Charles University in Prague, Faculty of Science
Department of Cell Biology

Study programme: Biology
Branch of study: Biology

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Processing of different substrates by mammalian Dicer
Štěpení různých substrátů savčím Dicerem

Bachelor′s thesis

Supervisor: **doc. Mgr. Petr Svoboda, Ph.D.**

Prague, 2013

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 16.5.2013

Acknowledgements

I would like to express my gratitude to my supervisor doc. Mgr. Petr Svoboda, Ph.D. for his dedicated approach, helpful advice and valuable comments. I would also like to thank my family and friends for their full support and encouragement.

Contents

Abstract
Small RNA pathways represent sequence-specific mechanisms regulating gene expression or mediating antiviral defence in eukaryotes. The common feature of these pathways are ~20-30-nucleotide small RNAs, which function as sequence-specific guides. Small RNA pathways differ from each other in their roles, biogenesis of small RNAs and mechanism of regulation their targets in different organisms. In mammals, there are three recognized small RNA pathways: RNA interference (RNAi), microRNA (miRNA) and PIWI-interacting RNA (piRNA) pathways. Biogenesis of small RNAs of RNAi and miRNA pathways is dependent on the Dicer protein, which generates small interfering RNAs (siRNAs) and miRNAs from long double-stranded RNAs (dsRNAs) and small hairpins, respectively. This bachelor thesis provides an insight into structure and function of mammalian Dicer, particularly into differences in Dicer processing of pre-miRNA and siRNA precursors.

Keywords: human Dicer, siRNA, miRNA, RNA interference, microRNA pathway

Abstrakt
Dráhy malých RNA představují u eukaryotických organismů sekvenčně specifické mechanismy regulující genovou expresi nebo zprostředkující antivirovou obranu. Společnou vlastností těchto drah jsou ~20 až 30nukleotidové malé RNA, které fungují jako sekvenčně specifičtí průvodci. Dráhy malých RNA se od sebe a mezi různými organismy liší v rolích, biogenezi malých RNA a způsobu regulace cílových molekul. U savců jsou 3 klíčové dráhy malých RNA: RNA interference, mikroRNA (miRNA) dráha a PIWI-interagující RNA (piRNA) dráha. Tvoření malých RNA pro RNA interferenci a miRNA dráhu je závislé na proteinu Dicer, který štěpí malé interferující RNA (siRNA) a miRNA z jejich prekurzorů: dlouhých dvouřetězcových RNA a vlásenek. Tato bakalářská práce poskytuje vhled do struktury a funkce savčího Diceru a do rozdílů ve zpracování pre-miRNA a prekurzorů siRNA.

Klíčová slova: lidský Dicer, siRNA, miRNA, RNA interference, mikroRNA dráha

1 Introduction

The small RNAs are involved in sequence-dependent gene silencing in eukaryotic organisms, which is generally called RNA silencing. The common feature of small RNA pathways are small RNA molecules of ~20-30 nucleotides, which form an effector ribonucleoprotein complex with a member of Argonaute protein family (reviewed in Ketting, 2011). The small RNA functions as a guide, which helps to effector complex to recognize its target by base-pairing. The effector complex subsequently mediates RNA silencing effect, which depends on specific Argonaute protein and small RNA. Although small RNA pathways are found in almost all eukaryotes, they differ in many aspects between species, including number and function of small RNA pathways acting in a species. In addition, the distinguishing small RNA pathways is sometimes difficult because of their crosstalks and shared protein components. In mammals, the main recognized small RNA pathways are the RNAi pathway, the miRNA pathway and the piRNA pathway.

The piRNA pathway employs ~24-31-nucleotide piRNAs which are bound to proteins from Piwi clade of Argonaute protein family (reviewed in Ketting, 2011; Kim et al, 2009). The piRNA-pathway is active predominantly in germline cells, where it is responsible for silencing of transposable elements. Biogenesis of piRNAs is not completely understood, although it is known to be independent on Dicer, a ribonuclease III (RNase III), which is a key component of other small RNA pathways. Therefore, I will not discuss the piRNA pathway in further details.

RNAi is mediated by ~20-23-nucleotide dsRNAs called siRNAs, which are generated from long dsRNAs or extended hairpins with perfect base-pairing (reviewed in Kim et al, 2009; Nejepinska et al, 2012). The mammalian siRNA precursors are derived from transcripts carrying inverted repeats, from sense-antisense pairs of transcripts stemming from convergent transcription or from sense and antisense transcription at separate loci, for example genes and their pseudogenes or interspersed repetitive elements. The siRNA precursors have a variable length and termini. Dicer processes the precursors into siRNAs of defined size (Figure 1a). As a components of RISC loading complex, Dicer and transactivation response RNA-binding protein (TRBP) assist loading siRNA duplex into an effector complex called RNA-induced silencing complex (RISC), which contains an Argonaute protein family member (Ago). The AGO2 cleaves a passenger strand of siRNA duplex, which is then ejected. A guide strand is used for targeting complementary mRNA, which is cleaved by AGO2 in the middle of the base-paired sequence (Figure 1a).

The role of the RNAi differs among organisms. In *Drosophila melanogaster* and in *Caenorhabditis elegans*, RNAi plays a role in gene regulation, silencing of transposable elements, and antiviral defence (reviewed in Nejepinska et al, 2012). In contrast, the role of RNAi is unclear in mammals, as long dsRNA molecules also trigger the interferon response in somatic cells. The interferon response is a pathway, in which dsRNA induces sequence-independent global repression of translation and is a part of mammalian antiviral defence (reviewed in Gantier & Williams, 2007). The substrate features and the physiological condititions leading to activation of either RNAi or the interferon response as well as how these pathways influence each other in mammalian somatic cells remain to be elucidated. Oocytes and cells of early embryo represent an exception; the interferon response is suppressed there (Stein et al, 2005) and long dsRNA is efficiently processed into siRNAs and triggers RNAi response in these cell types (Tam et al, 2008; Watanabe et al, 2008).

The miRNA pathway functions in post-transcriptional regulation of gene expression (reviewed in Kim et al, 2009; Yeo & Chong, 2011). Briefly, the miRNA pathway employs ~22-nucleotide miRNAs, which are encoded in the genome. Canonical miRNA encoding genes are transcribed by RNA polymerase II into primary trancripts called pri-miRNAs, which carry one or more stem-loop structures. The stem-loop structure is processed in the nucleus by the microprocessor (Drosha-DGCR8) complex, which cleaves ~11 base pairs (bp) from the base of the stem region and releases a ~65-nucleotide product containing a terminal loop and imperfectly paired stem region with 2-nucleotide 3′ overhangs, called precursor miRNA (pre-miRNA). The pre-miRNA is transported to cytoplasm and cleaved by Dicer into miRNA duplex, which is subsequently loaded onto an Argonaute protein. The passenger strand is removed and the guide strand binds target mRNA via imperfect base-pairing, which results in the repression of translation or mRNA degradation (Figure 1b). The non-canonical miRNAs bypass processing by either the microprocessor complex or Dicer in their biogenesis. Therefore, they can be found in the Drosha or Dicer mutants, respectively. The importance of microRNA pathway is ilustrated by an estimation that more than 60 % of all mRNAs might be regulated by miRNAs in humans (Friedman et al, 2009).

