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Recognition of expressed double-stranded RNA in mammalian cells

Rozpoznávanie dvojvláknovej RNA v cicavčích bunkách

Master's thesis

Supervisor: Doc. Mgr. Petr Svoboda, Ph.D.

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Prohlášení

Prohlašuji, že jsem diplomovou 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.

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CONTENTS

ABSTRACT

Long double-stranded RNA (dsRNA) is a unique structure formed during viral replication or transcription of repetitive elements. Mammalian cells evolved several mechanisms how to respond to dsRNA. dsRNA can be engaged in one of three pathways: interferon response, RNA editing, and RNA interference (RNAi). RNAi is evolutionary conserved effect of dsRNA, which results in sequence-specific messenger RNA degradation. However, in mammals, RNAi is functional only in mouse oocytes, which express truncated version of Dicer (Dicer^{O}). In somatic cells, dsRNA triggers sequence-independent interferon pathway.

The main aim of this Master's thesis was to examine how specific double-stranded RNA-binding proteins (DRBPs) influence distribution of long dsRNA into RNAi and sequence-independent pathways. We used a luciferase-based reporter RNAi assay to monitor sequence-specific and sequence-independent effects of dsRNA co-expressed with selected DRBPs. Our results suggest that none of the tested DRBPs is sufficient to stimulate RNAi in somatic cells. Interestingly, the overexpression of either TARBP2 or PACT suppressed RNAi in cells expressing Dicer^{O}. Moreover, microRNA pathway, which employs the same protein factors as RNAi, is not inhibited by TARBP2 or PACT. Therefore, we propose that DRBPs overexpression impairs substrate recognition by Dicer, but not Dicer activity *per se*.

Key words: double-stranded RNA, Interferon response, RNA interference, double-stranded RNA-binding proteins

ABSTRAKT

Dlhá dvojvláknová RNA je unikátnou štruktúrou, ktorá vzniká počas vírovej replikácie, alebo počas transkripcie repetitívnych sekvencií. Cicavčie bunky si preto vyvinuli niekoľko mechanizmov ako reagovať na dvojvláknovú RNA: interferonovú odpoveď, RNA editáciu, a RNA interferenciu. RNA interferencia je evolučne konzervovanou dráhou, ktorá vedie k sekvenčne-špecifickej degradácii cieľovej mediátorovej RNA. V cicavčích bunkách je však RNA interferencia aktívna iba v myších oocytoch, ktoré exprimujú skrátenú verziu proteínu Dicer, zatiaľ čo v somatických bunkách spúšťa dvojvláknová RNA sekvenčne-nezávislú interferonovú odpoveď.

Hlavným cieľom moje diplomovej práce bolo zistiť, akým spôsobom ovplyvňujú proteíny viažuce dvojvláknovú RNA smerovanie dvojvláknovej RNA buď do dráhy RNA interferencie alebo do sekvenčne-nezávislých dráh. Na monitorovanie sekvenčne-špecifických a sekvenčne-nešpecifických efektov dvojvláknovej RNA ko-exprimovanej s proteínmi viažucimi dvojvláknovú RNA sme použili luciferázovú reportérovú esej. Naše výsledky ukazujú, že žiadny z testovaných proteínov nie je schopný stimulovať RNA interferenciu v somatických bunkách. Nadmerná expresia proteínu TRBP2 alebo proteínu PACT prekvapivo potlačuje RNA interferenciu v bunkách produkujúcich skrátenú verziu proteínu Dicer. A čo viac, mikroRNA dráha, ktorá využíva rovnaké proteínové faktory ako RNA interferencia, nie je ovplyvnená expresiou proteínu TRBP2 ani proteínu PACT. Z toho vyplýva, že expresia proteínov viažucich dvojvláknovú RNA narušuje rozpoznávanie a spracovávanie dvojvláknovej RNA pomocou Diceru, ale aktivitu samotného Diceru neovplyvňuje.

