 ludzkiej Dicer, podobnie jak DUF283 DCL-4 *A. thaliana*, posiada motyw dsRBD [144, 145]. Zadano zatem pytanie, czy domena DUF283 ludzkiej Dicer może wiązać dwuniciowy RNA. W celu zbadania oddziaływań pomiędzy His6-DUF283 a kwasami nukleinowymi posłużono się metodą EMSA (ang. *electrophoretic mobility shift assay*), metodą różnicowej migracji w żelu poliakrylamidowym. Wyniki przeprowadzonych badań wykazały, że domena DUF283 ludzkiej Dicer, podobnie jak domena DUF283 DCL-4 *A. thaliana* [145], nie wiąże dsRNA (rysunek: Fig. 1a [2]). Postanowiono zatem zbadać, czy DUF283 ludzkiej Dicer może wiązać inne formy RNA, np. jednoniciowy RNA (ang. *single-stranded RNA*, ssRNA). W badaniach wykorzystano ssRNA o długości: 12, 22, 32, 52, 62 nt. Wykazano, że ludzka DUF283 wiązała wszystkie testowane ssRNA (rysunek: Fig. 2a-e [2]). W przypadku cząsteczek o długości 32, 52 i 62 nt nie obserwowano pojedynczych kompleksów DUF283 i ssRNA, tylko ich całe spektrum. Ze względu na pozostawanie kompleksów DUF283 i dłuższych ssRNA w kieszonkach żelu

oraz niską wydajność wiązania 12 nt RNA przez DUF283, stałą wiązania (K_d) wyznaczono jedynie dla DUF283 i 22 nt RNA. Wyniosła ona 9,5 ± 0,5 μM. Jest to podobna wartość do wartości K_d otrzymanej dla kompleksu domeny dsRBD Dicer z dsRNA o długości 20 pz (~6,5 μM [115], >8 μM [231]). Dalsze badania ujawniły, że DUF283 wiąże również krótkie cząsteczki ssDNA (rysunek: Fig. 2g [2]).

Reakcje kontrolne prowadzone były z wykorzystaniem albuminy wołowej (BSA, ang. *bovine serum albumin*) – białka niewiążącego kwasów nukleinowych, a także preparatu białkowego His6, otrzymanego w wyniku produkcji i oczyszczania peptydu powstającego na bazie plazmidu użytego do produkcji His6-DUF283, lecz pozbawionego cDNA DUF283. Ponadto, w przypadku kolejnej reakcji kontrolnej, po zakończeniu inkubacji RNA z białkiem do mieszaniny reakcyjnej podawano siarczan dodecylu sodu (SDS, ang. *sodium dodecyl sulfate*), czynnik denaturujący białka. W trakcie rozdziału mieszaniny reakcyjnej w natywnym żelu poliakrylamidowym, zdenaturowane białko nie pozostawało związane z kwasem nukleinowym. Co ciekawe, w przypadku reakcji kontrolnej, do której podany został SDS, zaobserwowano produkt nieobecny w innych reakcjach, w tym w innych reakcjach kontrolnych. Produkt ten migrował w żelu wolniej niż niezwiązany RNA i szybciej niż kompleks RNA:DUF283 (rysunek: Fig. 1d [2]). Wnikliwa analiza wykazała, iż użyta w badaniach cząsteczka R22 posiada sekwencję samo-komplementarną i może tworzyć homodimer (rysunek: Fig. 1d [2]). R22 nie tworzyła homodimeru w reakcjach prowadzonych bez udziału DUF283 (czyli w kontrolach negatywnych, K-), stąd wysnuto hipotezę, że dimeryzacja R22 może być wspierana przez białko DUF283. Uzyskane wyniki sugerowały, iż DUF283 może pełnić funkcję charakterystyczną dla białek opiekuńczych, polegającą na wspieraniu parowania komplementarnych sekwencji występujących w kwasach nukleinowych (ang. *nucleic acid annealing activity)* [232-234]. W dalszej części pracy niniejsza aktywność będzie nazywana: aktywnością wspierającą parowanie RNA lub DNA.

W celu potwierdzenia przyjętej hipotezy zbadano zdolność DUF283 do wspierania hybrydyzacji komplementarnych cząsteczek kwasów nukleinowych wykorzystując trzy pary oligomerów mających potencjał do tworzenia: (i) w pełni komplementarnego dupleksu RNA, (ii) dupleksu miRNA-21 z jednym niesparowaniem, jedną pętlą wewnętrzną i dwoma 1-nt niesparowanymi końcami 3' oraz (iii) w pełni komplementarnego dupleksu DNA (**rysunek 10**). W badaniach wykorzystano także ludzką Dicer typu dzikiego, która została wyprodukowana w bakulowirusowym systemie ekspresji genów (nazywaną w dalszej części pracy hDicer), we współpracy z zespołem prof. Krystyny Bieńkowskiej-Szewczyk (Zakład Biologii Molekularnej Wirusów, Międzyuczelniany Wydział Biotechnologii Uniwersytetu Gdańskiego i Gdańskiego Uniwersytetu Medycznego). Reakcje kontrolne zostały przeprowadzone z wykorzystaniem komercyjnego preparatu GiDicer - Dicer *Giardia intestinalis*; ta tzw. „minimalna Dicer" nie posiada domeny DUF283 (**rysunek 2, B**). Zastosowane stężenia

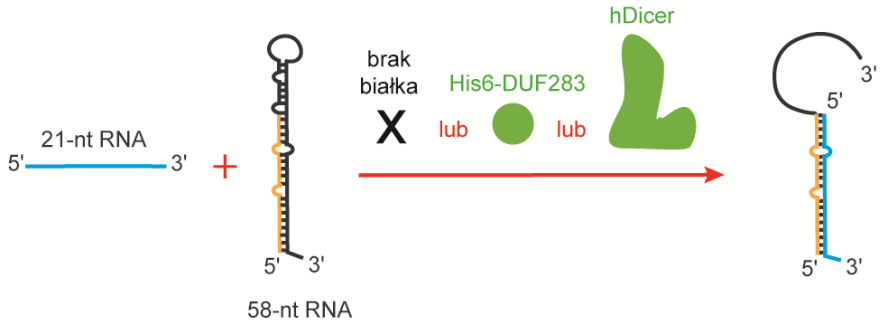
komplementarnych cząsteczek kwasu nukleinowego były na tyle niskie, że w kontrolach negatywnych, bez białka, nie dochodziło do ich hybrydyzacji. Jednocześnie, stężenia cząsteczek komplementarnych były wystarczające do utworzenia dupleksów w przypadku obecności czynnika wspierającego hybrydyzację kwasów nukleinowych, np. białek posiadających aktywność wspierającą parowanie RNA lub DNA (ang. *RNA/DNA annealers*). We wszystkich trzech układach zarówno His6-DUF283, jak i hDicer wspierały parowanie komplementarnych oligomerów, a wydajność tworzenia dupleksów wzrastała wraz ze stężeniem białka oraz czasem inkubacji (rysunki: Fig. 3-6 [2]). Zaobserwowano, że hDicer w mniejszym stopniu wpierała parowanie komplementarnych oligomerów, w porównaniu do His6-DUF283, co może być wytłumaczone obecnością innych domen wiążących kwasy nukleinowe w ludzkiej Dicer, które potencjalnie mogą konkurować z domeną DUF283 o wiązanie ssRNA lub ssDNA. GiDicer, w analogicznych warunkach reakcyjnych, nie wspierała parowania krótkich RNA lub DNA; wydajność powstawania dupleksów w reakcjach z GiDicer była porównywalna z wydajnością powstawania dupleksów w kontrolach negatywnych, bez udziału białka (rysunek: Fig. 7 [2]). Jak wspomniano wcześniej, GiDicer nie posiada domeny DUF283. Na podstawie wyników pochodzących z przeprowadzonych reakcji wywnioskowano, że domena DUF283 może mieć kluczowe znaczenie dla aktywności wspierającej parowanie RNA lub DNA, prezentowanej przez hDicer.



Rysunek 10 Schematyczne przedstawienie reakcji wspierania parowania komplementarnych RNA i DNA. Reakcje były prowadzone bez białka (kontrola negatywna) lub z dodatkiem His6-DUF283, hDicer lub GiDicer. Produktem reakcji był, w zależności od użytych oligomerów, (i) perfekcyjny dupleks RNA o długości 22 pz, (ii) dupleks miRNA-21 lub (iii) perfekcyjny dupleks DNA o długości 29 pz.

Następnie zadano pytanie, czy DUF283 i ludzka Dicer mogą wspierać parowanie dwóch cząsteczek RNA, jeśli jedna z cząsteczek przyjmuje stabilną strukturę drugorzędową, maskując w ten sposób sekwencję komplementarną wobec drugiej cząsteczki RNA. W reakcjach wykorzystaliśmy dwie cząsteczki RNA, krótszą (21-nt) i dłuższą (58-nt). 58-nt cząsteczka RNA tworzyła strukturę drugorzędową typu spinki, a sekwencja komplementarna wobec 21-nt RNA była obecna w trzonie tworzonej spinki, zgodnie ze schematem przedstawionym na **rysunku 11**. Przeprowadzone badania wykazały, że zarówno His6-DUF283 jak i hDicer wspierały parowanie komplementarnych RNA, natomiast

w kontrolach negatywnych nie zaobserwowano spontanicznego tworzenia dupleksów (rysunek: Fig. 7 [2]).



Rysunek 11 Schematyczne przedstawienie reakcji wspierania parowania cząsteczek RNA, w której wykorzystano 21-nt RNA (zaznaczony kolorem niebieskim) i 58-nt RNA przyjmujący strukturę spinki (sekwencję komplementarną wobec 21-nt RNA zaznaczono kolorem żółtym). Reakcję prowadzono bez białka (kontrola negatywna) lub z dodatkiem His6-DUF283, lub hDicer.

Aktywność wpierająca parowanie RNA lub DNA jest charakterystyczna zarówno dla białek typu ang. *nucleic acid annealers*, jak i białek opiekuńczych, tzw. chaperonów kwasów nukleinowych (ang. *nucleic acid chaperones*) [232-234]. Wiadomo również, że chaperony kwasów nukleinowych potrafią wspierać, w sposób niezależny od ATP, rozplatanie struktur dwuniciowych w RNA czy DNA (ang. *unwinding activity*) oraz wymianę jednej z nici w dupleksie (ang. *strand displacement activity*) [232-234]. Jednakże eksperymenty prowadzone z użyciem His6-DUF283 lub hDicer oraz dupleksów RNA nie wykazały aktywności rozplatania struktur dwuniciowych przez wspomniane białka, w zastosowanych warunkach reakcyjnych (rysunek: Supplementary Fig. S5 [2]). Wyniki przeprowadzonych przez nas badań sugerowały, że His6-DUF283 i hDicer posiadają tylko niektóre cechy typowe dla chaperonów kwasów nukleinowych. Przyjęliśmy zatem, że DUF283 i ludzka Dicer należą do grupy białek typu *ang. nucleic acid annealers*, czyli białek posiadających aktywność wspierającą parowanie RNA lub DNA.

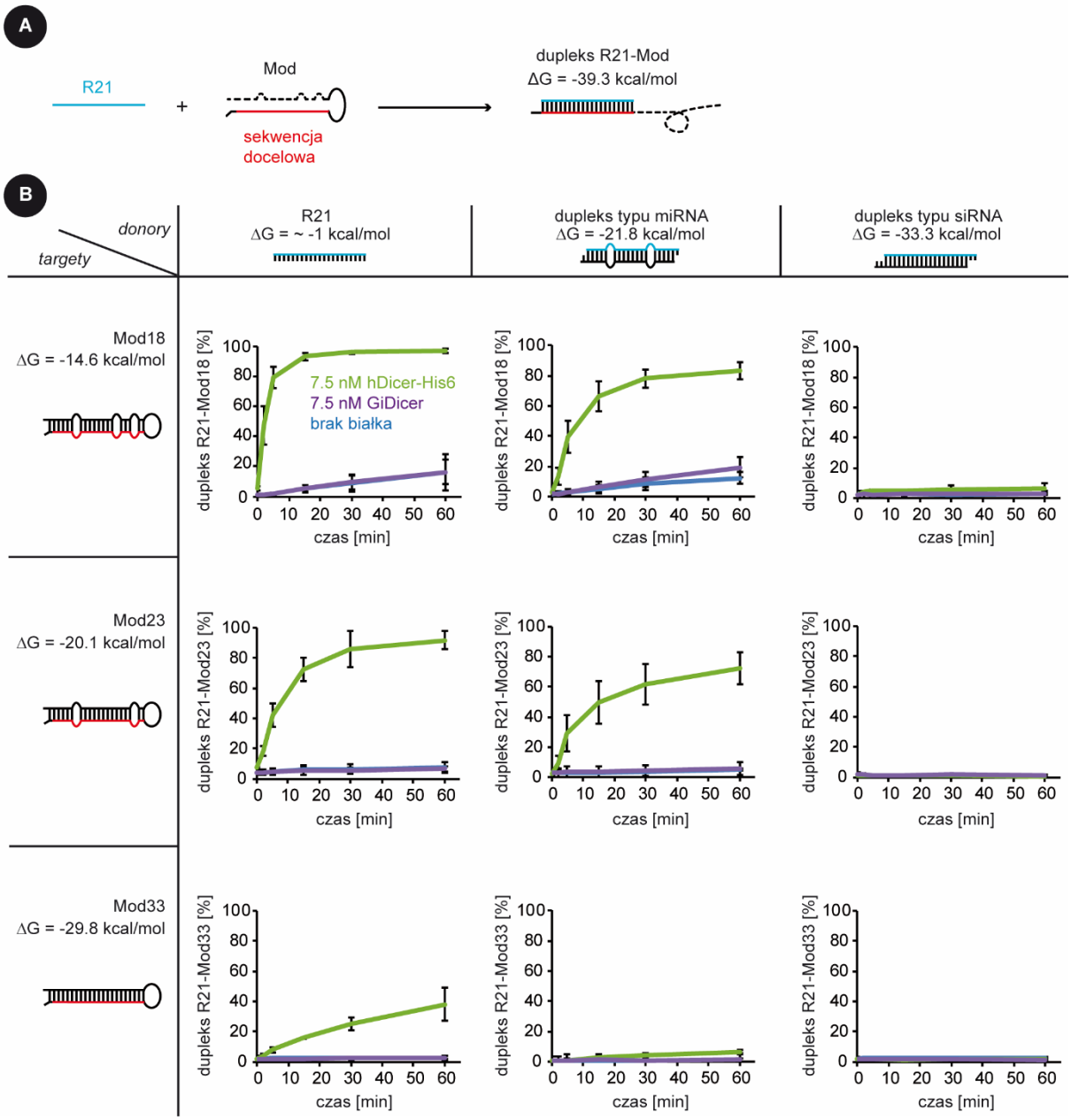
3.3 Pokornowska i wsp., The RNA-RNA base pairing potential of human Dicer and Ago2 proteins, Cell Mol Life Sci. 2019

W niniejszej pracy podjęto próbę odpowiedzi na pytanie: w jakim stopniu struktura RNA wpływa na wspierany przez ludzką Dicer proces parowania RNA, a następnie rozważano potencjalne znaczenie biologiczne omawianego procesu.

W badaniach wykorzystano ludzką rybonukleazę Dicer wyprodukowaną w bakulowirusowym systemie ekspresji genów (Bac-to-Bac, ThermoFisher Scientific). Wektor wejściowy (ang. *entry vector*) do produkcji białka, pDEST8-Dicer-HisC, został uzyskany dzięki uprzejmości profesora Witolda Filipowicza (Friedrich Miescher Institute for Biomedical Research) [26]. Wektor ten zawierał sekwencję kodującą ludzką Dicer (locus AB028449, GenBank) oraz sekwencję znacznika histydynowego (His6) dodaną do końca 3' cDNA Dicer

(w dalszej części pracy wyprodukowane białko będzie nazywane „hDicer-His6"). Wektorem transformowano bakterie *E.coli* DH10Bac (ThermoFisher Scientific), w których zachodziła transpozycja sekwencji kodującej hDicer-His6 do bakmidu. Kolonie bakteryjne zawierające rekombinowany bakmid identyfikowano za pomocą testu α-komplementacji (ang. *blue/white screening*). Z odpowiednich kolonii (kolonie białe) izolowano bakmidowy DNA i sekwencjonowano go metodą Sangera w rejonie kodującym sekwencję białka Dicer. Następnie, bakmidem zawierającym sekwencję Dicer zgodną z sekwencją referencyjną AB028449 transfekowano owadzie komórki Sf21, a po pięciu dniach hodowli zbierano nadsącz zawierający cząstki wirusa. Wirusa namnażano w komórkach Sf21 przez kolejne siedem dni, a następnie podawano go do hodowli zawiesinowej owadzich komórek HF (High Five) i inkubowano z wytrząsaniem przez 93 godziny. Uzyskiwane rekombinowane białko hDicer-His6 izolowano z komórek i oczyszczano z użyciem chromatografii powinowactwa na złożu niklowym. Oczyszczone białko cechowała wysoka homogenność (rysunek: Supplementary Fig. S1 [3]). Następnie, testowano aktywności tak otrzymanego preparatu białkowego: aktywność cięcia pre-miRNA, wiązania RNA i aktywność wspierającą parowanie RNA. Aktywność hDicer-His6 była porównywalna z aktywnością preparatu hDicer użytego w naszych wcześniejszych badaniach [2]. W badaniach, oprócz białka hDicer-His6, użyto także preparatu komercyjnego GiDicer. Jak pokazano w naszej poprzedniej pracy, GiDicer nie posiada aktywności wspierającej parowanie komplementarnych RNA (rozdział 3.2, [2]), dlatego białko to wykorzystane zostało w eksperymentach kontrolnych.

W celu zbadania wpływu struktury RNA na wydajność wspieranego przez ludzką Dicer procesu parowania RNA, wykorzystano układy (pary) złożone z tzw. *donorów* oraz *targetów*. Wszystkie *donory* zawierały 21-nt RNA (R21). Sekwencja komplementarna wobec R21 znajdowała się w obrębie 50-nt *targetów* (Mod) i pozostawała niezmieniona, zgodnie ze schematem przedstawionym na **rysunku 12A**. Łącznie zaprojektowano dziewięć układów, stanowiących kombinację trzech typów *donorów* i trzech typów *targetów*. *Donorami* były, odpowiednio: (i) R21, (ii) duplex typu miRNA oraz (iii) dupleks typu siRNA; w dupleksach tych jedną z nici stanowiła cząsteczka R21. *Targety* stanowiły cząsteczki: (i) Mod18, (ii) Mod23 i (iii) Mod33, które przyjmowały strukturę typu spinki, przy czym sekwencja docelowa dla R21 znajdowała się w trzonie spinki, a stabilność termodynamiczna przyjmowanych przez *targety* struktur układała się w następujący szereg: Mod18<Mod23<Mod33 (**rysunek 12, B)**. Każda z reakcji zawierała etap pre-inkubacji *donora* z białkiem, po którym do mieszaniny reakcyjnej dodawano *target*. Stężenia molowe *donora* i *targetu* były równe i wynosiły 5 nM.



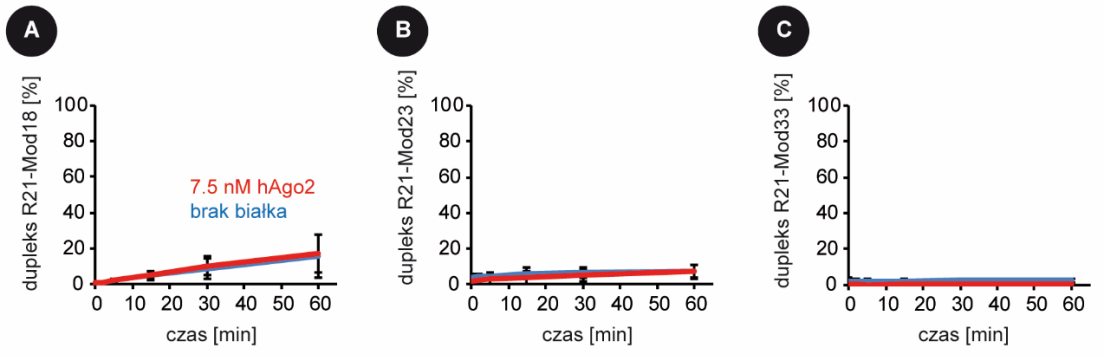
Rysunek 12 (A) Schemat reakcji parowania RNA dla cząsteczek: R21 i Mod. Cząsteczkę R21 zaznaczono kolorem niebieskim, natomiast komplementarną wobec niej sekwencję w obrębie Mod zaznaczono kolorem czerwonym. Sekwencje różnicujące cząsteczek Mod (Mod18, Mod23 i Mod33) zaznaczono linią przerywaną. (B) Reakcje parowania RNA z udziałem *donorów* i *targetów*. Reakcje prowadzone były bez białka (kontrola negatywna, kolor niebieski), z dodatkiem hDicer-His6 (kolor zielony) lub GiDicer (kolor fioletowy). W reakcjach zastosowano następujące punkty czasowe: 2, 5, 15, 30, 60 minut. Rysunek wykonano na podstawie Pokornowska i wsp., 2019 [3].

Wyniki przeprowadzonych badań wykazały, że białko hDicer-His6 wspierało parowanie komplementarnych RNA w przypadkach, w których oba substraty (*donor* i *target*) przyjmowały struktury drugorzędowe posiadające niesparowania w rejonach dwuniciowych (miRNA, Mod18, Mod23) oraz we wszystkich przypadkach, w których *donorem* był krótki jednoniciowy R21 (**rysunek 12, B** oraz rysunek: Fig. 2 A-E [3]). Gdy *donorem* był dupleks siRNA, reakcja parowania komplementarnych RNA nie była wspierana przez hDicer-His6, a przebieg reakcji,

w odpowiednich punktach czasowych, był podobny do reakcji prowadzonych bez udziału białek (**rysunek 12, B** oraz rysunek: Fig. 2 G-I [3]). Podobna sytuacja miała miejsce, gdy substratami w reakcjach były: dupleks miRNA oraz Mod33 (**rysunek 12, B** oraz rysunek: Fig. 2 F [3]). Ponadto, we wszystkich dziewięciu testowanych układach wydajności parowania RNA w reakcjach z udziałem GiDicer i w reakcjach bez udziału białka były podobne w tych samych punktach czasowych (**rysunek 12, B** oraz rysunek: Fig.2 [3]).

Spontaniczna dysocjacja dupleksów miRNA i siRNA w trakcie trwania reakcji może mieć wpływ na przebieg zaprezentowanych powyżej reakcji, ponieważ mogłaby być źródłem jednoniciowej cząsteczki R21. Reakcje kontrolne nie wykazały jednak spontanicznej dysocjacji dupleksów miRNA i siRNA; po godzinnej inkubacji w buforze reakcyjnym nie obserwowano rozpadu dupleksów (rysunek: Supplementary Figure S4 [3]). Ponieważ ludzka Dicer zawiera motyw wiążący ATP w domenie helikazy, sprawdzono również, czy ATP może wpływać na stabilność dupleksów w reakcji z hDicer-His6. W tym celu inkubowano dupleks miRNA lub siRNA z białkiem w buforze zawierającym ATP. Zaobserwowano, iż ATP nie miał wpływu na stabilność dupleksów w reakcjach z Dicer (rysunek: Supplementary Figure S4 [3]). ATP nie wpływał także na wydajność reakcji wspierania parowania RNA, w których obecna była Dicer (dane niepublikowane).

Cząsteczki wykorzystane w naszych badaniach oryginalnie zostały użyte przez Ameres i wsp. do zbadania w jaki sposób kompleks siRISC rozpoznaje i tnie transkrypty zawierające miejsca docelowe dla siRNA [214]. Niniejsze badania ujawniły, że w warunkach *in vitro* białko Ago2 obecne w kompleksie RISC przecina tylko te transkrypty, które nie tworzą stabilnych struktur drugorzędowych [214]. Wyniki uzyskane przez badaczy sugerowały brak zdolności białek Ago2 do rozluźniania stabilnych struktur drugorzędowych typu spinki przyjmowanych przez RNA. Jednakże grupa Ameres i wsp. nie zbadała zdolności białka Ago2 do wspierania parowania komplementarnych RNA. Biorąc pod uwagę nasze dotychczasowe wyniki postanowiliśmy zbadać, czy Ago2 może wspierać parowanie krótkiego RNA z sekwencją docelową znajdującą się w stabilnym trzonie struktur spinkowych przyjmowanych przez drugą, dłuższą cząsteczkę RNA. Ze względu na fakt, że dojrzały minimalny kompleks RISC zawiera rybonukleazę Ago i jednoniciową cząsteczkę srRNA, w badaniach wykorzystaliśmy trzy układy substratów, w których *donorem* był R21 (układy R21/Mod18, R21/Mod23 i R21/Mod33). W doświadczeniach użyto komercyjnego preparatu ludzkiej rybonukleazy Ago2 (hAgo2). Wyniki przeprowadzonych reakcji ujawniły, że jedynie w układzie R21/Mod18, hAgo2 nieznaczne wspiera parowanie komplementarnych RNA (**rysunek 13** oraz rysunek: Fig 3 [3]). Otrzymane wyniki były zgodne z danymi zaprezentowanymi przez Ameres i wsp., którzy wykazali, że kompleks RISC nie potrafi rozluźniać stabilnych struktur drugorzędowych RNA typu spinki [214].

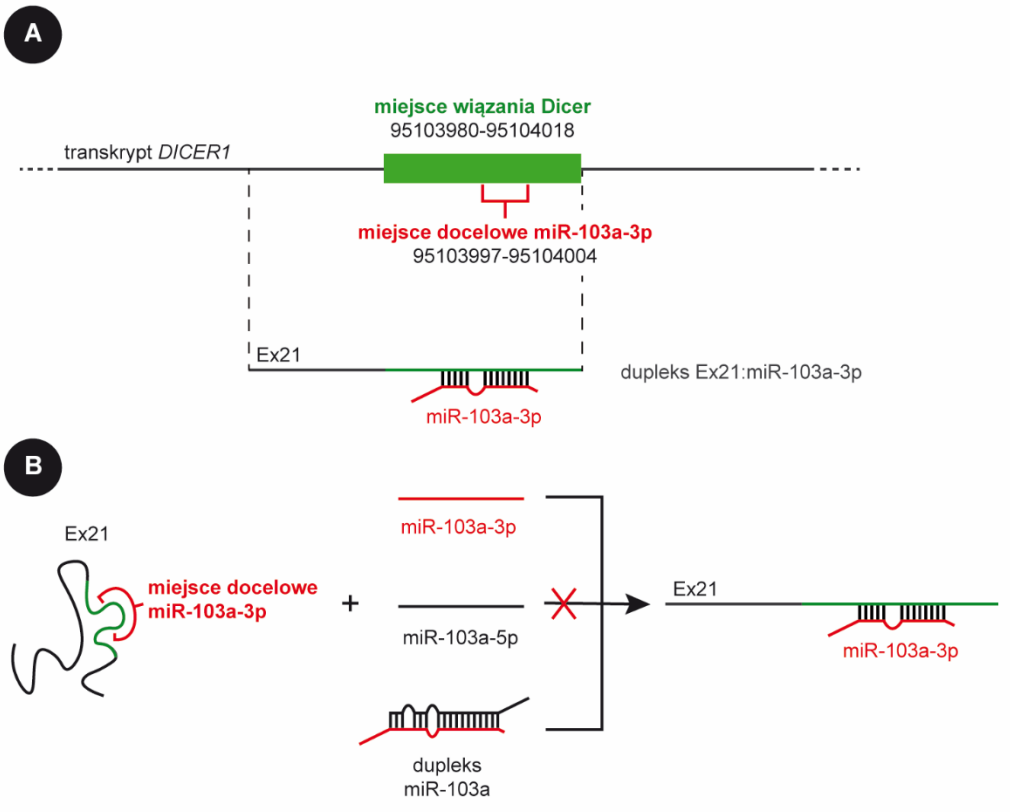


Rysunek 13 Reakcje parowania RNA z udziałem R21, jako *donora*, oraz trzech *targetów*: (A) Mod18, (B) Mod23 i (C) Mod33, prowadzone bez udziału białka (kolor niebieski) lub w obecności białka hAgo2 (kolor pomarańczowy). W reakcjach zastosowano następujące punkty czasowe: 2, 5, 15, 30, 60 minut. Rysunek wykonano na podstawie Pokornowska i wsp., 2019 [3].

W drugiej części pracy rozważaliśmy potencjalne znaczenie biologiczne aktywności wspierającej parowanie RNA lub DNA rybonukleazy Dicer człowieka. Naszą uwagę zwróciły badania grupy profesora Rajewsky'ego, opublikowane w 2014 roku, które przedstawiają zbiór (mapę) miejsc wiązania ludzkiej Dicer do trankryptomu komórek HEK293 [227]. W celu określenia sekwencji cząsteczek RNA wiązanych przez ludzką Dicer w warunkach *in cellulo* badacze wykorzystali metodę głębokiego sekwencjonowania wiązanych przez Dicer RNA izolowanych z komórek za pomocą zmodyfikowanej techniki immunoprecypitacji, PAR-CLIP. Metoda ta polegała na „wyławianiu" kompleksów Dicer:RNA powstałych *in cellulo*. Stabilne kompleksy uzyskiwane były w komórkach poprzez włączanie fotoreaktywnych analogów rybonukleotydowych do RNA podczas procesu transkrypcji, a następnie powstające kompleksy RNA i białek były poddane fotozszywaniu poprzez naświetlanie komórek promieniowaniem nadfioletowym. Kompleksy Dicer:RNA „wyławiano" z lizatów komórkowych przy użyciu techniki immunoprecypitacji, a RNA, znajdujący się w kompleksach, sekwencjonowano. Przedstawione dane wykazały, że Dicer szczególnie często wiąże się do transkryptu, z którego powstaje. Znaleziono aż 36 miejsc wiązania Dicer w obrębie rejonu mRNA Dicer kodującego białko (rysunek: Supplementary Fig. S6 [3]); ze względu na wykorzystanie zmodyfikowanych genetycznie komórek do produkcji Dicer, w badaniach tych nie uwzględniono rejonów 5' UTR i 3' UTR mRNA Dicer. Co ciekawe, Forman i wsp. znaleźli liczne miejsca wiązania się cząsteczek miRNA do rejonów kodujących białko transkryptów *DICER1* [235]. Biorąc pod uwagę zgromadzone dane postanowiliśmy porównać miejsca (sekwencje) wiązania Dicer (dane z [227]) z sekwencjami docelowymi dla miRNA (dane: http://mirdb.org/, [235]) w obrębie mRNA Dicer. Wnikliwa analiza bioinformatyczna wykazała, że w obrębie 35 z 36 miejsc wiązanych przez Dicer występują aż 304 potencjalne miejsca docelowe dla miRNA (rysunek: Supplementary Fig. S6 [3]). Aby sprawdzić, czy zaobserwowana prawidłowość jest charakterystyczna tylko dla transkryptu *DICER1*, czy również inne transkrypty posiadają miejsca wiązania Dicer, które pokrywają się

z miejscami docelowymi dla miRNA, przeprowadziliśmy analogiczne analizy dla innych transkryptów kodujących białka (dane: Supplementary Table S3 [3]). Uzyskane dane wykazały, że nie tylko transkrypt *DICER1*, ale również transkrypty innych genów, np. *AGO1*, *TRAF4* (ang. *TNF receptor-associated factor 4*), *DDX6* (ang. *DEAD-box helicase 6*), są wiązane przez Dicer w obrębie miejsc docelowych dla miRNA.

Za potranskrypcyjną regulację ekspresji genów poprzez oddziaływanie z transkryptami w dużym stopniu odpowiadają cząsteczki miRNA, ale też liczne białka wiążące RNA (ang. *RNA-binding proteins, RBPs*), inne niż białka Ago. Wiele z tych białek wiąże się w obrębie, bądź w pobliżu, miejsc docelowych dla cząsteczek miRNA [236]. Przykładem takiego białka jest Pumilio, o którym wspomniano w rozdziale 1.3.2. Pumilio poprzez specyficzne wiązanie sekwencji PRE wprowadza lokalne zmiany w strukturze RNA transkryptu i eksponuje miejsca docelowe dla miRNA, umożliwiając w ten sposób wiązanie się kompleksów RISC [215]. Wyniki prowadzonych przez nas badań sugerowały, iż Dicer mogłaby pełnić podobną funkcję poprzez wiązanie się do komplementarnych wobec związanej cząsteczki srRNA miejsc w obrębie transkryptów. W celu weryfikacji tej hipotezy, biorąc pod uwagę przedstawione powyżej informacje, przeprowadzono reakcje parowania RNA, w których użyto: 23-nt miR-103a-3p oraz RNA reprezentujący fragment mRNA *DICER1* zawierający miejsce wiązania Dicer i miejsce docelowe dla miR-103a-3p (fragment egzonu 21, rysunek: Supplementary Fig. S6 [3]); cząsteczkę tę nazwano „Ex21" (**rysunek 14A**). Cząsteczka miR-103a-3p występowała w reakcji w postaci jednoniciowej lub w dupleksie miR-103a. W celu zbadania specyficzności reakcji parowania RNA, przeprowadzono także reakcję kontrolną, w której zastosowano drugą nić dupleksu miR-103a, miR-103a-5p (**rysunek 14B**). Do mieszanin reakcyjnych dodawano hDicer-His6 lub hAgo2. Uzyskane wyniki wykazały, że hDicer-His6 wspiera parowanie miR-103a-3p do sekwencji docelowej zarówno jeśli miRNA występuje w postaci jednoniciowej (rysunek: Fig. 4B [3]), jak i w dupleksie miR-103a (rysunek: Fig. 4D [3]). Tymczasem hAgo2 tylko w niewielkim stopniu wspierała parowanie komplementarnych RNA (rysunek: Fig. 4B, D [3]). Podsumowując, badania przedstawione w publikacji Pokornowska i wsp. [3], wykazały, że w warunkach *in vitro* ludzka Dicer wpiera hybrydyzację krótkich RNA do ich miejsc docelowych (sekwencji komplementarnych), nawet jeśli krótkie RNA obecne są w dupleksach typu miRNA, a miejsca docelowe znajdują się w obrębie stabilnych struktur przyjmowanych przez dłuższe cząsteczki RNA. Ago2 takiej aktywności nie posiada.



Rysunek 14 (A) Schemat przedstawiający fragment egzonu 21 transkryptu *DICER1*, w obrębie którego zidentyfikowano miejsce wiązania Dicer oraz miejsce docelowe dla miR-103a-3p (czerwoną klamrą zaznaczono rejon komplementarny wobec *seed*). Lokalizacja zaznaczonych miejsc w obrębie transkryptu *DICER1* (NM_001271282) jest zgodna z *Genome Reference Consortium Human Build 38* (hg38). (B) Schemat reakcji parowania Ex21 i miR-103a-3p. W reakcji użyto: Ex21, oraz miR-103a-3p, miR-103a-5p (kontrola), lub dupleks miR-103a. Reakcja prowadzona była bez białka, z dodatkiem hDicer-His6 lub hAgo2. Produktem reakcji był dupleks Ex21:miR-103a-3p.

Analiza danych pochodzących z głębokiego sekwencjonowania pul RNA wiązanych przez Dicer, Ago2 i Ago3 w komórkach HEK293 [227] wykazała, że ponad połowa miejsc wiązanych przez Dicer jest również wiązana przez białka Ago2 i Ago3; naturalnie, część wspólnych miejsc wiązania dotyczyła tzw. „miejsc aktywnych", czyli miejsc stanowiących źródło pre-miRNA i miRNA (dane: Supplementary Table S2 [3]). Analizy dotyczące miejsc wiązania w obrębie mRNA, wykazały, iż w przypadku transkryptu *DICER1*, 12 (z 36 zidentyfikowanych) miejsc wiązanych przez Dicer pokrywa się z trzynastoma miejscami wiązanymi przez Ago2 i Ago3 (jedno miejsce wiązania Dicer pokrywa się z dwoma miejscami wiązania Ago2/3). W obrębie wspólnych sekwencji znaleziono 39 miejsc docelowych dla miRNA (rysunek: Supplementary Fig. S8 [3]). W oparciu o uzyskanie wyniki zaproponowano model bezpośredniego udziału Dicer w potranskrypcyjnej regulacji ekspresji genów (rysunek: Fig. 6 [3]). Model ten przedstawia dwa możliwe scenariusze. Pierwszy scenariusz zakłada, że wygenerowany przez Dicer dupleks miRNA nie zostaje przekazany do Ago2, w wyniku czego nie dochodzi do powstania aktywnego kompleksu RISC. Związany z Dicer dupleks miRNA zostaje skierowany do miejsc docelowych dla miRNA. Po związaniu z transkryptem, kompleks

miRNA:Dicer chroni transkrypt przed wiązaniem innych, obecnych w komórce kompleksów RISC, przez co ogranicza degradację transkryptu i, tym samym, stabilizuje jego poziom w komórce (rysunek: Fig. 6B [3]). Hipoteza ta jest poparta obserwacjami grupy prof. Rajewskiego, dotyczącymi „miejsc pasywnych" Dicer. Dokładniej, transkrypty wiązane przez Dicer, ale niewiązane przez Ago2 i Ago3, wykazywały wyższy poziom ekspresji w komórkach HEK293, niż transkrypty wiązane zarówno przez Dicer, jak i Ago2/3, czy też transkrypty niewiązane przez Dicer [227]. Drugi scenariusz zakłada, że przekazanie miRNA z Dicer do Ago następuje dopiero w momencie wiązania kompleksu RISC z transkryptem. Zgodnie z tym scenariuszem Dicer wspiera Ago2 w wiązaniu do miejsc docelowych dla miRNA zlokalizowanych w obrębie stabilnych struktur transkryptu, w konsekwencji czego dochodzi do degradacji transkryptu lub represji translacji (rysunek: Fig. 6C [3]). Planowane są dalsze doświadczenia mające na celu przetestowanie powyższych hipotez.