Dicer belongs to RNase III superfamily and represents a key component of RNAi and miRNA pathways (reviewed in Jaskiewicz & Filipowicz, 2008). In mammals, both are dependent on the same Dicer homolog, which generates siRNA and miRNA. Although miRNA and siRNA precursors differ in extent of perfect base-pairing and in the presence of a terminal loop, mammalian Dicer is able to process both types of substrate (Hutvagner et al, 2001; Provost et al, 2002). At the same time, both pathways clearly functionally differ: the

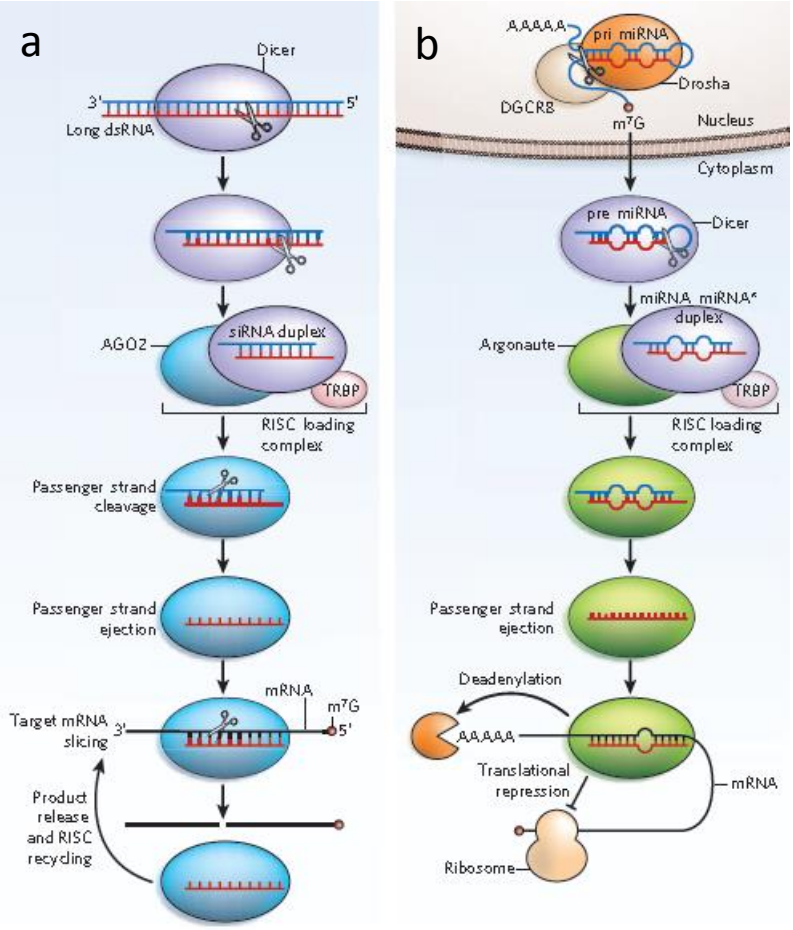

Figure 1: **RNA interference and microRNA pathways**
a) A schematic representation of the human RNA interference pathway.
b) A schematic representation of the human microRNA pathway.
Both pathways are described in the text. Proteins are distinguished by colours. Scissors indicate cleavage.
m^7G represents 7-methyl guanylate cap of mRNA.
Figures are taken from (Jinek & Doudna, 2009).

miRNA pathway constitutes an important regulatory mechanism of gene expression in somatic cells while RNAi, whose role in somatic cells is at least partly taken over by interferon response, is highly active and essential in oocytes (Suh et al, 2010; Tam et al, 2008; Watanabe et al, 2008). The difference in the roles between RNAi and the miRNA pathway in somatic and germ cells raises a question whether mammalian Dicer distinguishes between both types of precursors and processes them differently.

The aim of my bachelor thesis is to summarize Dicer structure and function with particular attention to processing of different substrates. The second chapter will provide an introduction to Dicer processing, domain arrangement and architecture of mammalian Dicer. The third chapter will summarize the structures of Dicer domains and their roles in Dicer processing. The fourth chapter will review the different processing of pre-siRNA and pre-miRNA precursors and mechanism, which might be responsible for this difference.

2 Structure of mammalian Dicer

The structure of a protein is important for understanding its domain arrangement as well as for revealing possible substrate-domain interactions and the mechanism of function. The mammalian Dicer is a multidomain protein of about ~220 kDa. It consists of the

following domains (listed from the N- to the C-terminus): the N-terminal helicase domain, the Piwi Argonaute Zwille (PAZ) domain, the domain of unknown function (DUF) 283, two RNase III domains (RNase IIIa and RNase IIIb) and the C-terminal double-stranded RNA-binding domain (dsRBD) (Figure 2). The functional model compares Dicer to a molecular ruler, which cleaves a constant distance from the end of dsRNA (Zhang et al, 2004).

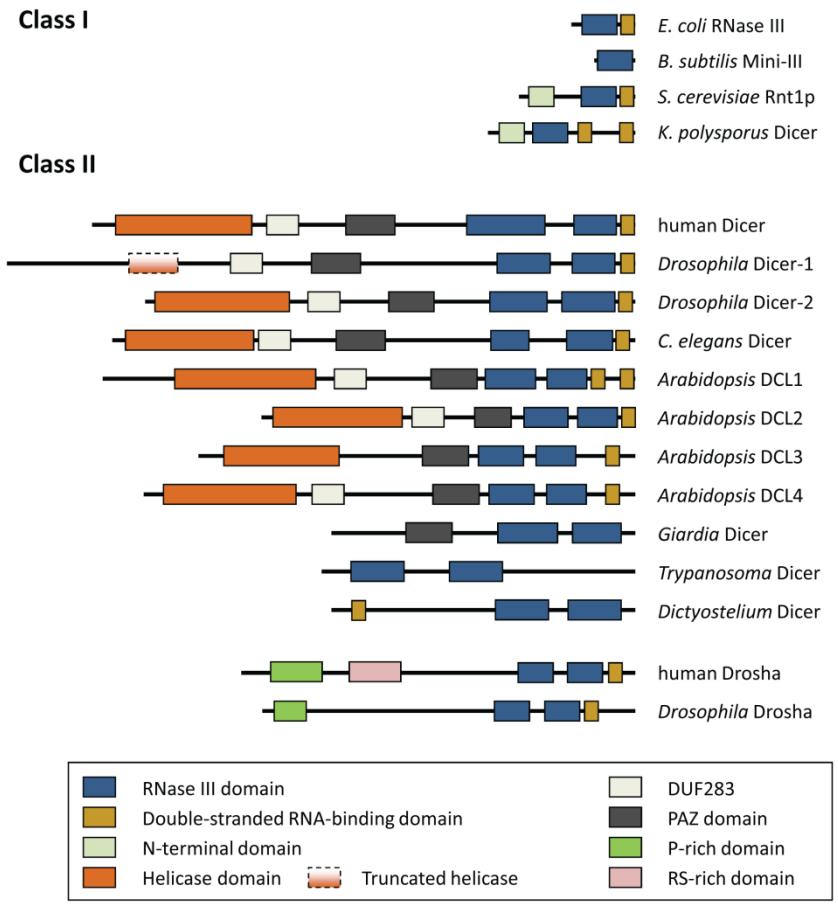

Figure 2: **The RNase III superfamily**
A schematic representation of domain organization of RNase III superfamily proteins. The RNase III proteins are divided into two classes of the RNase III superfamily (Jaskiewicz & Filipowicz, 2008). The class I includes bacterial and fungal RNase III orthologs, which have only one RNase III domain and function as a homodimer. The class II includes Dicer and Drosha orthologs with two tandemly arranged RNase III domains. The members of class II function as a monomer. Individual domains are distinguished by colours. Figure taken from (Doyle et al, 2012).

While the crystal structure of the entire mammalian Dicer protein is not available, there are several other relevant sources of information:

- biochemical studies on recombinant Dicer determining the mechanism of cleavage (Zhang et al, 2004), affinity to different substrates (Provost et al, 2002) and functions of domains (e.g. (Ma et al, 2008; MacRae et al, 2007; Provost et al, 2002)),
- crystallographic study on Dicer from *Giardia intestinalis* (MacRae et al, 2006),
- crystallographic studies on a fragment of mammalian Dicer (Du et al, 2008) or on its individual domains (Ma et al, 2004; Takeshita et al, 2007),
- electron microscopy studies of human Dicer (Lau et al, 2012) and its complexes with other proteins (Lau et al, 2009; Wang et al, 2009).