Kľúčové slová: dvojvláknová RNA, interferonová odpoveď, RNA interferencia, proteíny viažuce dvojvláknovú RNA

LIST OF ABBREVIATIONS

A	Adenosine
ADAR	Adenosine deaminase acting on RNA
Amp	Ampicillin resistance
APAF	Apoptotic protease activating factor
ATP	Adenosine triphosphate
A-to-I	Adenosine to inosine
bp	Base pair
C	Cytidine
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsRBD	Double-stranded RNA-binding domain
dsRNA	Double-stranded RNA
DRBDs	Double-stranded RNA-binding proteins
E2F1	E2F transcription factor 1
EGFP	Enhanced green fluorescent protein
elF2	Eukaryotic initiation factor 2
endo-siRNA	Endogenous small interfering RNA
FADD	Fas-associated death domain protein
FL	Firefly luciferase
G	Guanosine
I	Inosine
IFN	Interferon
isomiRs	Different-sized miRNAs
IR	Inverted repeat
JNK	Jun N-terminal protein kinase
LGP2	Laboratory of genetics and physiology 2
MDA5	Melanoma differentiation associated gene-5
miRNA	Micro RNA
Mos	V-mos Moloney murine sarcoma viral oncogene homolog
mRNA	Messenger RNA
nt	Nucleotide

OAS	IFN-inducible 2’-5’-oligoadenylate synthetase
OASL	2’-5’ oligoadenylate synthetase-like
PACT	Protein activator of interferon-induced protein kinase, Protein activator of PKR, PRKRA
piRNA	Piwi-interacting RNA
PKR	Protein kinase R, Interferon-induced, double-stranded RNA-activated protein kinase
pre-miRNA	Precursor microRNA
pre-mRNA	Precursor messenger RNA
pri-miRNA	Primary microRNA
RIG-1	Retinoic acid inducible gene I
RISC	RNA-induced silencing complex
RL	*Renilla* luciferase
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rRNA	Ribosomal RNA
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SMD	Staufen1-mediated mRNA decay
ssDNA	Single-stranded DNA
SSM	Staufen-swapping motif
ssRNA	Single-stranded RNA
STAT	Signal transducers and activators of transcription
STAU1	Double-stranded RNA-binding protein Staufen homolog 1
STAU2	Double-stranded RNA-binding protein Staufen homolog 2
TRBP2	HIV-1 TAR RNA-binding protein 2
TBD	Tubulin-binding domain
TLRs	Toll-like receptors
Tudor-SN	Tudor staphylococcal nuclease
UPF1	ATP-dependent RNA helicase up-frameshift 1
UTR	Untranslated region
2-5A	2’,5’-linked oligoadenylates

1. INTRODUCTION

Double-stranded RNA (dsRNA) is a unique structure originated mainly during viral replication or transcription of retrotransposons. dsRNA is formed by pairing of two antiparallel RNA strands and adopts right-handed A-form double helix (Rana, 2007). This structure is specifically recognized by double-stranded RNA-binding proteins (DRBPs). The binding of DRBPs to dsRNA is facilitated by evolutionary conserved motif called dsRNA binding domain (dsRBD) (St Johnston et al., 1992). First DRBPs were identified in early 1990s by St Johnston et al. (St Johnston et al., 1992), and since then, the number of identified DRBPs is still growing, which suggests that the recognition of dsRNA by DRBPs plays essential role in great variety of biological processes as diverse as regulation of gene expression, RNA silencing pathways, regulation of translation, mRNA splicing, editing, export and localization, but also host defence against virus infection and signalling (summarized in Saunders and Barber, 2003).

The following review of literature summarizes the current knowledge of mechanisms, key factors and functions of biological pathways, in which long dsRNA plays essential role.

1.1. Long dsRNA

Long dsRNA can originate from (1) exogenous sources (from the environment), as well as from (2) endogenous sources (the cell can produce dsRNA by itself).

The most common exogenous source of dsRNA naturally occurring in cells is a virus infection, because almost any virus produces dsRNA during the life cycle. ssRNA viruses often produce dsRNA as an intermediate during the replication; the genome of dsRNA viruses represents a source of dsRNA itself (summarized in Kumar and Carmichael, 1998). Moreover, in dsDNA viruses (e.g. adenoviruses) the dsRNA is often produced from bidirectional transcription of overlapping genes (reviewed in Kumar and Carmichael, 1998). However, one exception from viruses producing dsRNA during their life cycle is negative-strand RNA viruses (e.g. Sendai virus, or Newcastle disease virus), where the level of dsRNA after infection is undetectable in cells (Weber et al., 2006).

dsRNA can be also produced by cell itself by intramolecular pairing of complementary sequences or by pairing of complementary sequences on two different molecules. Such dsRNA can be generated after transcription of transposable elements and repetitive sequences, after pairing of antisense-transcribed pseudogenes with their complementary progenitors, or

by bidirectional sense and antisense transcripts, self-complementary mRNAs or inverted repeat transcripts (reviewed in Okamura and Lai, 2008).