4 Podsumowanie i perspektywy

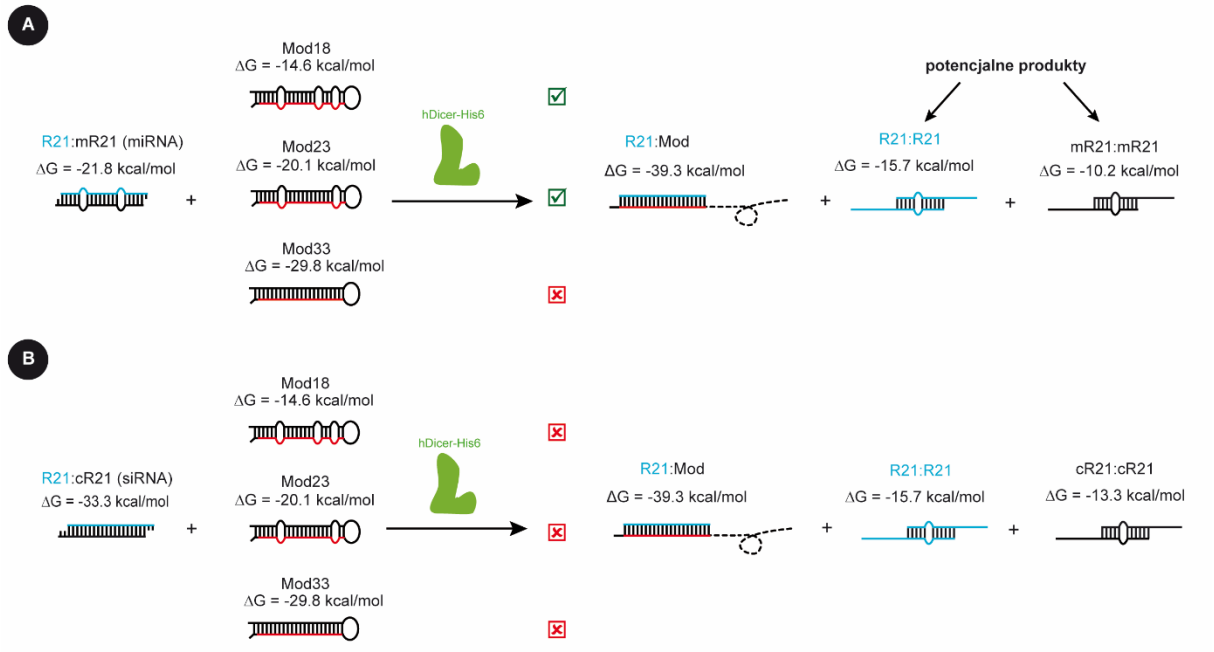
Dwie prace oryginalne wchodzące w skład niniejszej rozprawy doktorskiej [2, 3] opisują niepoznane dotychczas obszary aktywności rybonukleazy Dicer człowieka. Zaprezentowane badania poprzedzone zostały wnikliwą analizą oraz usystematyzowaniem ówczesnej wiedzy na temat rybonukleaz typu Dicer, w szczególności Dicer człowieka. Wyniki tych działań, a także nasze spostrzeżenia i przemyślenia zostały ujęte w pracy przeglądowej Kurzynska-Kokorniak i wsp., 2015 [1]. Najważniejszymi rezultatami badań przedstawionych w niniejszej rozprawie doktorskiej są:

i. wykazanie, że domena DUF283 ludzkiej Dicer oddziałuje z jednoniciowymi RNA i DNA [2];
ii. wykazanie, że domena DUF283 i Dicer człowieka wspierają parowanie cząsteczek kwasów nukleinowych w warunkach *in vitro*; [2];
iii. przypisanie Dicer do grupy białek typu ang. *nucleic acid annealer*, tj. białek o aktywności wpierającej parowanie RNA lub DNA [2];
iv. wykazanie, że w warunkach *in vitro* ludzka Dicer wpiera hybrydyzację krótkich RNA do komplementarnych miejsc docelowych w obrębie długich RNA, nawet jeśli krótkie RNA obecne są w dupleksach typu miRNA, a miejsca docelowe znajdują się w obrębie stabilnych struktur przyjmowanych przez dłuższe cząsteczki RNA [3];
v. wykazanie, że ludzka rybonukleaza Ago2, w warunkach *in vitro*, nie wspiera parowania dwóch cząsteczek RNA, jeśli sekwencja komplementarna jednego z substratów występuje w obrębie stabilnych struktur drugorzędowych typu spinki [3];
vi. zaproponowanie modelu bezpośredniego udziału Dicer w potranskrypcyjnej regulacji ekspresji genów, zakładającego współzawodniczenie bądź współpracę Dicer z białkami Ago [3];
vii. poszerzenie wiedzy dotyczącej rybonukleazy Dicer człowieka.

W zaprezentowanych badaniach wykorzystano preparaty His6-DUF283 oraz hDicer-His6, które zostały otrzymane przeze mnie odpowiednio, w bakteryjnym i bakulowirusowym systemie produkcji białek, a także preparat hDicer otrzymany we współpracy z zespołem prof. Krystyny Bieńkowskiej-Szewczyk (Zakład Biologii Molekularnej Wirusów, Międzyuczelniany Wydział Biotechnologii Uniwersytetu Gdańskiego i Gdańskiego Uniwersytetu Medycznego). Preparaty GiDicer (Dicer *G. intestinalis*) oraz ludzkiej Ago2 zostały zakupione komercyjnie.

W pracy Kurzynska-Kokorniak *et al.*, 2016 [2] wykazano, że zarówno domena DUF283, jak i kompletna ludzka Dicer posiadają aktywność wspierającą parowanie cząsteczek kwasów nukleinowych, która jest charakterystyczna dla białek typu ang. *nucleic acid annealer* i chaperonów kwasów nukleinowych. Białka typu *nucleic acid annealer*, w przeciwieństwie do chaperonów kwasów nukleinowych, pozostają związane z substratami dopóki proces

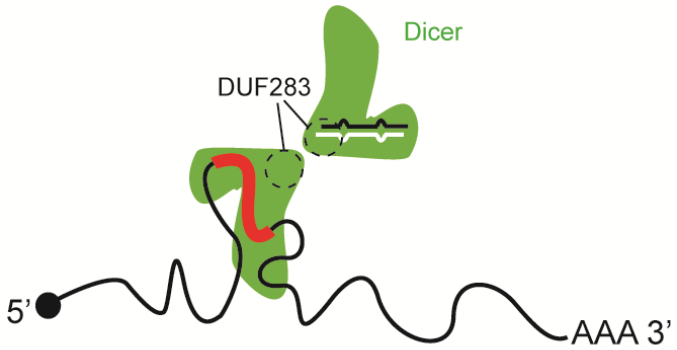
parowania komplementarnych zasad azotowych nie jest ukończony [232]. Podobnie, domena DUF283 Dicer człowieka wiąże ssRNA, lecz nie dsRNA [2]. Co więcej, badania wpływu struktury drugorzędowej RNA na aktywność wspierającą parowanie RNA, przedstawione w pracy Pokornowska *et al.*, 2019 [3], wykazały, że Dicer wspiera proces wymiany nici RNA w dupleksie, gdy substratami są dupleks miRNA i dłuższy RNA przyjmujący stabilną strukturę drugorzędową typu spinki zawierającej niesparowania w rejonie trzonu. W związku z tym pojawia się pytanie, w jaki sposób Dicer wspiera wymianę nici w dupleksie miRNA (**rysunek 15A**), jednocześnie nie potrafiąc rozplatać struktur typu dupleks? Możliwym jest, że domena DUF283 ludzkiej Dicer rozpoznaje niesparowania w dupleksach jako rejony jednoniciowe, dzięki czemu dochodzi do lokalnego rozplatania dupleksu miRNA, czy też nie w pełni sparowanego trzonu spinki (np. w *targetach*: Mod18 i Mod23). W przypadku takiego scenariusza, jednoniciowe fragmenty mają szansę hybrydyzować z odpowiednimi, „współzawodniczącymi" rejonami komplementarnymi (**rysunek 15A**). W rezultacie, krótka nić komplementarna (R21) mogłaby opuszczać dupleks miRNA i współtworzyć dupleks o stabilniejszej strukturze (R21:Mod). Reakcja wymiany nici w dupleksie nie przebiega natomiast w przypadku zastosowania Mod33. Trzon struktury spinki przyjmowanej przez Mod33 jest pozbawiony niesparowań, przez co, przypuszczalnie, DUF283 nie może wiązać tego rejonu substratu. Podobnie, w przypadku zastosowania dupleksu siRNA reakcja nie zachodzi, gdyż trzon struktury dupleksu jest całkowicie sparowany (**rysunek 15B**). Podobne wyniki otrzymano dla białka opiekuńczego ORF1 (ang. *open reading frame 1*) retrotranspozonu LINE-1 myszy (ang. *long interspersed nuclear element 1*). ORF1 wspiera reakcję wymiany nici dupleksu w sytuacji, w której struktura dupleksu przyjmowana przez produkt jest stabilniejsza niż struktura dupleksu substratu. Niniejsze wnioski korelują z kolejną obserwacją autorów tej publikacji, mianowicie, że ORF1 obniża temperaturę topnienia dupleksu posiadającego niesparowania i podwyższa temperaturę topnienia w pełni sparowanego dupleksu [237]. Wstępne wyniki analiz stabilności termodynamicznej dupleksów miRNA i siRNA w reakcjach zawierających His6-DUF283, lub bez białka, wskazują, że ludzka DUF283 posiada podobną aktywność. Badania zmierzające do wyjaśnienia molekularnych podstaw aktywności wspierającej parowanie cząsteczek kwasów nukleinowych, prezentowanej przez DUF283 oraz Dicer człowieka, są obecnie kontynuowane w Zakładzie Biochemii Rybonukleoprotein ICHB PAN.



Rysunek 15 Schematyczne przedstawienie reakcji parowania RNA z udziałem dupleksu (A) miRNA lub (B) siRNA (jako *donora*) oraz cząsteczki typu Mod (jako *targetu*), na podstawie Pokornowska i wsp., 2019 [3]. Schemat przedstawia wymianę nici w dupleksie RNA złożonym z nici wiodącej (R21, zaznaczona kolorem niebieskim) oraz nici częściowo komplementarnej wobec R21 (zaznaczona kolorem czarnym). Nić częściowo komplementarna zostaje wymieniona na dłuższą nić zawierającą sekwencję w pełni komplementarną (zaznaczona kolorem czerwonym) wobec R21.

W literaturze opisane zostały dwa mechanizmy działania białek wspierających parowanie RNA. Pierwszy, określany jako *ang. molecular crowding*, polega na jednoczesnym wiązaniu dwóch komplementarnych nici kwasu nukleinowego przez białko, co powoduje lokalne zagęszczenie tych cząsteczek w środowisku reakcyjnym i zwiększenie prawdopodobieństwa ich parowania [232]. Przykładem takiego białka jest StpA, które umożliwia wycinanie intronów wirusowych pre-mRNA (ang. *precursor mRNA*, *primary transcript*). Wykazano, że jednoczesne wiązanie dwóch krótkich RNA i ich parowanie zależy od dimeryzacji StpA [238]. Drugi mechanizm zakłada udział białek określanych mianem *ang. matchmakers*. Białka te, poprzez wiązanie cząsteczek RNA, doprowadzają do lokalnych zmian konformacyjnych w obrębie kwasu nukleinowego, które ułatwiają parowanie związanego RNA z sekwencjami komplementarnymi obecnymi w układach trans [232]. Przykładem takiego białka jest Hfq (ang. *host factor for Qβ replication*) *E. coli*, które wspiera interakcję między małymi niekodującymi RNA a mRNA [239, 240] lub kinetoplastydowe białko gBP21 (ang. *guide RNA-binding protein 21*). Białko gBP21 wspiera parowanie gRNA (ang. *guide RNA*) do miejsca docelowego w obrębie pre-mRNA, naznaczając w ten sposób miejsce edycji pre-mRNA. Wykazano, że gRNA i gBP21 tworzą kompleks, w którym koniec 5' gRNA jest wiązany przez gBP21. W wyniku oddziaływania gRNA i gBP21 zasady azotowe gRNA są eksponowane w kierunku rozpuszczalnika, a ujemnie naładowane grupy fosforanowe szkieletu cukrowo-fosforanowego są wiązane przez białko. W ten sposób białko gBP21 zmniejsza odpychanie

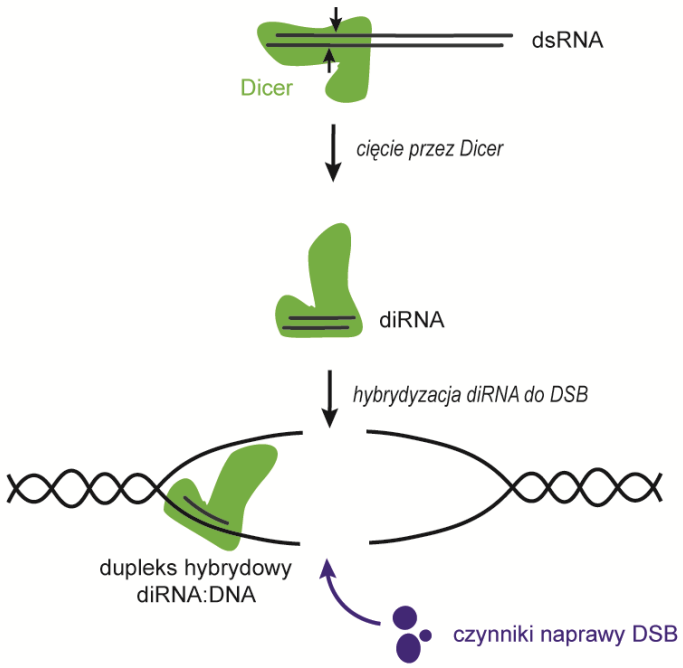
elektrostatyczne pomiędzy hybrydyzującymi RNA (poprzez tzw. ekranowanie ładunku) i dysocjuje, gdy proces parowania komplementarnych RNA jest ukończony, gdyż traci powinowactwo do dwuniciowego produktu [241, 242]. Biorąc pod uwagę fakt, że ludzka Dicer wspiera parowanie dwóch ustrukturyzowanych RNA, które nie hybrydyzują spontanicznie (rysunek: Fig. 2 B, C, E [3]), można przypuszczać, że Dicer wykorzystuje mechanizm charakterystyczny dla białek typu *matchmaker*. Ponadto, jak wspomniano w rozdziale 1.2.4., domena DUF283 jest domeną dimeryzacji i odpowiada za wiązanie białek partnerującym rybonukleazom typu Dicer [145, 146]. Wstępne badania *in vitro* wykazały, że domena DUF283 ludzkiej Dicer może tworzyć homodimery (dane nieopublikowane). Można zatem przypuszczać, że ludzka Dicer mogłaby brać udział w potranskrypcyjnej regulacji ekspresji genów (model przedstawiony na rysunku 6 [3]) jako homodimer. W takim przypadku jedna cząsteczka Dicer mogłaby wiązać dupleks miRNA, natomiast druga, miejsce docelowe w obrębie mRNA. Dimeryzacja Dicer mogłaby ułatwiać zachodzący w obrębie utworzonego kompleksu proces parowania komplementarnych RNA poprzez zwiększenie lokalnego stężenia RNA (mechanizm typu *molecular crowding*), a jednocześnie w związanych RNA dochodziłoby do zmian konformacyjnych eksponujących sekwencje komplementarne (mechanizm opisany dla białek typu *matchmakers*). Taki scenariusz został zilustrowany na **rysunku 16**.



Rysunek 16 Model udziału dimeru Dicer w potranskrypcyjnej regulacji ekspresji genów. Jedna cząsteczka Dicer wiąże dupleks miRNA, natomiast druga, miejsce docelowe dla miRNA (zaznaczone czerwonym kolorem), przez co dochodzi do lokalnego zwiększenia stężenia RNA (*molecular crowding*). Dimeryzacja Dicer przebiega poprzez domenę DUF283 (obszar otoczony linią przerywaną). Dodatkowo, zgodnie z mechanizmem charakterystycznym dla białek typu *matchmaker*, Dicer rozluźnia lokalną strukturę drugorzędową transkryptu, w którą uwikłane jest miejsce docelowe dla miRNA, oraz strukturę dupleksu miRNA.

Rozważając potencjalne znaczenie biologiczne aktywności wspierającej parowanie RNA Dicer, wysnuliśmy hipotezę, że Dicer może być bezpośrednio zaangażowana w potranskrypcyjną regulację ekspresji genów poprzez oddziaływanie z miejscami docelowymi dla miRNA (**rysunek 14**). Badania w warunkach *in cellulo* zmierzające do potwierdzenia powyższej hipotezy kontynuowane są obecnie w Zakładzie Biochemii Rybonukleoprotein ICHB PAN. Rozważania dotyczące potencjalnego znaczenia biologicznego odkrytej

aktywności Dicer można kontynuować w odniesieniu do procesów powiązanych z utrzymaniem integralności genomu jądrowego. Jak wcześniej wspomniano, ludzka Dicer może być zaangażowana w produkcję diRNA i naprawę dwuniciowych uszkodzeń DNA (rozdział 1.1.3.). Biorąc pod uwagę wyniki badań przedstawionych w niniejszej rozprawie doktorskiej można przypuszczać, że Dicer ma potencjał do wspierania parowania diRNA do jednoniciowego DNA, obecnego w miejscu uszkodzenia dupleksu DNA. W ten sposób mogłoby dochodzić do inicjacji naprawy DNA (**rysunek 17**). Wykazano, że hybrydy RNA:DNA tworzą się w miejscach uszkodzenia i są niezbędne w procesie naprawy uszkodzenia na drodze rekombinacji homologicznej w komórkach drożdży *Saccharomyces pombe* [243]. Dalsze eksperymenty, wykorzystujące układy modelowe imitujące miejsca uszkodzeń DNA i produkcję diRNA, z pewnością pogłębiłyby wiedzę na temat potencjalnego znaczenia biologicznego aktywności ludzkiej rybonukleazy Dicer wspierającej parowanie cząsteczek kwasów nukleinowych.



Rysunek 17 Uproszczony model udziału Dicer w inicjacji naprawy dwuniciowych uszkodzeń DNA (DSB). Dwa niezależne procesy transkrypcji, konstytutywnej oraz inicjowanej w miejscu uszkodzenia DNA, są źródłem dwóch wzajemnie komplementarnych cząsteczek mRNA. Komplementarne transkrypty hybrydyzują ze sobą (dsRNA) i są rozpoznawane przez Dicer oraz cięte do diRNA. Następnie Dicer wspiera parowanie diRNA do komplementarnego miejsca w pobliżu uszkodzenia DNA.

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Instytut Chemii Bioorganicznej
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w Poznaniu
Zakład Biochemii Rybonukleoprotein

mgr inż. Maria Pokornowska

Załączniki do pracy doktorskiej:

Aktywność ludzkiej rybonukleazy Dicer wspierająca parowanie cząsteczek kwasów nukleinowych: odkrycie, charakterystyka biochemiczna i potencjalne znaczenie biologiczne

wykonanej pod kierunkiem
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SURVEY AND SUMMARY

The many faces of Dicer: the complexity of the mechanisms regulating Dicer gene expression and enzyme activities

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ABSTRACT

There is increasing evidence indicating that the production of small regulatory RNAs is not the only process in which ribonuclease Dicer can participate. For example, it has been demonstrated that this enzyme is also involved in chromatin structure remodelling, inflammation and apoptotic DNA degradation. Moreover, it has become increasingly clear that cellular transcript and protein levels of Dicer must be strictly controlled because even small changes in their accumulation can initiate various pathological processes, including carcinogenesis. Accordingly, in recent years, a number of studies have been performed to identify the factors regulating Dicer gene expression and protein activity. As a result, a large amount of complex and often contradictory data has been generated. None of these data have been subjected to an exhaustive review or critical discussion. This review attempts to fill this gap by summarizing the current knowledge of factors that regulate Dicer gene transcription, primary transcript processing, mRNA translation and enzyme activity. Because of the high complexity of this topic, this review mainly concentrates on human Dicer. This review also focuses on an additional regulatory layer of Dicer activity involving the interactions of protein and RNA factors with Dicer substrates.

INTRODUCTION

Small regulatory RNAs, such as microRNAs (miRNAs) or small interfering RNAs (siRNAs), play essential roles in many important biological processes, including developmental timing, growth control, differentiation and apoptosis (1–3). In humans, the vast majority of small regulatory RNAs are miRNAs. To date, ∼2600 miRNAs encoded in the human genome that are derived from almost 1900 miRNA precursors (pre-miRNAs) have been identified (http://www.mirbase.org/). They have been found to control the expression of most human protein-coding genes through the miRNA pathway (4,5). Moreover, it has been demonstrated that miRNAs play a very important role in host-virus interactions in mammals (6–9). Therefore, the cellular levels of miRNAs and other components of miRNA pathways must be tightly controlled, both spatially and temporally. Aberrant regulation of miRNA levels can initiate pathological processes, including carcinogenesis as well as neurodegenerative, immune system and rheumatic disorders (10–13).

A fundamental role in the biogenesis of miRNAs in humans is played by a ribonuclease III (RNase III) enzyme termed Dicer, which recognizes and cleaves 50–70-nucleotide (nt) single-stranded pre-miRNAs with hairpin structures or double-stranded RNAs (dsRNAs) into functional 21–23-nt miRNAs or siRNAs, respectively (14). Human Dicer is a 220-kDa multidomain enzyme comprising an amino (N)-terminal putative helicase domain (homologous to DExD/H-box helicases), a DUF283 domain (domain of unknown function), a PAZ (Piwi-Argonaute-Zwille) domain, two RNase III domains (RNase IIIa and RNase IIIb) and a dsRNA-binding domain (dsRBD) (14–18). Because all metazoan Dicers are large and complex proteins, they are difficult to crystallize. A lack of structural data has limited the understanding of Dicer-mediated processes. The crystal structure of an intact Dicer enzyme has only been determined for *Giardia intestinalis*; however,

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Giardia Dicer is only comprised of the PAZ and tandem RNase III domains and lacks many of the domains and regions characteristic of Dicers in higher eukaryotes (16). A three-dimensional model of the human Dicer enzyme has been determined based on electron microscopy data (19–22). In addition, structures of several individual Dicer domains have been established, including the crystal structures of the platform-PAZ-connector helix cassette (23) and the carboxy (C)-terminal RNase III domain (RNase IIIb) (24). Moreover, a three-dimensional model of the DUF283 domain has been generated by computational methods (25).

The first model of Dicer ribonuclease activity was proposed in 2004 by Filipowicz *et al*. (18). According to this model, miRNA and siRNA precursors are recognized by the PAZ domain. The latter binds to the 3′ end of the substrate, with a preference for 2-nt-long overhangs (18,26–29). The dsRBD of Dicer has been shown to play only an auxiliary role in substrate binding and cleavage. This model also implies that Dicer contains a single dsRNA cleavage centre formed by the RNase IIIa and RNase IIIb domains, which are both located within the same molecule. This enzyme cuts both strands of dsRNA precursors at regions located ∼20 base pairs (bp) from their termini (18). This model has been further improved by the enhanced identification and characterization of the functions of other Dicer domains and motifs. Currently, it is assumed that Dicer has two pockets that bind to substrate ends, a 3′-end-binding pocket located within the PAZ domain (16,18) and a 5′-end-binding pocket located within the PAZ domain and the so-called platform domain (23,30). Importantly, docking to the 5′-end-binding pocket has been proposed to be efficient only when substrate ends are less stably base-paired, which is more characteristic of miRNA precursors (23). Accordingly, analyses of Dicer homologues have revealed that the 5′-end-binding pocket is highly conserved among most Dicers producing miRNAs but not siRNAs. Thus, this motif is not present in Dicers from lower eukaryotes (e.g. *Giardia* and fungi), which lack the miRNA pathway (30). Furthermore, recent data have suggested that the N-terminal helicase domain of Dicer is involved in the discrimination between miRNA and siRNA precursors by interacting with the hairpin loop structures of pre-miRNAs (21,31–33). Importantly, RNA binding by the helicase domain has been proposed to cause substrate-dependent changes in the Dicer structure (21,34). Nevertheless, the helicase domain has also been shown to be dispensable for Dicer cleavage activity (35,36). Further, this domain has been proposed to function as an autoinhibitor of Dicer (37). The function of the DUF283 domain remains unknown. Initially, it was suggested to be critical for pre-miRNA processing because Dicer mutants lacking it (in addition to the helicase domain) lost this activity (35,36). However, Doudna *et al*. have shown that the cleavage activity of a Dicer mutant with a deletion of the DUF283 domain, but possessing all other components, is only slightly affected (37). Individual Dicer domains have also been shown to interact with other proteins. This issue will be discussed later in this manuscript.

Based on the first models of Dicer proteins it was proposed that the length of the small RNAs produced by Dicer was determined by the distance between the PAZ domain and the cleavage centre, which depends on the length of the linker that connects them (16,19). Thus, Dicer was considered to be a molecular ruler that measured and cleaved 20–25-bp duplexes from dsRNA substrates. Interestingly, Doudna *et al*. have recently shown that active human Dicer can be reconstituted from two or three separately obtained fragments (31). More importantly, the two fragments, one comprising the DUF283 and PAZ domains (N-terminal) and the other comprising the RNase III and dsRBD domains (C-terminal), could still produce 22-bp products, although both fragments lacked a portion of the linker connecting the PAZ and RNase III domains (31). Thus, one can hypothesize that the lengths of Dicer-generated products are determined by the nature of PAZ and RNase III domain interactions, which may occur either directly or through an RNA substrate.

The ribonuclease Dicer is found throughout eukaryotes but is absent in bacteria and archaea. It has been suggested that the Dicer family has independently diversified in animal, plant and fungal lineages (38,39); however, it has been lost from some protozoan parasites (e.g. *Leishmania major* and *Trypanosoma cruzi*) and some fungi (e.g. the model organism *Saccharomyces cerevisiae* and other closely related yeasts) (40,41). Current evidence suggests that the Dicer gene underwent duplication early during animal and plant evolution, presumably coinciding with the origin of multicellularity, giving rise to two distinct groups in animals (Dicer-1 and Dicer-2) and to four groups in plants (Dicer-like (DCL) proteins DCL-1 to DCL-4). In insects, Dicer-1 and Dicer-2 have been shown to recognize distinct substrates and to generate different classes of small RNAs; i.e. miRNAs and siRNAs, respectively (42). Furthermore, Dicer-2 has been shown to cleave cell-derived dsRNA precursors to produce endogenous siRNAs (43), in addition to double-stranded virus-derived RNAs to produce exogenous siRNAs (44). Accordingly, an essential function of Dicer-2 in host defense against RNA viruses has been documented for *Drosophila* (44). Interestingly, Dicer-2 was subsequently lost from lineages that developed alternative antiviral strategies, such as vertebrates (39).

Since its discovery in 2000 (14), the ribonuclease Dicer has been considered to be one of the key factors responsible for the production of small regulatory RNAs. Consequently, a number of articles thoroughly discussing various aspects of Dicer involvement in the biogenesis of miRNAs and siRNAs have been published. However, an overall understanding of the gene expression and protein functions of this enzyme is still far from being elucidated. Thus, this review summarizes the current knowledge of various factors involved in the regulation of Dicer gene transcription, primary transcript processing, mRNA translation and enzyme activity. Because of the high complexity of the topics that are discussed, we mainly focus on human Dicer. Finally, as mutations in the Dicer gene may generate a range of phenotypes that are correlated with many types of cancer, we also discuss the association of Dicer with cancer.

ORGANIZATION OF THE DICER GENE AND FACTORS CONTROLLING ITS EXPRESSION

In all vertebrates, only one gene encoding the Dicer protein has been identified; however, its chromosomal

location, as well as the number of exons it contains, varies between species. The human Dicer-coding gene (*DICER1*) is located on chromosome 14 and contains 26 protein-coding exons and a few non-protein-coding exons. The latter form the 5′-untranslated region (5′-UTR); (http://www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000100697;r=14:95552565--95624347). The organization of *DICER1* is presented in Figure 1A. The dominant initiation codon (AUG) is found within exon 2 according to the nomenclature of exons adopted by Marsden *et al*. (indicated in black in Figure 1) (45) and it is found within exon 4 according to the nomenclature adopted by Irvin-Wilson and Chaudhuri (indicated in red in Figure 1) (46).

Many factors can regulate the transcription of *DICER1*. These factors can be universal or cell-, tissue- or stage-specific. For instance, SOX4, a transcription factor involved in the regulation of embryonic development and in the determination of cell fate, positively regulates the expression of the Dicer gene by binding to its promoter and enhancing its activity (47). SOX4 is also a well-known oncogene, and its overexpression is observed in many types of cancers (48–50), including prostate cancer (51). Accordingly, in human prostate cancer cells, the level of Dicer mRNA has been found to be upregulated (52). Another report on the transcriptional regulation of Dicer gene expression has shown that upon melanocyte differentiation, the melanocyte master transcriptional regulator, microphthalmia-associated transcription factor (MITF), binds to and activates a conserved regulatory sequence located upstream of the transcriptional start site of the Dicer gene, thereby stimulating its expression in these cells (53). It is also not surprising that within the promoter of the Dicer gene, target sequences for the ubiquitous tumour suppressors p53 and p63 have been identified (54). Nevertheless, the current knowledge of transcription factors influencing the expression of the Dicer gene is still very limited. The complexity of this process is highlighted by the fact that the level of Dicer transcripts is not always correlated with its protein level, implying that the regulation of its expression may occur at the post-transcriptional level (55). For example, Wiesen and Tomasi have used different human and mouse cell lines to demonstrate that histone deacetylase inhibitors only modestly alter Dicer mRNA levels but substantially decrease its protein levels, possibly by activating cellular stress-response pathways (55). These authors have also shown that at the protein level, Dicer expression may be downregulated by ∼4000 bp-long dsRNAs and interferon alpha (type I interferon) and upregulated by interferon gamma (type II interferon).

Currently, it is clear that from one Dicer gene, multiple Dicer transcript variants can be produced as a result of the initiation of transcription from alternative promoters and alternative splicing (Figure 1B). Four mRNA variants that encode full-length human Dicers (comprising 1922-amino acid residues) have been identified to date (http://www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000100697;r=14:95552565--95624347). These four variants differ in their 5′ and 3′ non-protein-coding sequences, which contain different regulatory elements, while their coding regions remain unchanged. In addition, numerous shorter alternative splice variants have been found. Some of these splice variants encode proteins retaining only the N- or C-terminus of Dicer, while some variants do not encode any protein. Four antisense transcripts associated with *DICER1* have also been identified, ranging from ∼720 to 2300 nt in length (http://www.ensembl.org/Homo_sapiens/Transcript/Summary?db=core;g=ENSG00000235706;r=14:95643820--95646262;t=ENST00000439999). Their functions have not yet been established. However, it has recently been reported that antisense transcripts may regulate the activity of the genes from which they are derived (56,57).

Two research groups, Marsden *et al*., working with various healthy human tissues (45), and Irvin-Wilson and Chaudhuri, working with human breast cells (46), have extensively analysed the organization of the 5′-UTRs of Dicer mRNAs. Both of these groups have demonstrated that the observed diversity within these 5′-UTRs is associated with the tissue- and developmental-specific expression of the Dicer gene. Moreover, they have found that its organization impacts the translational efficiency of Dicer mRNA. The results of the very first studies of Dicer transcripts, which were conducted by Hamaguchi *et al*., suggested that many potential upstream open reading frames are present within the 5′-UTR (58). This issue was also assessed by Marsden *et al*. (45) and by Irvin-Wilson and Chaudhuri (46). The latter authors have reported that Dicer transcripts isolated from human breast cells have a high number of upstream start codons in-frame with stop codons. Such a composition of the 5′-UTR has been shown to decrease the stability of Dicer transcripts. These authors have also found that in healthy human breast cells, two predominant 5′-UTR variants of *DICER1* mRNA are produced (compare transcript no. (1) and (2) in Figure 1B). The shorter variant, which lacks the two exons with additional AUG codons (exons 2 and 3, according to the nomenclature adopted by Irvin-Wilson and Chaudhuri), has been shown to be more efficiently translated in an *in vitro* reporter system (46). These authors have also shown that in human breast cells, instead of the previously identified promoters (58), an alternative promoter located far upstream is employed for Dicer gene expression (46) (compare transcript nos. (1–2) and (3) in Figure 1B). In addition, Marsden *et al*. have identified unique 5′-UTRs formed by a combination of three leader exons (1A, 1B and 1C) and three alternatively spliced exons (AS1, AS2 and AS3) (Figure 1A and B). Furthermore, these authors have demonstrated *in vitro* that the presence of any of the three alternatively spliced exons (AS1, AS2 or AS3) in the 5′-UTR decreases the translation of the reporter gene. However, the lowest translation efficiency has been detected for transcripts containing the leader exon 1B, which is expressed from the alternative long-distance CpG island promoter (45) (transcript nos. (4–6) in Figure 1B; exon 1B is boxed in bold).

Reports of the alternative processing of human Dicer pre-mRNA, as discussed above, have also revealed that shorter Dicer mRNA variants are produced in differentiated epithelial cells and in a number of cancer cell lines (59,60). An interesting splice variant of the human Dicer gene has been identified in neuroblastoma cells (61). In this variant, one of the protein-coding exons is skipped and as a result,

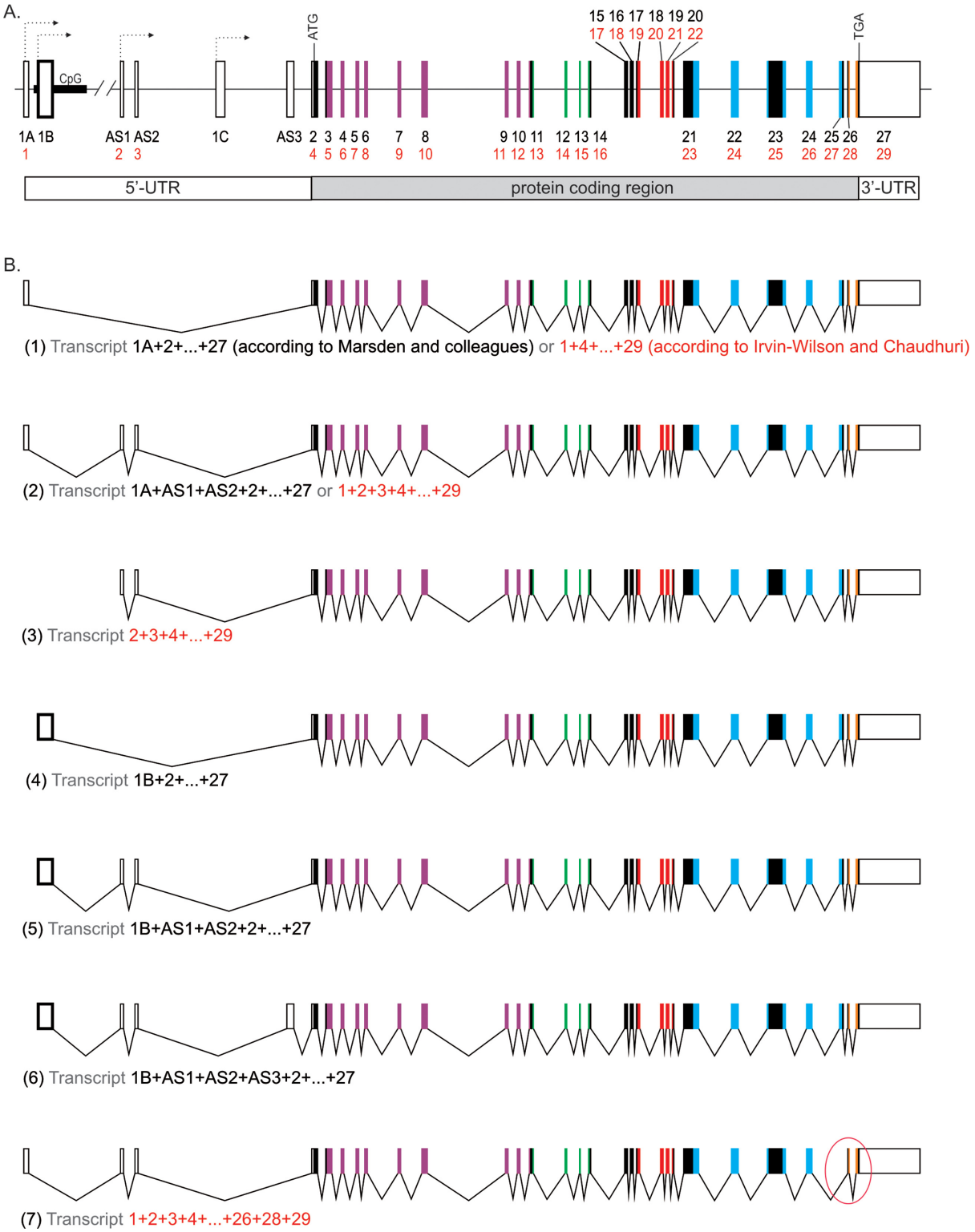

Figure 1. Schematic diagram of (**A**) the organization of the human Dicer gene and (**B**) exemplary Dicer transcript variants. The nomenclature of exons adopted by Marsden *etal*. (45) is indicated in black and that adopted by Irvin-Wilson and Chaudhuri (46) is indicated in red. The non-protein-coding exons, indicated by open boxes, form the 5′-UTR and are described by Marsden *etal*. as variants of exon 1 (leader exons 1A, 1B and 1C; and alternatively spliced exons abbreviated AS—AS1, AS2 and AS3), while those described by Irvin-Wilson and Chaudhuri are numbered 1, 2 and 3. The alternative transcription initiation sites are indicated by arrows. Exon 1B is boxed in bold and is located within a genomic sequence containing a CpG island. Fragments of exons coloured in purple encode a helicase domain, whereas the green fragments encode a DUF283 domain, the red fragments encode a PAZ domain, the blue fragments encode both RNase IIIa and RNase IIIb domains and the orange fragments encode a dsRBD domain. The transcript variant no. (7), for which one of the protein-coding exons is skipped (the region circled in red), encodes a Dicer protein lacking the majority of its RNase IIIb domain. A detailed description can be found in the text.

the reading frame is altered and a premature stop codon is gained (transcript no. (7); region circled in red in Figure 1B). Consequently, a protein that is 93 amino acid residues shorter than the full-length Dicer and differs in the last 41 C-terminal amino acids is produced (lacking one of the two RNase III domains and the dsRBD) (61). It is not clear whether this truncated protein is a hallmark of neuroblastoma development or whether it plays a role in differentiation and tumorigenesis. The influence of the dysfunction of a single RNase III domain on Dicer activity has been studied by Sharp *et al*. (62) and Amatruda *et al*. (63). These groups have observed that this enzyme can retain partial activity even if one RNase III domain is inactive. Specifically, Dicer with a dysfunctional RNase IIIa domain fails to produce miRNAs from the 3′-arm of pre-miRNA hairpins (62), whereas Dicer with an affected RNase IIIb domain does not produce miRNAs from the 5′-arm of pre-miRNA hairpins (62,63). Those results provide insights into the mechanisms by which mutations in Dicer may affect the level of miRNA expression and thus trigger tumorigenesis.

Some authors considered the Dicer gene to be a housekeeping gene (64); however, compared with other housekeeping genes, its 3′-UTR-encoding fragment has been shown to be unusually long (>4000 bp) (64). In Dicer mRNA, this region is involved in the post-transcriptional regulation of gene expression. The 3′-UTR of Dicer mRNA can be targeted by several miRNAs, e.g. miR-103/107 (59,60,64), miR-192 (65) or members of the let-7 miRNA family (60,66). Interestingly, target sequences for let-7 miRNAs have also been found within the coding regions of Dicer transcripts (66). Furthermore, Mayr and Bartel have identified various polyadenylation signals at the 3′ ends of Dicer pre-mRNAs in multiple cancer cell lines (67). These authors have shown that in cancer cells, the production of Dicer from a more abundant mRNA with a shorter 3′-UTR is several times greater than that from an mRNA with a longer 3′-UTR (67). Shorter mRNAs that lack large 3′-UTR fragments, which contain a number of miRNA-binding sites, cannot be targeted by RNA-silencing machinery. Thus, the regulation of the level of Dicer mRNA by at least some miRNAs is lost in cells carrying such shortened transcripts. The problem associated with the use of alternative polyadenylation sites within Dicer pre-mRNAs has also been discussed by Kanaoka *et al.* (68). These authors have demonstrated that in colorectal cancer, primary Dicer transcripts are polyadenylated at two alternative sites, giving rise to two transcript isoforms with either a long or truncated 3′-UTR (68).