Each of these studies provides a partial insight into structure and function of mammalian Dicer. To reconstruct the architecture and mechanism of function of mammalian Dicer, I will integrate information from different sources mentioned above.

The biochemical studies on recombinant human Dicer demostrated that Dicer processes small RNAs from ends of dsRNA or hairpin substrates (Zhang et al, 2002) and each RNase III domain cleaves one strand of a substrate (Zhang et al, 2004). The processing of mammalian Dicer is ATP independentand requires divalent cationts (Zhang et al, 2002).

The *G. intestinalis* Dicer crystal structure plays a key role in understanding how Dicer works (MacRae et al, 2006). In comparison to human Dicer, *G. instestinalis* Dicer is much smaller (~82 kDa) and simpler because it consists of two RNase III, platform and PAZ domains only. Therefore, it has been suggested to represent a conserved nuclease core structure (MacRae et al, 2006). The front view of *G. intestinalis* crystal structure has an overall shape of a hatchet (Figure 3). The blade is formed by an internal dimer of two RNase III domains. They are connected by a bridging domain, which constitutes the back end of the blade. The platform domain is adjacent to RNase IIIa and makes up the upper part of the handle. The PAZ domain is connected by long helix to RNase IIIa and forms the base of the handle (MacRae et al, 2006). The processing centre of the protein is responsible for dsRNA cleavage and is located on the interface between two RNase III domains. It consists of two active sites, where each belongs to one RNase III domain (Zhang et al, 2004) and contains two divalent cationts necessary for cleavage (MacRae et al, 2006; Provost et al, 2002; Zhang et al, 2002).

The crystal structure of *G. intestinalis* Dicer supports the molecular ruler model where the distance between the RNase IIIa active site and the PAZ domain RNA-binding pocket corresponds to the length of 25 bp dsRNA, which represents the length of *G. intestinalis* siRNA (MacRae et al, 2006). The finding led to construction of the structural model with bound dsRNA (MacRae et al, 2006), which revealed several positively charged patches on the surface of *G. intestinalis* Dicer. These patches were in contact with dsRNA and are located between the processing center and the RNA-binding pocket of the PAZ domain. The model was confirmed in biochemical study with Dicer mutants (MacRae et al, 2007).

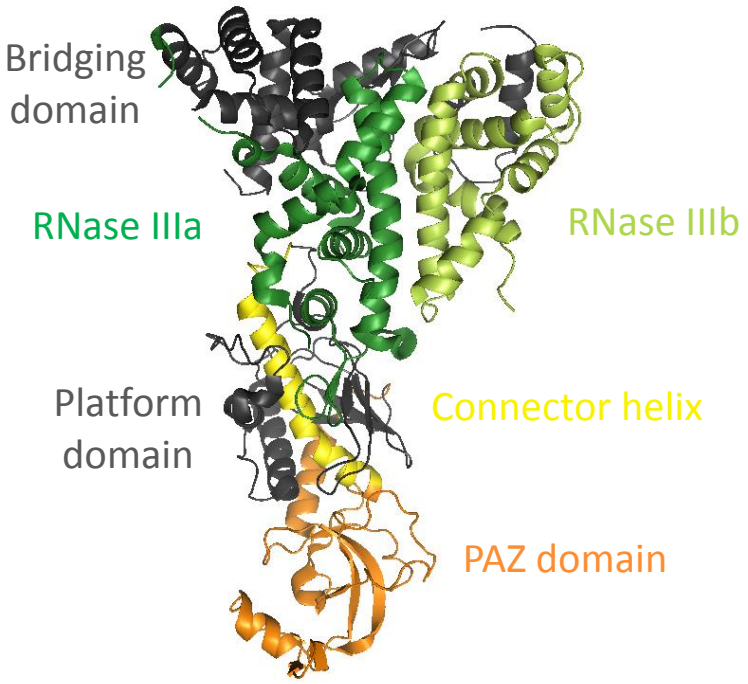

Figure 3: **The crystal structure of *G. intestinalis* Dicer**
The crystal structure of *G. intestinalis* Dicer (PDB 2FFL) containing two RNase III domains (dark and light green), the bridging domain (grey), the platform domain (grey) and the PAZ domain (pink). The connector helix coloured in yellow links the PAZ domain to RNase IIIa domain. Flexible regions are not shown. Image was generated using PyMOL.

The overall shape of human Dicer and position of some of its domains were determined by negative-stain electron microscopy and single particle analysis (Lau et al, 2012; Lau et al, 2009). In the latter case, the authors focused on PAZ, platform and RNase IIIb domains, in which they inserted an amino acid sequence serving as a biotin-protein ligase substrate. After biotinylation and binding of streptavidin, they observed Dicer with attached streptavidin on the surface of each particular domain. The positions of N-terminal helicase domain and C-terminal dsRBD were discovered by their deletion and comparing deletion mutants with full-length Dicer (Lau et al, 2012).

The overall shape of human Dicer resembles the letter L; the shape is further divided into head, body and base (Figure 4a). The PAZ domain is adjacent to the platform domain in the head of the protein. RNase IIIb is located in the body. The helicase domain constitutes the base of the letter L, but its function in mammalian Dicer remains uncertain and will be discuss in the following chapters (Figure 4b). The authors combined models of *G. intestinalis* Dicer and human Dicer so that their PAZ and platform domains overlapped (Lau et al, 2012). The position of the processing centre relative to their PAZ and platform domains differs by approximately 120° around the long axis of the human Dicer, which serves as the axis of rotation (Figure 4c). The difference is explained as a cause of diverse product lengths. The human Dicer produces siRNA about 4 nucleotides shorter than *G. intestinalis* Dicer, which corresponds to roughly one-third of a dsRNA helical turn. Therefore, the processing centre

has to access the cleavage site of dsRNA from the different angle relative to the dsRNA helical end in comparison with *G. intestinalis* Dicer (Lau et al, 2012).