In addition to sources of dsRNA mentioned above, long dsRNA can be introduced into cells artificially. There are several experimental techniques of introduction dsRNA into cells, for example injection of chemically synthesized dsRNA or transfection of a plasmid carrying inverted repeat (IR) of a specific gene (Stein et al., 2003).

Taken together, long dsRNA can occur in eukaryotic cells under different circumstances and the specific cellular localization or binding of specific DRBPs can affect the fate of specific dsRNA.

1.1.1. Recognition of dsRNA

dsRNA adopts right-handed A-form helix with diameter of 23 Å, one helical turn contain 11-12 bp in comparison to DNA, which typically adopts B-form helix with diameter of 20 Å and 10.5 bp per helical turn (reviewed in Rana, 2007). Structural differences between A- and B-form helix result in greater stability and tightly packaging of A-form helix of dsRNA (Rana, 2007); moreover, the space organization of major and minor grooves also differs in these two helices. Major groove of A-form helix is deeper and narrower, which makes functional group less accessible for protein interactions (Ryter and Schultz, 1998). Minor groove of A-form helix is broader and shallower, which makes it more accessible for protein recognition, compared to minor groove of B-form helix (Figure 1) (Ryter and Schultz, 1998).

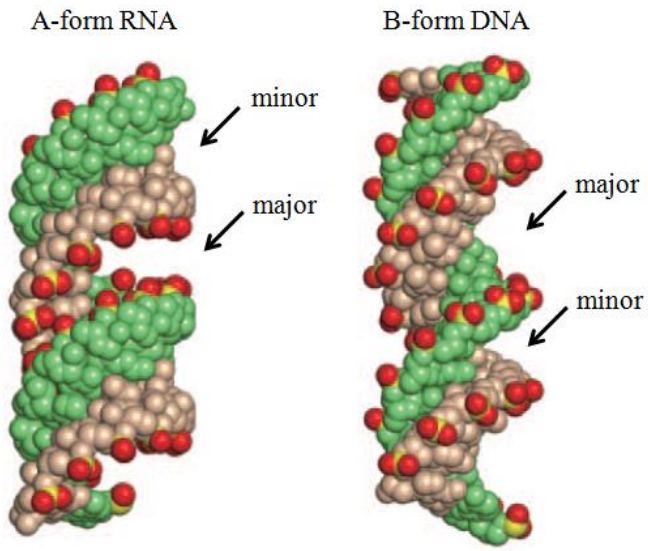

Figure 1 – Models of A- and B- form helices. Position of major and minor groove is indicated by arrows. A-form helix shows characteristic tight packaging and deep narrow major groove, which makes functional group less accessible. On the other hand, B-form helix consists of wide major groove, which makes the functional groove more accessible for protein interactions. (Adopted from Rana, 2007).

dsRNA conformation is recognized by DRBPs through dsRBD, which is approximately 65-70 amino acids long (St Johnston et al., 1992). dsRBD adopts αββββα fold, with two α helices packed along three stranded antiparallel β sheets (Kharrat et al., 1995; Bycroft et al., 1995; Nanduri et al., 1998); dsRBD interacts with a segment containing approximately 11-16 bp of dsRNA (Manche et al., 1992). This segment consists of two successive minor grooves with a major groove lying in between (Ryter and Schultz, 1998). Generally, the binding of dsRNA through dsRBD is considered as shape-dependent rather than sequence-specific (Stefl et al., 2005; Chang and Ramos, 2005); however, there is an evidence that the dsRBD recognizes not only the shape but also the sequence of dsRNA, for example in the case of selective adenosine deamination (Stefl et al., 2010).

In addition to dsRNA, dsRBD has also affinity to ssRNA with extensive secondary structures, but dsRBD does not recognize ssRNA, dsDNA or ssDNA (reviewed in Saunders and Barber, 2003).