Another report concerning Dicer transcripts has postulated that Dicer mRNA may specifically interact with exportin-5, a nuclear receptor involved in the export of certain classes of RNAs, including pre-miRNAs, viral hairpin RNAs and some tRNAs (69). All of these RNAs compete for binding to exportin-5. Thus, the saturation of this receptor with other RNAs decreases the export of Dicer mRNA to the cytoplasm and as a consequence, reduces further Dicer gene expression.

It has also been found that the level and/or activity of human Dicer can be regulated by its post-translational modification, e.g. phosphorylation (70) and SUMOylation (71). Relationships among phosphorylation, a major cell signalling pathway (the Ras pathway), and the core miRNA machinery have been previously demonstrated for a Dicer-interacting protein, the trans-activation response RNA binding protein (TRBP) (72). Very recently, Arur *et al*. have shown that Ras signalling also results in Dicer phosphorylation during oogenesis in *Caenorhabditis elegans* (73). These authors have demonstrated that extracellular signal-regulated kinase (ERK) phosphorylates Dicer (within the RNase IIIb and dsRBD domains) during most of oogenesis and that this phosphorylation is necessary and sufficient to trigger Dicer's nuclear translocation in worm, mouse and human cells (73). In addition to inducing nuclear localization, Dicer phosphorylation has also been found to inhibit its function (73). Interestingly, Dicer has been observed to be rapidly dephosphorylated just before fertilization (73).

Dicer also contains several potential lysine SUMOylation sites (71). It has been demonstrated that cigarette smoke may alter alveolar macrophage miRNA production by Dicer SUMOylation. The latter process has been found to decrease Dicer activity, resulting in the massive downregulation of miRNAs and presumably promoting smoking-related diseases (71). These findings demonstrate that environmental exposure may cause changes in miRNA levels via the post-translational modification of Dicer.

Dicer has also been proposed to be a glycoprotein (74). In addition to evidence that Dicer is translocated through the endoplasmic reticulum, it has been suggested that glycosylation of this enzyme may play a role in maintaining its intracellular level (74). The conjugation of carbohydrate chains to proteins may contribute to their proper folding; however, the functional implications of this modification in the case of Dicer remain elusive and require further investigation.

DICER EXERTS ITS FUNCTIONS AS PART OF LARGE MULTIPROTEIN COMPLEXES

Although Dicer alone is capable of cleaving pre-miRNA and long dsRNA to miRNA and siRNA, respectively, its catalytic activity is known to be modulated by associated proteins, particularly by two closely related proteins, TRBP and protein activator of protein kinase R (PACT) (35,75,76). These two Dicer-binding proteins are important regulators that contribute both substrate and cleavage specificity during small regulatory RNA production. In particular, it has been reported that Dicer, in association with PACT, preferentially cleaves pre-miRNAs rather than precursors of siRNAs (77). This discrimination is less pronounced when Dicer is coupled with TRBP (77). These findings are consistent with those of another study, showing that *in vitro* PACT binds siRNA at a lower affinity than TRBP (78). Moreover, both proteins not only influence substrate discrimination by Dicer but also may change the cleavage site, thereby triggering the generation of different sized iso-miRNAs (isomiRs) (77,79). Additionally, the direct binding of TRBP to pre-miRNA substrates has been proposed to increase the initial rate of substrate recognition by Dicer (80). TRBP has also been shown to increase the stability of Dicer/substrate complexes and all of these components together presumably stimulate dicing (80). Further-

more, evidence indicates that TRBP, in association with Dicer, contributes not only to RNA substrate binding and product length determination (79–83) but also to the assembly of larger multiprotein complexes, including the RNA-induced silencing complex (RISC) and RISC-loading complex (RLC) (22,75,76,84). Similar roles have been proposed for PACT (35); however, its specific function is still poorly understood. RISC is a central effector of RNA-silencing pathways (85–87). The functional core of every RISC is composed of a member of the Argonaute (Ago) protein family and a small regulatory RNA (88,89). The latter guides the RISC to a target RNA transcript, permitting its binding through Watson–Crick base pairing (87). Maniataki and Mourelatos have proposed that Dicer, in association with Ago2, binds to and cleaves pre-miRNA. Next, the resultant miRNA duplex is passed along to Ago2 via the cooperation of Dicer and TRBP (90). In humans, Dicer, TRBP and Ago2 form the core of the RLC (84,87). Electron microscopy and single particle image analysis of a reconstituted RLC have revealed that within the ternary complex, the Dicer N-terminal helicase domain interacts with TRBP, while the C-terminal catalytic domains of RNase III are proximal to Ago2 (22). Interestingly, Doudna *et al*. have shown that *in vitro*, the duplex generated by Dicer cleavage may be released from the Dicer/TRBP complex and may rebind in a different orientation in the helicase domain of Dicer before it is loaded onto Ago2 (91). The fate of the RLC following the loading of Ago2 with duplex RNA is elusive. Some research groups have suggested that the complex remains intact (35,87) and that the presence of Dicer and TRBP stimulates target RNA processing by Ago2 (75,87). Other groups either have reported the dissociation of Dicer from Ago2 after the latter is loaded with an RNA duplex (90) or have assumed that Dicer does not participate directly in the slicer activity of the RISC (84). Thus, the role of Dicer in coupling miRNA biogenesis and post-transcriptional gene silencing remains unclear and requires further investigation. Notably, it has been suggested that Dicer may not be required for the assembly of siRNA-containing RISCs *in vivo* in mice (92) and *in vitro* in humans (93). Hence, it is possible that the mechanisms of RISC assembly in the miRNA and siRNA pathways are different.

It has also been demonstrated that RISC or RISC-like complexes can operate in nucleus (94,95). However, the data collected indicate that nuclear RISC is much smaller than the cytoplasmic RISC complex. The estimated size of the former one is close to the size of the single Argonaute protein (94). Thus, one can assume that the nuclear RISC complex lacks Dicer.

Another protein partner that can modulate the activity of Dicer is adenosine deaminase acting on RNA 1 (ADAR1), which catalyzes the adenosine-to-inosine editing of dsRNA and pri- and pre-miRNAs (34,96–98). According to current data, the editing activity of ADAR1 is attributed to its homodimer form, whereas heterodimers of ADAR1 and Dicer do not exhibit editing activity (96). It has been further demonstrated that ADAR1 forms a complex with Dicer through a direct protein–protein interaction involving the DUF283 and DEAD-box RNA helicase domains of Dicer (96). As mentioned above, the helicase domain has also been reported to be involved in interacting with TRBP (22,76,99), indicating that TRBP and ADAR1 associate with at least one common Dicer domain. In addition, it has been postulated that ADAR1 increases the rate of substrate cleavage by inducing conformational changes in Dicer (96).

NON-MIRNA-RELATED INTERACTION NETWORK OF DICER

Early studies of RNA interference (RNAi) phenomena and miRNA biogenesis pathways have led to the prevailing concept that Dicer is localized solely to the cytoplasm (100). In addition, several research groups have demonstrated that a small pool of Dicer co-purifies with membranes (74,76,101). More recently, it has been shown that mammalian Dicer can also function in the nucleus, where it has been found to be associated with nuclear ribosomal DNA (rDNA) chromatin, precisely interacting with the transcribed and promoter regions of rDNA repeats (102). Nevertheless, in mammals, the roles of these interactions remain unclear. It is possible that the association of Dicer with rDNA repeats preserves the stability of this region (102). Such a role of Dicer analogues has been previously shown in flies (103) and yeast (104). In various species, Dicer has also been demonstrated to link the RNAi pathway to heterochromatin assembly (105–111). According to the model proposed by Grewal *et al*. for yeast, nuclear Dicer generates siRNA associated with a nuclear Argonaute complex, termed the RNA-induced transcriptional silencing complex (RITS) (108,109). In addition to Argonaute proteins, the RITS contains histone-binding and adaptor proteins. It recruits histones and histone-modifying enzymes to target chromatin and generates repressed chromatin structures in a process called transcriptional gene silencing (108,109). Furthermore, it has been demonstrated in mammalian cells that a reduction in the Dicer level results in a more open chromatin structure due to a decrease in methylation, an increase in the acetylation of histones, and the loss of the chromatin-bound Argonaute proteins (112,113). Likewise, a depletion of Dicer in a human embryonic cell line has been shown to activate chromatin at the *PHLDA2* locus, an important tumour suppressor gene region (114). The observed phenomenon was linked to the changes in the level of histone acetylation but not to the methylation state of the locus (114). It was later suggested that human Dicer, under normal conditions, is recruited to loci of endogenous overlapping transcription through association with RNA polymerase II and dsRNA (110). Dicer then co-transcriptionally cleaves dsRNA into siRNA, leading to Ago1 recruitment (110). All of these events prevent endogenous dsRNA formation from overlapping non-coding RNA transcription units, precluding an uncontrolled interferon response and cellular apoptosis (110).

Interestingly, Argonaute and Dicer proteins have also been shown to be able to affect splicing (112,115). Most splicing events occur co-transcriptionally (116,117). Batsche *et al*. have proposed a model in which nuclear Argonaute proteins are guided to the vicinity of potential splice sites by Dicer-generated small RNAs interacting with intragenic antisense transcripts synthesized by RNA polymerase II. Argonaute proteins, in association with guiding RNAs, are recruited to the spliceosome complex through interac-

tions with proteins bound to pre-mRNAs that are also produced by RNA polymerase II (112). As a result, transcriptase activity slows, facilitating spliceosome recruitment and inducing splicing events (112).

Recent studies have also revealed that Dicer is involved in the cell response to double-strand breaks (DSBs) in both animals and plants (118,119). Chromosome damage can occur, for example, due to oncogenic stress, ionizing radiation or the activities of site-specific endonucleases (118). In these situations, the efficient repair of DSBs is critical for the maintenance of genome integrity and cell survival. Dicer presumably processes RNAs formed by the transcription of broken DNA ends (120). It has been demonstrated in Arabidopsis and human cells that 21-nt small RNAs are produced from sequences flanking DSB sites (119). These small RNAs have been named diRNAs, which stands for DSB-induced small RNAs. It has been postulated that diRNAs function as guide molecules that direct chromatin modification or recruit protein complexes that facilitate DSB repair (119).

One recent report has also demonstrated that Dicer, together with PACT, TRBP and PKR, acts as a co-regulator of nuclear receptors (121), establishing a connection between core miRNA machinery and nuclear receptor signalling networks. These RISC components have been shown to interact with steroid receptor RNA activators, which recruit RISC proteins to steroid-responsive promoters, where they regulate the expression of downstream genes (121).

It is also important to mention that loss of Dicer in mouse oocytes has been shown to be associated with severe chromosome congression defects, presumably due to disorganized spindle formation (122,123). Moreover, Dicer depletion in mouse oocytes has been linked with upregulation of some retrotransposon families (122). Thus, Dicer seems to be a crucial player in the regulatory network that controls oocyte gene expression programs and integrity of the oocyte genome.

Dicer-interacting factors also include a group of viral proteins. Some RNA viruses, such as hepatitis C virus or human immunodeficiency virus, produce proteins that may inhibit the activity of Dicer; hence, these proteins have been termed viral suppressors of RNA silencing. These viral proteins may directly interact with Dicer (e.g. HCV core protein (124,125) or HIV-1 transactivator of transcription (126,127)), presumably with its N-terminal helicase domain, blocking its interactions with other protein partners, such as TRBP or ADAR1 (126,127). In addition, viral proteins may mediate the proteasomal degradation of Dicer, such as HIV-1 protein R (128).

Interactions between Dicer and other proteins can also influence the specificity of its action (129–131). For example, it has been demonstrated that the Dicer C-terminus functions as a 5-lipooxygenase (5LO)-binding domain (129). The association of these two proteins not only enhances the catalytic activity of 5LO but also modifies Dicer processing specificity towards pre-miRNAs, favouring the production of ∼55-nt and ∼10- to 12-nt-long RNA species (129). It is worth noting that years ago, Dicer cDNA clones were isolated from a yeast two-hybrid screen using 5LO as a bait (132). In humans, 5LO is mainly expressed in differentiated inflammatory cells, where it catalyzes the first two steps in the biosynthesis of potent inflammation mediators called leukotrienes (133). The exact nature and impact of Dicer-5LO interactions remain to be determined; nevertheless, the existing evidence provides a link between Dicer and inflammatory processes.

Several reports have shown that Dicer is subject to caspase-dependent degradation during apoptosis in human cells *in vitro* (130,134) and in *C. elegans* (131). The caspase-mediated cleavage of Dicer results in the release of a C-terminal Dicer fragment containing RNase IIIb and dsRBD. This C-terminal Dicer fragment cannot naturally produce functional miRNAs and lacks the majority of domains essential for binding to other proteins and factors. Instead, in *C. elegans*, this C-terminal fragment acts as a deoxyribonuclease to cause breaks in chromosomal DNA (131). Nevertheless, it is not clear whether the human ribonuclease Dicer also participates directly in chromosome fragmentation following caspase cleavage. Interestingly, the caspase-dependent cleavage of human Dicer has been observed during the late stages of HIV-1 infection *in vitro* (130).

Obviously, the Dicer–protein interaction network is still being delineated. The current list of involved proteins is summarized in Figure 2 and Supplementary Table S1.

FACTORS INTERACTING WITH DICER SUBSTRATES

Although the structure of pre-miRNA itself may influence the specificity of precursor cleavage by Dicer (135), an additional regulatory layer of Dicer activity is indicated by the interactions of factors with its substrates. Daley *et al.* have demonstrated that Lin28, a highly conserved RNA-binding protein, selectively blocks the processing of let-7 pre-miRNAs by Dicer through specific binding with these precursors (136,137). Furthermore, Gregory *et al*. have shown that a conserved cytosine residue in the loop of pre-let-7g is essential for Lin28 binding (138). In contrast, Kim *et al*. have emphasized a role of a tetranucleotide sequence motif (GGAG) present in the terminal loop of let-7 miRNA family precursors in their selective binding with Lin28 (139). These authors have demonstrated that Lin28 recruits a terminal uridylyl transferase (TUTase) to let-7 pre-miRNAs. This noncanonical polymerase adds an oligouridine tail to let-7 pre-miRNAs. These uridylated precursors cannot be efficiently processed by Dicer, and they are subsequently targeted for degradation. Lin28-dependent uridylation has also been reported for several other pre-miRNAs that contain the GGAG sequence motif in their apical loops (139,140). A more recent report has indicated that up to three Lin28 molecules assemble in a stepwise manner at the terminal loop region of let-7 miRNA family precursors, thereby efficiently inhibiting their processing by Dicer (141).

A novel mechanism that controls Dicer activity through the Lin28/let-7 axis has been recently described by Varelas *et al*. (142). This mechanism involves two major effector proteins of the Hippo kinase signalling pathways, TAZ and YAP (142). The Hippo pathway is an evolutionally conserved signalling cascade that controls organ size and stem cell fate through the regulation of cell prolifer-

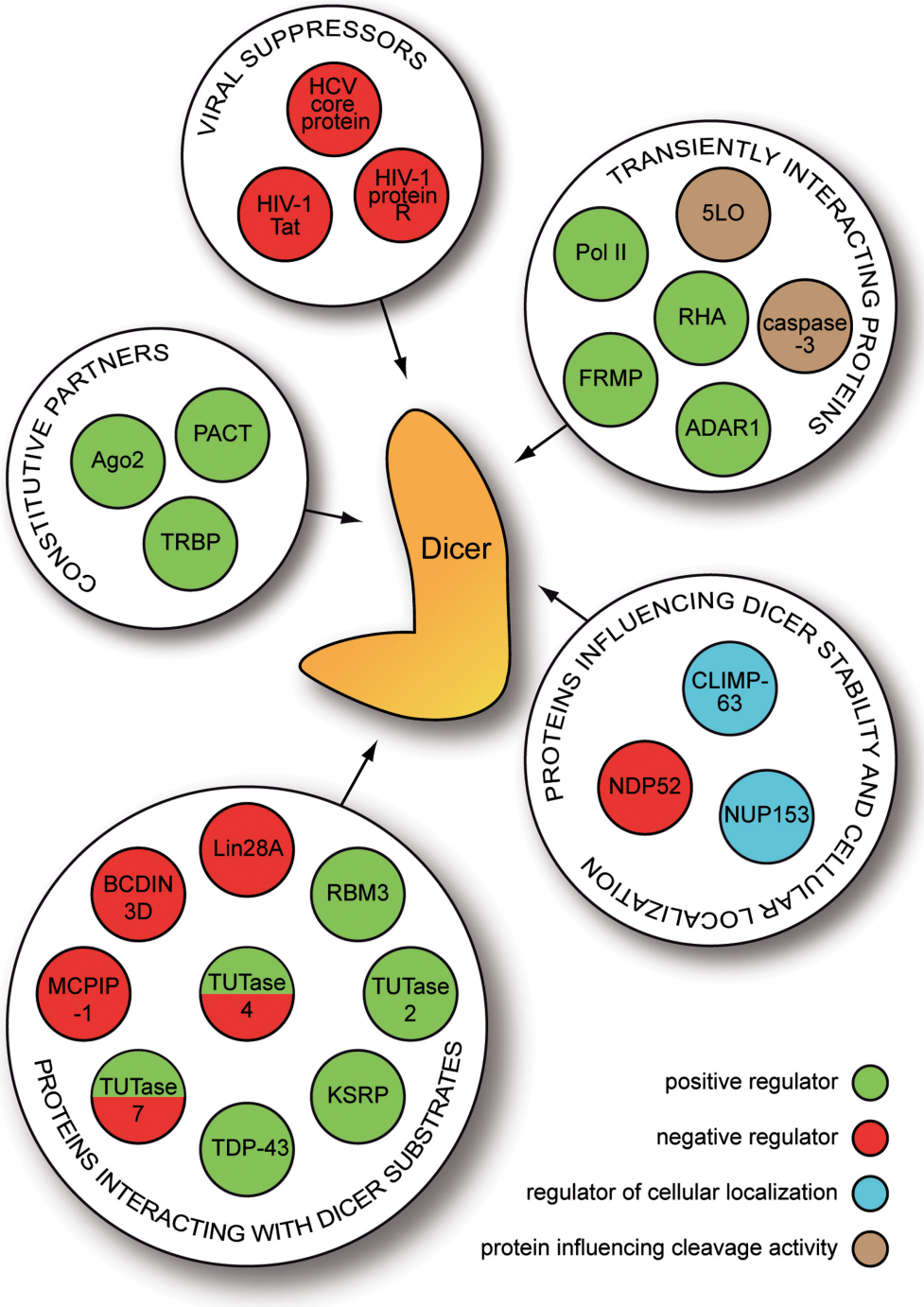

Figure 2. Proteins interacting with human ribonuclease Dicer and its substrates. 5LO—5 lipoxygenase, ADAR1—adenosine deaminase acting on RNA 1, Ago2—Argonaute protein 2, BCDIN3D—BCDIN3 Domain-Containing Protein, CLIMP-63—cytoskeleton-linking membrane protein 63 kDa, FMRP—fragile X mental retardation protein, HIV-1 Tat—HIV-1 transactivator of transcription, KSRP—KH-type splicing regulatory protein, MCPIP-1—monocyte chemoattractant protein-induced protein 1, NDP52—nuclear dot protein 52 kDa, NUP153—nucleoporin 153, PACT—protein activator of interferon-induced protein kinase, Pol II—RNA polymerase II, RBM3—RNA binding motif protein 3, RHA—RNA helicase A, TDP-43—TAR DNA-binding protein-43, TRBP—HIV-1 trans-activation response element RNA-binding protein, TUTase 2/4/7—3′ terminal uridylyl transferase 2/4/7. Summary description of Dicer interacting proteins can be found in Supplementary Table S1.

ation and apoptosis (143,144). The depletion of nuclear TAZ/YAP through the activation of the Hippo pathway results in the reduction of Lin28 and the increased expression of let-7 miRNAs (142). Consequently, let-7 miRNAs can target Dicer mRNA by the mechanism discussed earlier and downregulate its expression, affecting global levels of miRNAs (142).

In contrast with Lin28, a KH-type splicing regulatory protein (KSRP) has been determined to positively influence let-7 miRNA biogenesis (145–147). KSRP is a multifunctional protein that plays roles in the decay (148,149), splicing (150) and localization (151) of certain mRNAs. It has also been reported as a component of Drosha and Dicer complexes in cultured cells (146). It can interact with guanosine-rich motifs located in the apical loops of some miRNA precursors to promote their processing (145,146,152–154), possibly by optimizing the positioning and/or recruitment of miRNA-generating complexes (146).

It has also been demonstrated that the processing of certain pre-miRNAs occurs under the control of TAR DNA-binding protein-43 (TDP-43). TDP-43 interacts with Dicer and promotes the processing of some pre-miRNAs by binding to their terminal loops (155). A recent study has shown that TDP-43 preferentially binds to UG- and pyrimidine-rich sequences (156–158). However, it has also been reported that UG repeats are neither necessary nor sufficient for TDP-43 binding (159) and that TDP-43 cannot bind to UG repeats in the dsRNA region of pre-miR-574. These findings indicate that the RNA secondary structure also plays an important role in target recognition by TDP-43 (155).

The importance of the pre-miRNA structure in the regulation of Dicer activity has been further highlighted in a recent study performed by Wang *et al*. (34). These authors have shown that the products of pre-miR-151 editing by the ADAR family protein (varying in their apical loop structures) induce different conformational changes in the Dicer helicase domain upon binding. The characteristics of these changes are correlated with the dicing activity of the enzyme (34).

Another protein targeting the terminal loop of pre-miRNAs is the mammalian immunoregulator MCPIP1 (monocyte chemoattractant protein [MCP]-1-induced protein 1) (160). Miyazono *et al*. have demonstrated that MCPIP1 suppresses miRNA biosynthesis by cleaving the apical loops of pre-miRNAs. These authors have proposed that the loops of some miRNA precursors contain regulatory elements that can activate their degradation (160). The broader list of proteins that interact with Dicer substrates is presented in Supplementary Table S2.

Among the elements that affect Dicer activity through interaction with its substrates there is also a group of non-protein factors. For example, it has been reported that the *in vitro* dicing of guanosine-rich short-hairpin RNAs can be inhibited by quadruplex-binding compounds, such as certain phorphyrazines and bis-quinolinium (161). Dicer activity can also be influenced by the binding of RNA molecules other than substrates to this enzyme. Very recently, human transcriptome-wide analysis has identified so-called 'passive' Dicer binding sites (162). These sites are preferentially located in coding sequences and 3′-UTRs that adopt stem-loop structures. The latter, however, are different from the structures of typical pre-miRNAs. Dicer has been shown to be capable of binding but not of cutting passive sites. Interactions with Dicer stabilize RNAs carrying passive sites. Passive binding has also been proposed to serve as an anchoring mechanism for the efficient assembly of protein complexes. In addition, passive sites may function as a buffering system to control the catalytic activity of the enzyme by sequestering it from other targets. A similar strategy, based on Dicer sequestering, is utilized by viruses to mislead host defence mechanisms. For example, adenoviruses protect their RNAs by producing high amounts of long self-complementary transcripts that effectively compete for Dicer binding with other endogenous Dicer substrates. As a result, pivotal viral transcripts are not cleaved (163). Likewise, *in vitro* studies conducted by our group have indicated that the activity of human Dicer can be affected by short RNA molecules that are bound to it (164). Detailed studies have revealed that short RNAs can not only act as competitive or allosteric inhibitors of Dicer but can also influence this enzyme by base pairing with its substrates (165). We have found that RNA oligomers that can simultaneously bind both Dicer and its substrates are selective and effective inhibitors of pre-miRNA processing. Furthermore, we have demonstrated that RNAs as short as 12 nt promote the selective inhibition of complementary pre-miRNA cleavage by Dicer (165). The results of several recent studies support our observations that RNA may function as both a substrate and a regulator of miRNA pathway components. For example, Pasquinelli *et al*. have identified an interesting auto-regulatory loop that controls let-7 miRNA biogenesis in *C. elegans* (166) involving a protein called ALG-1 (Argonaute-like protein-1) that binds to a specific site at the 3′ end of let-7 primary transcripts (let-7 pri-miRNAs), promoting the processing of these precursors. The interaction between ALG-1 and let-7 pri-miRNA is mediated by mature let-7 miRNA through a conserved complementary site in let-7 pri-miRNA. Therefore, mature miRNAs may target and regulate the processing of their precursor non-coding RNAs. This finding of the auto-regulation of let-7 miRNA biogenesis provides novel insights into the mechanisms controlling miRNA production. Interestingly, Provost *et al*. have reported the discovery of 12-nt-long RNA species corresponding with the 5′ regions of miRNAs termed semi-miRNAs (smiRNAs) (167). The data collected by these authors suggest that smiRNAs can compete with miRNAs for binding sites within target UTRs. Thus, smiRNAs may represent a novel class of small non-coding RNAs generated along the miRNA pathway that are capable of regulating the activities of the miRNAs from which they are derived.

The cytoplasm of cells contains RNA molecules of different sizes and types, including a fraction of small regulatory RNAs. Accordingly, short sequence motifs influencing Dicer activity may occur in large functional RNAs, e.g. in mRNAs or in the stable intermediates formed during their degradation. The theory that stable intermediates of RNA degradation can accumulate in the cell and function as signalling molecules or participate in mechanisms that control cellular pathways has been discussed extensively by Figlerowicz *et al*. (168). Moreover, it has been demonstrated that products of RNA degradation may be

involved in regulatory processes occurring in cells (169,170). Interestingly, we have also identified transcripts whose fragments display substantial similarity to oligomers that bind to human Dicer and affect its activity (164). Consequently, recent findings have indicated that mutual interactions between miRNA precursors and other RNAs may form a very complex regulatory network that controls miRNA biogenesis and subsequent gene expression.

HUMAN DICER AND CANCER

The human Dicer gene is located within the subtelomeric region 14q32.13 that has been reported to be significantly affected by various mutations (common mutations, epimutations and copy number variations) (171,172). Ample evidence shows that mutations of these types may alter *DICER1* expression and/or resultant protein activity and consequently initiate pathological processes (173–175). Although a number of such mutations have been identified to date, pathomechanisms of Dicer-mutation-mediated diseases are still poorly understood. This fact is well exemplified by neoplastic diseases and has been recently thoroughly discussed by Foulkes, Priest and Duchaine (176). Thus, here we would like to point out only the most important issues.

There is no clear correlation among *DICER1* expression, cancer type and disease progression. For example, significant changes in *DICER1* expression have been detected during different stages of lung adenocarcinoma (177). A transient upregulation in Dicer gene expression has been observed during the early stages of lung adenocarcinoma, whereas it is downregulated during the more advanced stages of this cancer (177). In addition, the reduced expression of *DICER1* may be associated with poor prognosis in some types of lung cancers (178). In contrast, its expression has been shown to be increased in prostate adenocarcinoma cancer (179) and Burkitt's lymphoma (180). The levels of Dicer mRNA/protein accumulation in select cancers are shown in Supplementary Table S3.

There are also many controversies whether *DICER1* acts as a tumour suppressor or an oncogene (181). In humans, both constitutional (Supplementary Figure S1) and somatic (Supplementary Table S4) *DICER1* mutations have been identified The former are inherited (germline mutations) and present in every cell and the latter are not inherited and occur in some, usually small, fraction of cells or even in a single cell. Early studies involving mouse models suggested that *DICER1* functions as a haploinsufficient tumour suppressor (182,183). The haploinsufficiency mechanism proposed for *DICER1* postulates that the constitutional mutation inactivating one allele of *DICER1* (a heterozygous germline mutation), is initiatory and predisposes a cell to neoplastic disease; however, some other events are also required to induce tumorigenesis (184). Such constitutional, loss-of-function mutations have been found throughout *DICER1* (176,184) and they have been proposed to lead to the so called *DICER1* syndrome associated with various, usually early childhood cancers (184). Recently, several reports that support another model of Dicer-mutation-mediated tumourigenesis, so called 'two-hit' tumor suppressor model, have been published (173,175). This model is based on a classic Knundon's hypothesis assuming that two hits are needed to inactivate a tumor suppressor gene (185). The first hit inactivates one allele and occurs either in somatic cell (sporadic cancer) or in germline cell (hereditary cancer). The second hit, affecting a remaining wild-type allele, is always a somatic mutation. Interestingly, in case of *DICER1*, most of the second hit mutations have been found in the RNase IIIb domain (173,175,186–192). These mutations usually inactivate RNase IIIb only and specifically change Dicer activity. As mentioned earlier, Dicer lacking the functional RNase IIIb domain fails to cleave miRNAs located in the 5′-arms of pre-miRNA hairpins. As a result, miRNAs located in the 3′-arms of pre-miRNA hairpins are mainly produced (62,174).

The recent findings are consistent with the observations that certain families of miRNAs are indispensable for tumor cell development (11,193,194) that is why the inactivation of both *DICER1* alleles suppresses cancer (183). Thus, one can hypothesize that a selective pressure operating in cancer cells acts against complete loss of *DICER1* and supports the appearance of specific mutations that are not deleterious for the encoded protein. These mutations rather modify Dicer activity toward restricting the formation of tumor suppressor miRNAs and/or promoting the production of oncogenic miRNAs. Considering all these data, one can further speculate that *DICER1* can function both as a tumor suppressor and an oncogene. Finally, it should also be noted that there are many cancers for which neither germline nor somatic *DICER1* mutations have a major etiological role.

CONCLUSIONS AND PERSPECTIVES

The precise regulation of Dicer activity is critical for the proper functioning of all eukaryotic organisms. As discussed in this paper and summarized in Figure 3, Dicer abundance, activity and specificity can be regulated by various types of factors and at multiple levels. However, despite intensive studies, many basic questions regarding the mechanisms regulating the activity of this enzyme remain unanswered.

In the past few years, several reports have indicated the existence of interplay between Dicer and factors involved in various important cellular pathways. One can assume that the term 'Dicer interaction network' may refer exclusively to the Dicer protein, e.g. the contribution of the Dicer protein to chromatin structure remodelling, apoptosis or inflammation, as reviewed above. However, the involvement of Dicer mRNA in this interplay has also been documented. As discussed earlier, competition for nuclear exportin binding among Dicer mRNAs, pre-miRNAs and viral RNAs may affect Dicer protein levels (69). In addition, viral RNAs may influence the Dicer protein directly by its sequestration, which also prevents the maturation of host pre-miRNAs to functional miRNAs (163), as described in this review. Considering other conditions and details of cell functioning, the issue of the Dicer interaction network becomes much more complicated. Certainly, solving one problem leads to further questions. Thus, to obtain a more comprehensive image of the Dicer interaction network, systems biology, a new and increasingly popular biological discipline, must be considered. Nevertheless, there is no doubt that the elucidation of any aspects of the mechanisms underlying Dicer activity

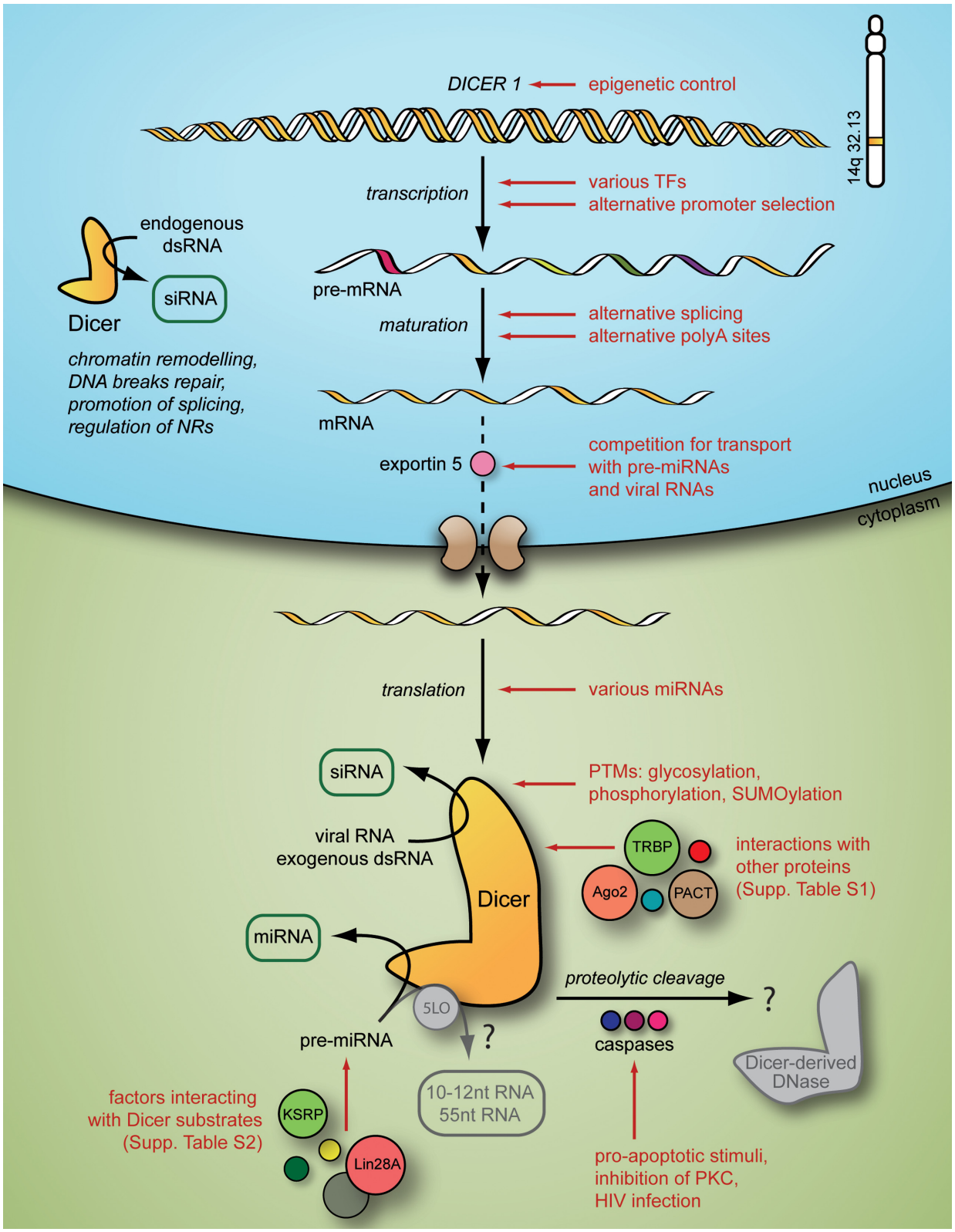

Figure 3. The widespread regulation of Dicer biosynthesis and function in human cells. 5LO—5 lipoxygenase, Ago2—Argonaute protein 2, KSRP—KH-type splicing regulatory protein, NRs—nuclear receptors, PACT—protein activator of interferon-induced protein kinase, PKC—protein kinase C, PTMs—post-translational modifications, TFs—transcription factors and TRBP—HIV-1 trans-activation response element RNA-binding protein. The hypothetical functions of Dicer, which have not been proven for the human enzyme, are presented in translucent grey and are indicated with a question mark.

will represent valuable contributions to the understanding of numerous phenomena, including developmental timing, growth, differentiation, apoptosis and viral infections.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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The many faces of Dicer: the complexity of the mechanisms regulating Dicer gene expression and enzyme activities

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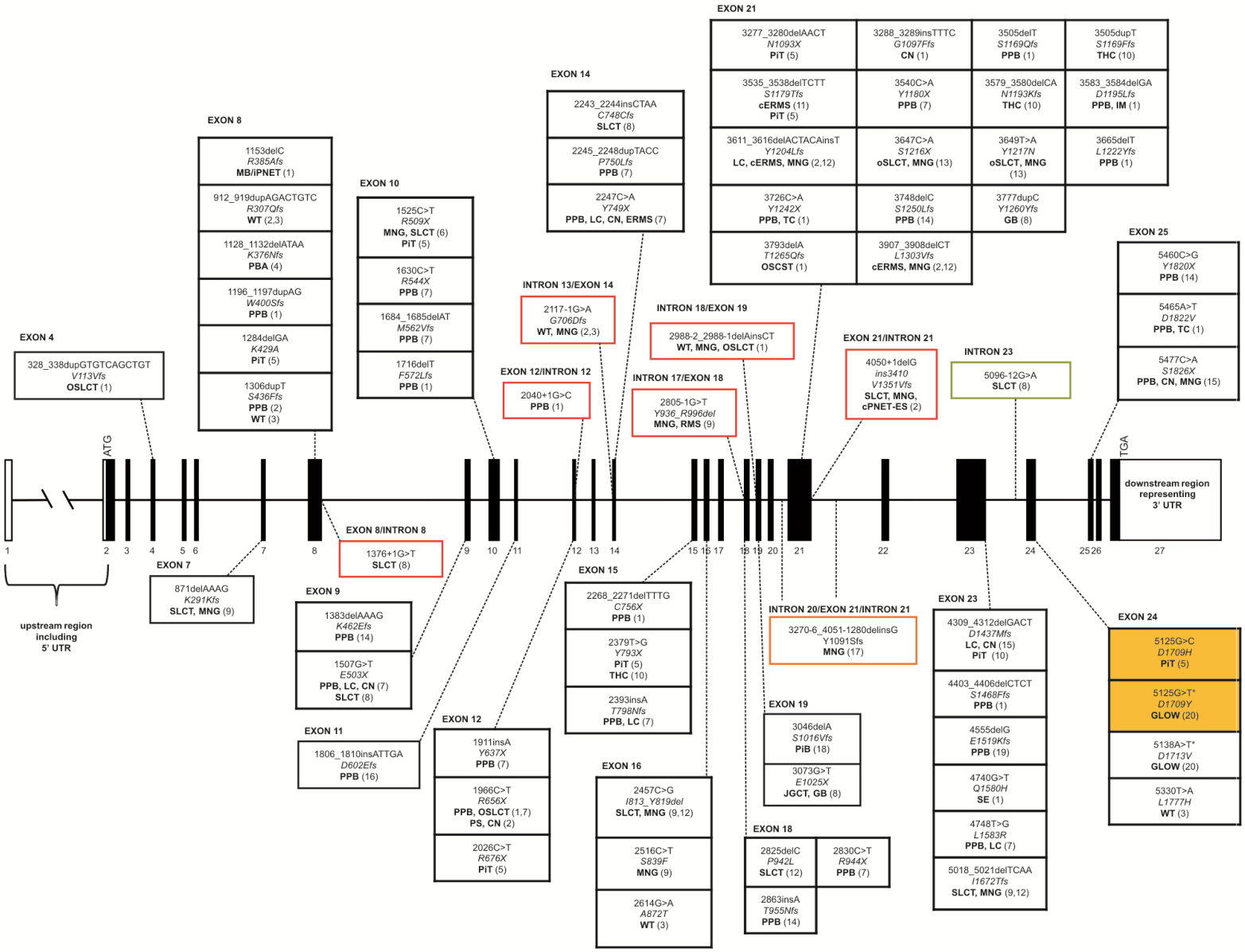
EXON 21
3277_3280delAACT N1093X PiT (5)
3288_3289insTTTC G1097Ffs CN (1)
3505delT S1169Qfs PPB (1)
3505dupT S1169Ffs THC (10)
3535_3538delTCTT S1179Tfs cERMS (11) PiT (5)
3540C>A Y1180X PPB (7)
3579_3580delCA N1193Kfs THC (10)
3583_3584delGA D1195Lfs PPB, IM (1)
3611_3616delACTACAinsT Y1204Lfs LC, cERMS, MNG (2,12)
3647C>A S1216X oSLCT, MNG (13)
3649T>A Y1217N oSLCT, MNG (13)
3665delT L1222Yfs PPB (1)
3726C>A Y1242X PPB, TC (1)
3748delC S1250Lfs PPB (14)
3777dupC Y1260Yfs GB (8)
3793delA T1265Qfs OSCST (1)
3907_3908delCT L1303Vfs cERMS, MNG (2,12)
EXON 14
2243_2244insCTAA C748Cfs SLCT (8)
2245_2248dupTACC P750Lfs PPB (7)
2247C>A Y749X PPB, LC, CN, ERMS (7)
EXON 8
1153delC R385Afs MB/iPNET (1)
912_919dupAGACTGTC R307Qfs WT (2,3)
1128_1132delATAA K376Nfs PBA (4)
1196_1197dupAG W400Sfs PPB (1)
1284delGA K429A PiT (5)
1306dupT S436Ffs PPB (2) WT (3)
EXON 10
1525C>T R509X MNG, SLCT (6) PiT (5)
1630C>T R544X PPB (7)
1684_1685delAT M562Vfs PPB (7)
1716delT F572Lfs PPB (1)
INTRON 13/EXON 14
2117-1G>A G706Dfs WT, MNG (2,3)
INTRON 18/EXON 19
2988-2_2988-1delAinsCT WT, MNG, OSLCT (1)
EXON 21/INTRON 21
4050+1delG ins3410 V1351Vfs SLCT, MNG, cPNET-ES (2)
INTRON 23
5096-12G>A SLCT (8)
EXON 25
5460C>G Y1820X PPB (14)
5465A>T D1822V PPB, TC (1)
5477C>A S1826X PPB, CN, MNG (15)
EXON 4
328_338dupGTGTCAGCTGT V113Vfs OSLCT (1)
EXON 12/INTRON 12
2040+1G>C PPB (1)
INTRON 17/EXON 18
2805-1G>T Y936_R996del MNG, RMS (9)
ATG
TGA
downstream region representing 3' UTR
EXON 8/INTRON 8
1376+1G>T SLCT (8)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
EXON 7
871delAAAG K291Kfs SLCT, MNG (9)
EXON 9
1383delAAAG K462Efs PPB (14)
1507G>T E503X PPB, LC, CN (7) SLCT (8)
EXON 15
2268_2271delTTTG C756X PPB (1)
2379T>G Y793X PiT (5) THC (10)
2393insA T798Nfs PPB, LC (7)
INTRON 20/EXON 21/INTRON 21
3270-6_4051-1280delinsG Y1091Sfs MNG (17)
EXON 23
4309_4312delGACT D1437Mfs LC, CN (15) PiT (10)
4403_4406delCTCT S1468Ffs PPB (1)
4555delG E1519Kfs PPB (19)
4740G>T Q1580H SE (1)
4748T>G L1583R PPB, LC (7)
5018_5021delTCAA I1672Tfs SLCT, MNG (9,12)
EXON 24
5125G>C D1709H PiT (5)
5125G>T* D1709Y GLOW (20)
5138A>T* D1713V GLOW (20)
5330T>A L1777H WT (3)
upstream region including 5' UTR
EXON 11
1806_1810insATTGA D602Efs PPB (16)
EXON 12
1911insA Y637X PPB (7)
1966C>T R656X PPB, OSLCT (1,7) PS, CN (2)
2026C>T R676X PiT (5)
EXON 16
2457C>G I813_Y819del SLCT, MNG (9,12)
2516C>T S839F MNG (9)
2614G>A A872T WT (3)
EXON 19
3046delA S1016Vfs PiB (18)
3073G>T E1025X JGCT, GB (8)
EXON 18
2825delC P942L SLCT (12)
2830C>T R944X PPB (7)
2863insA T955Nfs PPB (14)

Supplementary Figure S1. Heritable mutations in *DICER1* gene

The genomic (NG_016311) and transcript (NM_177438.2) reference sequences were used to notate human Dicer cDNA and protein (*in italics*) sequences. The phenotype of patients bearing the mutations studied is described **in bold**. Mutations framed in black boxes constitute non-synonymous or frame-shift exonic mutations. Mutations framed in red boxes constitute mutations located in the intron-exon splice sites. The mutation framed in the orange box is a whole exon deletion. The mutation framed in the green box is an intronic mutation with unknown influence on transcript or protein sequences. Mutations colored in yellow affect the amino acid residue responsible for metal binding in the catalytic center of RNase IIIb domain.