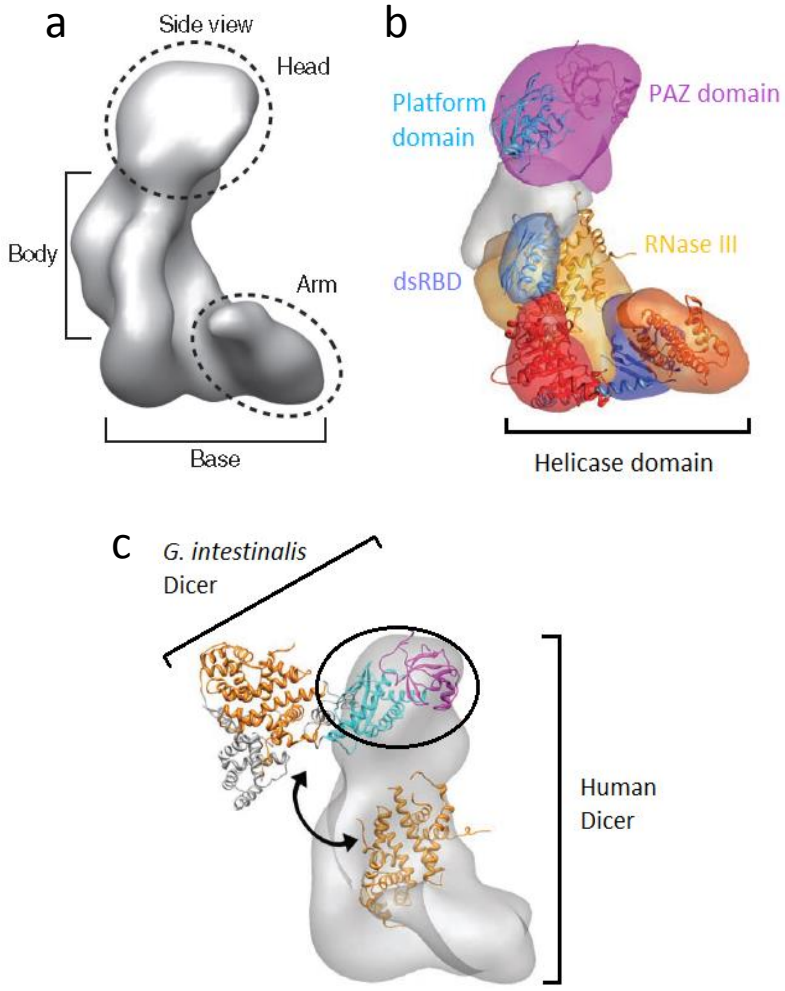

Figure 4: **The architecture of human Dicer**
a) The model of human Dicer showing the overall shape of human Dicer with its distinction into several regions.
b) The model of human Dicer with indicated positions of its domains: the PAZ domain (purple), the platform domain (turquoise), the dsRBD (blue), RNase III domains (yellow) and the helicase domain (red, dark blue and orange, indicated by clamp).
c) The combined model of *G. intestinalis* Dicer and human Dicer. The circle indicates the overlapping PAZ and platform domains of both Dicers. The double-headed arrow depicts the modification necessary for alignment RNase III domains of both Dicers. Figures adapted from Lau et al. (2012).

3 The role of Dicer domains in substrate processing

3.1 PAZ domain (Piwi Argonaute Zwille domain)

The PAZ domain is found in Argonaute and Dicer, proteins participating in dsRNA-dependent silencing pathways. The PAZ domain of Dicer is responsible for measurement of proper product size (MacRae et al, 2007; MacRae et al, 2006; Zhang et al, 2004). Two features of the PAZ domain are important: (1) substrate binding, (2) position within Dicer relative to other domains.

Zhang et al. performed the substitution of conserved amino acids residues (F960 or YY971/2) in the PAZ domain of human Dicer (Zhang et al, 2004). This mutation led to reduced processing of dsRNA, but the product size was not affected. This result correlates with the crystal structure of the PAZ domain from human Argonaute bound to dsRNA (Ma et

al, 2004). The structure reveals several amino acid residues participating in RNA binding, which might indicate robust substrate binding. Therefore, eliminating one or two of the participating amino-acid residues might influence the binding strength or the kinetic of binding, but does not function as an OFF switch.

The crystal structure of PAZ domain from mammalian Dicer has been apparently solved, but it has not been published yet (Park et al, 2011). The crystal structure of the PAZ domain from human Argonaute eIF2c1 with a bound 9-mer RNA (Ma et al, 2004) shows that the PAZ domain binds mainly 2-nucleotide 3′ overhang of dsRNA (Figure 5). The binding of RNA occurs in the RNA-binding pocket. The RNA-binding pocket has an elongated shape and is lined by aromatic and hydrophobic residues in order to maintain the hydrophobic environment of the pocket. Most amino acid residues of the RNA-binding pocket interact with the terminal nucleotide of the 3′ end. Other interactions direct to penultimate 3′-end nucleotide and several phosphodiester groups of this RNA strand. Only one amino-acid residue interacts with the complementary RNA strand and its substitution does not lead to loss of binding (Ma et al, 2004).

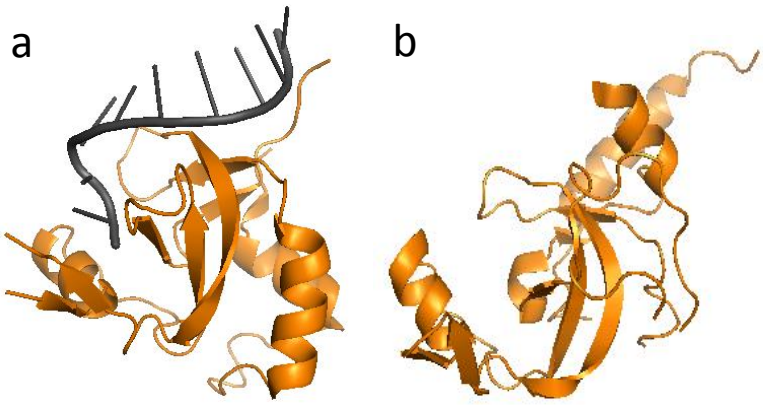

Figure 5: **The crystal structures of the PAZ domain**
a) The crystal structure of the PAZ domain from human Argonaute eIF2c1 (PDB 1SI3; orange) with bound 9-nucleotide RNA (grey). The 3′ end of RNA molecule is bound to 3′ RNA-binding pocket.
b) The crystal structure of the PAZ domain from *G. intestinalis* Dicer (PDB 2FFL). The 3′ RNA-binding pocket is denoted by an arrow. Images were generated using PyMOL.

The amino-acid residues of the RNA binding pocket of the PAZ domain from human AGO target general parts of nucleotides and the RNA-binding pocket is spacious enough to accomodate all bases in the terminal and penultimate position (Ma et al, 2004). Therefore, the binding of the PAZ domain from human AGO to dsRNA is sequence independent, i.e. it should not prefer any particular combination of two nucleotides at the 3′ end of dsRNA. However, the studies on human Dicer revealed that sequence of 2-nucleotide 3′ overhangs influenced the efficiency of processing as well as the length of products (DiNitto et al, 2010; Vermeulen et al, 2005). Although it was reported for the entire Dicer and not for the sole PAZ domain, it indicates that the PAZ domain of human Dicer might have base preferences in the positions of 2-nucleotide 3′ overhangs.

Dicer products are cleaved from termini of dsRNA (Zhang et al, 2002); the PAZ domain functions as the proximal part of a molecular ruler which binds the end of substrate. The processing centre of RNase III domains is the distal part of a ruler. The distance between the PAZ domain and the processing centre then corresponds to the length of small RNA products (MacRae et al, 2006). The role of the PAZ domain in product size measurement is well documented (MacRae et al, 2007; MacRae et al, 2006; Zhang et al, 2002); the key evidence came from the deletion of PAZ domain in *G. intestinalis* Dicer. Loss of the PAZ domain led to production of siRNA of variable length, which indicates the cleavage in random positions along the dsRNA substrate (MacRae et al, 2007). The PAZ domain's role was further confirmed by its substitution for RNA-binding spliceosomal protein U1A, which binds the loop of specific sequence (MacRae et al, 2007). The engineered protein cleaved a hairpin substrate at a stable distance from the loop in contrast to wild type Dicer, which measured the cleavage position from the open helical end (MacRae et al, 2007);

The mammalian Dicer measures the product size from either 3′ end or 5′ end of dsRNA substrate bound to the PAZ domain. At first, studies on human Dicer and *G. intestinalis* Dicer revealed that the size of the products was measured from the 3′ end of the substrates (MacRae et al, 2007; Vermeulen et al, 2005). This mechanism is called 3′ counting rule. Nevertheless, the 3′ counting rule did not held true completely for two reasons. First, the processing of substrate with one type of ends yielded mixture of products of different lengths. The longer were 3′ overhangs of substrates, the shorter were mixtures of products (MacRae et al, 2007; Vermeulen et al, 2005). Second, substrates with 3′ overhangs longer than 3 nucleotides yielded the products of the same length (Vermeulen et al, 2005). The explanation of the latter exception provided 5′ counting rule (Park et al, 2011). The PAZ domain of mammalian Dicer contains also 5′ RNA-binding pocket, which binds the 5′ end of dsRNA substrate and functions as alternative end of molecular ruler. The decision between two counting rules is dependent on the particular miRNA. However, the 5′ counting rule is used for pre-miRNA with relatively less stable structures at the base of the stem region (mismatch or A-U pair). On the contrary, the 3′ counting rule is used for pre-miRNA with stable base of stem region or for pre-miRNA with missing 5′ phosphate group (Park et al, 2011). The 5′ binding pocket is not found in the PAZ domain of *G. intestinalis* Dicer.