1.2. Pathways activated by dsRNA

Long dsRNA enters three different pathways: interferon (IFN) response, RNA editing and RNA interference (RNAi) (Figure 2).

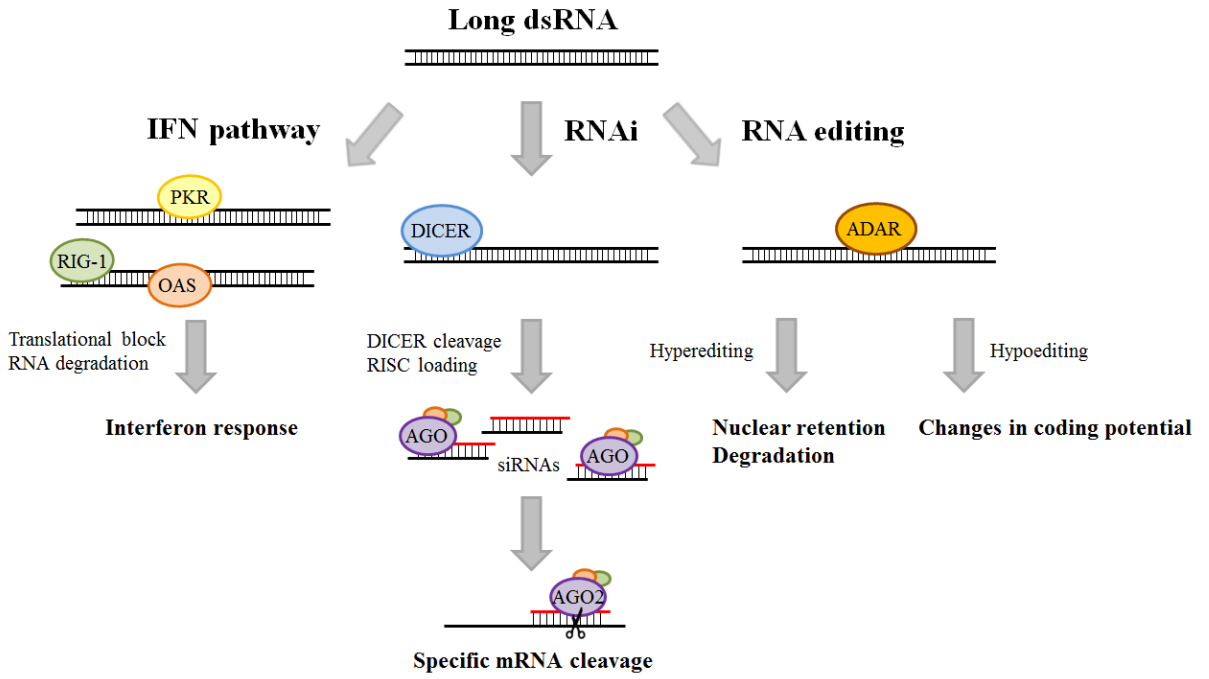

Figure 2 – Responses to dsRNA. Long dsRNA can trigger several biological pathways: IFN response, RNA interference or A-to-I RNA editing.

All these three pathways engage recognition and processing of dsRNA; however, the resulting effects varied in dependence on the organism, cell type, different DRBPs binding, or localization and length of dsRNA.

1.3. Interferon response

As mention above, dsRNA is a common by-product of viral replication or transcription (Kimura-Takeuchi et al., 1992), thus it is not surprising that cells adopted dsRNA as signal for miscellaneous threats. dsRNA is recognized by various DRBPs and subsequently activates cellular processes, whose aim is to signal infection, limit viral growth and restrain spreading of virus (Gantier and Williams, 2007). Once the dsRNA is recognized, a chain of events, involving inhibition of protein synthesis and transcriptional induction of interferon and other cytokines, can in the end leads to cell death. Thus, the response to dsRNA represents a crucial part of IFN response and can be considered as the first line of defence against viruses (reviewed in Gantier and Williams, 2007).

The well known DRBP, which plays important roles in sensing dsRNA, is protein kinase R (PKR). PKR pathway is activated upon binding to dsRNA and triggers the general inhibition of translation (Meurs et al., 1990). Another remarkable regulated RNA cleavage pathway activated upon sensing of dsRNA is 2-5A/RNase L system, which triggers the non-specific degradation of viral and cellular RNAs (summarized in Silverman, 2007; Player et al. 1998). In addition to PKR pathway and 2-5A/Rnase L system, other DRBPs, for example RIG-I and MDA5 can recognize dsRNA and trigger IFN expression (Yoneyama et al., 2004; Kato et al., 2006).