Abbreviations: cERMS – cervical embryonal rhabdomyosarcoma, CN – cystic nephroma, cPNET-ES cervicalprimitive neuroepithelial tumor-Ewing sarcoma, del – deletion, dup – duplication, ERMS – embryonal rhabdomyosarcoma, fs – frame-shift, GB – gynandroblastoma, GLOW – tumors associated with GLOW syndrome, IM – intraocular medulloepithelioma, ins – insertion, JGCT – juvenile granulosa cell tumor, LC – lung cysts, MB/iPNET – medulloblastoma/infratentorial primitive neuroectodermal tumor, MNG – multinodular goiter, OSCST – ovarian sex cord stromal tumor, oSLCT – ovarian Sertoli-Leydig cell tumor, PBA – pineoblastoma, PiT – pituitary blastoma, PPB – pleuropulmonary blastoma, PS – pulmonary sequestration, RMS – rhabdomyosarcoma, SE – seminoma, SLCT – Sertoli-Leydig cell tumor, TC – thyroid cysts, THC – thyroid carcinoma, WT – Wilms tumor

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Supplementary Table S1. The Dicer – protein interaction network

CATEGORY	PROTEIN	CELLULAR ROLE	NATURE OF THE INTERACTION	RELEVANCE TO DICER	REF.
CONSTITUTIVE PARTNERS OF DICER	Ago2 (argonaute protein 2)	A central effector protein of RNAi and an essential member of RISC; cleaves mRNA under guidance of associated miRNAs.	Direct interaction, involving their PIWI and RNase III domains, assisted by Hsp90.	Takes over RNA products from Dicer.	(1,2)
	PACT (protein activator of the interferon-induced PKR)	A major role in the antiviral activity of interferon as an activator of the interferon-induced PKR; protein involved in RNAi as a Dicer cofactor and a component of human RISC.	Direct interaction, involving the third dsRNA-binding domain (dsRBD) of PACT and the N-terminal region of Dicer.	Strengthens Dicer preference towards pre-miRNA, rather than siRNA precursors; influences miRNA production by changing the cleavage site choice by Dicer; contributes to RISC assembly.	(3-5)
	TRBP (TAR RNA-binding protein)	An integral part of RISC, first identified as a cellular protein that facilitates the replication of human immunodeficiency virus by inhibiting the interferon-induced protein kinase R (PKR) and by increasing translation of viral mRNA.	Direct interaction, involving TRBP Medipal domain (298–366 aa) and Dicer domain located between the ATPase and the helicase motifs (267–431 aa).	Recruits dsRNA substrates to Dicer, and remains associated with RNA to affect its structure and relative positioning on Dicer's active site (4-5-fold increase in activity of Dicer upon binding TRBP); contributes to substrate, and product length determination (isomiRs production); stabilizes Dicer and contributes to RISC formation.	(1,3,6-11)
TRANSIENTLY INTERACTING PROTEINS	ADAR1 (adenosine deaminase acting on RNA 1)	Nuclear deaminase, catalyzes A-to-I editing of dsRNA.	Direct interaction, involving DUF238 and DEAD-box RNA helicase of Dicer.	Increases the maximum rate of pre-miRNA cleavage by Dicer; facilitates loading of miRNA onto the RISC.	(12)
	Caspase-3	Protease, plays a central role in the execution phase of cell apoptosis; interacts with other caspases.	Direct interaction resulting in proteolytic cleavage of Dicer.	Cleaves Dicer; truncated Dicer (C-terminal fragment) acts as a DNase and functions in the propagation of apoptosis.	(13-15)

CATEGORY	PROTEIN	CELLULAR ROLE	NATURE OF THE INTERACTION	RELEVANCE TO DICER	REF.
TRANSIENTLY INTERACTING PROTEINS	RHA (RNA helicase A)	ATP-dependent RNA helicase that unwinds RNA-RNA and RNA-DNA duplexes in 3'->5' direction.	Direct interaction with Dicer, but also TRBP, Ago2 and RNA.	RISC-associated protein, promotes the interaction of guide-strand siRNA and miRNA with Ago2, postulated role in unwinding siRNA and miRNA/miRNA* duplexes.	(16)
	FMRP (fragile X mental retardation protein)	RNA-binding protein involved in mRNA transport and translation, etiologic factor of the fragile X syndrome.	Direct interaction, negatively modulated by FMRP phosphorylation.	Takes over RNA products from Dicer.	(17)
	Pol II (RNA polymerase II)	Catalyzes transcription of DNA to synthesize precursors of mRNAs and most snRNAs and miRNAs.	RNA-mediated interaction.	Recruits Dicer to loci of endogenous overlapping transcription, where it can cleave dsRNA into siRNA.	(18)
	5LO (5 lipoxygenase)	The key enzyme in leukotriene biosynthesis; catalyzes oxidation of arachidonic acid to hydroperoxyeicosatetraenoic acids (5-HpETE), and converts 5-HpETE to leukotriene A4.	Direct interaction with Dicer C-terminus.	Presumably influences Dicer cleavage pattern of pre-miRNAs, favoring production of ~55-nt and ~10 to ~12-nt long RNA species.	(19,20)
PROTEINS INVOLVED IN DICER TRANSPORT AND CONFERRING DICER STABILITY	CLIMP-63 (cytoskeleton-linking membrane protein 63 kDa, also known as p63)	Transmembrane protein of the endoplasmic reticulum (ER); mediates interaction between the ER and the cytoskeleton; plays a structural role for the ER morphology.	Direct interaction between N-terminal fragment of Dicer (242-430 aa) and a luminal domain of CLIMP-63.	Stabilizes newly synthesized Dicer, assists transition of Dicer through the ER and supports cellular localization of the protein (e.g., by anchoring Dicer to the nuclear periphery or in close proximity to the ribosomes on the ER).	(21)
	NDP52 (nuclear dot protein 52 kDa , also known as CALCOCO2 – calcium binding and coiled-coil domain 2)	The autophagy receptor; binds cytosolic substrates and interacts with autophagosome membrane-protein Atg8.	No data.	Mediates Dicer degradation in its RNA-free state.	(22)

CATEGORY	PROTEIN	CELLULAR ROLE	NATURE OF THE INTERACTION	RELEVANCE TO DICER	REF.
	NUP153 (nucleoporin 153)	The nuclear pore complex (NPC) protein; regulates the movement of macromolecules between the nucleus and cytoplasm.	Direct interaction.	Assists in shuttling Dicer to the NPC. Dicer associates with mobile NUP153 protein in the cytoplasm, and on the periphery of the NPC but not inside the nucleus.	(23)
VIRAL SUPPRESSORS OF SILENCING	HCV core protein	Structural protein of the viral capsid.	Direct interaction, possibly involving the helicase domain of Dicer and the N-terminal portion of the viral protein.	Inhibits Dicer processing activity.	(24,25)
	HIV-1 Tat (HIV-1 transactivator of transcription)	Plays a pivotal role in HIV-1 replication; activates transcription from the viral long terminal repeat (LTR) promoter by binding to the TAR hairpin in the nascent RNA transcript.	RNA-mediated interaction, involving helicase domain of Dicer (585-1913 aa).	Inhibits Dicer processing activity through direct interaction with Dicer and sequestration of TRBP.	(26,27)
	HIV-1 protein R (Vpr)	Plays a critical role in virus replication in non-dividing cells, induces G2 cell cycle arrest and apoptosis in proliferating cells.	Direct interaction.	Mediates proteasomal degradation of Dicer through the ubiquitin-ligase complex.	(28)

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Supplementary Table S2. Proteins influencing Dicer activity through the interactions with pre-miRNAs

TARGETED REGION OF pre-miRNA	PROTEIN NAME	CELLULAR FUNCTION	EFFECT ON DICER ACTIVITY	REF.
5′-END	BCDIN3D (BCDIN3 domain containing)	O-methyltransferase that specifically dimethylates the 5′ monophosphate of pre-miRNAs.	Negatively regulates Dicer processing, as 5′ monophosphate of pre-miRNAs is recognized by Dicer and is required for proper pre-miRNA processing.	(1)
LOOP	KSRP (KH-type splicing regulatory protein)	RNA-binding protein implicated in transcription, alternative pre-mRNA splicing, decay of labile mRNAs and certain miRNAs maturation; a part of Drosha and Dicer complex in cultured cells; binds specifically to 5′guanosine-rich motifs within the loop region of several miRNA precursors.	Promotes pre-miRNA processing by Dicer possibly by optimization of positioning and/or recruitment of miRNA-generating complexes.	(2-6)
	Lin28A	RNA-binding protein that increases the efficiency of protein synthesis by stabilizing mRNAs and driving them to polysomes; a specific suppressor of biogenesis of certain miRNAs, recognizes the tetra-nucleotide sequence motif (GGAG) in pre-miRNA apical loop; recruits terminal uridylyltransferases 4/7 (TUTase4/7).	Antagonizes Dicer processing of the certain group of pre-miRNAs by direct binding to precursors, and triggering their uridynylation and degradation.	(7-16)
	MCPIP-1 (monocyte chemoattractant protein-induced protein 1)	RNase involved in the modulations of the inflammatory response and immune homeostasis; regulates migration and infiltration of macrophages; triggers apoptosis and promotes angiogenesis.	Antagonizes Dicer processing; cleaves the apical loops of pre-miRNAs thus leading to the rapid degradation of precursors.	(17)
	RBM3 (RNA binding motif protein 3)	Cold-inducible mRNA binding protein that enhances global protein synthesis in mild hypothermic temperatures.	Positively regulates Dicer activity; binds directly to pre-miRNAs and facilitates/de-represses their ability to associate with Dicer.	(18)

TARGETED REGION OF pre-miRNA	PROTEIN NAME	CELLULAR FUNCTION	EFFECT ON DICER ACTIVITY	REF.
	TDP-43 (TAR DNA-binding protein-43)	Transcriptional repressor, splicing factor and translational regulation involved in metabolism of various RNAs; component of Drosha and Dicer complexes.	Positively regulates Dicer activity; facilitates binding of the Dicer complex to a subset of pre-miRNAs and promotes the cleavage of the specific pre-miRNAs.	(19)
3′-END	TUTase2 (3' terminal uridylyl transferase 2), also known as PAPD4	Non-canonical, cytoplasmic poly(A) RNA polymerase.	Positively regulates Dicer processing of certain group of pre-miRNAs by monouridylation of RNA and restoring pre-miRNA's 2-nt 3′ overhang.	(20)
	TUTase4 (3' terminal uridylyl transferase 2), also known as ZCCHC11	3′ uridylyltransferase that acts on certain pre-miRNAs and miRNAs.	Negatively regulates Dicer processing of pre-let-7 by Lin28A-dependent polyuridylation. Positively regulates Dicer processing of a certain group of pre-miRNAs by monouridylation of RNA and restoring pre-miRNA's 2-nt 3′ overhang.	(7,12,21)
	TUTase7 (3' terminal uridylyl transferase 2), also known as ZCCHC6	3′ uridylyltransferase that acts on certain pre-miRNAs.	Negatively regulates Dicer processing of pre-let-7 by Lin28A-dependent polyuridylation. Positively regulates Dicer processing of a certain group of pre-miRNAs by monouridylation of RNA and restoring pre-miRNA's 2-nt 3′ overhang.	(12,20)

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Supplementary Table S3. The Dicer mRNA/protein expression level in different human cancer tissues

ORGAN OR TISSUE		TYPE OF CANCER		EXPRESSION LEVEL		REF.
				mRNA	protein	
Nasopharynx		Nasopharyngeal carcinoma		↓	↓	(1)
Lungs		Non-small-cell lung carcinoma	bronchioloalveolar carcinoma and atypical adenomatous hyperplasia	↑	↑	(2)
			advanced adenocarcinoma	~	↑	(2)
			shorter postoperative survival	↓	N/A	(3)
Stomach		Gastric cancer		↓	↓	(4)
Colon or rectum		Colorectal adenocarcinoma		N/A	↑	(5)
Bladder		Urothelial carcinoma		↑	N/A	(6)
Smooth muscle		Leiomyosarcoma and leiomyoma		N/A	↑	(7)
Blood or bone marrow		Primary cutaneous T-cell lymphoma		N/A	↑	(8)
		Acute myeloid leukemia		↑	N/A	(9)
Breast		Malignant breast cancer		N/A	↓	(10)
				↓	NC	(11)
		Lymph node metastases		N/A	↑	(12)
		Aggressive basal-like, HER2+ and luminal B type tumors in breast cancer		↓	N/A	(13)
Ovaries		Ovarian carcinoma		↓	N/A	(14)
		Ovarian serous adenocarcinoma		↑	↑	(15)
		Metastatic ovarian carcinoma		↑	↑	(16)
		Invasive epithelial ovarian cancer associated with advancer tumor stage		↓	↓	(17)
Uterus		Endometrial adenocarcinoma		↓	N/A	(18)
Prostate		Prostate adenocarcinoma and prostatic intraepithelial neoplasia		N/A	↑	(19)
		Metastatic prostate adenocarcinoma		↑	N/A	
Skin and epidermis	Melanocytes	Cutaneous and acrolentiginous melanoma		N/A	↑	(20)
	Basal cells	Basal cell carcinoma		↓	N/A	(21)
	Squamous cells	Squamous cell carcinoma		↑	N/A	

N/A – not applicable, NC – no correlation, ~ similar to normal tissue, ↑ upregulated, ↓ downregulated

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Supplementary Table S4. The somatic mutations in *DICER1* occurring in different types of tumor

SOMATIC MUTATION	AMINO-ACID RESIDUE AFFECTED AND ITS LOCALIZATION IN DICER		TUMOR TYPE	REF.
1304C>T	P453L	Helicase C-terminal domain	GLOW	(1)
3237_3238insCCAGCAT	V1080Pfs	Ruler domain	SLCT	(2)
4031C>T	S1344L	RNase IIIa	WT	(3)
5113G>A	E1705K[1, 2]	RNase IIIb	SLCT unclassified SCST THC	(2) (4) (5)
5125G>A	D1709N[1, 2]	RNase IIIb	SLCT, PGCT (YSC) SLCT PPB PiB	(2) (4) (6) (7)
5125G>T	D1907Y[1,2]	RNase IIIb	PiB	(7)
5126A>G	D1709G[1, 2]	RNase IIIb	SLCT, JGCT	(2)
5127T>A	D1709E[1, 2]	RNase IIIb	SLCT, PGCT (YSC)	(2)
5138A>C	D1713A[2]	RNase IIIb	WT	(3)
5174G>A	R1725Q[2]	RNase IIIb	TGCT (seminoma)	(8)
5425G>A	G1809R	RNase IIIb	PPB	(6,9)
5425G>T	G1809W	RNase IIIb	PiB	(7)
5428G>C	D1810H[1]	RNase IIIb	SLCT	(2)
5428G>T	D1810Y[1]	RNase IIIb	SLCT, TE YSC, immature TE PPB	(2) (4) (6)
5428G>A	D1810N[1]	RNase IIIb	SLCT	(2)
5429A>G	E1788fs[3]	RNase IIIb	WT Mixed GBE, DGE	(3) (4)
5429A>T	D1810V[1]	RNase IIIb	SLCT with components of JGCT	(4)
5437G>C	E1813Q[1]	RNase IIIb	SLCT	(2,4)
5437G>A	E1813K[1]	RNase IIIb	SLCT cERMS PiB	(2,4) (10) (7)
5438A>G	E1813G[1] E1788fs[3]	RNase IIIb	SLCT PPB THC WT YSC, SLCT, DGE, eCA, immature TE, CHC	(2) (6,11) (5) (3) (4)
5438A>T	E1813V[1]	RNase IIIb	PiB	(12) (7)
5438A>C	E1813A[1]	RNase IIIb	NHL (dlBCL)	(13)
5439G>T	E1813D[1]	RNase IIIb	PiB THC	(7) (5)
5439G>C		RNase IIIb	SLCT	(4)
5452G>A	A1818T	RNase IIIb	WT	(3)
5492G>A	W1831X	RNase IIIb	PGCT (YST)	(2)
5529T>C	R1898G	dsRBD	GLOW	(1)

Mutations in the RNase IIIb domain: [1]metal-binding site affected; [2]conserved region affected; [3]additional exonic silencing site generated, predicted to exon 25 skipping and the expression of Dicer lacking the majority of its RNase IIIb domain.

Abbreviations: cERMS – cervical embryonal rhabdomyosarcoma, CHC – choriocarcinoma, DGE – dysgerminoma, eCA – embryonal carcinoma, fs – frame-shift of the open reading frame, GBE – gonadoblastoma, GLOW - tumors associated with GLOW syndrome, JGCT – juvenile granulosa cell tumor, NHL (dlBCL) – non-Hodgkin lymphoma (diffuse large B-cell lymphoma), PiB- pituitary blastoma, PGCT – primitive germ-cell tumor, PPB – pleuropulmonary blastoma, SCST – sex cord-stromal tumor, SLCT – Sertoli-Leydig cell tumor, TE- teratoma, TGCT – testicular germ cell tumor, THC – thyroid carcinoma, WT – Wilms tumor, YSC – yolk sac tumor

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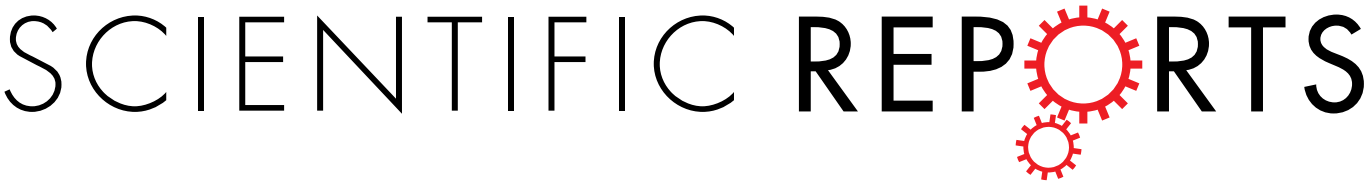

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Revealing a new activity of the human Dicer DUF283 domain *in vitro*

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The ribonuclease Dicer is a multidomain enzyme that plays a fundamental role in the biogenesis of small regulatory RNAs (srRNAs), which control gene expression by targeting complementary transcripts and inducing their cleavage or repressing their translation. Recent studies of Dicer's domains have permitted to propose their roles in srRNA biogenesis. For all of Dicer's domains except one, called DUF283 (domain of unknown function), their involvement in RNA substrate recognition, binding or cleavage has been postulated. For DUF283, the interaction with Dicer's protein partners has been the only function suggested thus far. In this report, we demonstrate that the isolated DUF283 domain from human Dicer is capable of binding single-stranded nucleic acids *in vitro*. We also show that DUF283 can act as a nucleic acid annealer that accelerates base-pairing between complementary RNA/DNA molecules *in vitro*. We further demonstrate an annealing activity of full length human Dicer. The overall results suggest that Dicer, presumably through its DUF283 domain, might facilitate hybridization between short RNAs and their targets. The presented findings reveal the complex nature of Dicer, whose functions may extend beyond the biogenesis of srRNAs.

The ribonuclease III (RNase III) Dicer is one of the key enzymes involved in the biogenesis of small regulatory RNAs (srRNAs). Dicer processes stem-loop precursors (pre-miRNAs) into short RNA duplexes containing functional 21-23-nt microRNAs (miRNAs) and double-stranded RNAs (dsRNAs) into small interfering RNAs (siRNAs)[1]. The Dicer-generated short RNA duplex is loaded into a multi-protein complex referred to as the RNA-induced silencing complex (RISC)[2]. During RISC activation, one strand of the RNA duplex is released and degraded, and the second strand remains in the complex and acts as a sequence-specific probe guiding RISC to complementary transcripts[2–4]. Depending on the degree of complementarity between the small RNA and the target molecule, RISC binding results in either mRNA cleavage and degradation or translational repression[5]. Target mRNA cleavage frequently occurs in plants, whereas translational repression is usually observed in animals. The minimal functional RISC consists of a member of the Argonaute (Ago) protein family and a small regulatory RNA, i.e., miRNA or siRNA[6–8]. However, many reports have indicated that RISC also includes Dicer, the trans-activation response RNA-binding protein (TRBP)[9,10], and presumably other auxiliary proteins, such as chaperones[11].

Dicers are multidomain enzymes composed of an N-terminal putative helicase domain (homologous to DExD/H-box helicases), a DUF283 domain (domain of unknown function), a PAZ (Piwi-Argonaute-Zwille) domain, two RNase III domains (RNase IIIa and RNase IIIb) and a dsRNA-binding domain (dsRBD)[1,12–15]. To date, functions for almost all of Dicer's domains in srRNA biogenesis have been proposed. The PAZ domain has been found to bind to the 3′ end of a substrate[15–18]. The N-terminal helicase domain is thought to be involved in discriminating between miRNA and siRNA precursors by interacting with the hairpin loop structures of pre-miRNAs[19–22]. The RNase IIIa and RNase IIIb domains form a single dsRNA cleavage center that binds miRNA/siRNA precursors and cleaves ~20 base pairs (bp) from their termini[15]. Finally, dsRBD has been shown to play only an auxiliary role in substrate binding and cleavage[15,19]. In contrast, the function of DUF283 is not well described. Initially, DUF283 was suggested to be critical for pre-miRNA processing because cleavage activity is lost in Dicer mutants lacking both DUF283 and the helicase domain[23,24]. Later, it was shown that the deletion of

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only DUF283 increases binding but decreases cleavage of dsRNA substrates by Dicer without affecting the binding and cleavage of pre-miRNAs[25]. Furthermore, structural studies have revealed that DUF283 adopts a fold typical for proteins that bind dsRNAs[26,27]. Nevertheless, *in vitro* studies of plant DUF283 from *Arabidopsis thaliana* Dicer-like protein 4 (At-DCL4) have indicated that this domain does not exhibit any detectable dsRNA-binding activity[27]. Instead, DUF283 has been found to be responsible for interacting with Dicer's protein partners in both plants[27] and mammals[28].

In this report, we demonstrate that the DUF283 from human ribonuclease Dicer (hDicer), similarly to its plant homolog[27], does not bind dsRNAs even though both domains show structural features characteristic of the dsRNA-binding proteins[26,27]. We found, however, that human DUF283 is capable of binding single-stranded nucleic acids *in vitro*. More detailed analyses revealed that DUF283 acts as a nucleic acid annealer that facilitates hybridization between RNA or DNA complementary strands. We found that full length hDicer also shows an annealing activity *in vitro*. Furthermore, we demonstrated that hDicer supports base-pairing between short RNA and a complementary region present in a longer RNA. The presented findings reveal the complex nature of Dicer, whose functions may extend beyond the biogenesis of srRNAs[29–32].

Results and Discussion

DUF283 can bind single-stranded RNAs. hDicer DUF283 was produced in bacteria with the pMCSG7 expression vector (Supplementary Fig. S1)[33]. Previous experiments have revealed that At-DCL4 DUF283 does not bind dsRNA, though it does possess a dsRNA-binding fold[27]. To test whether the human homolog is capable of binding dsRNAs, we performed an electrophoretic mobility shift assay (EMSA) using a 22-bp RNA duplex (R22-cR22). Because DUF283 has been shown to bind Zn^{2+} ions[27], we performed three sets of binding reactions: in buffer containing monovalent ions only (buffer complemented with EDTA), and in the same buffer supplemented with either Zn^{2+} or Mg^{2+} but lacking EDTA. The ^{32}P-labeled dsRNA was incubated with increasing amounts of DUF283, and the reaction mixtures were separated by native polyacrylamide gel electrophoresis (PAGE) and visualized by phosphorimaging. The data collected showed that hDicer DUF283 did not bind dsRNA regardless of the buffer used (Fig. 1a).

Because only the dsRNA substrate was applied in the experiment described above, we performed analogous experiments in which the dsRNA was replaced with single-stranded RNA (ssRNA) or single-stranded DNA (ssDNA) to determine whether DUF283 is able to bind other forms of nucleic acids. In the first experiment, we used an siRNA/miRNA-sized, 22-nucleotide (nt)-long ssRNA (R22). The ^{32}P-labeled R22 was denatured and then incubated with increasing amounts of DUF283 for 15 min at 4 °C (Fig. 1b). In addition, several sets of control reactions were performed (Fig. 1c,d), in which DUF283 was either substituted with bovine serum albumin (BSA) or exchanged with a protein preparation from *E. coli* cells transformed with the empty pMCSG7 expression vector (EP) purified using the same procedure as that for recombinant DUF283 (Fig. 1c). The results shown in Fig. 1b demonstrated that DUF283 was capable of binding the 22-nt ssRNA. To determine whether the capacity of DUF283 to bind ssRNA depends on the length of the ssRNA, we performed additional experiments involving 12-, 32-, 52- and 62-nt-long ssRNA species (Fig. 2a–e). Moreover, we tested whether the formation of the DUF283-ssRNA complex depends on the presence of monovalent or divalent ions (Na^+, Mg^{2+}, Zn^{2+}) (Supplementary Fig. S2). We found that DUF283 was able to bind all of the tested ssRNAs in a concentration-dependent manner. Moreover, DUF283-ssRNA binding was not influenced by the presence or absence of divalent metal ions. For the 12-, 22- and 32-nt-long ssRNAs, we observed one band corresponding to the DUF283-ssRNA complex, though the 12-nt ssRNA binding was very weak. For the 52-nt ssRNA, in addition to a band corresponding to the DUF283-ssRNA complex, we also found smeared bands and well-complexes (material that did not migrate out of the wells). With regard to the reaction involving the 62-nt ssRNA and DUF283, we observed mostly smeared bands along the entire lane and well-complexes (Fig. 2e). The experiments with longer ssRNAs were repeated several times; however, we never observed clear bands when we analyzed the reaction mixtures containing DUF283 incubated with $>$60-nt ssRNAs by native PAGE. Thus, we speculate that under the applied experimental conditions, DUF283 and longer RNA molecules formed a range of complexes. In general, we found that: (i) DUF283-ssRNA binding is not efficient – for each tested ssRNA, less than half of the substrate was bound at the highest DUF283 concentration used (approximately 8.0 μM); and (ii) for short ssRNAs (12-, 22-nt) only, we did not observe DUF283 aggregation (smeared bands or well-complexes). Considering the results above, we attempted to determine the K_d value for the DUF283-ssRNA complex in the experiment involving mi/siRNA-sized 22-nt ssRNA (R22) and DUF283 at concentrations ranging from 0.5 to 20.0 μM. As shown in Fig. 2f, the K_d value for the DUF283 and 22-nt ssRNA complex was 9.5 ± 0.5 μM. Previously, Wostenberg *et al.*[34] and Doyle *et al.*[35] have reported that the isolated dsRBD from hDicer binds ~20-bp RNA duplex with $K_d \approx 6.5$ μM and >8.0 μM, respectively. In addition, the K_d values for the full length hDicer and different ~20-nt ssRNAs have been established[36]. The values of these K_d range from approximately 0.13 to 3.0 μM. Altogether, our data indicate that DUF283 binds 22-nt ssRNA with a similar affinity as dsRBD binds ~20-bp dsRNAs, and with much lower affinity as full length hDicer binds ~20-nt ssRNAs.

Finally, we determined that DUF283 also binds a 29-nt-long ssDNA with an efficiency comparable to that observed for ssRNAs of similar lengths (Fig. 2g).

DUF283 accelerates the annealing of complementary RNA or DNA strands. The results of the experiments presented above indicated that DUF283 interacts with single-stranded nucleic acids. Interestingly, the results obtained in one control experiment revealed the formation of an additional product migrating slightly more slowly than R22 (Fig. 1d; the product indicated with the arrow). In this experiment, R22 was incubated with DUF283; however, in contrast to the results presented in Fig. 1a–c, the reaction products were separated in a buffer containing sodium dodecyl sulfate (SDS), which denatures proteins but not nucleic acids. A more detailed analysis showed that R22 molecules are capable of forming imperfectly complementary duplexes (Fig. 1d); the

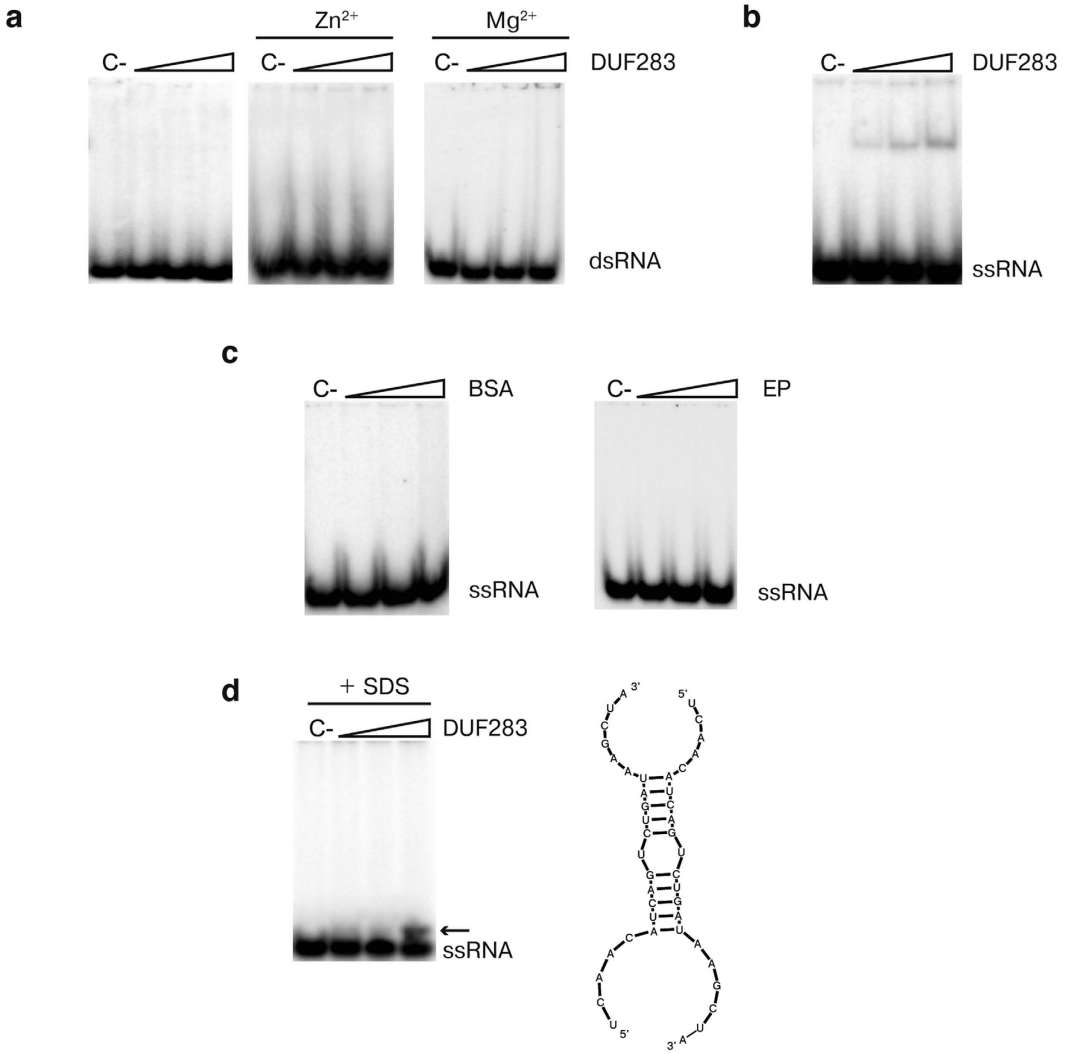

Figure 1. DUF283 interacts with single- but not double-stranded RNAs. Native PAGE gels showing the results of binding assays involving (**a**) 22-bp dsRNA and DUF283, (**b**) 22-nt RNA (R22) and DUF283, (**c**) R22 and BSA (left), R22 and the preparation obtained from bacteria transformed with the expression plasmid lacking the DUF283 sequence [EP] (right), (**d**) R22 and DUF283, which were resolved in loading buffer containing SDS at final concentration of 0.2%. The predicted secondary structure of the R22 dimer is shown in panel D. [C-] denotes controls with no protein. Triangles represent increasing amounts of DUF283, BSA or EP.

putative duplexes were not formed when R22 oligomers were incubated in buffer only or when R22 oligomers were incubated with BSA (Fig. 1c). Thus, one could speculate that under the given reaction conditions, DUF283 functioned as a nucleic acid annealer. To test this hypothesis, three pairs of complementary oligomers were used. The first pair included perfectly complementary 22-nt ssRNAs (R22-cR22), the second pair included imperfectly complementary 22- and 21-nt ssRNAs (cR22-cR21), and the third included perfectly complementary 29-nt ssDNAs (D29-cD29) (Fig. 3a–c; respectively). One oligonucleotide of each pair was always ^{32}P-labeled at the 5′ end; the corresponding molecules were mixed in annealing buffer, in molar ratio of approximately 1:50 between ^{32}P-labeled and unlabeled oligomers, and incubated for 5, 15 or 30 min with increasing amounts of DUF283 at room temperature. Control reactions either lacked DUF283 (Fig. 3; lines marked with [C-]) or contained BSA instead of DUF283 (Supplementary Fig. S3a–c). In the control experiments, the duplexes were generated most effectively for D29-cD29 (Fig. 3c and Supplementary Fig. S3c), less effectively for R22-cR22 (Fig. 3a and Supplementary Fig. S3a), and slightly above the detection level for cR22-cR21 (Fig. 3b and Supplementary Fig. S3b). The observed efficiencies of hybridization between the complementary oligonucleotides correlated well with the free energies calculated for the respective duplexes: −35.0 kcal/mol for D29-cD29; −33.9 kcal/mol for R22-cR22; and −24.2 kcal/mol for cR22-cR21. We also found that in all reactions containing DUF283, the fractions of annealed oligonucleotides were significantly increased in both a protein concentration- and time-dependent manner (Fig. 3).