Remarkably, the PAZ domain is not found in some Dicer homologs, for instance in Dcr1 or Dcr2 from *Tetrahymena thermophila* or Dcr1 from budding yeast *Kluyveromyces polysporus* (Mochizuki & Gorovsky, 2005; Weinberg et al, 2011). In the latter case, the product size is determined by dimerization of Dcr1 (Weinberg et al, 2011).

3.2 N-terminal helicase domain

The helicase domain is located at the N-terminus of Dicer sequence. The helicase domain constitutes the base of the enzyme, which is adjacent to catalytical domains (Lau et al, 2012). Therefore, it is likely in contact with the substrate. The helicase domain consists of two conserved domains: the N-terminal DExD/H domain and the helicase superfamily c-terminal domain. It belongs to RIG-I like helicase family (Zou et al, 2009), which is a member of the helicase superfamily 2 (SF-2). The helicase domain of mammalian Dicer contains seven conserved motifs (Nicholson & Nicholson, 2002). The motif I (Walker A motif) and the motif II (Walker B motif) are important for ATP binding and hydrolysis (reviewed in Caruthers & McKay, 2002). Therefore, these motifs should be crucial for the ATP-dependent helicase function. The crystal structure of the N-terminal helicase domain of Dicer had not been obtained yet.

The helicase domain is not found in *G. intestinalis* Dicer, hence it is not essential for Dicer cleavage. Biochemical studies showed that its function differs in vertebrates and invertebrates Dicers (Bernstein et al, 2001; Provost et al, 2002; Welker et al, 2011; Zhang et al, 2002). The processing by invertebrates Dicer is dependent on ATP (Bernstein et al, 2001). The absence of ATP leads to reduced dicing activity. The same effect has the mutation of the conserved amino-acid residue K34, which is found in the Walker A motif of the helicase domain in *Drosophila melanogaster* Dicer-2 (Welker et al, 2011). This motif is thought to be responsible for binding of ATP. However, the mechanism is more complex because the requirement of ATP for invertebrate Dicer processing is dependent on the type of dsRNA end (Welker et al, 2011). dsRNA with 3′ overhangs was processed by *D. melanogaster* Dicer-2 even without ATP or with mutation in Walker A motif, although the efficiency was higher in the presence of ATP. Processing of dsRNA with blunt ends or 2-nucleotide 5′ overhangs was severely impaired in the absence of ATP and the effect of mutation in K34 was more pronounced. The processing of long dsRNA with blunt ends was also more processive in comparison with 2-nucleotide 3′ overhangs ended dsRNA in the presence of ATP; it resulted in accumulation of siRNAs from an internal part of the substrate (Welker et al, 2011). The exact role of ATP in invertebrate Dicer cleavage as well as the role of the helicase domain has not been elucidated yet, although the role of the helicase domain in unwinding the dsRNA substrate in order to allow proper substrate or in translocation along dsRNA has been proposed (Bernstein et al, 2001; Welker et al, 2011).

In human Dicer, dsRNA substrate processing is ATP independent (Provost et al, 2002; Zhang et al, 2002). This was demonstrated by the same processing efficiency in presence or absence

of ATP. Moreover, the rate of cleavage was not influenced by addition of other nucleotides, noncleavable ATP derivates or mutation in Walker A motif of ATPase/helicase domain (Provost et al, 2002; Zhang et al, 2002). These experiments were performed using long dsRNA substrate with blunt ends, whose processing by invertebrates Dicer is ATP dependent (Zhang et al, 2002). Therefore, the role of the N-terminal helicase domain of mammalian Dicer is distinct from its role in invertebrates Dicers; it is probably involved in substrate recognition, which will be discussed in further detail in the third chapter.

3.3 C-terminal double-stranded RNA-binding domain (dsRBD)

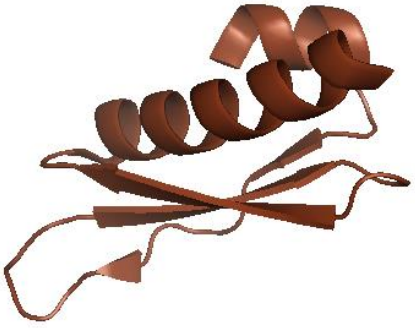

Figure 6: **The C-terminal dsRBD** The crystal structure of the C-terminal dsRBD from mouse Dicer (PDB 3C4B) with an α-β-β-β-α topology. Image was generated using PyMOL.

The dsRBD is located at the C-termini of most Dicer homologs. The structure of dsRBD of mammalian Dicer exhibits the common dsRNA binding motif, which is made of three antiparalel β-sheets and two α-helixes (Figure 6) (Du et al, 2008). The dsRNA binding motif is considered to bind dsRNA in a sequence-independent manner since it binds mainly sugar-phosphate backbone of dsRNA. However, the structural analysis of dsRNA binding motifs from adenosin deaminase acting on RNA 2 (ADAR2) suggests that some sequence preferences might exist (Stefl et al, 2010).

Data from biochemical studies provided varying evidence about dsRBD function in mammalian Dicer. The early biochemical studies indicated that dsRBD of mammalian Dicer binds dsRNA (Provost et al, 2002; Zhang et al, 2004). The substrate affinity of sole dsRBD was strong enough to interfere with processing of full-length mammalian Dicer when dsRBD was added to the reaction (Provost et al, 2002). The dsRBD deletion mutant processed a 30 bp dsRNA substrate less efficiently than full-length human Dicer (Zhang et al, 2004). In addition, cleavage of dsRNA with blunt ends was more affected than cleavage of dsRNA with 2-nucleotide 3′ end overhangs in a dsRBD mutant (Zhang et al, 2004). However, the Dicer substrate cleavage might be affected on many levels of processing, not only at the level of substrate binding. The results of other experiments with dsRBD deletion mutants of human Dicer support this notion, as they suggest that dsRBD is involved in catalysis but not in substrate binding (Ma et al, 2008). However, the experiments with the C-terminal fragment of human Dicer consisting of both RNase III domains and dsRBD provide evidence, that dsRBD is indeed important for substrate binding. The C-terminal

fragment of human Dicer was able to process substrate or bind dsRNA only, when the dsRBD domain was present. Its deletion from this fragment impaired both abilities (Ma et al, 2012).

The dsRBD of human Dicer does not distinguish perfect duplexes from pre-miRNAs. The experiments concerning binding affinity indicated that the strength of binding of sole dsRBD of human Dicer was almost the same for a pre-miRNA and for a 44 bp long perfect duplex. The subtle difference was caused by the terminal loop structure of the pre-miRNA, as it contains a junction between single stranded and double stranded RNA, which is very slightly prefered by dsRBD of human Dicer (Wostenberg et al, 2012). However, the possible effect of imperfect base pairing, which is characteristic for pre-miRNA, have not been thoroughly tested.

Wostenberg et al. suggested that the binding affinity grows with the length of the substrate dsRNA. The length of the perfect duplex dsRNAs used in the study varied from 12 to 44 bp and the binding affinity was assessed by electrophoretic mobility shift assay (EMSA) (Wostenberg et al, 2012). It can be assumed that longer substrates provide more potential binding sites and hence are more strongly bound than shorter dsRNAs in the EMSA. Therefore, the length of dsRNA does not influence the strength of binding rather than the probability that the dsRBD from mammalian Dicer will bind to it.