1.3.1. PKR pathway

In the 1990s, studies concerning dsRNA-induced translation inhibition revealed a novel protein kinase in cell lysates from IFN-treated cells (Hovanessian, 1991). This dsRNA-activated protein kinase, commonly known as PKR (alternative names Interferon-induced double-stranded RNA-activated protein kinase, or eukaryotic translation initiation factor 2-alpha kinase 2) is a dsRNA-activated Ser/Thr protein kinase, which inhibits mRNA translation (Clemens, 1997). PKR is expressed constitutively at low levels and the PKR expression increases upon activation of IFNs (reviewed in Garcia et al., 2006). In IFN-treated cells, PKR is predominantly localized in the cytoplasm and associated with ribosomes, however, a small fraction of PKR is found in nucleoli indicating that PKR has great variety of functions, which remains to be elucidated (reviewed in Wang and Carmichael, 2004).

PKR consists of a kinase domain at the C-terminus and two dsRBDs at the N-terminus (Meurs et al., 1990). dsRBDs are accessible for several DRBPs, which can regulate PKR activity. In nonstressed cells, PKR exists as a monomer, which is inactive as a result of sterical blockage of the kinase domain by dsRBD. The conformational change of PKR after dsRNA binding leads to homodimerization and unmasking of the kinase domain (Nanduri et al., 1998). These changes result in autophosphorylation, which further stabilizes dimerization and increases catalytic activity of PKR (Nanduri et al., 1998). Stable and activated PKR is able to phosphorylate different substrates (reviewed in Williams, 2001).

The main substrate for PKR is eIF-2α (alpha subunit of eukaryotic initiation factor 2), which consists of α, β and γ subunits. eIF-2 promotes delivery of Met-tRNAi (initiator methionine tRNA) to 40S ribosome, resulting in the initiation of the protein synthesis (summarized in Garcia et al., 2006). The activity of eIF-2 is regulated by phosphorylation of its α subunit at serin 51 (Samuel, 1993). Phosphorylation of eIF-2α results in an increased affinity to eIF-2B, leading to competitive inhibition of eIF-2B and inhibition of translation initiation (Samuel, 1993). Even a small increase in phosphorylation of eIF-2α can have dramatic effect of protein synthesis (Sudhakar et al., 2000).

In addition to eIF-2α, PKR can affect several signalling molecules including STAT (signal transducers and activators of transcription), p53, JNK (Jun N-terminal protein kinase), p38, ATF-3, interferon regulatory factors (IRF-1,3,7) and NF-κB (summarized in Williams, 2001). These factors are involved in mediating reactions to cell stress or cell damage, and can lead to inhibition of growth or cell cycle arrest.

Moreover, there is also evidence suggesting, that activation of PKR by dsRNA can lead to apoptosis (reviewed in Gil and Esteban, 2000). The first mechanism of PKR-triggered apoptosis is phosphorylation of eIF-2α, which leads to general inhibition of translation (Gil et al., 1999). Persistent absence of the proteosynthesis can lead to cell death. The second mechanism of apoptosis induced by PKR involves activation of NF-κB (Gil et al., 1999; Donze et al., 2004). Despite of the general concept that NF-κB is prosurvival factor, which prevents apoptosis, there is also evidence that under some circumstances, activation of NF-κB can lead to apoptosis (Taddeo et al., 2003). The third mechanism of PKR-induced apoptosis involves direct activation of apoptotic genes and activation of FADD/caspase 8 and APAF/caspase 9 pathways (summarized in Garcia et al., 2006).