To determine the rate of annealing reactions, we performed time-dependent assays involving DUF283 at a 500 nM concentration and three pairs of the tested oligonucleotides (Fig. 4a–c). To reduce the spontaneous

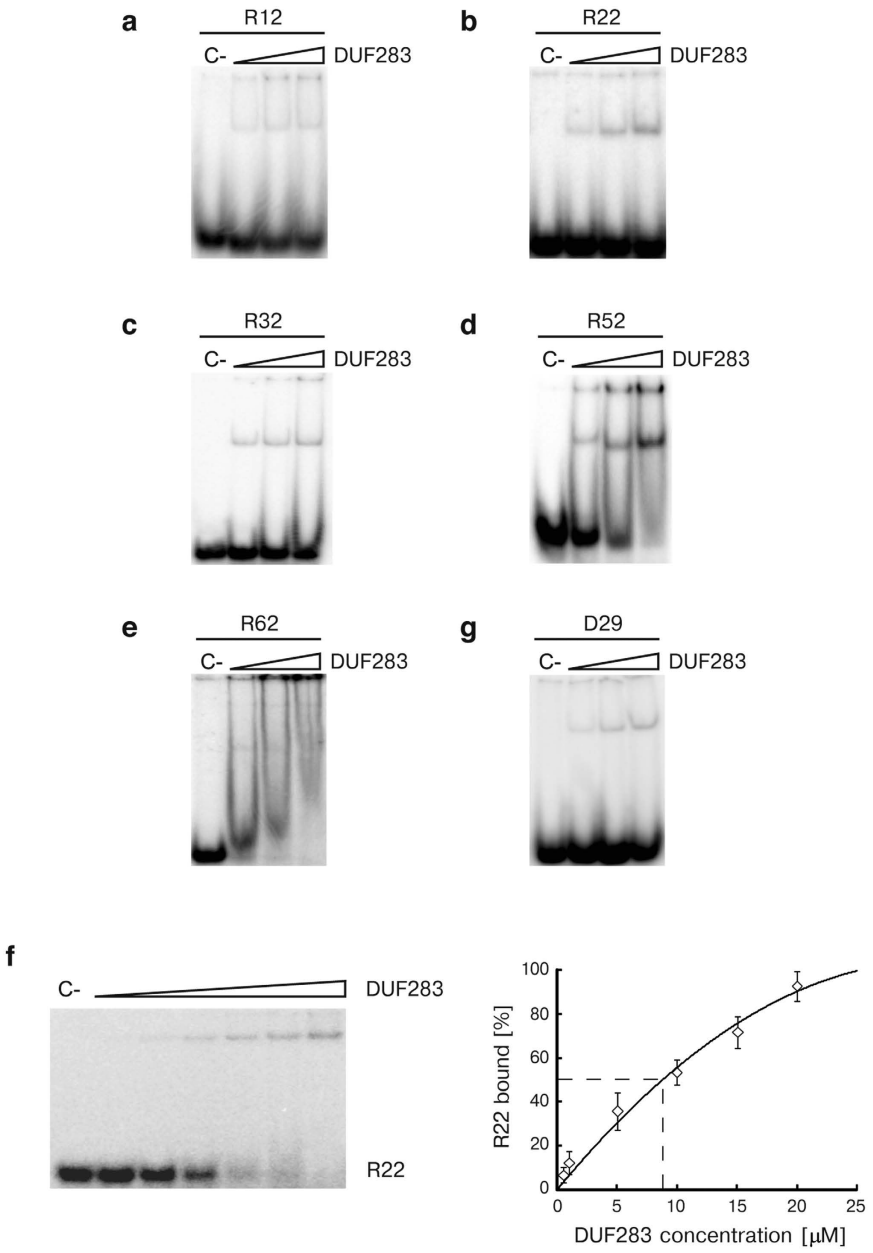

Figure 2. DUF283 binds single-stranded nucleic acids of different lengths in a concentration-dependent manner. Native PAGE gels showing the results of binding assays involving DUF283 and (**a**) 12-nt ssRNA, (**b**) 22-nt ssRNA, (**c**) 32-nt ssRNA, (**d**) 52-nt ssRNA, (**e**) 62-nt ssRNA. [C-] denotes controls with no DUF283. Triangles represent increasing amounts of DUF283 (0.5; 4.0; 8.0 μM). (**f**) DUF283 binds 22-nt ssRNA with $K_d = 9.5 \pm 0.5$ μM. Native PAGE gel showing the results of binding assay involving DUF283 and R22 (left). [C-] denotes a control with no DUF283. A triangle represents increasing amounts of DUF283 (0.5; 1; 5; 10; 15; 20.0 μM). A binding curve of DUF283 and 22-nt ssRNA (right). The curve was derived from densitometric quantification of the autoradiogram. The K_d values were calculated from the curves and presented results are the mean of three independent assays. (**g**) Native PAGE gel showing the results of binding assays involving DUF283 and 29-nt ssDNA.

annealing between complementary oligomers that was observed earlier (see Fig. 3 and Supplementary Fig. S3), we changed the molar ratio between ^{32}P-labeled and unlabeled oligomers to 1:20. As expected, spontaneous annealing of the complementary oligomers was significantly decreased for each pair. The duplexes were formed upon the addition of DUF283 with an initial velocity (V_0) of 0.36 ± 0.17 nM min^{-1} for R22-cR22 (Fig. 4a), 0.21 ± 0.10 nM min^{-1} for cR22-cR21 (Fig. 4b) and 0.42 ± 0.18 nM min^{-1} for D29-cD29 (Fig. 4c).

These results indicate that the initial reaction rate measured at saturating DUF283 concentration (~500 nM) was: the highest for perfectly complementary 29-nt ssDNAs; slightly lower for perfectly complementary 22-nt ssRNAs; and the lowest for imperfectly complementary 22- and 21-nt ssRNAs. Importantly, the determined V_0 values well correlated with free energies calculated for each pair of the tested oligonucleotides; i.e., the highest

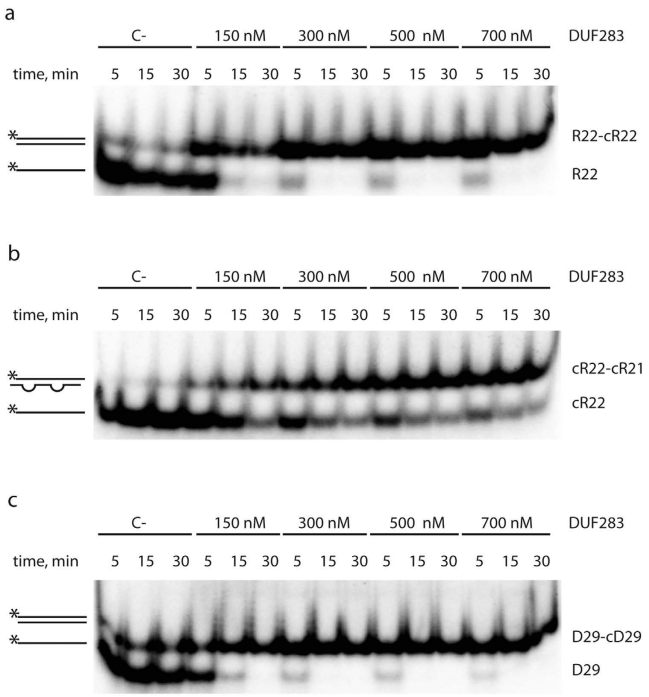

Figure 3. DUF283 accelerates annealing of complementary oligonucleotides. Native PAGE gels showing the results of annealing reactions involving DUF283 and the following nucleotide pairs: (**a**) R22 and cR22, (**b**) cR22 and cR21, (**c**) D29 and cD29. Reaction mixtures were resolved in buffer containing SDS at a final concentration of 0.2%. Schematic representations of substrates and products are shown on the left in this and in other figures. The asterisk indicates the ^{32}P 5′-end label. [C-] denotes a control reaction with no protein.

initial reaction rate was observed for the duplex with the lowest free energy (D29-cD29) and the lowest, for the duplex with the highest free energy (cR22-cR21). Although the initial reaction rates determined for three pairs of the tested oligonucleotides differed, the overall annealing efficiency after 30 min incubation with DUF283 was similar for all of them.

Ribonuclease Dicer acts as a nucleic acid annealer. The experiments described above involved only DUF283, a single hDicer domain. We sought to determine whether the complete hDicer would also support the annealing of complementary strands *in vitro*. Thus, the three pairs of complementary oligonucleotides used in the previous experiments were also applied in annealing assays with full length hDicer produced in a baculovirus system (Supplementary Fig. S4). Each pair of complementary oligonucleotides was incubated in annealing buffer with increasing amounts of hDicer for 30 min at room temperature; the negative control reactions lacked hDicer (Fig. 5). We observed that, similarly to DUF283, hDicer facilitated hybridization of complementary oligomers, and an increase in hDicer concentration was accompanied by an increase in the double-stranded product. Interestingly, this tendency was observed up to an hDicer concentration of approximately 200 nM. In contrast to DUF283 (Fig. 3), the further increase of the hDicer concentration reduced the effectiveness of the annealing process (Fig. 5). Because we did not detect duplex unwinding activity for either DUF283 or hDicer under the applied conditions (Supplementary Fig. S5), the observed effect could be explained by the fact that full length hDicer, in addition to DUF283, also contains other RNA/DNA-binding domains. These domains may bind oligonucleotides present in the reaction mixtures, precluding their effective base-paring. This observation is consistent with the K_d values for hDicer-ssRNA and DUF283-ssRNA complexes presented earlier, which indicated that hDicer binds ssRNAs more efficiently than DUF283.

To compare the annealing activities of DUF283 and hDicer, we performed annealing assays using serial protein dilutions. Based on the results obtained from three independent experiments, for each reaction, we calculated the percentage ratios between the double-stranded (duplexed) and single-stranded (non-duplexed) fractions of the labeled strand. The average percentage content of the double-stranded fraction was plotted against the molar concentrations of DUF283 or hDicer; dashed lines were drawn for the values obtained in control experiments with BSA (Fig. 6). For each tested pair, we calculated the maximum increase in the double-stranded fraction (Δf_{ds}) induced by the addition of either DUF283 or hDicer. DUF283 displayed a similar annealing efficiency at corresponding concentrations regardless of which pair of duplexes was used in the assay. The same effect was observed for hDicer.

A comparison of DUF283- and hDicer-assisted annealing showed that in the range from 0 to 100 to 150 nM concentration of either DUF283 or hDicer, annealing proceeded with similar efficiencies. For all tested oligomer pairs (applied at the concentrations of ~2 nM for the ^{32}P-labeled oligomer and ~100 nM for the complementary non-labeled oligomer), the maximum efficiency of annealing was observed at an hDicer concentration of

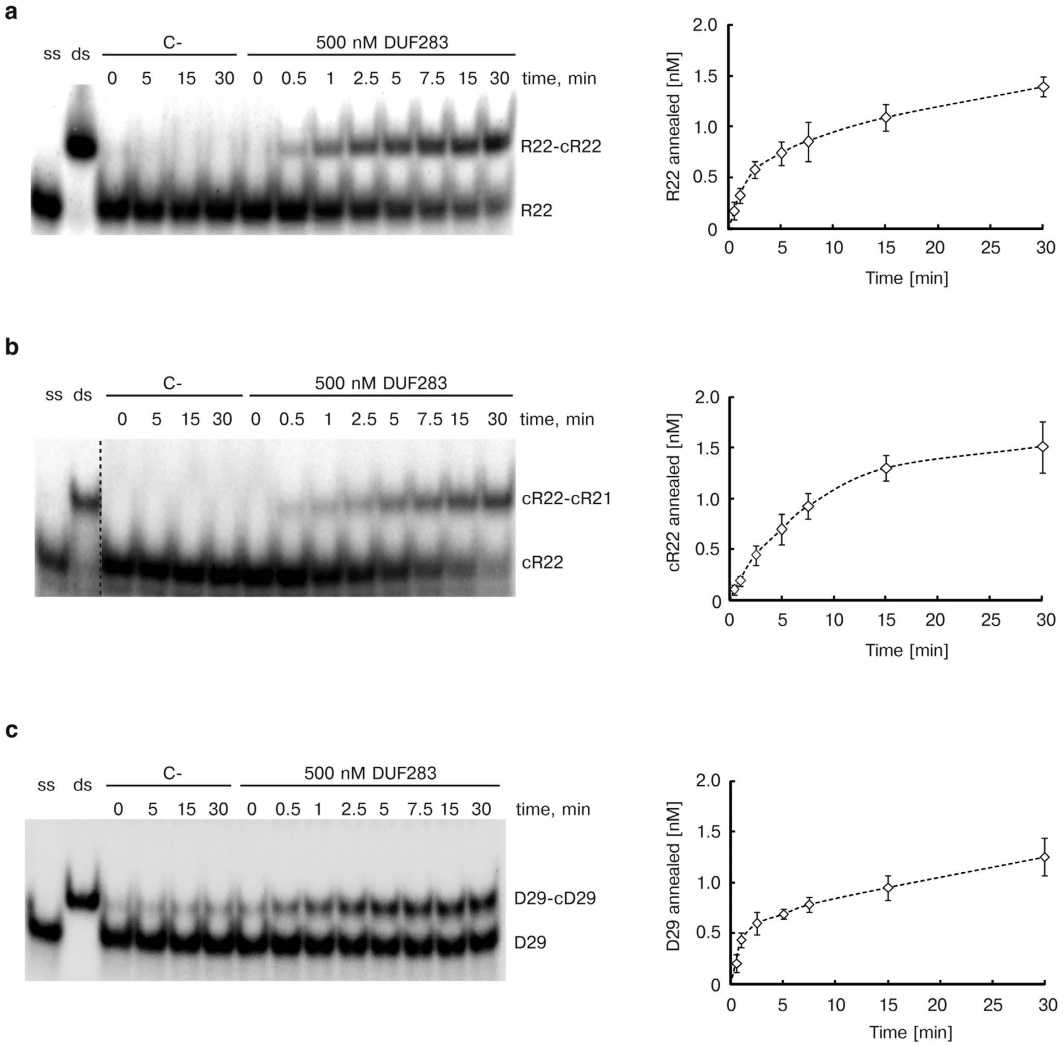

Figure 4. Time-dependent annealing activity of DUF283. Native PAGE gels showing the results of annealing reactions involving the following nucleotide pairs: (**a**) R22 and cR22, (**b**) cR22 and cR21, (**c**) D29 and cD29 (left). The reaction mixtures were incubated for the indicated period of time with no protein [C-] or with 500 nM of DUF283 and were then resolved in buffer containing SDS at a final concentration of 0.2%; [ss] denotes a single strand whereas [ds] a double strand control. Double strand controls contain a pair of the complementary oligonucleotides, in molar ratio of approximately 1:150 between ^{32}P-labeled and unlabeled oligomers, which were mixed in annealing buffer and hybridized by heating and slow cooling from 90 °C to 4 °C. Graphs showing representative time courses of the annealing reactions obtained by densitometric quantification of the autoradiograms (right).

approximately 100–150 nM. At hDicer concentrations above 150 nM, for all tested pairs, we observed a gradual decrease of annealing efficiency until near-baseline levels were reached (Figs 5 and 6). In contrast, for DUF283 at 150 nM and higher concentrations, annealing efficiency increased continuously until reaching a plateau at a DUF283 concentration of ~500 nM (Fig. 6). Although the maximum efficiency of annealing was achieved with a much lower concentration of hDicer compared with that of DUF283, we found that the DUF283 annealing capability was almost twice that of hDicer (see the Δf_{ds} parameters for DUF283 and for hDicer in Fig. 6). This observation is consistent with the earlier reports indicating that hDicer contains several domains diversely involved in single- and double-stranded RNA/DNA binding. Accordingly, one can speculate that at least some of these domains might compete with DUF283 for ssRNA binding and this way reduce the level of ssRNA available for DUF283. This competition might not significantly affect the hDicer-mediated annealing at low hDicer concentrations because the high excess of the complementary oligomer was used in our assay. In line with the above hypothesis, the increase in hDicer concentration above a certain threshold resulted in a gradual reduction of the annealing efficiency.

So far, the tertiary structure of the DUF283-RNA complex remains unknown. Thus, one cannot predict which amino acid residues of DUF283 are involved in its annealing activity. Therefore, we attempted to determine whether the annealing activity is also displayed by the Dicer homolog from *Giardia intestinalis*, which lacks the DUF283 domain. *Giardia* Dicer (GiDicer; often called minimal Dicer) is composed of the N-terminal domain,

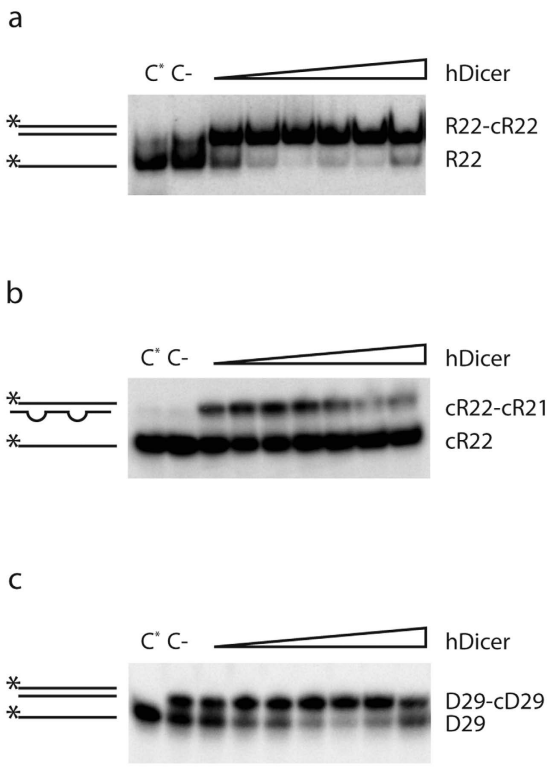

Figure 5. hDicer accelerates annealing of complementary oligonucleotides. Native PAGE gels showing the results of annealing reactions involving hDicer and the following nucleotide pairs: (**a**) R22 and cR22, (**b**) cR22 and cR21, (**c**) D29 and cD29. Reaction mixtures were incubated for 30 min with increasing amounts of hDicer or with no protein [C-] and were then resolved in buffer containing SDS at a final concentration of 0.2%. [C*] denotes the single-strand control containing the ^{32}P-labeled oligomer.

called platform[18,37], PAZ and two RNase III domains, but it lacks some of the domains and regions characteristic of Dicers in higher eukaryotes[13]. The annealing reaction involved the perfectly complementary RNA pair (R22-cR22). The ^{32}P-labeled R22 was mixed with cR22 in annealing buffer and incubated for 5, 15 or 30 min with ~150 nM of GiDicer or ~150 nM of hDicer (Fig. 7). The control reactions lacked either protein. This experiment revealed that, in contrast to hDicer, GiDicer did not exhibit annealing activity. This result does not prove that the annealing activity of hDicer is solely triggered by DUF283; nevertheless, one can hypothesize that annealing activity has emerged in Dicers of higher eukaryotes.

Both isolated DUF283 and complete hDicer support base-paring of short RNA with a complementary fragment of longer RNA *in vitro*. How RISC finds its target RNA remains enigmatic. Extensive studies of RISC-mediated siRNA-target interaction have revealed that these interactions are more complex than simple nucleic acid hybridization and that, presumably, some factors within RISC facilitate target recognition through as-yet-unknown mechanisms[38,39]. Although it has been shown that Ago2 may drive miRNA/siRNA duplex unwinding[40–42], data collected by other groups have suggested that RISC possesses no ability to unfold RNA secondary structures and that transcript cleavage by RISC is limited by the reduced accessibility of the target site in mRNA for the guiding siRNA[43]. Nevertheless, in these previous studies, the authors used a minimal RISC consisting of Ago2 and the guiding siRNA. Although some research groups have suggested that Dicer dissociates from Ago2 after the latter is loaded with an RNA duplex[44], other groups have reported that Dicer is present in RISC and may stimulate processing of target RNA by Ago2[9,10]. Thus, the role of Dicer in coupling mi/siRNA biogenesis and post-transcriptional gene silencing remains elusive. Interestingly, we noted that oligonucleotide annealing occurred with the highest efficiency at similar hDicer concentrations, at which the maximum rate of hDicer substrate cleavage was achieved (approximately 100–150 nM) (Supplementary Fig. S6). This result suggests that these two activities might be functionally correlated.

To explore the possibility that Dicer might be involved in post-transcriptional gene silencing, we sought to determine whether DUF283 and full length hDicer were able to support base-paring of a short ssRNA with a complementary region of a longer ssRNA. In this experiment, we used 21-nt ssRNA (cR21) and a 58-nt ssRNA (R58) with a stem-loop structure. The stem region of R58 contained 19-nt sequence complementary to cR21 (Fig. 8a). Prior to the reaction, R58 was incubated at 95 °C for 5 min and then slowly cooled to room temperature to ensure proper folding. Then, ^{32}P-labeled cR21 was mixed in annealing buffer with an equimolar amount of R58. Increasing amounts of either DUF283 or hDicer were added to the reaction mixture, which was further incubated for 30 min at room temperature. DUF283 or hDicer was replaced with BSA in the control reactions (Fig. 8b–d). Both DUF283 and hDicer facilitated hybridization between cR21 and R58 in a concentration-dependent manner

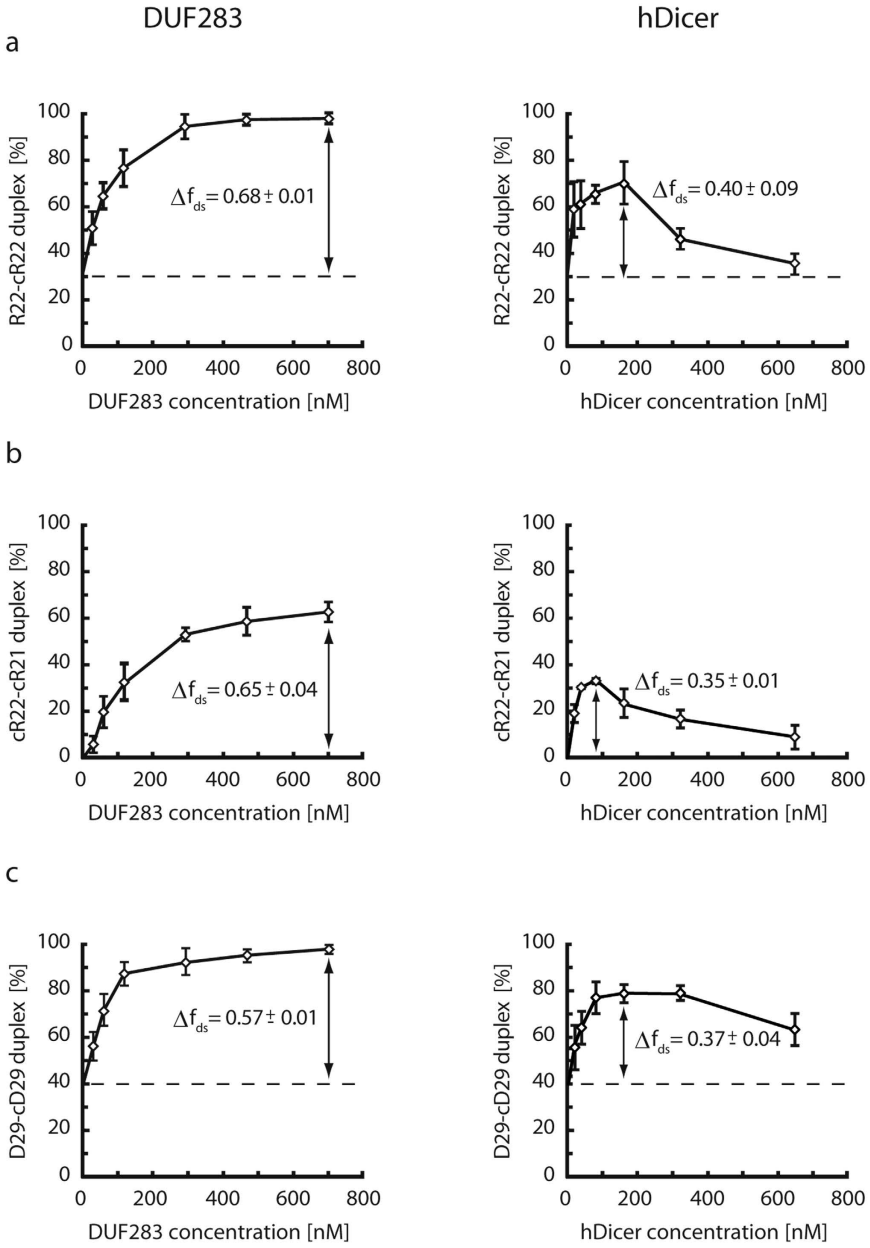

Figure 6. Comparison of DUF283 and hDicer annealing capacities. Graphic presentation of the results obtained from three independent annealing assays involving DUF283 (left) or hDicer (right) and three oligonucleotide pairs, as follows: (**a**) R22 and cR22; (**b**) cR22 and cR21; (**c**) D29 and cD29. The x-axis represents the DUF283/hDicer molar concentrations and the y-axis the percentage content of the double-stranded fraction [f_{ds}]. Dashed lines are drawn for the values obtained for control experiments with BSA (baselines). For each tested pair, the maximum increase in the double-stranded fraction [Δf_{ds}], driven either by DUF283 or hDicer, was calculated.

(Fig. 8b,c, respectively). Importantly, cR21-R58 binding was not observed in the control reactions (Fig. 8d). At high hDicer concentrations, in addition to the bands corresponding to cR21 and the cR21-R58 duplex, we also observed products stacked in the wells (Fig. 8c). These products were presumably super-shifted complexes composed of cR21, R58 and hDicer that were not denatured under the applied PAGE conditions. Such well-complexes were not observed for DUF283. In general, hDicer facilitated cR21-R58 duplex formation less efficiently than DUF283 alone (Fig. 8b,c). As discussed above, this result may be explained by hDicer activity possibly being split between two competing processes, namely the annealing by DUF283 and the binding by other hDicer domains. Nonetheless, more detailed studies are needed to determine whether hDicer domains other than DUF283 (e.g., the helicase domain) might also contribute to this annealing activity. According to the literature, annealing may be considered to be an ATP-dependent or -independent reaction[45–47]. Although we showed that DUF283/hDicer-assisted annealing occurred independently of ATP, the influence of ATP on the observed activity should be further explored. It is also notable that all reactions were performed in annealing buffers containing

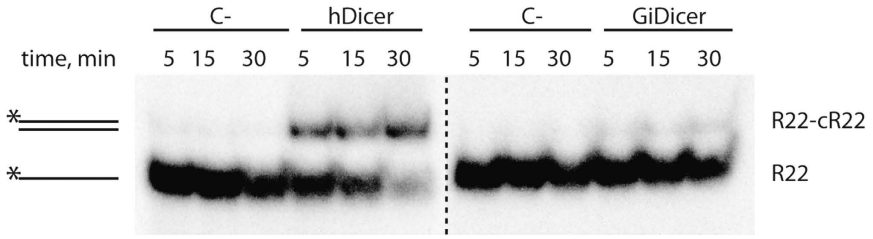

Figure 7. Comparison of hDicer and *Giardia* Dicer annealing capacities. Native PAGE gels showing the results of annealing reactions involving hDicer (left) or *Giardia* Dicer (GiDicer) (right) and the R22 and cR22 oligonucleotide pair. Reaction mixtures were incubated for 5, 15 or 30 min with ~150 nM of hDicer or with ~150 nM of GiDicer or with no protein [C-] and were then resolved in buffer containing SDS at a final concentration of 0.2%.

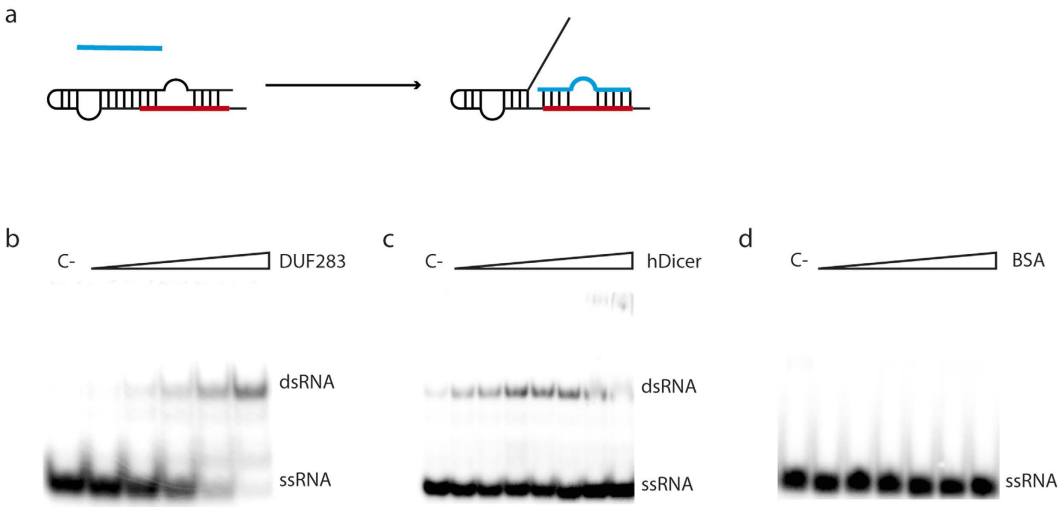

Figure 8. DUF283 and hDicer support base-pairing between a short RNA and the complementary sequence present within a longer RNA. (**a**) Schematic representation of the experiment. The colored lines represent complementary sequences. (**b–d**) Native PAGE gels showing the results of annealing reactions involving equimolar amounts of 21- and 58-nt RNAs and increasing amounts of (**b**) DUF 283, (**c**) hDicer or (**d**) BSA. The reactions were resolved in buffer containing SDS at a final concentration of 0.2%.

Zn^{2+} ions, which have been shown to inhibit Mg^{2+}-dependent substrate cleavage by RNase III-type enzymes (Supplementary Fig. S7) without blocking substrate binding[48]. Thus, one can hypothesize that Zn^{2+} ions may switch Dicer between the cleavage- and annealing-competent states.

In this report, we demonstrated that the DUF283 domain of hDicer binds single-stranded but not double-stranded nucleic acids. We also found that the isolated DUF283 domain as well as hDicer may act *in vitro* as a nucleic acid annealer that accelerates base-pairing between complementary fragments of two nucleic acids. The obtained results also suggested that DUF283/hDicer may relax the local secondary structure. However, we cannot at present explain the mechanism underlying the observed phenomenon and can only hypothesize that DUF283/hDicer might influence the structure of single-stranded nucleic acids via transient interactions, resulting in an annealing-competent state. Such a mode of action has been proposed for several nucleic acid annealers with dsRNA-binding motifs[46,49] similar to the motif identified in DUF283. It has been shown that such proteins exhibit strand-annealing activity with complementary RNAs that do not anneal spontaneously[50–52]. Considering the available tertiary structures of DUF283[26,27], this domain may interact with various single-stranded nucleic acids through a group of positively charged amino acids present on the surface of the β-sheets that constitute a portion of the α-β-β-β-α fold, which is referred to as a dsRNA binding motif. Nevertheless, as stated above, it remains unclear which particular amino acid residues might contribute to the RNA/DNA binding/annealing activity of DUF283.

Our *in vitro* results suggest that Dicer might function as a chaperone-like protein. Interestingly, chaperone machinery has been shown to be indispensable for effective RISC functioning[11]. Clearly, more detailed studies are needed to ascertain whether Dicer can function as a typical chaperone protein *in vitro* and *in vivo*. Additionally, it has been recently shown that Dicer can bind to specific stem-loop structures present within coding sequences and 3′ untranslated regions of various transcripts without performing dicing[53]. These stem-loop structures have been termed "passive Dicer-binding sites". Conceivably, srRNA-associated Dicer may target complementary sequences

present in such passive sites, thereby controlling the translational machinery as well as the fate of targeted transcripts. Given the stoichiometric model of miRNA function reported by Janas *et al.*, a large portion of miRNA molecules are not bound by Ago proteins[54]. Thus, it is possible that Dicer may also function independently of RISC and that miRNAs may bind to mRNAs in the absence of Ago but with the assistance of Dicer.

Methods

Oligonucleotides. Oligonucleotides were purchased from FutureSynthesis; the sequences are provided in Supplementary Table S1. The 5′-^{32}P oligonucleotide labeling by T4 Polynucleotide Kinase (Promega) was performed as described in Kurzynska-Kokorniak *et al.*[30,31]. The ^{32}P-labeled oligonucleotides were PAGE-purified with 8% denaturing polyacrylamide gels and resuspended in water to final concentrations of approximately 10,000 cpm/μL. For the RNA duplex used in the binding or duplex unwinding assays, ^{32}P-labeled R22 (or cR22) was hybridized with 10 pmoles of water-diluted cR22 (or cR21) by heating and slow cooling the mixtures from 90 °C to 4 °C. Next, the reaction mixtures were PAGE-purified with 12% native polyacrylamide gels to obtain pure, double-stranded fractions free of single-stranded species.

DUF283 production and purification. The DUF283 cDNA, which corresponds to the 128-amino acid (aa) sequence located between 625 and 752 aa of hDicer, was amplified by PCR using a purchased plasmid encoding a complete *Homo sapiens* Dicer1 ribonuclease type III sequence (PubMed, NM_030621) (GeneCopoeia). The fragment obtained was cloned into the pMCSG7 vector (courtesy of Laboratory of Protein Engineering, Institute of Bioorganic Chemistry, Polish Academy of Sciences), which introduces a His6 tag at the N-terminus of the protein. DUF283 was expressed in *E. coli* strain BL21Star (Thermo Fisher Scientific) in standard Luria-Bertani (LB) medium. The cells were induced with 0.4 mM IPTG and cultured for 17 hours at 18 °C with shaking. The cell pellets were lysed and purified with Ni^{2+}-Sepharose High Performance beads (GE Healthcare) with an imidazole gradient (0.02 M–1 M) in 0.05 mM Tris buffer (pH 8.0) supplemented with 0.5 M NaCl, 0.1% Triton X-100, and 5% glycerol. The next step of purification was performed using a HiTrap Q HP column (GE Healthcare). DUF283 was eluted and then concentrated in a buffer containing 0.05 M Tris (pH 8.0) 0.25 M NaCl, 0.1% and Triton X-100. The protein purity was assessed by SDS-PAGE, and the band corresponding to a putative DUF283 was cut out of the gel (Supplementary Fig. S1) and then analyzed by mass spectrometry (Supplementary Materials).

hDicer production and purification. A cDNA encoding the complete *Homo sapiens* Dicer1 ribonuclease type III sequence (PubMed accession number NM_030621) was purchased from GeneCopoeia. The full length human Dicer (hDicer) coding sequence was cloned into the pFastBac vector, which introduces a His6 tag at the N-terminus of the protein. Bacmids for insect cell transfection were generated using the Bac-to-Bac® Baculovirus Expression System (Life Technologies). For protein expression, Sf9 insect cells were infected with a recombinant baculovirus and collected after 3 days. The cells were lysed, and hDicer was purified by Ni^{2+} affinity chromatography (Ni-NTA Agarose, Qiagen) followed by ion-exchange chromatography (HiTrap Q HP, GE Healthcare). Finally, the sample was concentrated using Amicon filters (Merck). The protein purity was assessed by SDS-PAGE followed by western blot analysis with an anti-His-tag or anti-hDicer antibody (Supplementary Fig. S4). The protein was concentrated and stored at −20 °C in 20 mM Tris-HCl (pH 7.5) supplemented with 50 mM NaCl and 50% glycerol. The ribonuclease activity of hDicer was assessed in a standard cleavage assay.

hDicer immunoblot analysis. The protein suspensions were analyzed by SDS-PAGE followed by immunoblotting. For immunoblotting, two types of antibodies were used: mouse monoclonal (13D6) against human Dicer (Abcam) or rabbit polyclonal against the His6 tag (Abcam). Immunoreactive proteins were visualized using horseradish peroxidase (HRP) conjugates and enhanced chemiluminescence (ECL).

DUF283 binding assay. The reactions were carried out in 10-μL volumes. DUF283 (0.5; 4.0; 8.0 μM) was added to 10,000 cpm of ^{32}P-labeled RNA, DNA or dsRNA and incubated in binding buffer (150 mM NaCl, 20 mM HEPES (pH 8.0), 0.05% Triton X-100, 15% glycerol) for 15 min on ice. Before being added to the reaction mixtures, RNA or DNA oligomers were denatured for 3 min at 90 °C and rapidly cooled on ice. Control reactions were prepared with BSA (1.0, 5.0 and 10 μM) or a protein preparation obtained from *E. coli* cells transformed with the pMCSG7 plasmid (EP, empty plasmid) expressing only a 26-aa peptide comprising the His6-tag and the TEV protease cleavage site sequence (volumes equal to the volumes of the DUF283 preparations). Control reactions were carried out in supplemented buffers contained either 0.2 mM $ZnCl_2$ or 2.5 mM $MgCl_2$, 50 mM EDTA, or 10 mM phenanthroline (phen). The reactions were separated on 5% native polyacrylamide gels at 4 °C in 1 × TBE running buffer. The data were collected using a Fujifilm FLA-5100 Fluorescent Image Analyzer and quantified using MultiGauge 3.0 (Fujifilm).

Annealing assay. The reactions were carried out in 10-μL volumes. In each reaction set, except as otherwise stated, the nucleotide pair contained 10,000 cpm (approximately 20 fmol) of the ^{32}P-labeled molecule and ~1 pmol of the complementary strand. Corresponding molecules of each pair were mixed in DUF283/BSA annealing buffer (75 mM NaCl, 25 mM Tris-HCl (pH 8.1), 0,05% Triton X-100, 15% glycerol, 0.2 mM $ZnCl_2$) or hDicer annealing buffer (50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.2 mM $ZnCl_2$) and incubated for 30 min, unless stated otherwise, at room temperature with serial dilutions of DUF283 (10–700 nM), hDicer (10–650 nM), BSA (10 nM–1 μM) or *Giardia* Dicer (PowerCut Dicer, Thermo Scientific). The reactions were stopped by the addition of SDS to a final concentration of 0.2% and separated by native gel electrophoresis on 12% polyacrylamide gels at 4 °C in 1 × TBE running buffer. The data were collected using a Fujifilm FLA-5100 Fluorescent Image Analyzer and quantified using MultiGauge 3.0 (Fujifilm). To prove that the upper bands observed in the gels represented the duplexes formed by the tested oligonucleotides, several sets of experiments were carried out (see Supplementary Materials).

Duplex unwinding assay. The reactions were carried out in 10-μL volumes. The RNA duplex (cR22-cR21) was incubated in annealing buffer with increasing amounts of DUF283 (10; 300; 600 nM) or hDicer (10; 300; 600 nM) for 30 min at room temperature. The reactions were stopped by the addition of SDS to a final concentration of 0.2% and separated by native gel electrophoresis on 12% polyacrylamide gels at 4 °C in 1 × TBE running buffer. The data were collected using a Fujifilm FLA-5100 Fluorescent Image Analyzer and quantified using MultiGauge 3.0 (Fujifilm) (Supplementary Fig. S5).

hDicer cleavage assay. The hDicer cleavage assay was performed in a 10-μL volume in buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM NaCl and 2.5 mM $MgCl_2$; 10,000 cpm of ^{32}P-labeled pre-miR-210 was incubated with hDicer (10–650 nM) at 37 °C for 30 min. A control reaction contained 50 mM EDTA. In addition, a negative control reaction with no added enzyme was carried out under the same conditions to test the integrity of the substrate during the incubation. The reactions were stopped by the addition of 1 volume of 8 M urea loading buffer and heating for 5 min at 95 °C; the samples were separated on a 15% polyacrylamide/8 M urea gel. The data were collected using a Fujifilm FLA-5100 Fluorescent Image Analyzer and quantified using MultiGauge 3.0 (Fujifilm) (Supplementary Fig. S6).