3.4 Domain of Unknown Function 283 (DUF283)

DUF283 is a domain, which is found in Dicer in most higher eukaryotes. However, its structure and function had been addressed only recently. MacRae et al. suggested that the DUF283 domain shares consistent sequence similarity with the platform domain of *G. intestinalis* Dicer, and therefore might constitute a similar structure (MacRae et al, 2006). However, a thorough bioinformatic study showed that the predicted structure of DUF283 of *D. melanogaster* Dicer-1 has an α-β-β-β-α fold, which corresponds to dsRNA binding fold, typical for dsRBDs. The comparison of conserved amino-acid residues of DUF283 and the dsRBD indicated a similar pattern of surface conservation (Dlakic, 2006). Therefore, DUF283 may bind dsRNA. On the other hand, the deletion of DUF283 in human Dicer led to lower value of dissociation constant (K_d) of the complex of a DUF283 deletion mutant and 35 bp dsRNA in comparison with the K_d value of full-length Dicer bound to 35 bp long dsRNA (Ma et al, 2008). The K_d value of the complex of a DUF283 deletion mutant and pre-miRNA remained the same as the K_d value of the complex of wild type human Dicer and pre-miRNA (Ma et al, 2008). Here, the dissociation constant reflects a tendency of protein/ligand complex to disintegrate, which correlates negatively with stability of the

complex and hence with affinity of a protein to its ligand. Therefore, the lower is the K_d value, the higher is the affinity between molecules. One would expect an opposite result of DUF283 deletion if DUF283 was responsible for substrate binding. Nevertheless, the deletion of DUF283 reduced the difference in Dicer binding affinity between pre-hlet-7a-1 and 35 bp long perfect duplex (Ma et al, 2008). Therefore, DUF283 might be responsible for dinstinguishing between perfect duplex dsRNA and pre-miRNA due to, for instance, obstruction of substrate binding in case of a perfect duplex; this effect would consequently decrease the cleavage rate of a perfect duplex. However, the full-length Dicer cleavage rate of 35 bp perfect duplex dsRNA is higher than the DUF283 deletion mutant cleavage rate of the same substrate. This result contradicts the role of DUF283 in distinguishing between perfect duplex dsRNA and pre-miRNA. Taken together, the role of DUF283 is still unclear as the experiments provided inconclusive results.

3.5 RNase III domains

RNase IIIa and RNase IIIb are responsible for cleavage of dsRNA substrate. The homologs of these domains are found in all proteins of ribonuclease III family, which generate dsRNA products containing a characteristic 2-nucleotide overhang with hydroxyl group at the 3′ end and phosphate group at the 5′ end (reviewed in MacRae & Doudna, 2007). The bacterial RNase III enzymes contain one RNase III domain and function as a homodimers. In comparison with bacterial RNase III enzymes, mammalian Dicer contains two RNase III domains and functions as a monomer; its two RNase III domains form an intramolecular pseudodimer (Zhang et al, 2004). The crystal structure of *G. intestinalis* Dicer confirmed the intramolecular dimerization of RNase III domains (MacRae et al, 2006). The crystal structure of a catalytically active homodimer of human RNase IIIb showed that the RNase III domains associate to each other due to hydrophobic interactions on the dimer interface (Takeshita et al, 2007). As these hydrophobic amino-acid residues are conserved among Dicer homologs, these observed interactions might accomplish the formation of an internal heterodimer in full-length Dicer as well.

The crystal structure of RNase IIIb from human Dicer includes eight α-helices and one 3_{10} helix (Figure 7a) (Takeshita et al, 2007), which contains only 3 amino acids per turn and is less stable in comparison with an α-helix (Pauling et al, 1951). RNase IIIa and RNase IIIb from Dicer proteins contain a long inserted sequence of variable size, which is not found in other RNase III domains. In RNase IIIb from mammalian Dicer, the part of this region forms an α-helix, the rest of it constitutes a loop and a flexible region. The role of the inserted

sequence is unknown, however deletion of 13 amino-acid residues from the flexible region has no apparent effect on RNase IIIb processing (Takeshita et al, 2007). Moreover, the superposition of RNase IIIb from mouse and *G. intestinalis* Dicer, and *Aquifex* RNase III showed that the α-helix from this region is located in the RNA binding motif 4 (RBM4) of *Aquifex* RNase III, which interacts via hydrogen bonds with dsRNA (Gan et al, 2006). This inserted region might impair the function of corresponding region in RNase IIIb of mammalian Dicer and therefore might partly account for inefficient processing of mammalian Dicer in comparison with bacterial RNAse III (Zhang et al, 2002; Zhang et al, 2004).

The dsRNA cleavage requires two cleavage events, which raised questions whether

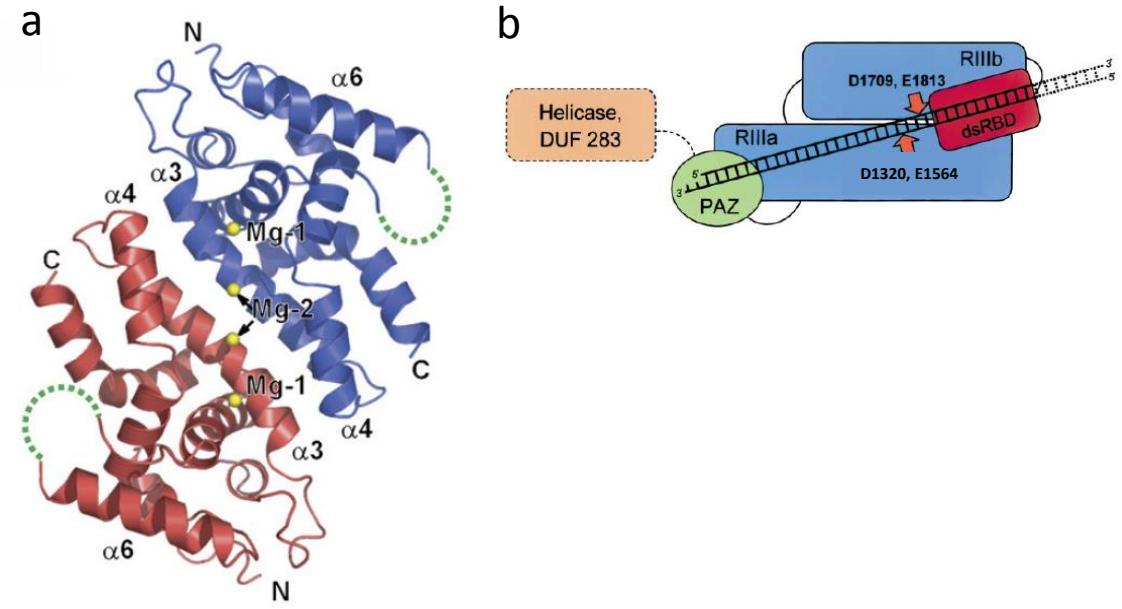

Figure 7: **RNase III domains**
a) The crystal structure of RNase IIIb of human Dicer forming a homodimer. Magnesiom ions are depicted as yellow spheres; the flexible regions are denoted by broken lines. The figure is taken from (Takeshita et al, 2007).
b) A schematic representation of human Dicer in complex with bound dsRNA. The domains are distinguished by colours and abbreviations: the helicase domain and DUF283 (orange), the PAZ domain (PAZ; green), the dsRBD (red), RNase IIIa (RIIIa; blue) and RNase IIIb (RIIIb; blue). Orange arrows denote the cleavage by the individual RNase III domain. The amino-acid residues of RNase III domains which are important for cleavage are indicated. The figure is adapted from (Zhang et al, 2004).

each RNAse III domain is responsible for cleavage of one strand or whether they cleave each strand together. In the first case, the processing centre would be divided into two active sites, each active site would be formed by one RNase III. In the latter case, each active site would be formed by two RNase III domains. The answer came from a biochemical study on human Dicer with mutations in conserved amino-acid residues of RNase III domains (Zhang et al, 2004). Authors prepared eight Dicer variants, each mutated in one of the following amino-acid residues: Glu1313, Asp1320, Glu1340, and Glu1564 of RNase IIIa and Gln1702,

Asp1709, Pro1729, and Glu1813 of RNase IIIb. Assays with 30 bp dsRNA substrate with 2-nucleotide 3′ overhangs and one labeled 5′ end showed that only mutations in Asp1320, Glu1564, Asp1709 and Glu1813 impaired the processing of dsRNA (Figure 7b). The wild type Dicer processed 30 bp dsRNA from both sides, which yielded labeled fragments of roughly two lengths: ~21 and ~11 nucleotides. Four above mentioned mutants yielded only one of these fragments, either 21-nucleotide (RNase IIIa: Asp1320, Glu1564), or 11-nucleotide (RNase IIIb: Asp1709, Glu1813). Authors further prepared double mutants with combinations of these 4 mutations. When both mutations were in the same RNase III domain, the mutants produced only one fragment. The mutants with mutations in both RNase III domains were inactive. These data demostrated that each active site is formed by one RNase III domain. Thus, each RNase III domain is responsible for cleavage of one RNA strand. The RNase IIIa cleaves a strand with 3′ end bound to PAZ domain, the RNase IIIb cleaves the complementary strand (Zhang et al, 2004). The arrangement of active sites in relation to RNase III domains was confirmed by crystallographic study on *G. intestinalis* Dicer (MacRae et al, 2006).