In general, PKR can be activated by interaction with dsRNA and ssRNA with extensive secondary structures (Saunders and Barber, 2003). It was found, that fragments of dsRNA shorter than 30 bp are not able to stably bind and activate PKR (Manche et al., 1992). This length requirement can emerge from spatial organization of dsRBD, which enables dsRBD to wrap around dsRNA molecule (Nanduri et al., 1998). Thus, 80 bp and longer dsRNA are considered optimal for PKR activation. Interestingly, high concentration of dsRNA does not activate PKR. In fact, high concentration of dsRNA can result in inhibition of enzymatic activity of PKR (Manche et al., 1992). However, the minimal 30 bp requirement for activation seems not to be very reliable. It was shown that PKR can be activated also by shorter molecules, which form imperfect 16 bp stem with ~ 10-15 nt single-stranded flanking ends in the presence of 5'-triphosphate (Zheng and Bevilacqua, 2004; Nallagatla et al., 2007). Later on, it was found that also small-interfering RNAs (20-27 nt) can activate PKR *in vitro*, but its activation *in vivo* is poor (Marques et al., 2006). At the same time the role of the RNA helicase RIG-1 in recognition of siRNAs was elucidated (Marques et al., 2006). In addition to activation of PKR by interaction with dsRNA, PKR can be also activated by other stimuli, e.g. cytokines, growth factors, proinflammatory stimuli like toll-like receptors or oxidative stress (summarized in Garcia et al., 2006).

Existence of multitude regulators of PKR, with cellular and viral origin, confirms the great importance of PKR function. Positive regulators of PKR are PACT, MDA7 and E2F1. Also several inhibitors of PKR were identified: $p58^{IPK}$, TRBP, Hsp90/Hsp70 proteins, glycoprotein p67 and nucleophosmin (reviewed in Garcia et al., 2006).

Taken together, this suggests that the PKR activation is regulated mostly by availability of the substrate and the presence of binding partners.

1.3.2. 2-5A/RNase L system

Another pathway activated by dsRNA is the 2-5A/Rnase L system (Schroder et al., 1994). Two key factors in this system, OAS (2'-5'-oligoadenylate synthetase) and RNase L, are expressed in cells at low level, and similarly to PKR, their expression is induced by IFNs (Sadler and Williams, 2008). These properties enable 2-5A/RNase L system to work in detection of viral dsRNA (Sadler and Williams, 2008).

In humans, there are four genes coding 2'-5'-oligoadenylate synthetase (OAS1, OAS2, OAS3 and OASL), producing ~ 8-10 spliced protein variants (Mashimo et al., 2003); in mice, in

addition to OAS2 and OAS3, there are 7 separate OAS1 genes, however, not all of them are active (Kakuta et al., 2002).

The binding of dsRNA longer than 15 bp to OAS leads to conformational changes and subsequent activation of the enzyme (Hartmann et al., 2003). Activated OAS subsequently converts ATP (adenosine triphosphates) to pyrophosphates and 2',5'-linked oligoadenylates (2-5A) (Hartmann et al., 2003). The well-established function of 2-5A is activation of endoribonuclease RNase L (Zhou et al., 1993), which dimerizes upon binding of 2-5A. Activated RNase L catalyses the degradation of viral and cellular RNAs, including mRNAs and rRNAs; leading to the inhibition of protein synthesis (reviewed in Silverman, 2007; Player and Torrence, 1998)). Furthermore, RNase L is also involved in the induction of apoptosis (Li et al., 2004), and it is proposed that 2-5A/RNase L system plays important role also in control of cell growth and tumorigenesis (Eskildsen et al., 2003). However, the evidence that RNase L inhibits expression of PKR, probably by cleavage of PKR mRNA (Wang and Carmichael, 2004), suggests that the interplay between 2-5A/Rnase L system and PKR remains to be elucidated.

1.3.3. LTRs, RIG-1, MDA5 and others

In addition to PKR and 2-5A/RNase L systems, other factors can regulate the IFN response. Different potential of these factors to affect IFN response varies based on different conditions, e.g. dsRNA origin, length, structure or localization (summarized in Gantier and Williams, 2007).

Toll-like receptors (TLRs) are a great family of innate immune recognition cell surface receptors recognizing different microbial products, e.g. peptidoglycans, lipopolysaccharide, CpG motifs characteristic of bacterial DNA or dsRNA (reviewed in Garcia et al., 2006). Recognition of dsRNA by TLR-3 triggers signalling through transduction cascades, including JNK, p38, IRF-3 and NF-κB (Alexopoulou et al., 2001).