Comparison of the influence of Mg^{2+} and Zn^{2+} on hDicer cleavage activity. The reactions were prepared in 10-μL volumes. hDicer (2 pmol) was pre-incubated in buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM NaCl and either 2.5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, or no divalent cations for 5 min at 4 °C. Next, the reaction mixtures containing Mg^{2+} were supplemented with $ZnCl_2$ to a final concentration of 0.1, 0.2, or 0.5 mM. Analogously, the reaction mixtures containing Zn^{2+} were supplemented with $MgCl_2$ to final concentrations of 1.0, 2.0, or 5.0 mM. All reaction mixtures were incubated for an additional 5 min at 4 °C. Cleavage reactions were initiated by the addition of 0.2 pmol of ^{32}P-labeled pre-miR-210 and were further incubated for 30 min at 37 °C. The reactions were stopped by the addition of 1 volume of 8 M urea loading buffer and heating for 5 min at 95 °C; the samples were then separated on a 15% polyacrylamide/8 M urea gel. Data were collected using a Fujifilm FLA-5100 Fluorescent Image Analyzer and quantified using MultiGauge 3.0 (Fujifilm) (Supplementary Fig. S7).

Data analysis. The amount of the ^{32}P-labeled substrate (ssRNA or ssDNA) and the double stranded product (dsRNA or dsDNA) were determined from the intensity of the respective bands in the gels measured by MultiGauge 3.0 software (Fujifilm). Time courses for strand annealing were fitted by numerical integration. The initial velocities were obtained as, $V_0 = (d\,[dsRNA]/dt)t = 0$ from the slopes of the fitting curves at their zero time.

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Author Contributions

A.K.K. conceived and developed the concept, designed all experiments, analyzed and discussed all results and wrote the paper. M.P. generated DUF283. M.P. and N.K. designed and carried out the experiments, analyzed and discussed the results, and helped prepare the manuscript. W.H. generated hDicer. W.H. and K.B.S. discussed the results and helped with the experiments involving hDicer. M.F. conceived and supervised these studies, analyzed and discussed their results and edited the manuscript.

Additional Information

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Revealing a new activity of the human Dicer DUF283 domain *in vitro*

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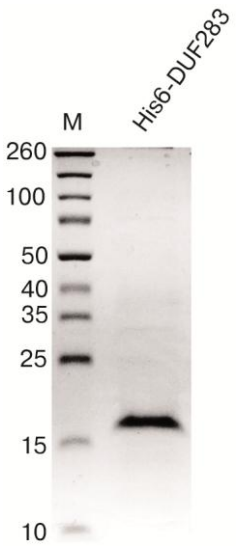
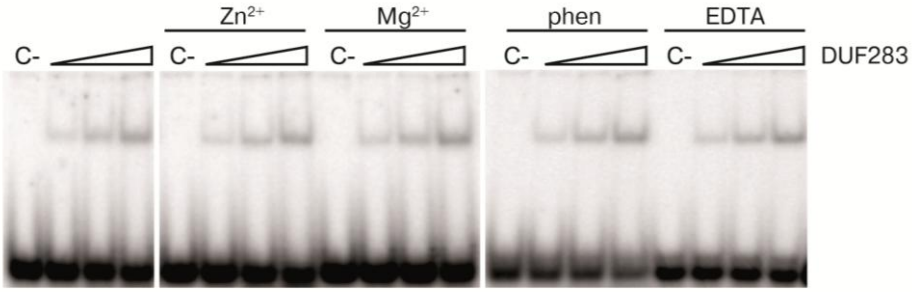
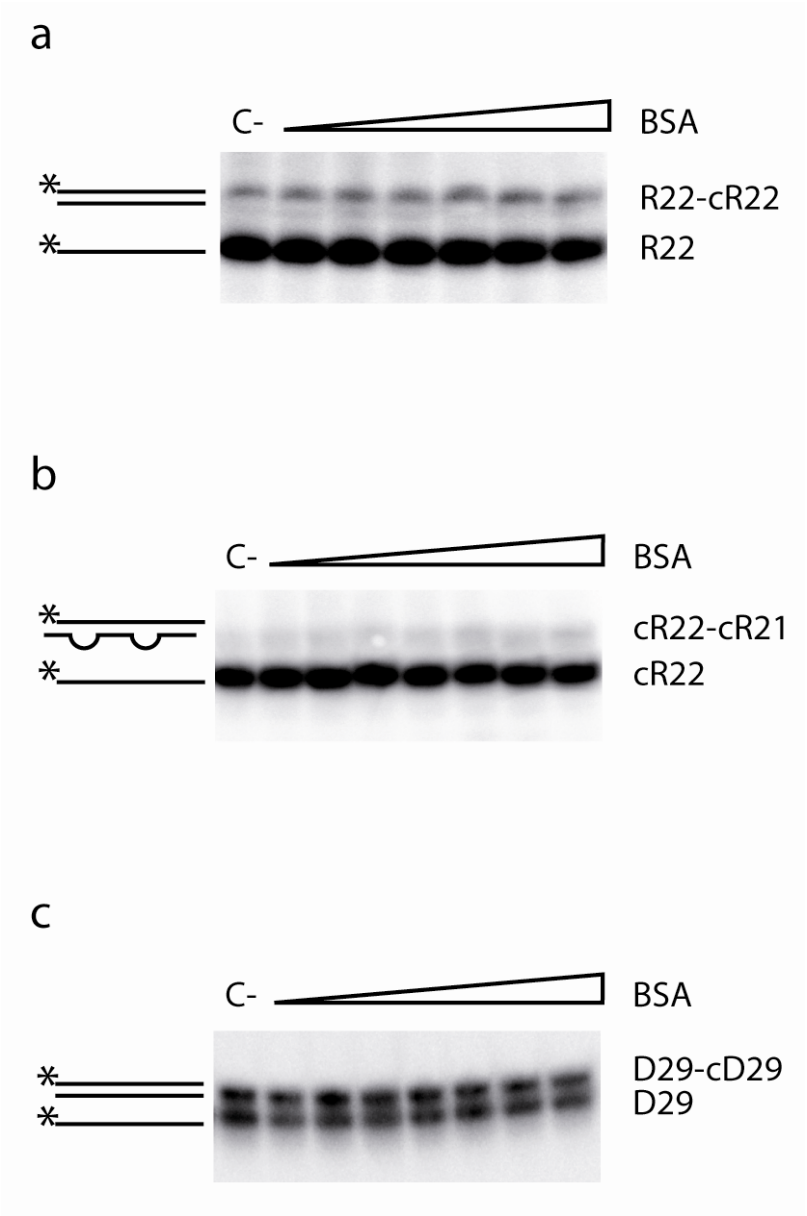


Supplementary Figure S1. SDS-PAGE analysis of the DUF283 preparation.

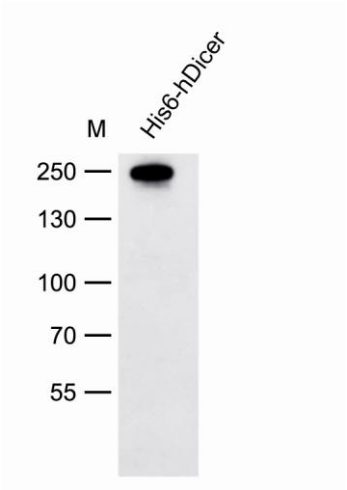
Polyhistidine-tagged DUF283 (~17 kDa; 6 µg) was analyzed by SDS-PAGE, and the proteins were stained with Coomassie brilliant blue. [M] denotes protein size marker (10 – 260 kDa, Thermo Scientific). The band corresponding to approximately 17 kDa was cut from the gel and analyzed by mass spectrometry.



Supplementary Figure S2. DUF283 binds ssRNAs independent of the presence of divalent cations. Native PAGE gels showing the results of binding assays involving DUF283 and 22-nt ssRNA; the binding buffer lacked divalent cations, contained Mg^{2+}, contained Zn^{2+}, contained the Zn^{2+} chelating agent phenanthroline (10 mM), or contained the Mg^{2+} chelating agent EDTA (50 mM), as indicated.

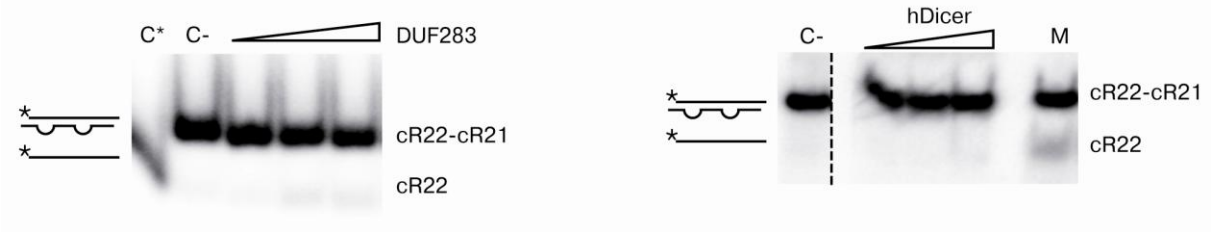


Supplementary Figure S3. BSA does not accelerate base-pairing of complementary oligonucleotides. Native PAGE gels showing the results of annealing reactions involving BSA and the following nucleotide pairs: (a) R22 and cR22, (b) cR22 and cR21, (c) D29 and cD29. The reaction mixtures were incubated for 30 min with increasing amounts of BSA (10 nM – 1 μM) or with no protein [C-] and then resolved in buffer containing SDS at a final concentration of 0.2%.

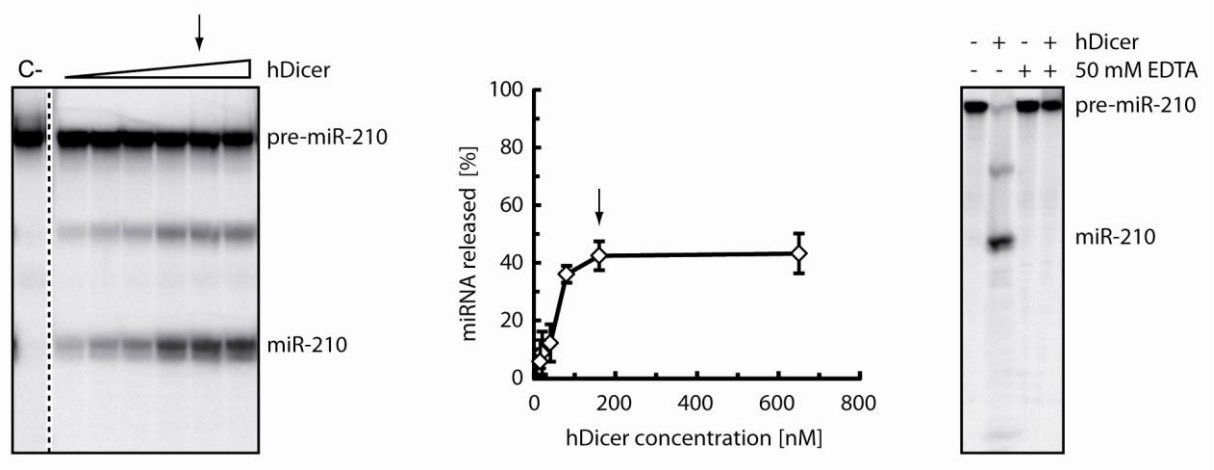


Supplementary Figure S4. Western-blot analysis of the hDicer preparation.

The polyhistidine-tagged hDicer preparation (~220 kDa) was analyzed by Western blotting with an anti-Dicer monoclonal antibody (13D6, Abcam). Molecular-weight size standards [M] are marked on the left (55 – 250 kDa).

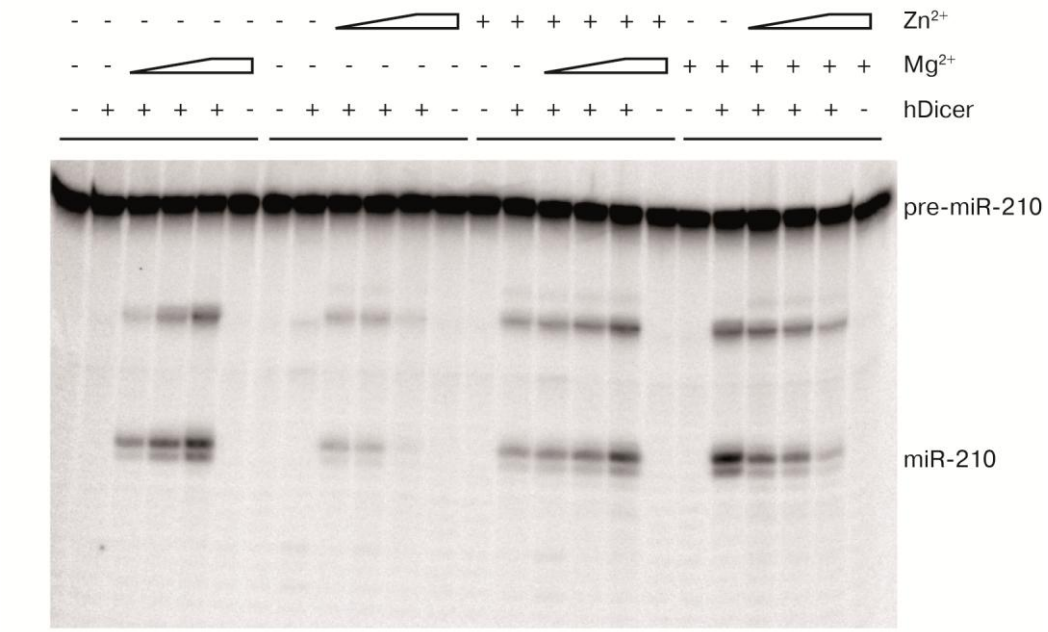


Supplementary Figure S5. DUF283 and hDicer do not show duplex unwinding activity.
The RNA duplex (cR22-cR21) was incubated in annealing buffer with increasing amounts of DUF283 (left) or hDicer (right) for 30 min at room temperature. Reaction mixtures were resolved in buffer containing SDS at a final concentration of 0.2%. [C-] denotes a control reaction with no protein. Schematic representations of single-stranded and double-stranded forms are shown on the left. The asterisk indicates the ^{32}P 5′-end label. [C*] denotes the single-strand control containing the ^{32}P-labeled oligomer only. [M] denotes the single-strand [cR22] and double-stranded [cR22-cR21] control.



Supplementary Figure S6. hDicer cleaves pre-miRNA in a concentration-dependent manner.

The ^{32}P-labeled pre-miR-210 was incubated with increasing amounts of hDicer (10, 30, 50, 100, 150, 650 nM; presented as a triangle) or with no protein [C-] for 30 min at 37°C. The samples were separated on a 15% polyacrylamide/8 M urea gel (left). Based on the results obtained from three independent experiments, we calculated for each reaction the ratios between the substrate (pre-miR-210) and product (miR-210). The average percentage of the product released was plotted against the molar concentrations of hDicer (middle). The arrow indicates the hDicer concentration at which the maximum efficiency of the substrate cleavage was achieved. 50 mM EDTA inhibits Mg^{2+}-dependent cleavage of the ^{32}P-labeled pre-miR-210 by hDicer (right).



Supplementary Figure S7. Biochemical analysis of hDicer used in the experiments.

Processing of pre-miRNAs by hDicer is Mg^{2+} dependent but is inhibited by Zn^{2+}. The ^{32}P-labeled pre-miR-210 was incubated with hDicer in buffer containing increasing amounts of either Mg^{2+} (1.0 – 5.0 mM) or Zn^{2+} (0.1 - 0.5 mM); alternatively, the reaction buffer contained either 0.1 mM $ZnCl_2$ or 2.5 mM $MgCl_2$ and was subsequently supplemented with increasing amounts of Mg^{2+} (1.0 – 5.0 mM) or Zn^{2+} (0.1 - 0.5 mM), respectively.

Supplementary Table S1. Oligonucleotide sequences.

Name	Sequence (5'→3')
R12	GAAUCUUAACGC
R22	UCAACAUCAGUCUGAUAAGCUA
cR22	UAGCUUAUCAGACUGAUGUUGA
cR21	CAACACCAGUCGAUGGGCUGU
R32	GUGCAUUGUAGUUGCAUUGCAUGUUCUGGUCA
R52	GGGAGAAUCAUAAGUAGCCCCUCGUUCACUCCCCCCAUGUUAACAGUUAGCC
R58	AGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGU
R62	UAGCAGCACGUAAAUAUUGGCGUUAAGAUUCUAAAAUUAUCUCCAGUAUUAACUGUGCUG CU
D29	AAAAAGTACACAGTCTAACATCAACTCGC
cD29	GCGAGTTGATGTTAGACTGTGTACTTTTT
pre-miR-210	GCCCCUGCCCACCGCACACUGCGCUGCCCCAGACCCACUGUGCGUGUGACAGCGGCUG

Supplementary Results

Preparation of single strand and double strand controls.

The ^{32}P-labeled cR22 oligomer (cR22*) was mixed with the high excess of the unlabeled complementary cR21 oligomer in the annealing reaction buffer. Then, the reaction mixture was heated to 90°C and slowly cooled down to 4°C to enforce cR22* hybridization with cR21. As a control, a similar reaction without cR21 addition was performed. Finally, the reaction mixtures were incubated for 15 min at room temperature and analyzed in 12% native polyacrylamide gel in a buffer containing 0.2% SDS (see below Figure 1SR, lanes 1 and 2). An analogous experiment with ^{32}P-labeled cR21 oligomer (cR21*) and unlabeled cR22 was performed (see below Figure 1SR, lanes 3 and 4).

Additionally, similar reactions involving cR22* and cR21 as well as cR21* and cR22 were performed, however, in these reactions lower excess of the unlabeled oligomer was applied and hybridization between complementary oligomers were mediated by the addition of DUF283, not by heating to 90°C and cooling down to 4°C. The reaction mixtures were incubated for 15 min at room temperature and analyzed in 12% native polyacrylamide gel in a buffer containing 0.2% SDS (Figure 1SR, lanes 5, 6 and 7, 8).

In the last set of experiments labeled cR22* and labeled cR21* were mixed with unlabeled cR22 and cR21, respectively, and then DUF283 was added. In control experiments DUF283 was added to reaction mixtures containing either labeled cR22* or labeled cR21* (without unlabeled cR22 and cR21, respectively). The reaction mixtures were incubated for 15 min at room temperature and analyzed in 12% native polyacrylamide gel in a buffer containing 0.2% SDS (Figure 1SR, lanes 9, 10 and 11, 12).

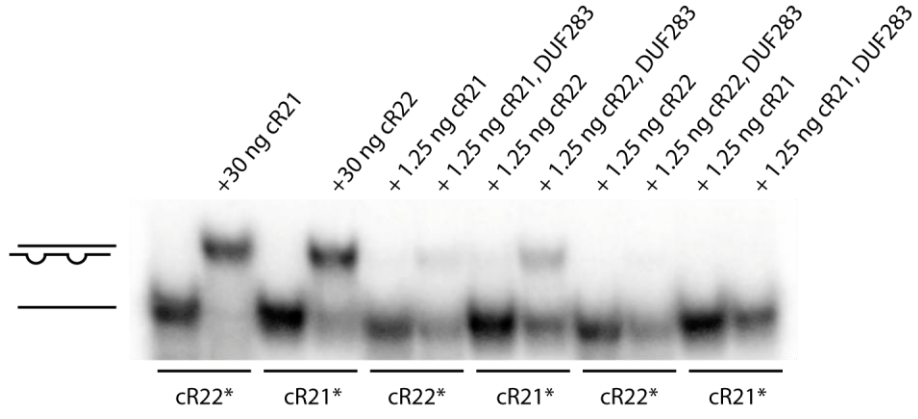


Figure 1SR. Native PAGE gel showing the results of annealing assays involving cR22 and cR21 nucleotide pair (the imperfect duplex). In each reaction ~0.1 ng (approximately 20 fmol) of the ^{32}P-labeled oligonucleotide was used.

Altogether, the data presented in Figure 1SR clearly indicate that DUF283 mediates hybridization between complementary oligomers. Moreover, these experiments permitted to distinguish between single stranded substrates and double stranded products (single and double strand controls).

mMass Report: *1*

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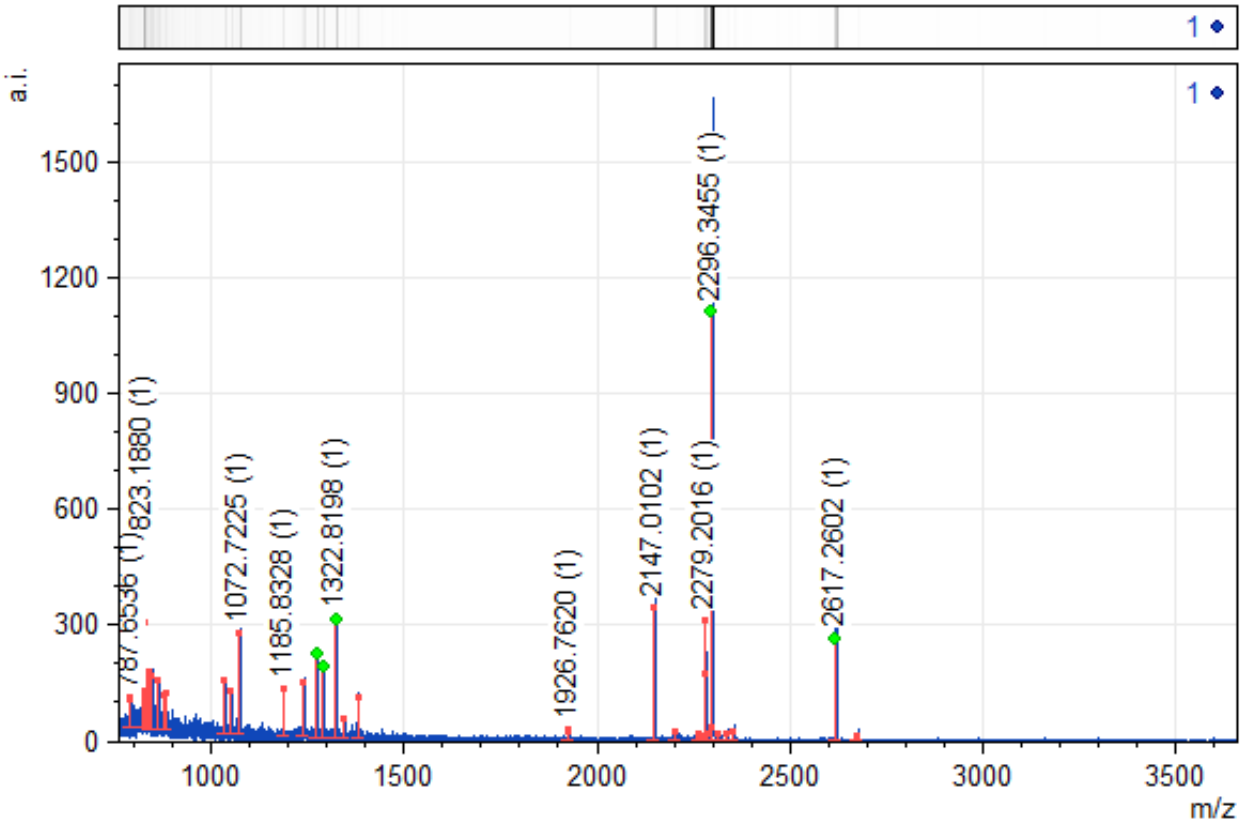

Sequence - *Dicer*

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	151	17343.5913	17354.4528	44.4 %	33.7 %

HHHHHHSSGV DLGTENLYFQ SNAVTINTAI GHINRYCARL PSDPFTHLAP KCRTRELPDG TFYSTLYLPI NSPLRASIVG PPMSCVRLAE RVVALICCEK LHKIGELDDH LMPVGKETVK YEEELDLHDE EETSVPGRPG STKRRQCYPK A

Position	Modification	Type	Mo. Mass	Av. Mass	Formula
All C	Carbamidomethyl	fixed	57.0215	57.0514	CH2CONH2 - H
All M	Oxidation	variable	15.9949	15.9994	O

Meas. m/z	Calc. m/z	δ (Da)	δ (ppm)	Rel. Int. (%)	z	Annotation	Formula
1273.7880	1273.6391	0.1488	116.8	19.24	1	[76-87] r.ASIVGPPMSCVR.I [1xCarbamidomethyl]	C53H92N16O16S2
1289.7315	1289.6341	0.0975	75.6	16.30	1	[76-87] r.ASIVGPPMSCVR.I [1xCarbamidomethyl; 1xOxidation]	C53H92N16O17S2

1322.8198	1322.7103	0.1095	82.8	27.63	1	[40-51] r.LPSDPFTHLAPK.c	C62H95N15O17
2296.3455	2296.1860	0.1595	69.5	100.00	1	[56-75] r.ELPDGTFYSTLYLPINSPLR.a	C107H162N24O32
2617.2602	2617.1900	0.0702	26.8	24.04	1	[121-143] k.YEEELDLHDEEETSVPGRPGSTK.r	C110H169N29O45

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ORIGINAL ARTICLE

The RNA–RNA base pairing potential of human Dicer and Ago2 proteins

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Abstract

The ribonuclease Dicer produces microRNAs (miRNAs) and small interfering RNAs that are handed over to Ago proteins to control gene expression by targeting complementary sequences within transcripts. Interestingly, a growing number of reports have demonstrated that the activity of Dicer may extend beyond the biogenesis of small regulatory RNAs. Among them, a report from our latest studies revealed that human Dicer facilitates base pairing of complementary sequences present in two nucleic acids, thus acting as a nucleic acid annealer. Accordingly, in this manuscript, we address how RNA structure influences the annealing activity of human Dicer. We show that Dicer supports hybridization between a small RNA and a complementary sequence of a longer RNA in vitro, even when both complementary sequences are trapped within secondary structures. Moreover, we show that under applied conditions, human Ago2, a core component of RNA-induced silencing complex, displays very limited annealing activity. Based on the available data from new-generation sequencing experiments regarding the RNA pool bound to Dicer in vivo, we show that multiple Dicer-binding sites within mRNAs also contain miRNA targets. Subsequently, we demonstrate in vitro that Dicer but not Ago2 can anneal miRNA to its target present within mRNA. We hypothesize that not all miRNA duplexes are handed over to Ago proteins. Instead, miRNA-Dicer complexes could target specific sequences within transcripts and either compete or cooperate for binding sites with miRNA-Ago complexes. Thus, not only Ago but also Dicer might be directly involved in the posttranscriptional control of gene expression.

Keywords RNA-binding proteins · RNA annealers · RNA-annealing activity · miRNA/siRNA pathways · Translational regulator · mRNA fate

Introduction

The ribonuclease Dicer plays a fundamental role in the biogenesis of small regulatory RNAs, such as microRNAs (miRNAs) and small interfering RNAs (siRNAs). Dicer recognizes and cleaves single-stranded miRNA precursors (pre-miRNAs) adopting stem-loop structures and double-stranded RNAs (dsRNAs) into functional 21–23-nucleotide (nt) miRNAs and siRNAs, respectively [1]. Dicer proteins are multidomain enzymes. Human Dicer is composed of an (N)-terminal putative helicase domain, a DUF283 domain (domain of unknown function), Platform, a PAZ (Piwi–Argonaute–Zwille) domain, two RNase III domains (RNase IIIa and RNase IIIb) and a dsRNA-binding domain (dsRBD). The N-terminal helicase domain has been shown to specifically interact with single-stranded hairpin loops of pre-miRNAs [2–4]. The DUF283 domain has been demonstrated to bind single-stranded nucleic acids [5], which may

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suggest its involvement in interactions with hairpin loops of pre-miRNAs as well [4]. Two adjacent domains, Platform and PAZ, anchor the 5′ phosphate and 2-nt 3′ overhang of a substrate [2, 6]. The RNase IIIa and RNase IIIb domains form a single-dsRNA cleavage center that cuts approximately 20 base pairs from the termini of a miRNA or siRNA precursor [6, 7]. The C-terminal dsRBD plays only an auxiliary role in RNA binding [8]. Dicer-generated miRNAs or siRNAs are handed over to Argonaute (Ago) proteins to control gene expression by targeting complementary sequences within mRNA transcripts. Most miRNAs, by base pairing with mRNA targets through a 7–8 nt seed sequence, induce translation inhibition of the target gene [9]. On the contrary, siRNAs which are fully complementary to the target, trigger mRNA cleavage by Ago proteins [10]. Nevertheless, even a near-perfect base pairing of miRNA to its target has been shown to induce mRNA cleavage [11, 12].

Apart from being involved in miRNA and siRNA biogenesis, human Dicer is also known for its participation in multiple cellular events (reviewed in [13]). Additionally, the results of our latest studies have revealed that recombinant human Dicer (called later in the text "hDicer") is capable of supporting base pairing between complementary RNA molecules, which suggests that this enzyme might function as a nucleic acid annealer [5]. RNA annealers, like RNA chaperones and RNA helicases, facilitate RNA folding and help RNA molecules adopt their functional structures in vivo [14–16]. Moreover, RNA chaperones and RNA annealers have been demonstrated to facilitate interactions between complementary sequences present in two separate RNA molecules. Examples of protein-assisted annealing phenomena can be observed during pre-mRNA editing in kinetoplastid organisms [17, 18], biogenesis of 18S rRNA in *S. cerevisiae* [19, 20], and RNA interference [14]. In all three cases, a guide RNA is tightly bound to an annealer such that the bases are exposed for binding with a complementary sequence within a target RNA. In the case of RNA interference, the effector complex called RNA-induced silencing complex (RISC), composed of a small RNA (miRNA or siRNA) and the Ago protein, binds to mRNA and induces its translational repression or degradation [21]. Nevertheless, RISC has been shown to face difficulties when a complementary sequence is located within stable secondary structures present in target RNAs [21]. Importantly, the results of our previous studies have demonstrated that hDicer can facilitate base pairing between complementary fragments of two nucleic acids, even when one RNA molecule adopts a stable structure [5]. In addition, Dicer has been shown to bind not only miRNA or siRNA precursors but also mRNAs and long noncoding RNAs, without processing them into small RNAs [22]. Since base pairing between the small regulatory RNAs and their targets on mRNAs is of a critical importance for the fate of the mRNA, in this manuscript, we sought to gain a deeper insight into the RNA–RNA base pairing potential of human Dicer and Ago2, the two proteins found to bind both small RNAs and mRNA transcripts. The present study is, to our knowledge, the first to demonstrate a comprehensive analysis on how RNA structure influences the RNA-annealing activity of essential proteins of the miRNA/siRNA pathways, Dicer and Ago2.

Materials and methods

Oligonucleotides

RNA and DNA oligonucleotides (Table 1) were purchased from FutureSynthesis. Ex21 DNA was transcribed in vitro with an AmpliCap-Max T7 High Yield Message Maker Kit (CELLSCRIPT). 5′-^{32}P oligonucleotide labeling by T4 Polynucleotide Kinase (Promega) was performed as described earlier [5]. The Ex21 transcript was labeled at the 3′ terminus by T4 RNA ligase (Thermo Fisher Scientific) and ^{32}P-Cp.

Table 1 Oligonucleotide sequences

Name	Sequence (5′→3′)
R21	UCGAAGUAUUCCGCGUACGUG
mR21	GCGUAAGCGGAAUAAUUCGAU
cR21	CGUACGCGGAAUACUUCGAAA
miR-103a-5p	GGCUUCUUUACAGUGCUGCCUUG
miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA
pre-miR-21	UGUCGGGUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUCUGACA
pre-miR-33a	CUGUGGUGCAUUGUAGUUGCAUUGCAUGUUCUGGUGGUACCCAUGCAAUGUUUCCACAGUGCAUCACAG
Mod18	GGUUGAACUAUUUCGUCUAUCUGGAAACACGUACGCGGAAUACUUCGAUU
Mod23	GGUUGAACUAUUUCGUGUAUCUGGAAACACGUACGCGGAAUACUUCGAUU
Mod33	GGUUGAAGUAUUUUGUGUAUGUGGAAACACGUACGCGGAAUACUUCGAUU
Ex21	CTATTAGCACCTTGATGTGCAGCATTTTCAGGGACAATTGTGCTGTGCTTACAGTAATTATCTATAGTGAGTCGTATTA

Mod oligonucleotides and the Ex21 transcript, to adopt their native structures, were denatured in 50 mM NaCl for 3 min at 90 °C, immediately transferred to 75 °C and slowly cooled down to 10 °C. Duplexes were prepared as described earlier [5].

Endonucleases used in the studies

Recombinant human Ago2 (hAgo2) protein was purchased from Active Motif, Giardia intestinalis endoribonuclease Dicer-like recombinant protein (GiDicer) was from MyBiosource, and recombinant human Dicer (hDicer) was produced in our laboratory. The baculovirus expression system entry plasmid-encoding human Dicer with a His-tag at the C terminus was kindly provided by Witold Filipowicz. hDicer was prepared as described by Zhang et al. [23], however, a final dialysis against EDTA-containing buffer was omitted. For purification and storage, non-reducing conditions were applied. The SDS-PAGE gels of hDicer, GiDicer and hAgo2 preparations are presented in Supplementary Fig. S1.

Annealing assay

The reactions were carried out in 20-µL volumes. Each reaction set, unless otherwise noted, contained 10,000 cpm (approximately 5 nM) of the 5′-end ^{32}P-labeled RNA molecule (R21, miR-103a-3p, miR-103a-5p) or RNA duplex (R21-cR21, R21-mR21, miR-103a) and 5 nM of long complementary RNA (Mod18, Mod23, Mod33, Ex21). Alternatively, in the case of experiments including Ex21, the long RNA (Ex21) was 3′-end ^{32}P labeled. The corresponding molecules were mixed in annealing buffer (50 mM NaCl, 20 mM Tris–HCl (pH 7.5), 0.05% Triton X-100, 5% glycerol) and incubated for 30 min (unless stated otherwise) at 37 °C with dilutions of hDicer (0, 0.75, 3, 7.5, 12, 15 nM), GiDicer (0, 5, 10, 12.5, 20, 25 nM) or hAgo2 (0, 2.5, 5, 12.5, 25, 37.5, 50 nM). In the time-course annealing experiments, 7.5 nM of a protein was applied. In each annealing assay, the protein was preincubated with a short RNA for 15 min at 4 °C before the complementary long RNA was added, unless otherwise stated in the text. In the case of the control annealing experiment, buffer B1 (100 mM KCl, 2 mM $MgCl_2$, 30 mM HEPES (pH 7.4), 0.5 mM DTT, 3% glycerol) or buffer B2 (100 mM KCl, 5 mM $MgCl_2$, 30 mM HEPES (pH 7.4), 0.5 mM DTT, 3% glycerol, 7 mM EDTA) was applied. The reactions were stopped by the addition of SDS to a final concentration of 1% and separated by native gel electrophoresis in 10% polyacrylamide gels at 4 °C in 1 × TBE running buffer.

Duplex stability assay

miRNA-like or siRNA-like duplexes were incubated at 37 °C in annealing buffer with or without 7.5 nM hDicer for 0, 2, 5, 15, 30 and 60 min in the presence or absence of 1 mM ATP. The reactions were stopped and analyzed as described in the annealing assays.

Dicer cleavage assay

The cleavage assay was performed in 10-µL volumes in buffer containing 50 mM NaCl, 20 mM Tris (pH 7.5) and 2.5 mM $MgCl_2$. The reaction mixture included ^{32}P-labeled substrate and either hDicer (10 nM) or GiDicer (25 nM). The incubation was carried out at 37 °C for 1 h with hDicer or 16 h with GiDicer. The reaction mixtures were denatured and subsequently loaded on 15% polyacrylamide gel supplemented with 7 M urea and 1 × TBE. Electrophoresis was run for 2 h under 1200 V in 1 × TBE buffer. The cleavage assays are presented in Supplementary Fig. S1A and C.

Ago2 cleavage assay

The assay was performed in 10-µL volumes using ^{32}P-labeled Mod18 (1000 cpm/µL) as a target RNA, 100 nM R21 as a guide, and 100 nM hAgo2. The incubation buffer contained 50 mM NaCl, 20 mM Tris (pH 7.5), 1 mM ATP and 5 mM $MgCl_2$. Human Ago2 was preincubated with R21 for 15 min at 4 °C before Mod18 was added. The reaction was performed for 1 h at 37 °C and subsequently stopped before analysis as described for the Dicer cleavage assay. The cleavage assay is presented in Supplementary Fig. S1B.

Gel imaging and analysis

The data were collected using a Fujifilm FLA-5100 Fluorescent Image Analyzer. The amounts of ^{32}P-labeled substrate and double-stranded product were determined from the intensity of the respective bands in the gels measured by MultiGauge 3.0 software (Fujifilm). Time courses for annealing assays were fitted by numerical integration. The initial velocities were obtained as $V_0 = (d\,[\mathrm{dsRNA}]/\mathrm{d}t)t = 0$ from the slopes of the fitting curves at their zero time.

Free energy calculations

In the case of monomolecular folding, the free energies were calculated by RNAstructure from the ViennaRNA Package (https://rna.urmc.rochester.edu/RNAstructure.html) [24]. In the case of bimolecular interactions, the free energies were calculated by IntaRNA (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp) [25], the software which uses energy parameters from the ViennaRNA Package, making

the calculation results compatible with those made with RNAstructure.

Data analysis

Genomic coordinates and annotations of Dicer-binding sites were obtained from Rybak-Wolf et al. [22] (Supplementary Table S1, Sheet 1). Genomic coordinates and annotations of Ago2 and Ago3-binding sites were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), accession numbers GSM1334330 and GSM1334331, respectively. The sequence of the hg19 human genome was obtained from UCSC (http://hgdownload.soe.ucsc.edu/goldenPath/hg19/bigZips/). Locations of predicted interaction sites of miRNA molecules within the selected transcripts were obtained from mirDB (http://mirdb.org/). Genomic coordinates were converted from hg19 notation to hg38 notation using LiftOver software 1 (https://genome.ucsc.edu/cgi-bin/hgLiftOver). Tables of miRNA target sites within the Dicer protein coding sequence and their intersections with binding sites of Dicer or Ago2/3 (Supplementary Table S1) and the intersecting binding sites of Dicer and Ago2/3 (Supplementary Table S2) were made with in-house Python scripts.

Results

Influence of the RNA structure on the annealing activity of hDicer

To explore the influence of RNA structure on the annealing activity of hDicer, we applied a set of pairs of complementary RNAs originally designed by Ameres et al. to investigate an association of human RISC with target RNAs [21]. Each pair consisted of a short 21-nt RNA, termed "R21" or "guide RNA", and a longer 50-nt target RNA adopting a hairpin structure and containing the fully complementary R21 target site, schematically described in Fig. 1a. Within all pairs, R21 remained unchanged, whereas the sequence composition outside of the 21-nt target site in the longer RNA was changed so that the accessibility of the target site for R21 was gradually reduced due to an increase in the secondary structure stability of the hairpin formed by the longer RNA [21]. In total, we used three target RNAs, named by Ameres et al. as follows: Mod18, Mod23 and Mod33. The free energy values, calculated by RNAstructure software [24], were as follows: − 14.6 kcal/mol for Mod18; − 20.1 kcal/mol for Mod23; and − 29.8 kcal/mol for Mod33. The lowest free energy value indicates the most stable structure. Secondary structures of Mod RNAs are presented in Supplementary Fig. S2.

In the preliminary assays, ^{32}P-labeled R21 was mixed in annealing buffer with Mod18, Mod23, or Mod33 at a molar ratio of approximately 1:1 between ^{32}P-labeled and unlabeled oligomers and incubated for 30 min with increasing amounts of hDicer at 37 °C (Fig. 1b). Spontaneous annealing was determined by excluding the enzyme in the assay mixture. In addition, we performed a control reaction with R21, Mod18 and, as in our previous assays, *Giardia intestinalis* Dicer (GiDicer), which has been shown to lack annealing activity (Fig. 1c) [5]. As expected, the collected results demonstrated that the most efficient annealing occurred when R21 and Mod18 were applied, and this efficiency decreased with an increase in the secondary structure stability of the target RNA (Fig. 1a, b). Incubation of R21 and Mod18 with GiDicer did not enhance annealing between R21 and Mod18 compared to the control reaction without the protein (Fig. 1c).