Dicer requires divalent cationts for cleavage (Provost et al, 2002; Zhang et al, 2002). The divalent cationts form an integral part of the active site. The optimal activity is performed in the presence of Mg^{2+}. It can be replaced by Mn^{2+} or Co^{2+}, although the activity of enzyme is reduced (Zhang et al, 2002). Their absence in the reaction separates the two processing steps of Dicer: substrate binding and cleavage (Provost et al, 2002; Zhang et al, 2002). The same effect has the low temperature (4° C) or presence of EDTA (ethylenediaminetetraacetic acid), a chelator of divalent cationts (Zhang et al, 2002).

4 Processing of different substrates by mammalian Dicer

The mammalian Dicer processes two distinct groups of substrates. The first group is represented by precursors of siRNA.The precursors of siRNA are long dsRNA with long regions of perfect pairing. siRNA precursors might have different length and type of ends. The second group is represented by pre-miRNAs, which are approximately 70 nucleotides long and form hairpin structure with an imperfectly paired stem region and a terminal loop. Pre-miRNAs have predominantly 2-nucleotide 3′ overhangs as a result of Drosha cleavage (Kim et al, 2009).

The important question is whether mammalian Dicer is able to distinguish between both substrate groups. The experiments with siRNA precursor and pre-miRNA showed that there is a significant difference in a rate of cleavage between both substrates (Figure 8)

(Chakravarthy et al, 2010). The cleavage rate of both substrate was monitored in single and multiple turnover dicing assays in order to establish mechanism responsible for different processing. The amount of protein was in excess over the amount of substrate in a single turnover dicing assay, hence each enzyme cleaved only one substrate molecule in theory. Therefore, a release of product would not affect the cleavage rate. In a multiple turnover dicing assay, amount of substrate was in excess over amount of protein; the release of product would affect the Dicer cleavage rate of substrate. The rate of substrate cleavage was faster for pre-hlet-7a-1 substrate in both assays. Human Dicer cleaved half of the substrate in less than 5 minutes in case of pre-hlet-7a-1 and in ~75 minutes in case of siRNA precursor in a single turnover assay. In a multiple turnover assay, human Dicer needed ~15 minutes for cleavage of half of the pre-hlet-7a-1 molecules, but less than 1 % of 35 bp perfect duplexes were cleaved after 40 minutes (Chakravarthy et al, 2010). These results are in agreement with other biochemical studies (Ma et al, 2008; Ma et al, 2012). Therefore, mammalian Dicer discriminates between both substrates, but which step of Dicer processing is responsible for the difference in cleavage rate? The possible candidates are for example different substrate binding, distinct catalysis or distinct product release.

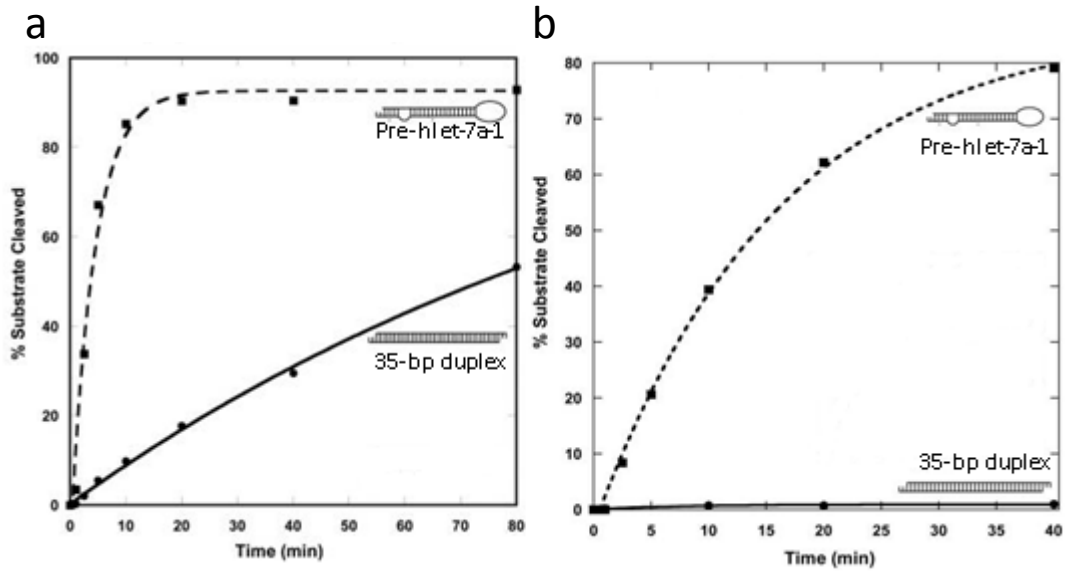

Figure 8: **Difference in Dicer rate of cleavage between pre-miRNA and siRNA precursor**
a) Quantification of single turnover assays with human Dicer and a pre-miRNA (pre-hlet-7a-1) or a perfect duplex (35-bp duplex). Assays were performed with 25 nM human Dicer and <1 nM pre-hlet-7a-1 or <1 nM 35-bp duplex.
b) Quantification of multiple turnover assays with human Dicer and a pre-miRNA (pre-hlet-7a-1) or a perfect duplex (35-bp duplex). Assays contained 5 nM human Dicer and 50 nM pre-hlet-7a-1 or 50 nM 35-bp duplex.
A name and a schematic representation of each substrate is depicted near the corresponding plot. Figures are adapted from (Chakravarthy et al, 2010).

The Dicer binding affinity was slightly stronger for miRNA than for siRNA precursors; the K_d value of human Dicer-pre-miRNA complex was at most 2.5 times smaller than the K_d value of human Dicer-siRNA precursor (Chakravarthy et al, 2010; Ma et al, 2008; Ma et al, 2012). However, the difference in binding affinity alone is not pronounced enough to be responsible for so markedly distinct cleavage rate.

Nevertheless, the pronounced difference in cleavage rate between both substrates might be due to an inhibitory effect of N-terminal helicase domain on catalysis (Ma et al, 2008). The experiments with mutants of mammalian Dicer showed that deletion of helicase domain reduced the difference in Dicer cleavage rate of both substrates under single turnover conditions (Ma et al, 2008; Ma et al, 2012). The loss of helicase led to increased cleavage of both substrates. Remarkably, the change in the cleavage rate of siRNA precursor was so high that it almost equalized the wild type Dicer cleavage rate of pre-miRNA. The increase in pre-miRNA cleavage rate after deletion of the helicase domain was only ~10% of the original value. Further experiments revealed that the loss of helicase domain affected the cleavage rate of siRNA precursor mainly due to change in catalysis of the enzyme. Therefore, the N-terminal helicase domain might be responsible for distinguishing between both substrates, because its deletion markedly affected only cleavage of siRNA precursor (Ma et al, 2008).