However, the observation that the response to dsRNA is retained also in TLR-3 null cells indicates the substitutability of TLRs in response to dsRNA by other cytoplasmic factors involved, which can be involved in IFN pathway (Diebold et al., 2003). It was shown, that RNA helicases RIG-1 (retinoic acid inducible gene I), MDA5 (melanoma differentiation associated gene-5) and LGP2 (laboratory of genetics and physiology 2) are involved in sensing viral RNAs and triggering the IFN response (summarized in Gantier and Williams, 2007). Interferon inducible RIG-1 is a cytoplasmic RNA helicase, which activates IRF-3 and

NF-κB signalling pathways (Yoneyama et al., 2004). MDA5 shares the sequence homology with RIG-1 and it was shown, that also MDA5 is able to activate IFN-β expression and it is considered as key mediator of IFN signalling induced by viruses (Kato et al., 2006). Moreover, several findings indicate that RIG-1 can differentiate between non-self dsRNA originated from virus replication and self dsRNA, for example pre-miRNAs, based on the configuration of dsRNA ends (Marques et al., 2006).

1.4. RNA editing

In addition to sequence-independent IFN response in cytoplasm, long dsRNA present in nucleus can undergo adenosine deamination mediated by adenosine deaminase acting on RNA (ADAR) enzymes.

A-to-I RNA editing converts adenosine to inosine in dsRNA; this hydrolytic deamination of the adenine base is catalyzed by ADARs (Figure 3) (summarized in Nishikura, 2010). Translation and splicing machinery recognize newly formed inosine as guanosine, moreover, inosine does not pair with uridine (initial pair A-U), but preferentially pairs with cytidine (reviewed in Wang and Carmichael, 2004). Thus, RNA editing can influence protein coding potential, as well as RNA structure.

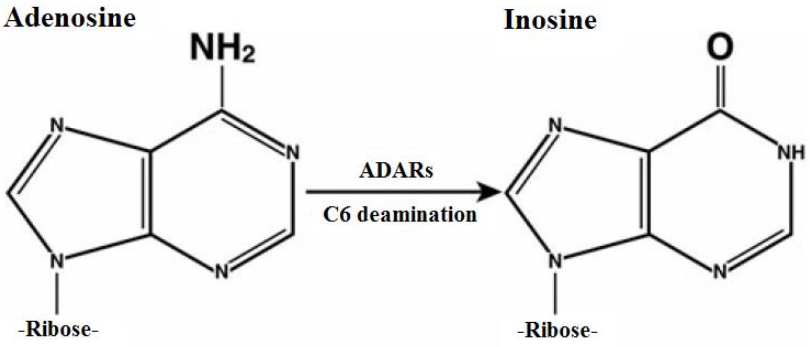

Figure 3 – RNA editing converts adenosine to inosine. Hydrolytic C6 deamination is catalyzed by ADARs. (Adopted from George et al., 2011).

1.4.1. Adenosine deaminase acting on RNA

Mammals have two active ADAR enzymes – ADAR1 and ADAR2; the third ADAR3 enzyme is enzymatically inactive. Paralogues of ADARs are present in all animals, but they are absent in protozoa, yeast and plants (Jin et al., 2009). In vertebrates, ADAR1 is expressed in two forms, a short form (ADAR1-S, p110) and a long form (ADAR1-L, p150) (Patterson and Samuel, 1995). ADAR1-S is constitutively expressed and localized predominantly to the nucleus (Patterson and Samuel, 1995), while ADAR1-L is produced from an interferon-

inducible promoter (George and Samuel, 1999) and is localized in the cytoplasm and the nucleus (Patterson and Samuel, 1995). Based on cytoplasmic localization and ability to recognize and deaminate dsRNAs, it was proposed that ADAR1-L can play role in defence against viruses replicating in the cytoplasm (Bass, 2002).

All members of ADAR family contain 1-3 dsRBD that form α-β-β-β-α conformation. These dsRBDs interact directly with dsRNA and their presence is necessary for dsRNA binding (Valente and Nishikura, 2007). Structural features of N-terminal region differ in particular ADAR family member, for example ADAR1 contains 2 Z DNA binding domains (Herbert et al., 1997), in contrast to ADAR3, which lacks Z DNA domain, but contains arginine rich R domain (Chen et al., 2000). Common feature of all ADARs is also presence of catalytic deaminase domain at their C-terminal region (Liu and Samuel, 1996).