Next, to assess the rate of hDicer-facilitated annealing, we performed time-course assays that involved R21 and Mod18, Mod23, or Mod33. In these assays, in addition to the above-characterized three pairs of substrates, we also used two short RNAs that were differentially base paired with guide RNA, R21. One 21-nt RNA, called "mR21", was designed using EvOligo software computation core [26] to form a double-mismatched duplex with R21, that mimicked a miRNA duplex. The other 21-nt RNA, called "cR21", formed a perfect duplex with R21 that mimicked an siRNA duplex (based on Ameres et al. [21]). In this way, we obtained nine possible combinations of three different 'donors' and three different 'targets'. In the group of 'donors' there were: R21, R21-mR21 (a miRNA-like duplex) and R21-cR21 (an siRNA-like duplex), while in the group of 'targets' there were: Mod18, Mod23 and Mod33 (Fig. 2). The free energies calculated by RNA structure [27] or IntaRNA [25] for all 'donors' and 'targets' are shown next to the predicted structures in Fig. 2. The predicted free energy values for all resultant R21-target complexes (products), i.e., R21-Mod18, R21-Mod23 and R21-Mod33, were very similar (≈ − 39.3 kcal/mol), so that the base pairing efficiency between R21 and the individual Mods should depend only on the secondary structures adopted by substrates, i.e., 'donors' and 'targets'. The corresponding 'donors' and 'targets', as presented in Fig. 2, were mixed in annealing buffer in a molar ratio of approximately 1:1 and incubated for 2, 5, 15, 30 or 60 min with 7.5 nM hDicer at 37 °C. Control reactions either lacked hDicer or contained 7.5 nM GiDicer instead of hDicer. Based on the results obtained from three independent experiments, for each reaction, we calculated the percentage ratios between the fraction containing the R21-Mod duplex and the free 'donor' fraction, as indicated in Supplementary Fig. S3. The average percentage content of the R21-target duplex was plotted against the incubation time (Fig. 2).

In a time-course experiment involving substrates with the least stable secondary structures, i.e., R21 and Mod18

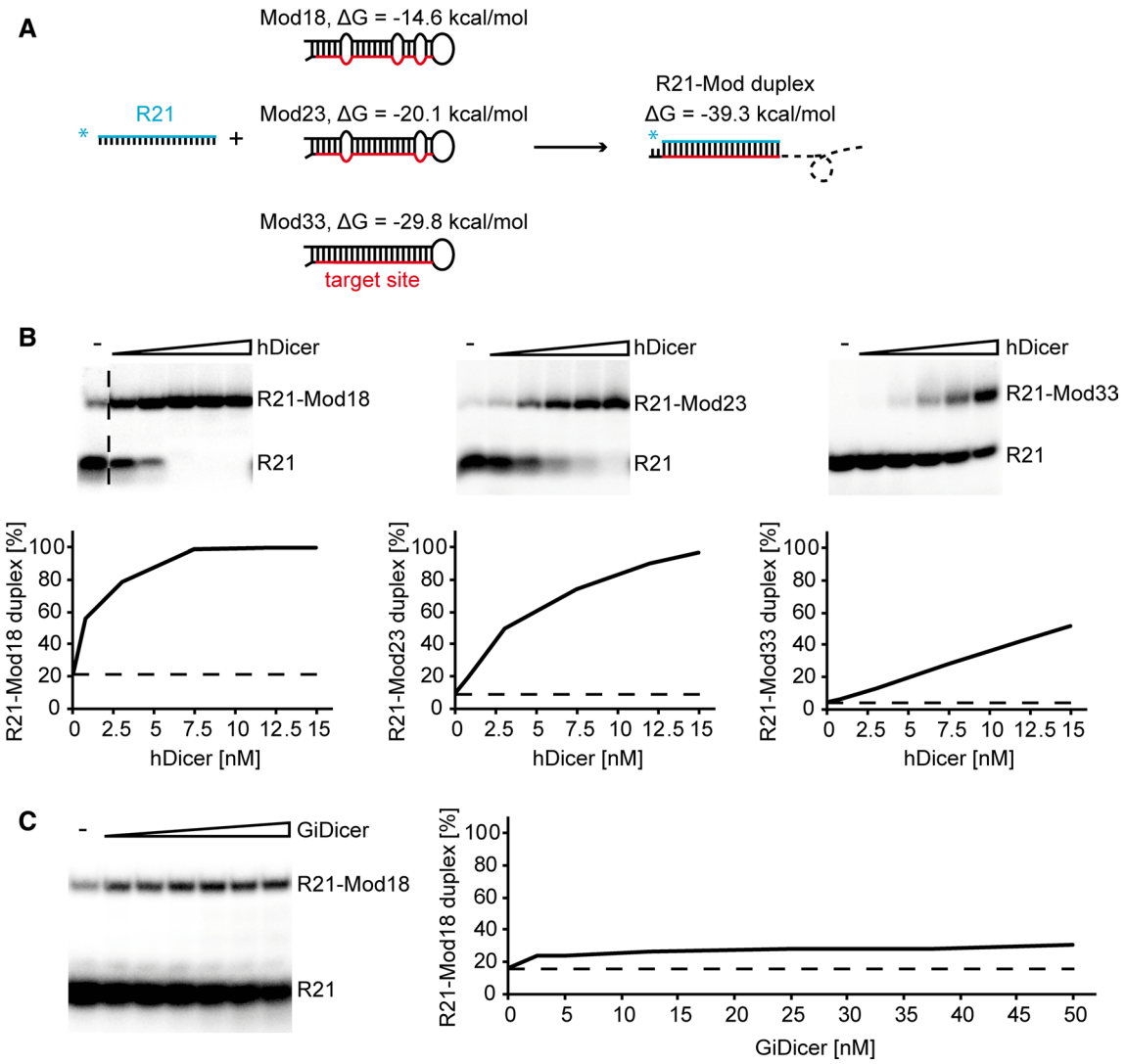

Fig. 1 Human Dicer promotes annealing of short RNA to its target sequence within a longer hairpin RNA. **a** Schematic representation of templates used in preliminary annealing assays. Guide RNA, R21 (in blue), anneals with its target site (in red) within Mod18, Mod23 or Mod33 to form a perfectly complementary duplex. The free energy values are shown next to the predicted structures. **b** Native PAGE gels showing the results of annealing reactions involving R21 and Mod18 (left), R21 and Mod23 (middle), and R21 and Mod33 (right). Reaction mixtures were incubated with increasing amounts of hDicer or with no protein ("hyphen"). Graphs showing representative annealing reactions obtained by densitometric quantification of the autoradiograms (bottom panel). Horizontal dashed lines are drawn for the values obtained for control experiments with no protein (baselines). The vertical dashed line indicates that two fragments of gel were used to compose one image. **c** The native PAGE gel showing the results of annealing reactions involving R21 and Mod18 and increasing amounts of GiDicer. Labeling was the same as for **b**

(Fig. 2a), the amount of R21-Mod18 duplex increased, reaching a maximum (nearly 100%) after a 15-min incubation with hDicer. The spontaneous annealing of complementary RNAs, with no protein, after a 60-min incubation was ~20%. The efficiency of annealing was the same for GiDicer as for the reactions with no protein for the corresponding time points, which further confirmed that GiDicer does not accelerate annealing of complementary RNA strands. For the pair R21 and Mod23 (Fig. 2b), the amount of R21-Mod23 duplex reached the maximum (nearly 100%) after a 30-min incubation with hDicer, whereas for R21 and Mod33 (Fig. 2c), the amount of R21-Mod33 duplex reached up to 50% after a 60-min incubation with hDicer. The next three cases involved the miRNA-like duplex and Mod18, Mod23, or Mod33 (Fig. 2d–f). In a time-course experiment with the miRNA-like duplex and Mod18 (Fig. 2d), the amount of R21-Mod18 complex increased, reaching the maximal observed annealing (~85%) after a ~30-min incubation with hDicer. For the pair miRNA-like duplex and Mod23 (Fig. 2e), the amount of R21-Mod23 complex

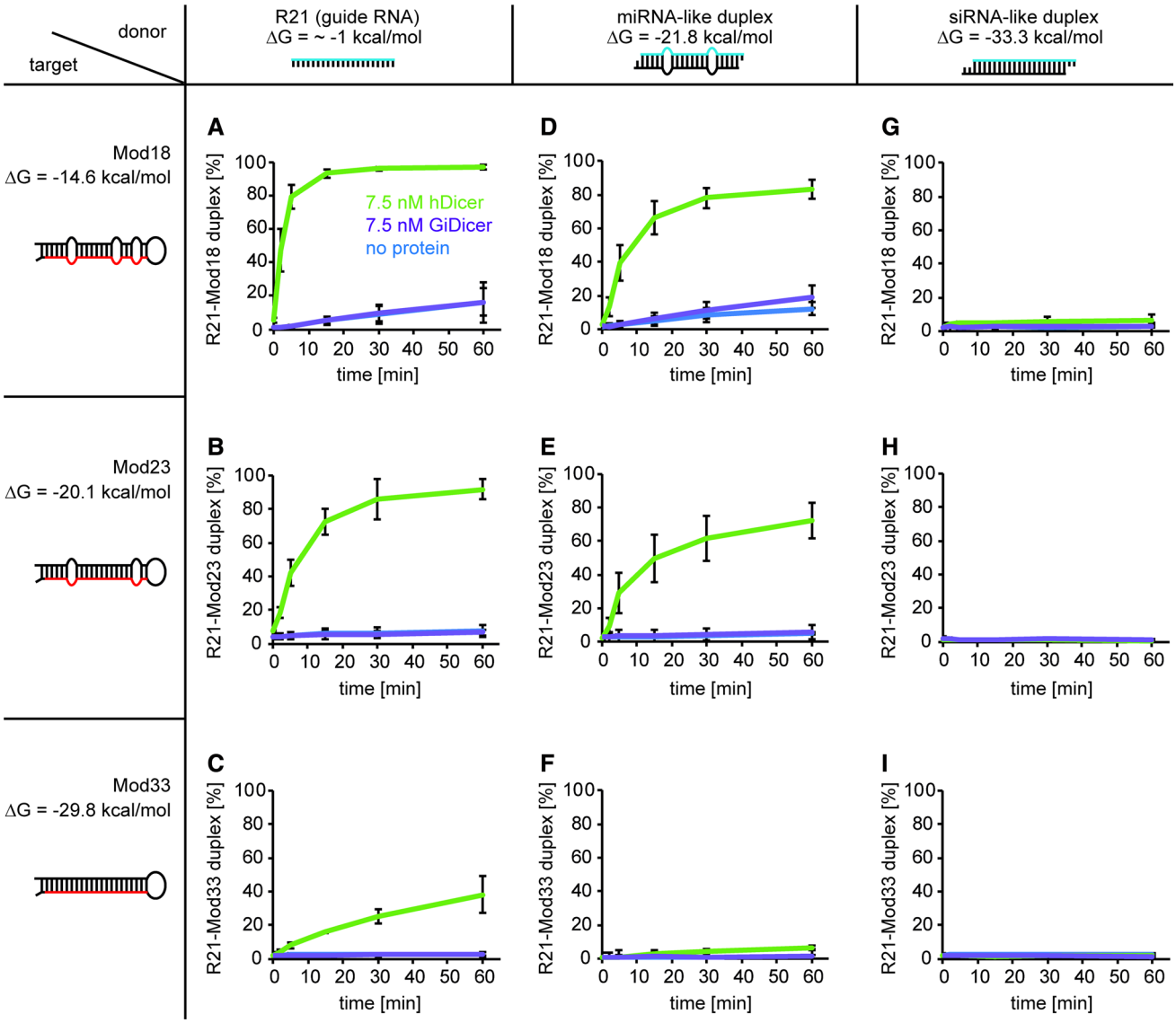

Fig. 2 Time-dependent annealing activity of hDicer involving a pair of complementary RNAs, 'donor' and 'target'. Graphic presentation of the results obtained from three independent annealing assays involving hDicer (in green), GiDicer (in purple), or no protein (in blue) and nine 'donor' and 'target' pairs, as follows: R21 and **a** Mod18, **b** Mod23, or **c** Mod33; miRNA-like duplex and **d** Mod18, **e** Mod23, or **f** Mod33; siRNA-like duplex and **g** Mod18, **h** Mod23, or **i** Mod33. The *x*-axis represents the incubation time expressed in minutes, and the *y*-axis represents the percentage content of the R21-Mod duplex fraction

reached ~ 75% after a 60-min incubation with hDicer. In the case of miRNA-like duplex and Mod33, we observed a very poor base pairing between R21 and Mod33 when hDicer was added (Fig. 2f, Supplementary Fig. S3F). Finally, the last three cases involved the siRNA-like duplex and Mod18, Mod23 or Mod33 (Fig. 2g–i). The results obtained revealed barely observable annealing of R21 with Mod18 when hDicer was applied (Supplementary Fig. S3G) and no base pairing of R21 with either Mod23 or Mod33 (Supplementary Fig. S3H and I).

Next, based on the data presented in Fig. 2, we calculated the initial velocity (V_0) values that reflected the efficiency of duplex formation within the first minute of reaction upon the addition of hDicer (Table 2). These results showed that the initial rate of hDicer-assisted annealing between R21

Table 2 Initial velocities (V_0) [nM/min] of R21-Mod duplex formation calculated for the hDicer-assisted annealing reactions

	R21	miRNA-like duplex	siRNA-like duplex
Mod18	0.74 ± 0.06	0.37 ± 0.08	< 0.01
Mod23	0.35 ± 0.07	0.27 ± 0.11	0
Mod33	0.05 ± 0.01	< 0.01	0

and Mods was the highest for R21 and Mod18, and this rate decreased with an increase in secondary structure stability of 'donors' and 'targets'. For those pairs for which annealing was not observed, the V_0 values were designated 0. In the case of all control reactions, i.e., the reactions with no protein or with GiDicer, annealing was not observed during the first minute of reaction (Supplementary Fig. S3). In conclusion, we observed that hDicer increased the rate at which two separate complementary RNAs base paired, which is characteristic of nucleic acid annealers.

As the presented annealing reactions may be influenced by the stability of miRNA and siRNA duplexes ('donors'), we also investigated the dissociation potential of both these duplexes with or without hDicer as incubation time increased up to 60 min (Supplementary Fig. S4). We observed that both duplexes were stable over time. However, when the miRNA duplex and the 'target' were present in the reaction mixture, a modest unwinding of the duplexes occurred (Supplementary Fig. S3D–F). Although spontaneous dissociation of a miRNA duplex might be a source of a free R21 molecule, that minor process did not seem to significantly affect the total efficiency of hDicer-facilitated RNA annealing. Human Dicer contains the N-terminal helicase domain with ATP-binding motif. However, as yet, ATP hydrolysis has not been found to apply into the cleavage activity of human Dicer [23, 28]. Here, we tested whether ATP may influence the stability of miRNA and siRNA duplexes upon incubation with hDicer. Nevertheless, we found that the addition of ATP to the reaction mixture did not trigger unwinding of these two duplexes (Supplementary Fig. S4).

Altogether, the collected results indicate that the outcome of the hDicer-facilitated in vitro annealing reaction depends on the structure stability of the substrates and products and that hDicer promotes the formation of the most stable base-paired structures in an ATP-independent manner.

The limited RNA-annealing activity of hAgo2

The minimal functional RISC consists of a member of the Ago protein family and a small regulatory RNA, i.e., miRNA or siRNA [29]. Studies on the influence of the mRNA secondary structure on target recognition and cleavage by RISC, such as that by Ameres et al., have shown that hAgo2-mediated target mRNA cleavage is far more complex than simple hybridization between a small regulatory RNA and mRNA [21, 30, 31]. Those results suggested that hAgo2 could display RNA-annealing activity; nevertheless, the putative RNA-annealing potential of this protein has never been studied in detail. Accordingly, to investigate the possible base pairing activity of hAgo2, we applied the commercially available preparation of hAgo2 and the following three RNA pairs, R21 and Mod18, R21 and Mod23, and R21 and Mod33. In preliminary assays, similar to hDicer, ^{32}P-labeled R21 was mixed in annealing buffer with Mod18, Mod23 or Mod33 and incubated for 30 min with increasing amounts of hAgo2 (5, 10, 12.5, 20, and 25 nM) at 37 °C (Fig. 3a–c). We found that hAgo2 very inefficiently supported the base pairing of the complementary RNAs applied in the studies; this efficiency reached ~30% for the pair R21 and Mod18, while it was slightly below 20% in the reaction lacking the protein (Fig. 3a). We also carried out time-course assays with 7.5 nM hAgo2 (Fig. 3d–f). The annealing between R21 and Mod18 after a 60-min incubation with hAgo2 did not exceed 30%, which was equivalent to the level of spontaneous base pairing between R21 and Mod18 (Fig. 3d). Furthermore, we did not observe annealing products when R21 and Mod23 or R21 and Mod33 were applied regardless of the presence or the absence of hAgo2 in the reaction mixture (Fig. 3e, f).

Next, we tested the RNA-annealing potential of hAgo2 by applying R21, Mod18 and various buffer conditions, including the buffer originally used by Ameres et al. [21] with slight modifications concerning lowered magnesium ion concentration or addition of EDTA to prevent product cleavage (Supplementary Fig. S5). Under these conditions, we also found no spectacular increase in hAgo2-mediated base pairing between R21 and Mod18, which was ~40% with protein and ~30% without protein. In the case of hDicer, we observed twofold increase in base pairing efficiency between R21 and Mod18, comparing to control reactions (Supplementary Fig. S5). Overall, the collected results revealed a very limited RNA-annealing activity of hAgo2, in comparison to hDicer, under the applied in vitro conditions.

Dicer-binding sites found within mRNAs contain targets for miRNAs

We next examined the biological relevance of our findings. Analysis of the transcriptome-wide map of human Dicer targets generated by Rybak-Wolf et al. [22] reveals that human Dicer, among other transcripts, binds to its own mRNA. Deeper analysis of those data shows 36 separate records reflecting distinct Dicer-binding sites within the human Dicer transcript NM_001271282, named variant 4. We found that 8 out of the 36 Dicer-binding sites are located within exon 21. Furthermore, six Dicer-binding sites are located within exon 23, two sites are located within exons 7, 8, 11, 16, 18 and 24, and one Dicer-binding site is found in exons 2, 4, 5, 6, 12, 13, 15, 20, 22, and 27 (Supplementary Fig. S6). Interestingly, data published by Forman et al. demonstrated that within the human Dicer transcript protein-coding sequence, there are numerous sites that can be targeted by miRNAs [32]. Taking into consideration the above-presented information, we asked whether miRNA target sequences can be found within Dicer-binding sites. Consequently, using the miRDB database [33], we looked for predicted miRNA targets within the human

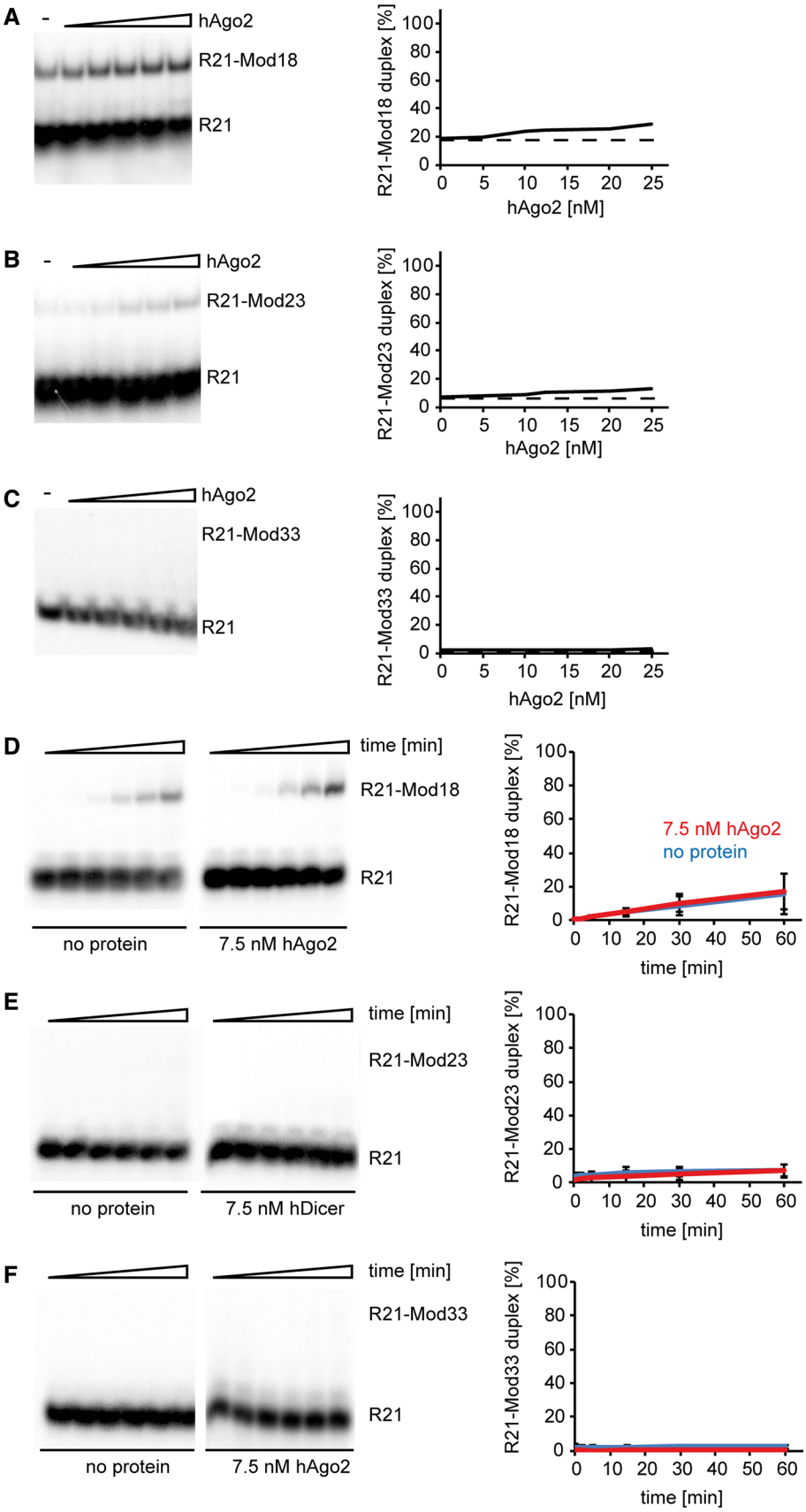

Fig. 3 hAgo2 displays limited RNA-annealing activity. **a**–**c** Native PAGE gels showing the results of annealing reactions involving **a** R21 and Mod18, **b** R21 and Mod23, and **c** R21 and Mod33. Reaction mixtures were incubated with increasing amounts of hAgo2 or with no protein ("hyphen"). Graphs showing representative annealing reactions obtained by densitometric quantification of the autoradiograms (right panel). Horizontal dashed lines are drawn for the values obtained for control experiments with no protein (baselines). **d**–**f** Time-dependent annealing assays involving **d** R21 and Mod18, **e** R21 and Mod23, and **f** R21 and Mod33. Graphic presentation of the results obtained from three independent annealing assays involving hAgo2 (in orange) or no protein (in blue). The *x*-axis represents the incubation time expressed in minutes, and the *y*-axis represents the percentage content of the R21-Mod duplex fraction

Dicer-binding sites identified by Rybak-Wolf et al, with a focus on the DICER1 transcript NM_001271282 protein-coding region. We found 304 records (Supplementary Table S1); their distribution among Dicer-binding sites is presented in Supplementary Fig. S6. The identified miRNA targets were located within almost all (~97%) of the sites bound by Dicer in its transcript. However, importantly, the number of Dicer-binding sites within the Dicer transcript might be overrepresented due to Dicer overexpression in the cell system used by Rybak-Wolf et al. [22]. Therefore, to establish whether the observed phenomenon is specific to the experimental setup or is a more general rule, we explored whether miRNA target sequences could also be found within Dicer-binding sites located in transcripts other than DICER1. A brief search, with the use of the miRDB database [33], revealed that miRNA targets are found within multiple Dicer-binding sites present within miscellaneous mRNAs, for example, Ago1, TNF receptor-associated factor 4 (TRAF4), DEAD-box helicase 6 (DDX6), glutamine synthetase (GLUL), guanine nucleotide-binding protein G(k) subunit alpha (GNAI3), malectin (MLEC), methylsterol monooxygenase 1 (MSMO1) and SUMO1-activating enzyme subunit 1 (SAE1) (Supplementary Table S3).

hDicer supports annealing of miRNA with its target sequences within mRNA

Next, we sought to determine whether hDicer would support base pairing of a miRNA to its target located within the Dicer transcript. In this experiment, we used a well-characterized miRNA, miR-103a-3p [34], which has been found to be abundantly expressed in the human embryonic kidney 293 (HEK293) cell line [35]. This cell line was used by Rybak-Wolf et al. for the preparation of the transcriptome-wide map of human Dicer targets as a result of cross-linking and immunoprecipitation (CLIP) of in vivo Dicer-RNA complexes [22]. A target sequence for miR-103a-3p was found to be located within exon 21 (Supplementary Fig. S6). Predictions of interactions between miR-103a-3p and the selected fragment of exon 21, made with the IntaRNA tool [25, 36, 37], revealed that 13 out of 23 nt of miR-103a-3p base pair with the target site (Fig. 4a). Since the DICER1 transcript is over 10,000 nt in length, observing the in vitro annealing process using such a long RNA would be extremely challenging. Therefore, we used in vitro transcription, with 7-methylguanosine 5′ CAP addition, to obtain a short fragment of DICER1 transcript containing a 39-nt hDicer-binding site and the miR-103a-3p target sequence (Supplementary Fig. S7); we referred to this product as "Ex21". In the annealing assay, we applied miR-103a-3p, i.e., the guide strand, miR-103a-5p, i.e., the passenger strand and the miR-103a duplex. The assay was performed with either hDicer or hAgo2 (Fig. 4b–d); the protein was

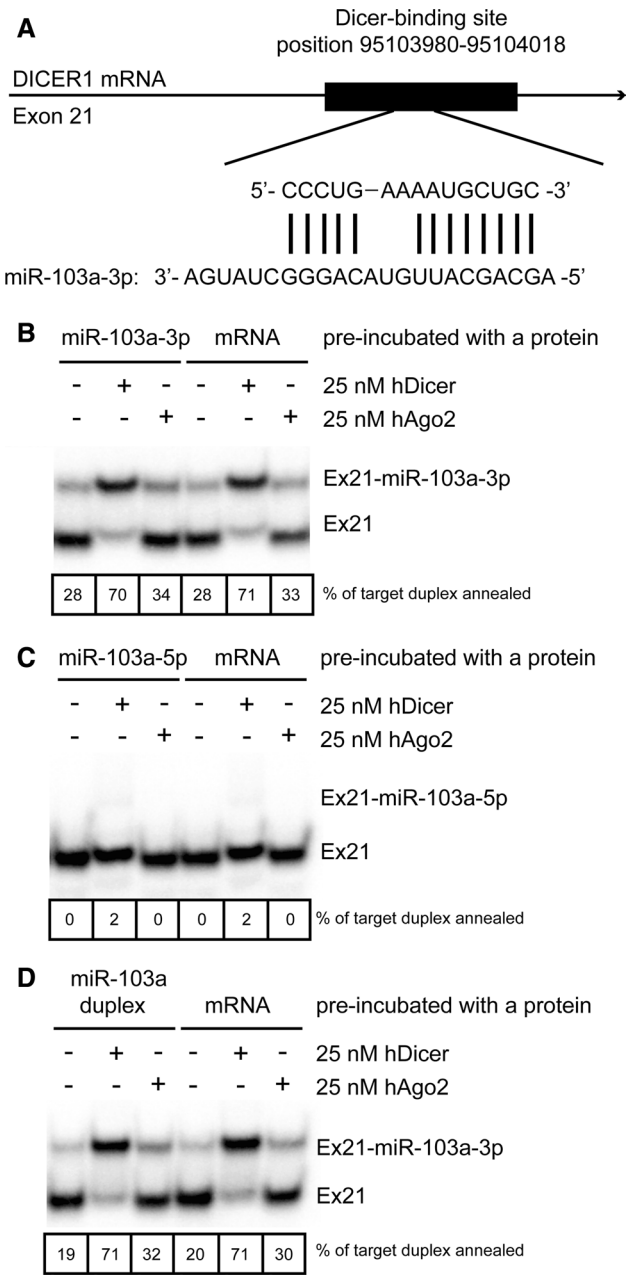

Fig. 4 hDicer accelerates the annealing of miRNA to its target site present within the *DICER1* transcript. **a** A scheme representing the base pairing of miR-103a-3p with its target site within the *DICER1* transcript. **b–d** Native PAGE gels showing the results of annealing reactions involving 5 nM of 3′-^{32}P-labeled Ex21 and 50 nM of either **b** miRNA-103a-3p, **c** miRNA-103a-5p or **d** miRNA-103a duplex. Reaction mixtures were incubated with either 25 nM hDicer or 25 nM hAgo2, or with no protein for 30 min at 37 °C. Prior to the addition to the reaction mixtures, the proteins were preincubated either with miRNA or Ex21 (mRNA) for 15 min at 4 °C

preincubated with either miRNA or Ex21. The base pairing potential between miRNA and Ex21 was evaluated in control reactions containing no protein. The results obtained showed that hDicer significantly facilitated base pairing

of miR-103a-3p to its target within Ex21, irrespective of whether miR-103a-3p was alone (Fig. 4b) or present in the miR-103a duplex (Fig. 4d). Moreover, we did not observe a difference in hDicer-assisted annealing efficiency between the reactions in which hDicer was preincubated with miRNA and the reactions in which hDicer was preincubated with Ex21. Moreover, we noticed inefficient base pairing between miR-103a-3p and Ex21 in control reactions with no protein and in those with hAgo2 (Fig. 4b, d). However, preincubation of hAgo2 with miR-103a-3p or the miR-103a duplex slightly enhanced base pairing of this small RNA to its target, compared to the respective control reactions without protein. For the pair miR-103a-5p and Ex21, we observed a very weak base pairing product in the reactions carried out with hDicer (Fig. 4c). The in-depth analysis of this case revealed, however, that miR-103a-5p includes a short sequence that is complementary to Ex21 (data not shown).

Dicer and Ago may bind to the same sequences within exonic protein-coding regions

We then asked whether Dicer and Ago can bind to the same sequences within RNA. To answer this question, we compared the human Dicer-CLIP and Ago2/3-IP data generated by Rybak-Wolf et al. [22], and we distinguished 4594 (for Dicer) and 5565 (for Ago2/3) intersecting (common) sequences, which constituted more than half of all sites bound by Dicer (8469) (Supplementary Table S2). The detailed analysis revealed that as many as 39% of all the binding sites common for Dicer and Ago2/3 were located within mRNAs (exonic regions), where protein-coding sequences (CDSs) constituted ~25%, 3′ untranslated regions (UTRs) ~12% and 5′-UTRs ~2% (Fig. 5). A deeper insight into the Dicer-CLIP and Ago2/3-IP records collected for the human Dicer transcript revealed that 12 Dicer-bound sequences overlapped with 13 Ago2/3-bound sequences (Supplementary Fig. S8 and Supplementary Table S2). Additionally, almost all distinguished Dicer-bound sequences were also found to be putative targets for miRNAs (Supplementary Fig. S8). Among these records, we found the case described above, the 39-nt Dicer-binding site targeted by miR-103a-3p. Importantly, the construct applied by Rybak-Wolf et al. stably expressing FLAG/HA-tagged human Dicer in HEK293 cells lacked the 5′- and 3′-UTRs characteristic of the Dicer transcript [22]. Consequently, the referred data collected for the human Dicer transcript could be reduced by the records comprising UTRs, which excludes also records for the 3′-UTR region containing a great number of miRNA-binding sites. In addition, we must consider that the physiological ratio between Dicer and Ago might be distorted due to overexpression of FLAG/HA-tagged proteins for immunoprecipitation purposes. Nevertheless, the collected data show an interesting interplay between Dicer and Ago, suggesting that these two proteins may act as cooperators or competitors.

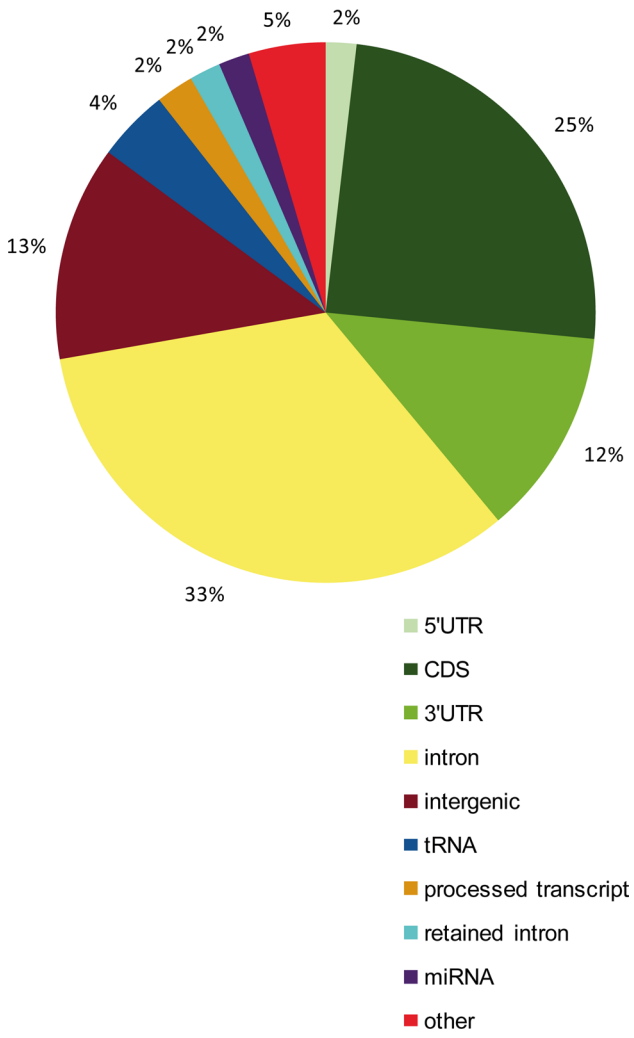

Fig. 5 Genomic features of transcripts for which genomic locations overlapped between shared binding sites of Dicer and Ago2. The values in the chart show the percentage of frequency for distinct genomic features in relation to the total number of shared binding sites (5565). For locations with more than one corresponding transcript, the genomic feature common for the highest number of corresponding transcripts was chosen for calculations

Although the current knowledge of Dicer and Ago-related mechanisms and pathways, as well as the results of our present studies, do not allow us to answer whether Dicer and Ago could compete or cooperate for binding to the same sites within transcripts, they let us to hypothesize that not every miRNA duplex is handed over to the Ago protein. When the miRNA duplex generated by Dicer stays bound with this ribonuclease, miRNA might guide Dicer to a specific complementary target sequence present within a transcript. Accordingly, Dicer, by blocking the access of Ago to mRNA, might prevent transcript degradation (compare Fig. 6a, b). Alternatively, Dicer, by facilitating the

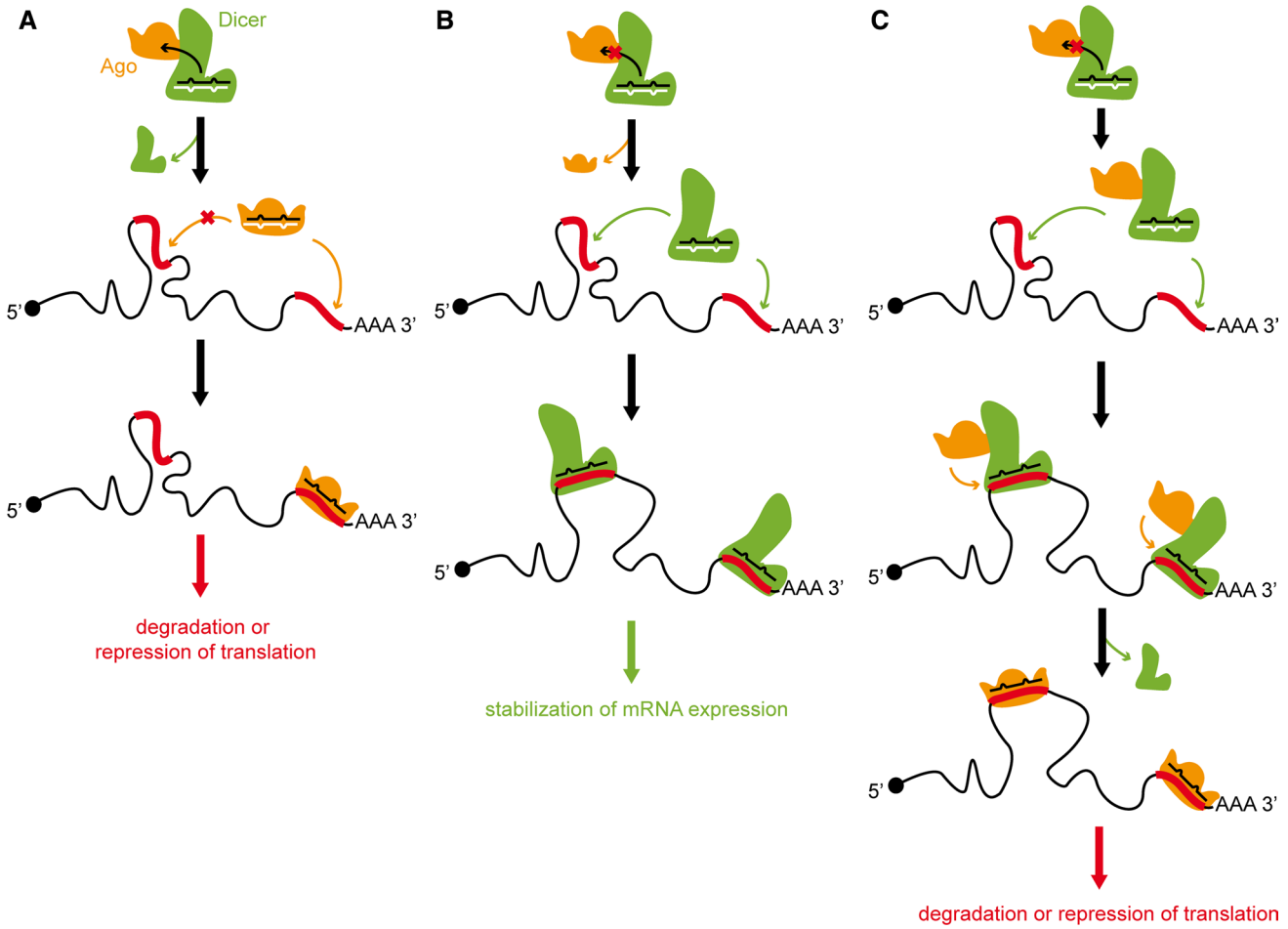

Fig. 6 Proposed scenarios of Dicer direct involvement in the posttranscriptional regulation of gene expression. **a** Canonical miRNA silencing pathway: miRNA generated by Dicer is loaded onto the Ago protein to target specific mRNAs for cleavage or translational repression. Stable secondary structured fragments of transcripts are not targeted by miRNA-loaded Ago complexes. **b** miRNA generated by Dicer is not passed to Ago, instead miRNA-Dicer complex is guided to specific complementary target sequences present within a transcript. Consequently, Dicer, by blocking the access of Ago to mRNA, prevents transcript degradation. **c** miRNA-loaded Dicer, by facilitating the accessibility to the double-stranded fragments of transcripts, supports Ago in targeting transcripts, thus inducing their degradation or repression of translation

accessibility to transcripts through alterations in their secondary structures, might assist Ago with reaching miRNA targets (Fig. 6c). Such scenarios indicate that Dicer has a potential to act in competition with (Fig. 6b) or in cooperation with (Fig. 6c) Ago proteins when both target the same sequence within a transcript.