A biochemical study using different substrate molecules revealed that a terminal loop of pre-miRNA is a key feature which neutralizes the inhibitory effect of the helicase domain. The authors of the study prepared four substrates in order to determine, which part of pre miRNA contributes to Dicer processing. These constructs were: (1) pre-hlet-7a-1 (pre-miRNA), (2) 35 bp long dsRNA, (3) short hairpin with stem region from 35 bp dsRNA and the terminal loop from pre-hlet-7a-1, and (4) 37 bp long dsRNA containing stem region of pre-hlet-7a-1 (Figure 9a). The single turnover dicing assay with wild type human Dicer showed that a terminal loop of pre-miRNA is responsible for higher cleavage rate of pre-miRNA; both constructs containing a terminal loop were cleaved with approximately the same rate regardless of their stem regions. The 35 bp long dsRNA and 37 bp long construct containing pre-hlet-7a-1 stem region were cleaved less efficiently (Figure 9b). On the other hand, the cleavage rates of all constructs were similar in single turnover assay with N-terminal helicase deletion mutant; the efficiency of cleavage was even higher than the efficiency of cleavage of pre-hlet-7a-1 in previous assay (Figure 9c) (Ma et al, 2012). This result confirmed the data from previous study, that the N-terminal helicase domain plays an important role in different processing of siRNA and pre-miRNA precursors via inhibition of cleavage of siRNA precursors (Ma et al, 2008). The difference in substrate cleavage under single turnover

conditions is caused by a terminal loop of pre-miRNA, which is recognized by the N-terminal helicase domain. An inhibitory effect of this domain on Dicer processing might represent an adaption of mammalian Dicer to a key role of the miRNA pathway in somatic cells or a safety precaution, which secures that mammalian Dicer would cleave correct substrates (Nicholson, 2012).

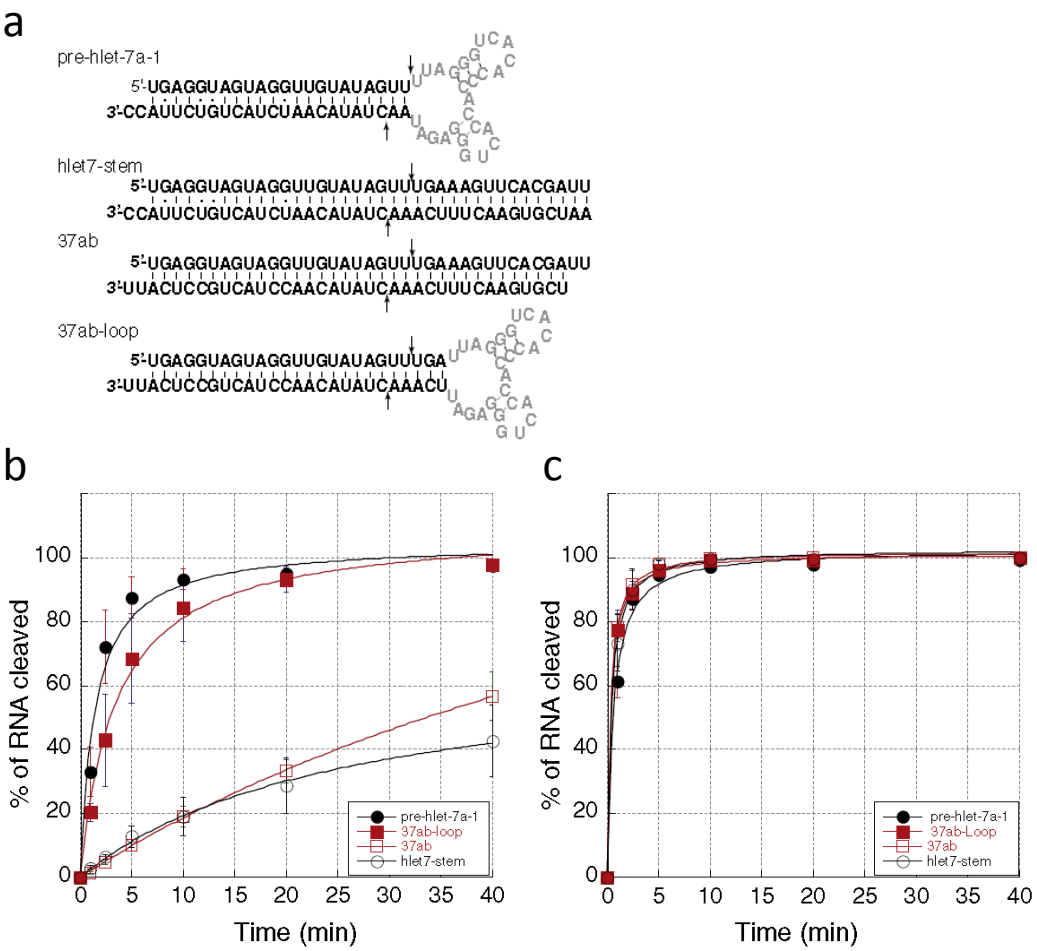

Figure 9: **The impact of the terminal loop of pre-miRNA and the helicase domain on Dicer processing**

a) Schemes of four substrates used in the assays. pre-hlet-7a-1 represents a pre-miRNA. 37ab is a siRNA precursor, which is mentioned in the text as 35-bp duplex. hlet7-stem and 37ab-loop are combined constructs of pre-hlet-7a-1 and 35-bp duplex. hlet7-stem contains a stem region from pre-hlet-7a-1 with and a part of 35-bp duplex. 37ab-loop contains terminal loop of pre-hlet-7a-1 and a stem region is formed by a part of 35-bp duplex. In the structures, Watson-Crick base pairs are indicated by lines; G-U pairs are denoted by dots.

b) Quantification of single turnover assays with full-length human Dicer and one of four substrates. The rate of cleavage is highest in the case of 37ab-loop, which contains a terminal loop and perfectly paired stem region.

c) Quantification of single turnover assays with human Dicer lacking N-terminal domain and one of four substrates.

Figures taken from (Ma et al, 2012).

The result of multiple turnover assay strongly indicated that different product release played an important role in processing of substrate, as the difference in cleavage rate between pre-miRNA and pre-siRNA was more pronounced than in the single turnover assay (Chakravarthy et al, 2010). Moreover, amount of cleaved substrate grew in time in case of pre-miRNA, but in case of siRNA precursor, the amount of cleaved substrate grew only for first 15 minutes and then stayed roughly the same for the rest of the time (Chakravarthy et al, 2010). This result indicated that sole Dicer might form a complex with siRNA products and this behaviour consequently reduces the cleavage rate of the reaction, as was suggested previously (Zhang et al, 2002). It needs to be determined, which structure of pre-miRNA or siRNA precursor is responsible for different product release. However, Dicer helps to load siRNA and miRNA on the RISC complex in vivo, so the observed effect of different product release might not play a role in vivo, where the products might be quickly taken over by downstream effector proteins.

5 Conclusions

Mammalian Dicer distinguishes between pre-miRNAs and siRNA precursors and cleaves pre-miRNAs more efficiently. The main difference in processing of pre-miRNA and siRNA precursors is caused by the N-terminal helicase domain of mammalian Dicer, which has an inhibitory effect on Dicer processing, especially towards siRNA precursors. Furthermore, mammalian Dicer forms complexes with its interacting partners, TRBP and PACT. The *in vitro* experiments revealed that the presence of the TRBP enhances the rate of substrate cleavage for both precursors (Chakravarthy et al, 2010) and that in case of miRNAs, intensity and the direction of change is dependent on the structure of an individual pre-miRNA (Lee & Doudna, 2012). The effect of these interacting partners therefore constitutes an additional level of regulation, which needs to be further addressed, as the concentration of these interacting partners may change in response to different physiological conditions.

Taken together, mammalian Dicer seems a more adapted to prefer pre-miRNA over siRNA precursors. Consistently, as the miRNA pathway plays a more important role in mammalian cells in comparison with RNAi. Further research needs to determine whether Dicer cleaves pre-miRNA more efficiently also *in vivo* and whether this is the cause of different levelst of siRNAs and miRNAs in mammalian cells.

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