Discussion

There is no doubt that the mechanisms involved in posttranscriptional regulation of gene expression by small regulatory RNA are complex and as yet not fully recognized. In the miRNA pathway, mature miRNA duplexes generated by Dicer are loaded onto the Ago proteins to target specific mRNAs for cleavage or translational repression [38]. There are several lines of evidence that suggest that, beyond the canonical determinants of miRNA base pairings to their targets, an essential function in regulating the effectiveness of gene silencing by miRNA species is played by RNA-binding proteins (RBPs) (reviewed in [39]). Considering mRNA fate, both positive and negative regulatory actions of such RBPs have been identified [39]. Moreover, the mechanisms of mRNA regulation, which include interplay between miRNAs and RBPs, could be either cooperative or competitive in nature. RBPs, such as Pumilio or splicing factor proline/glutamine-rich protein (Sfpq), influence the secondary structure of the mRNA fragment bound and thereby support miRNA-mediated gene expression silencing. Human Pumilios, Pumilio1 (PUM1) and Pumilio2 (PUM2), are sequence-specific RBPs whose targets often encode proteins acting in cancer-related pathways [40].

Most Pumilio-binding sites have been found to be located within stable secondary structures of 3′-UTRs. PUM1, by binding to its target sites, has been shown to induce a local change in RNA structure, which, in consequence, exposes specific miRNA binding sequence within 3′-UTRs [41]. Sfpq, a functionally versatile DNA- and RNA-binding protein, preferentially binds to long 3′-UTRs harboring multiple copies of Sfpq-binding motifs and promotes, presumably by modulating the secondary structure of a miRNA target, optimal positioning of miRNA-loaded RISC [42]. Nevertheless, other RBPs, such as Deadend 1 [43, 44], RNA-binding motif protein 38 [45] and coding region determinant-binding protein [46, 47], may limit the accessibility of miRNA targets by competitive binding within mRNAs. Importantly, a single RBP can function either in competition or cooperation with miRNAs. For example, Hu-Antigen R (HuR) was initially found to stabilize cationic amino acid transporter 1 mRNA containing AU-rich elements (AREs) by binding to ARE sequences present within 3′-UTRs [48] and by blocking the access of miRNAs to their targets [49]. Conversely, HuR has been reported to help in miRNA targeting and the repression of c-Myc [50] and Ras homolog B (RhoB) [51] expression. The binding sites for miRNAs and HuR have been reported to, rather than overlap, be located either in the intermediate vicinity of one another [50–52] or significantly far away [49, 53] from one another. Proposed models of competitive and cooperative interactions between miRNAs and HuR on shared target mRNAs postulate HuR-imposed conformational changes of mRNA structure, which may result in either hiding or exposing individual miRNA-binding sites for RISC [54].

Likewise, the results of studies carried out by Rybak-Wolf et al. suggested a role of translational regulator for Dicer [22]. The authors demonstrated that Dicer, by binding to some sites present within transcripts referred to as "passive sites", i.e., the sites that are not cut by Dicer, can stabilize expression of the targeted transcripts. Upon Dicer depletion in HEK293 cells, the expression of transcripts containing passive sites was found to be significantly destabilized [22]. Our detailed analysis of Dicer-CLIP and Ago2/3-IP data generated by Rybak-Wolf et al. [22] revealed that Dicer and Ago can bind to the same sites within various types of RNAs, mostly protein-coding transcripts (Fig. 5 and Supplementary Fig. S8). These findings suggest a possible competition or cooperation between these two proteins. This observation is also interesting as Dicer is considered a protein that does not recognize or bind specific sequences or unique sequence motifs [55, 56], which stands in contrast, for example, to Pumilio, Sfpq or HuR proteins. Importantly, the results presented in this report suggest that the sequence specificity of Dicer binding can be triggered by small RNAs that are bound to Dicer and are complementary to targeted RNAs, which is also characteristic for Ago proteins [57]. Deliberating about the competitive or cooperative relation between these two proteins, miRNA-bound Dicer [22], similarly to Ago, may target mRNAs; however, such miRNA-driven mRNA targeting by Dicer would probably differ from the miRNA–mRNA interaction within RISC [58]. Such a difference, for example, might result from the great RNA-annealing potential of Dicer (Figs. 2, 4) [5] compared to the limited RNA-annealing potential of Ago proteins (Figs. 3, 4) [21]. Since numerous reports have suggested that Dicer is present within RISC [57, 59, 60], we can assume that, regarding the annealing activity, Dicer may support Ago with targeting secondary structured, double-stranded fragments of transcripts. This assumption finds strong support in studies showing that endogenous Ago proteins may be recruited to miRNAs that are already pre-annealed to mRNAs [61, 62]. In addition, in vitro studies have suggested that interactions between Dicer and Ago2 may block the cleavage activity of Dicer [63], which indeed seems to be dispensable for Dicer when targeting transcripts. Accordingly, Ago may switch Dicer cleavage activity off.

It is also interesting to speculate about the fate of miRNAs generated by Dicer. After pre-miRNA cleavage, miRNA duplexes have been suggested to be released and rebound by Dicer for proper Ago loading [64]. In addition, some miRNA duplexes have been found to be bound by Ago2 in two different orientations, depending on Ago2 partnering proteins, such as Dicer, TRBP (trans-activation response RNA-binding protein) and PACT (protein activator of interferon-induced protein kinase R) [65]. Obviously, the two-way possible loading of Ago2 with a miRNA duplex may result in different mRNA targeting. In addition, an efficient loading of a miRNA duplex onto Ago requires chaperone machinery [66], which implies that this process may be not entirely efficient. Thus, one can imagine that when RISC lacks some specific proteins, a miRNA duplex would not be passed to Ago proteins and would remain bound with Dicer. Indeed, Dicer-CLIP experiments revealed hundreds of putative miRNAs not loaded into Ago proteins [22]. Recently, it has also been shown that most adult tissues contain reservoirs of miRNAs in low molecular weight not bound to mRNA RISC [67]. Such miRNAs are presumably not actively engaged in target repression. Hence, the efficiency of miRNA-mediated target repression depends not only on the miRNA levels themselves but also on the level of RISC assembly and mRNA targeting. One can also imagine that Dicer may arrest the process of miRNA handover to Ago within the RISC loading complex, thereby reducing the level of active miRNAs (Fig. 6).

In summary, the present study demonstrate a first comprehensive analysis of the RNA–RNA base pairing potential of human Dicer and Ago2 in the context of the secondary structures adopted by individual RNA substrates. We show that Dicer, compared to Ago2, displays much greater annealing

activity with RNAs having their complementary sequences trapped within stably secondary structures. We believe that such RNA-annealing activity of Dicer might be appreciated by RISC when the latter targets complementary sequences located within stable secondary structures of mRNA transcripts. Consequently, Dicer might be directly involved in translational control of gene expression.

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The RNA-RNA base pairing potential of human Dicer and Ago2 proteins

Cellular and Molecular Life Sciences

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LEGENDS FOR SUPPLEMENTARY FIGURES

Supplementary Figure S1 Cleavage activities and protein preparations of GiDicer, hAgo2 and hDicer. (A) An upper panel: the PAGE gel showing the results of a cleavage assay performed with pre-miR-33a and GiDicer (25 nM). A bottom panel: SDS-PAGE of GiDicer (MyBiosource). (B) An upper panel: the PAGE gel showing the results of a cleavage assay performed with hAgo2 (100 nM), Mod18 as a target and R21 as a guide. A bottom panel: the SDS-PAGE of hAgo2 (Active Motif). (C) An upper panel: the PAGE gel showing the results of a cleavage assay performed with pre-miR-33a and hDicer (10 nM). A bottom panel: the SDS-PAGE of hDicer preparation. Red arrows indicate cleavage products, while black arrows indicate proteins.

Supplementary Figure S2 Secondary structures of Mod RNAs used in the study. The predicted structures for (A) Mod18, (B) Mod23 and (C) Mod33, generated by RNAstructure Fold online tool, are presented. The structures are color-annotated according to base pairing probability. The free energy values expressed in kcal/mol are shown at the bottom. The target site within each Mod RNA is indicated by a rectangle. Nucleotides are numbered starting from the 5'-end.

Supplementary Figure S3 A time-dependent RNA annealing activity of hDicer involving a pair of complementary RNAs, ′donor′ and ′target′. Native PAGE gels show the results of annealing reactions involving nine ′donor′ and ′target′ pairs, as follows: R21 and (A) Mod18, (B) Mod23 or (C) Mod33; miRNA-like duplex and (D) Mod18, (E) Mod23 or (F) Mod33; siRNA-like duplex and (G) Mod18, (H) Mod23 or (I) Mod33. The reactions were incubated with the protein for 0, 2, 5, 15, 30 or 60 min at 37°C (indicated by a triangle). Control reactions contained no protein (left panels) or were prepared with GiDicer (right panels).

Supplementary Figure S4 The influence of incubation time and ATP on stability of RNA duplexes. RNA duplexes, (A) miRNA-like R21-mR21 or (B) siRNA-like R21-cR21, were incubated in annealing buffer with or without hDicer as incubation time increased up to 60 min. (C-) and (R21) denote RNA duplex or single-stranded R21 controls, respectively, that were applied on the gel without incubation. The vertical dashed line indicates that two fragments of gel were used to compose one image.

Supplementary Figure S5 The influence of different buffer conditions on annealing activity of hAgo2 and hDicer. Native PAGE gels show the results of annealing reactions involving 7.5 nM R21, 5′-^{32}P-labeled Mod18 and either no protein or hDicer, or hAgo2. Reaction mixtures were incubated in buffer containing (A) 2 mM $MgCl_2$ or (B) 5 mM $MgCl_2$ and 7 mM EDTA.

Supplementary Figure S6 The distribution of Dicer-binding sites and predicted miRNA targets within human *DICER1* transcript encoding full length enzyme. Tall bars represent coding exons (CDS), while small bars represent non-protein coding exons (3′-UTR and 5′-UTR regions) of *DICER1* primary transcript. All exon numbers refer to the transcript NM_001271282. The arrow indicates the translation start site. The *DICER1* transcript is bound by Dicer at 36 sites (indicated by circles), of those, 35 (indicated by green circles) contain multiple miRNA targets, as predicted by miRDB. A single Dicer-binding site has been found to contain no miRNA targets (indicated by a white circle). The Dicer-binding site which is indicated by an asterisk (*) is situated within the 3′-UTR region. The numbers within circles indicate number of Dicer-binding sites found

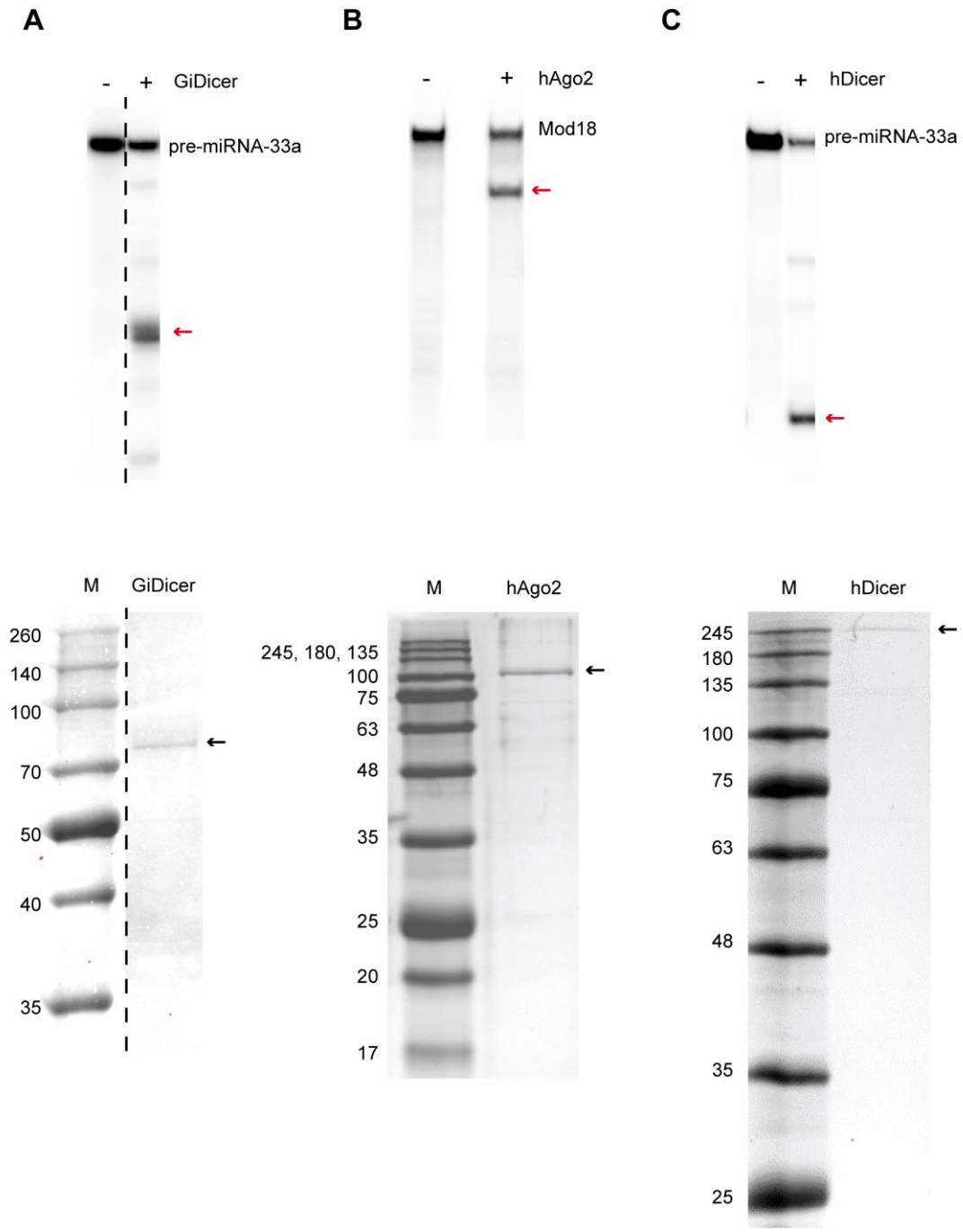
within the respective exon (based on Rybak-Wolf *et al.* (1)). miRNAs, predicted by miRDB, are listed and assigned to the respective sites below. "(2)" next to the miRNA indicates that two targets for a particular miRNA were found within Dicer-binding sites situated in the exon.

Supplementary Figure S7 A close insight into miR-103a-3p and Ex21 target base pairing. The short vertical lines indicate base pairing, predicted with the IntaRNA software. The sequence bound by Dicer (based on Rybak-Wolf *et al.* (1)) is underlined green.

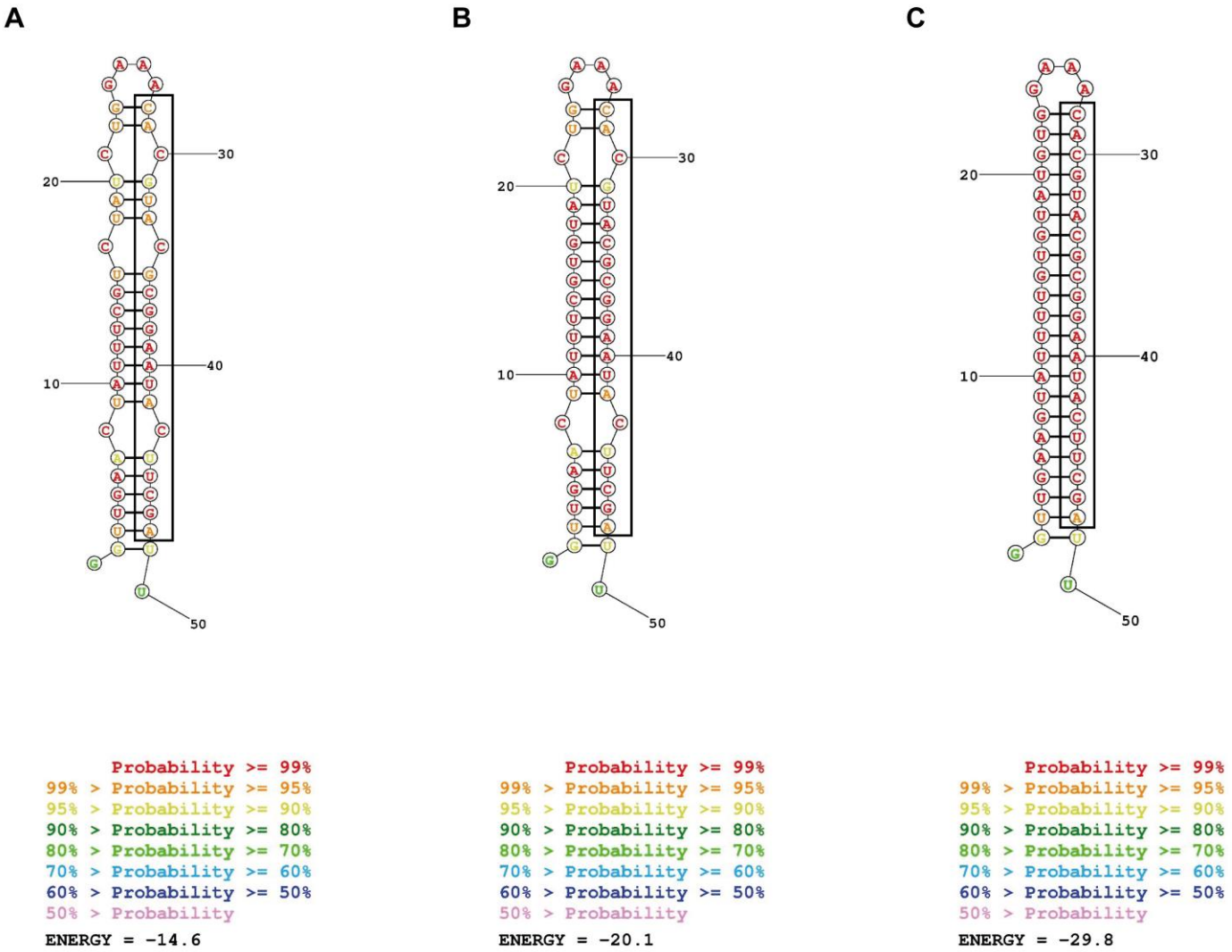
Supplementary Figure S8 The distribution of overlapping binding sites for Dicer and Ago2/3. (A) A scheme of the *DICER1* primary transcript. Tall bars represent coding exons (CDS), while small bars represent nonprotein coding exons (3′-UTR and 5′-UTR regions). All exon numbers refer to the transcript NM_001271282. The arrow indicates the translation start site. (B) Distribution of Dicer-binding sites that overlap with Ago2/3-binding sites. The site indicated by a white circle contains no miRNA targets. The sites that contain miRNA targets found within Dicer-binding sites only are colored green, while the sites that include miRNA targets common for both Dicer- and Ago2/3-binding sites are colored red. The site indicated by an asterisk (*) is situated within the 3′-UTR region. (C) Distribution of Ago2/3-binding sites that overlap with Dicer-binding sites. The sites indicated by white circles contain no miRNA targets. The sites that contain miRNA targets found within Ago2/3-binding sites only are colored yellow, while the sites that include miRNA targets common for both Dicer- and Ago2/3-binding sites are colored red. The site indicated by an asterisk (*) is situated within the 3′-UTR region. (D) Distribution of miRNA targets common for both Dicer and Ago2/3-binding sites. miRNAs, predicted by miRDB, are listed and assigned to the respective sites.

SUPPLEMENTARY FIGURES

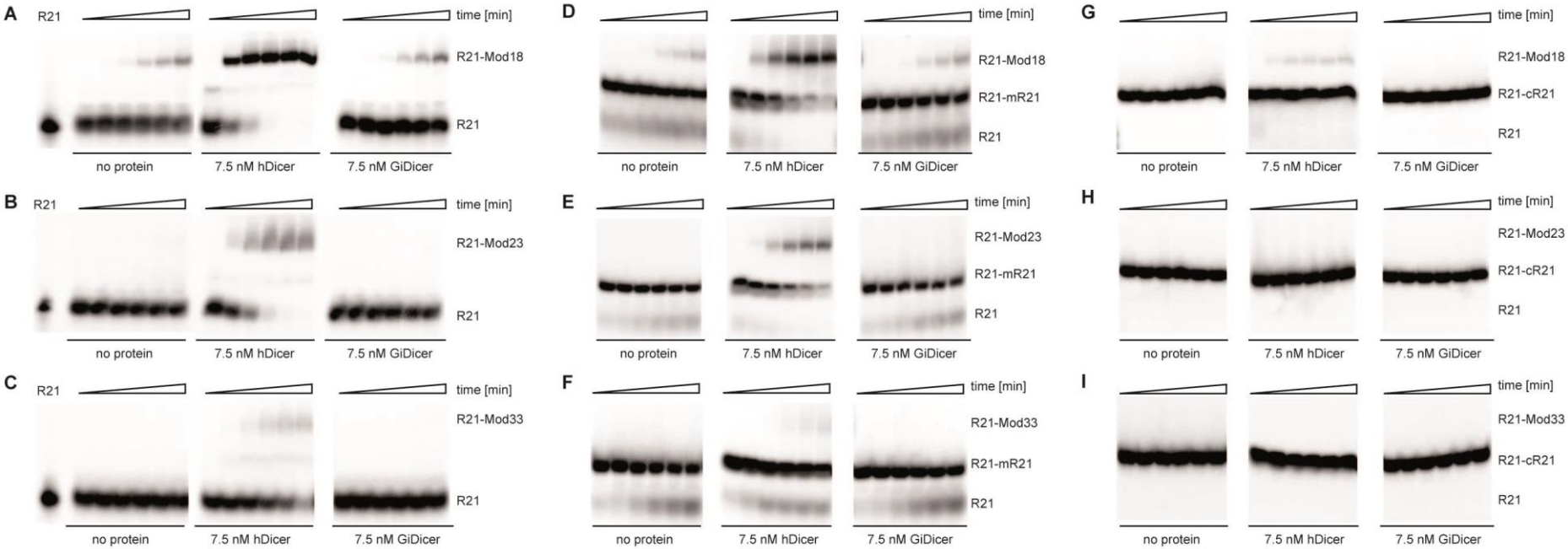
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



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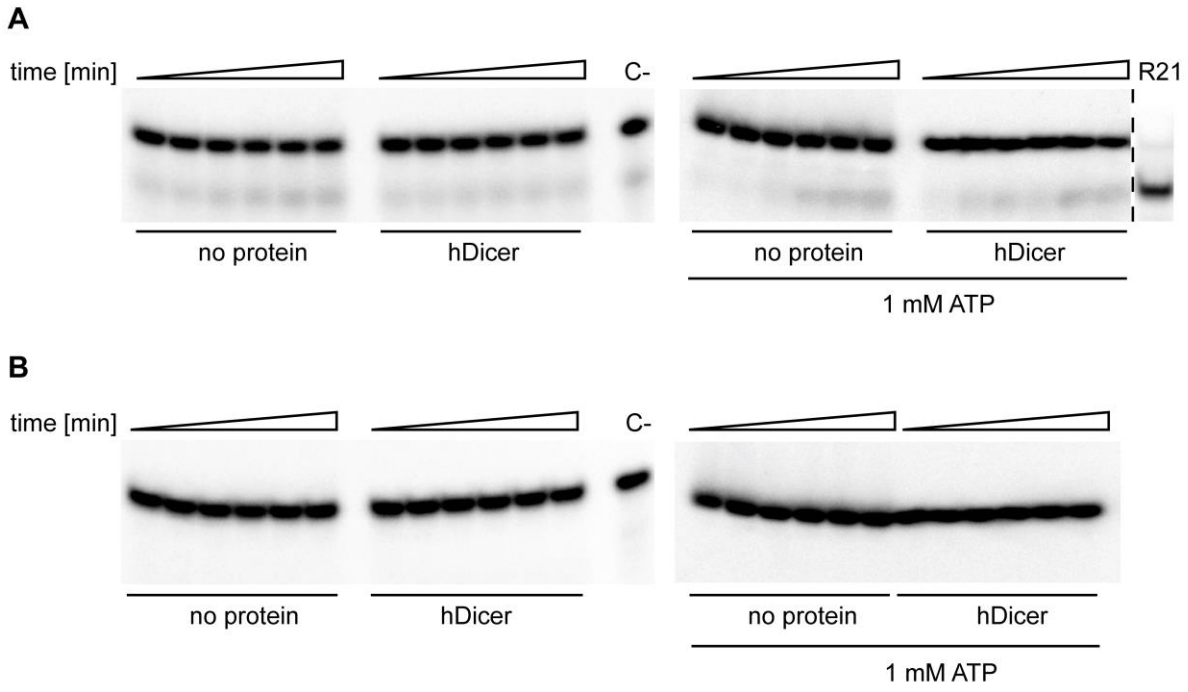

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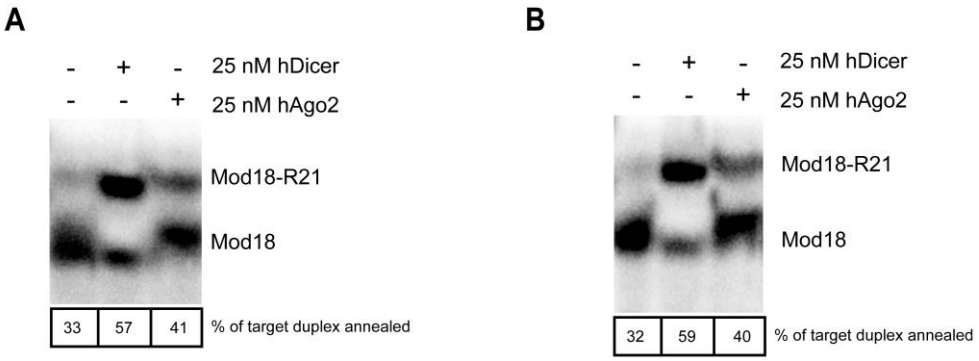
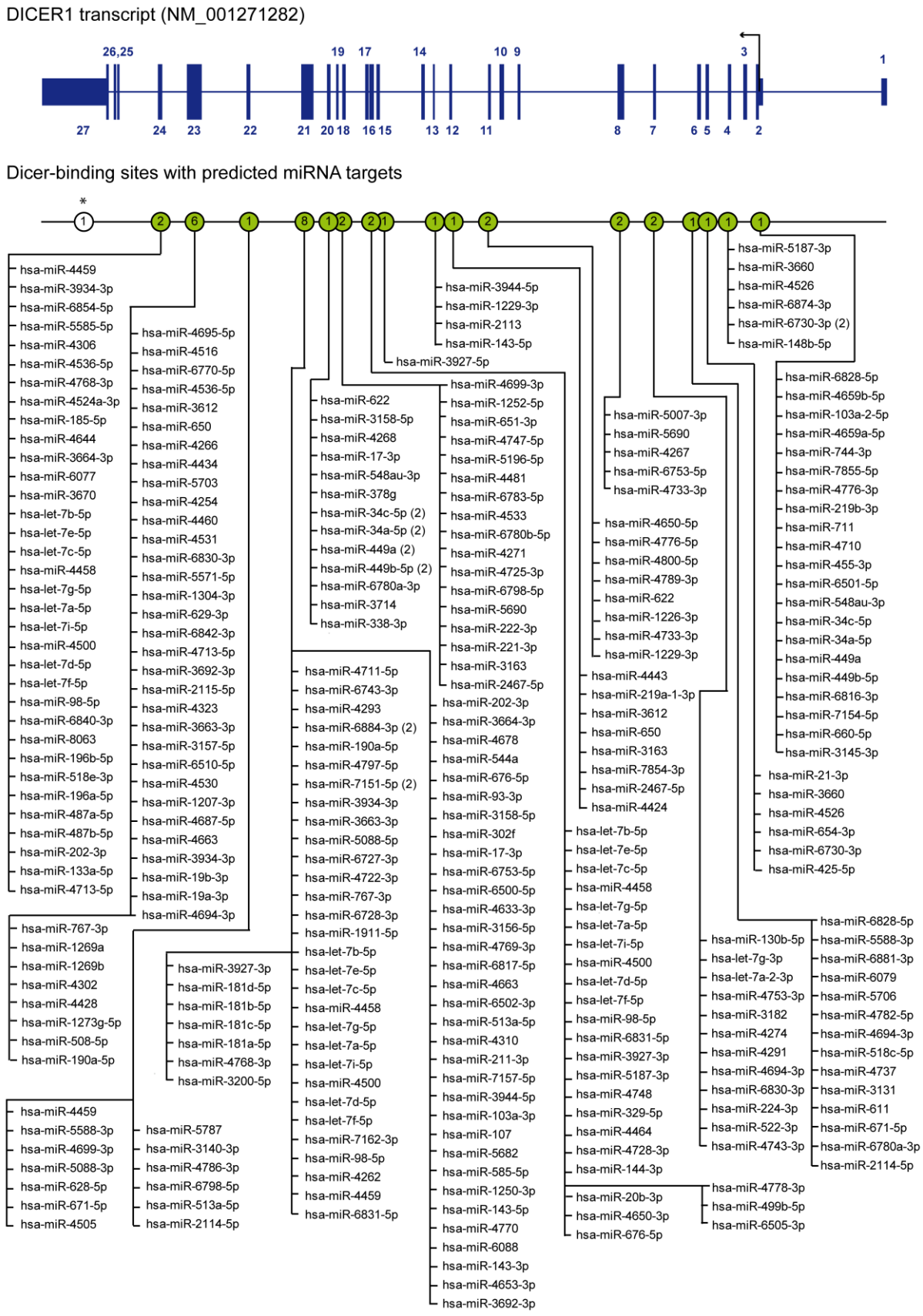
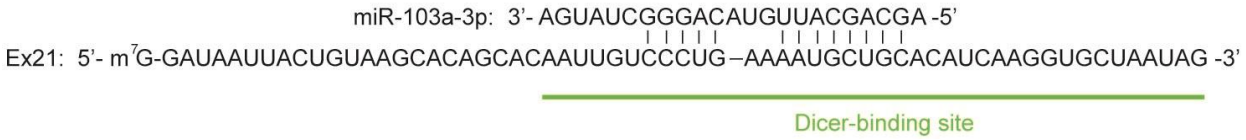


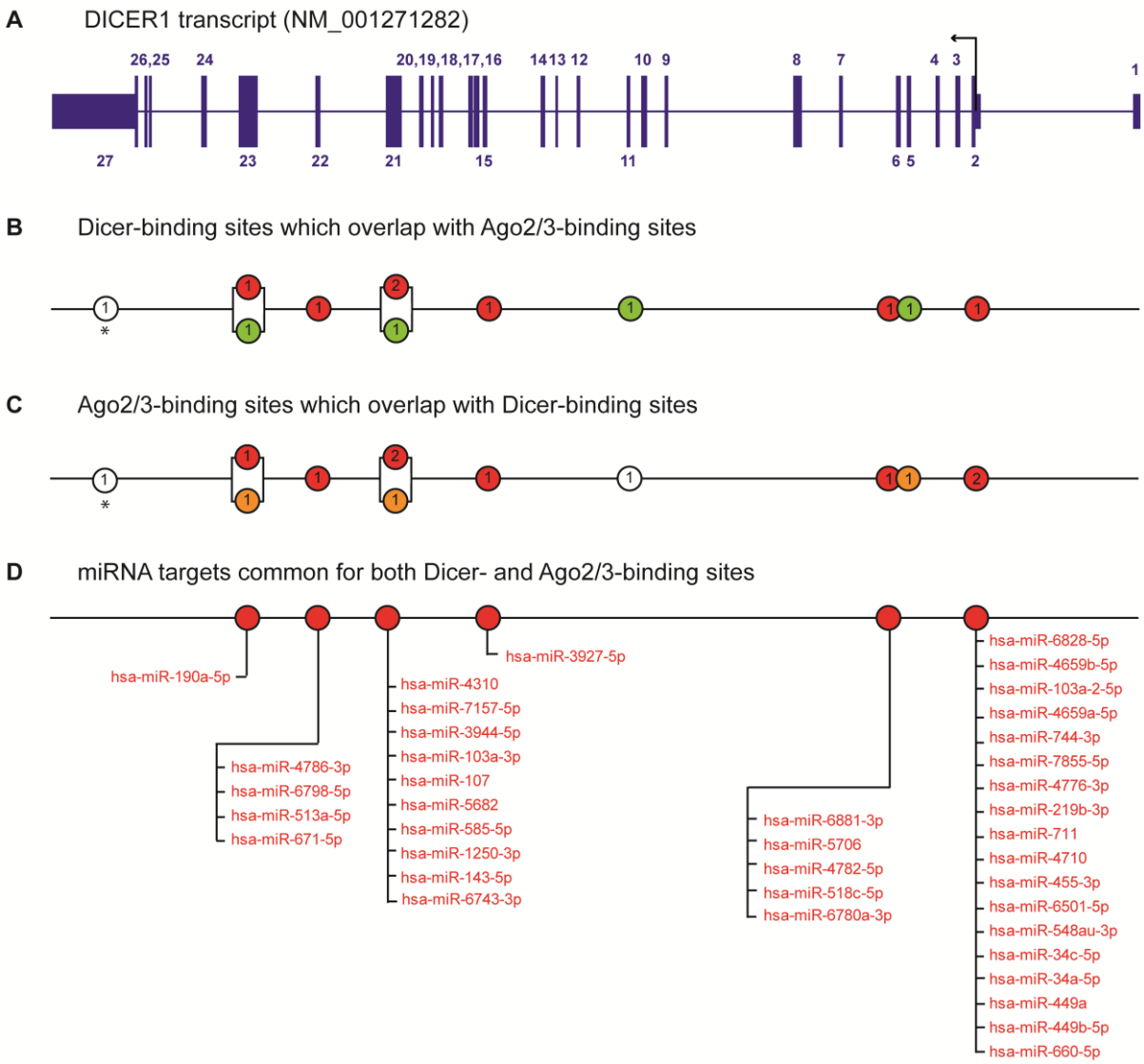
Supplementary Figure S6



Supplementary Figure S7



Supplementary Figure S8



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Hiperłącze do pobrania tabel: Supplementary tables S1-S3

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Poznań, 29 lutego 2020

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Kurzynska-Kokorniak A, Koralewska N, Pokornowska M, Urbanowicz A, Tworak A, Mickiewicz A, Figlerowicz M[§] (2015) ***The many faces of Dicer: the complexity of the mechanisms regulating Dicer gene expression and enzyme activities***. Nucleic Acids Res. 19;43(9):4365-80,

mój wkład polegał na przygotowaniu wstępnej wersji rysunku 1 oraz rysunku S1. Ponadto, przygotowałam opracowania, które zostały umieszczone w tabelach S3 i S4, a także zebrałam dane, które zostały wykorzystane w rozdziałach *Organization of the Dicer gene and factors controlling its expression* oraz *Human Dicer and Cancer*.

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mój wkład polegał na otrzymaniu preparatu białkowego DUF283. Ponadto, wykonałam doświadczenia, których rezultaty zostały zaprezentowane na rysunkach 1-4, 6, 8, S1, S2, S5 i 1SR. Brałam również udział w interpretacji wyników i w przygotowaniu rozdziału *Methods* (*Metody*) oraz w edycji manuskryptu.

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mój wkład polegał na otrzymaniu preparatu białkowego ludzkiej Dicer oraz przygotowaniu pierwszej wersji manuskryptu i jego późniejszej edycji. Ponadto, wykonałam doświadczenia, których rezultaty zostały zaprezentowane na rysunkach 1, 3, 4, S1, S3, S4 i S5 oraz tabelach 2 i S3.

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Oświadczam, że wkład mgr inż. Marii Pokornowskiej w powstanie wymienionej poniżej publikacji polegał na zebraniu danych i przygotowaniu pierwszej wersji rozdziałów *Organization of the Dicer gene and factors controlling its expression* oraz *Human Dicer and Cancer*. Była Ona również odpowiedzialna za przygotowanie wstępnych wersji Rysunków 1 i S1 oraz Tabel S3 i S4.

Tytuł publikacji: The many faces of Dicer: the complexity of the mechanisms regulating Dicer gene expression and enzyme activities

Autorzy: Kurzynska-Kokorniak A, Koralewska N, Pokornowska M, Urbanowicz A, Tworak A, Mickiewicz A, Figlerowicz M[§]

Rok opublikowania: 2015

Czasopismo: Nucleic Acids Research (2015) 19;43(9):4365-80. doi: 10.1093/nar/gkv328

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Oświadczamy, że wkład mgr inż. Marii Pokornowskiej w powstanie wymienionej poniżej publikacji polegał na otrzymaniu preparatu białkowego DUF283 oraz wykonaniu większości doświadczeń; rezultaty tych doświadczeń zostały przedstawione na rysunkach: 1-4, 6, 8, S1, S2, S5 i 1SR. Brała Ona również udział w interpretacji otrzymanych wyników, a także w przygotowaniu wstępnej wersji wszystkich rysunków i w edycji manuskryptu.

Tytuł publikacji: Revealing a new activity of the human Dicer DUF283 domain in vitro

Autorzy: Kurzynska-Kokorniak A[§], Pokornowska M, Koralewska N, Hoffmann W, Bienkowska-Szewczyk K, Figlerowicz M[§]

Rok opublikowania: 2016

Czasopismo: Scientific Reports (2016) Apr 5;6:23989, doi: 10.1038/srep23989

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Oświadczam, że wkład mgr inż. Marii Pokornowskiej w powstanie wymienionej poniżej publikacji polegał na otrzymaniu preparatu białkowego Dicer człowieka, zaplanowaniu i wykonaniu większości doświadczeń; rezultaty tych doświadczeń zostały przedstawione na rysunkach: 1-4, 6, S1 i S3-5 oraz w tabelach: 2 i S3. Brała Ona również udział w interpretacji otrzymanych wyników, przygotowaniu wstępnej wersji wszystkich rysunków, a także w przygotowaniu pierwszej wersji manuskryptu i jego późniejszej edycji.

Tytuł publikacji: The RNA–RNA base pairing potential of human Dicer and Ago2 proteins

Autorzy: Pokornowska M, Milewski MC, Ciechanowska K, Szczepańska A, Wojnicka M, Radogostowicz Z, Figlerowicz M, Kurzynska-Kokorniak A[§]

Rok opublikowania: 2019

Czasopismo: Cellular and Molecular Life Sciences (2019) Oct 26 , doi: 10.1007/s00018-019-03344-6

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