1.4.2. RNA editing by ADARs

A-to-I RNA editing shows high sensitivity to length and structure of targeted duplex. dsRNA of at least 25-30 bp (two turns of the dsRNA helix), but preferably greater than 100 bp in length is optimal for ADAR activity (Bass and Weintraub, 1988; Nishikura et al., 1991); RNA duplexes shorter than 15 bp are edited inefficiently (Nishikura, 1992). Up to 50 % of adenosine residues are edited in a long dsRNA in an almost random manner (hyperediting) (Polson and Bass, 1994). In ~ 20-30 bp dsRNAs or long dsRNAs with imperfect basepairing (mismatched bases, loops and bulges), only few specific adenosines are edited (hypoediting), indicating importance of dsRNA secondary structure in recognition and editing by ADARs (Lehmann and Bass, 1999). However, there is also evidence that not only shape, but also the sequence of dsRNA is recognized and the binding of ADAR2 dsRBD is achieved by direct readout of RNA sequence (Stefl et al., 2010).

Hyperedited dsRNA binds a specific protein complex (p54nrb, splicing factor PSF, matrin 3) resulting in retention and possible degradation of dsRNA in the nucleus (Zhang and Carmichael, 2001). There is also an evidence showing that hyperedited dsRNA is preferentially cleaved at sites consisting IU and UI base pairs by Tudor staphylococcal nuclease (Tudor-SN) (Scadden and Smith, 2001b; Scadden, 2005), moreover the cleavage occurs on both strands of dsRNA and requires 2’ OH group (Scadden and O'Connell, 2005).

A-to-I RNA editing in protein coding sequences can lead to a codon change, elimination or creation of splicing sites, or alterations of coding sequences of selected genes, which results in diversification of protein functions (summarized in Nishikura, 2010). Moreover, A-to-I RNA

editing can occur also in noncoding sequences, such as 3' untranslated regions, introns or non-coding RNAs (Morse and Bass, 1997; Morse and Bass, 1999) and the presence of edited bases found in repetitive elements raises the possibility that ADAR editing might be engaged in regulation repetitive elements and transposons expression (Morse et al., 2002).

1.5. RNA silencing

In addition to IFN response and ADAR editing, dsRNA can trigger RNA silencing pathways. RNA silencing is a common term for related pathways that employ small RNAs and proteins from Argonaute family (Ago proteins). In general, RNA silencing includes RNA interference (RNAi), small RNA-mediated chromatin silencing and DNA rearrangements, PIWI interacting RNA pathway, and microRNA (miRNA) pathway (reviewed in Filipowicz et al., 2005). All these pathways utilize small RNAs as sequence-specific guides for regulation of gene expression. RNA silencing has generally inhibitory functions, including regulation of chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability, and translation (summarized in Carthew and Sontheimer, 2009).

1.5.1. miRNA pathway

Generally, micro RNAs (miRNAs) are a large group of factors regulating gene expression and they are involved in many cellular and developmental processes in eukaryotic organisms (Krol et al., 2010). Moreover, it is predicted, that activity of more than 50% of all protein-coding genes is regulated by miRNAs in mammals (Krol et al., 2010).

miRNAs are a large group of post-transcriptional regulators of gene expression involved in many cellular and developmental processes in eukaryotic organisms. The biogenesis of miRNAs and molecular mechanisms of miRNA pathway was summarized in number of reviews (Filipowicz et al., 2005; Filipowicz et al., 2005; Kim et al., 2009; Bartel, 2009; Carthew and Sontheimer, 2009; Jinek and Doudna, 2009; Krol et al., 2010). Briefly, miRNAs are generated from short hairpin precursors, these endogenous transcripts with ~ 65-70 nt stem-loop structures are polyadenylated and capped (Figure 4). The imperfect stem-loops present on primary miRNAs (pri-miRNAs) are recognized and excised in the nucleus by RNase III enzyme called Drosha in a complex with DGCR8. Newly-generated pre-miRNAs are subsequently translocated into the cytoplasm, where Dicer cleaves them producing ~ 22 nt miRNA-miRNA duplexes with 2-nt 3' overhangs. One of the miRNAs strands is incorporated into the RNA-induced silencing complex (RISC), which comprises of an Argonaute protein (AGO 1-4), Dicer and TRBP, and mature miRNA are formed. miRNAs are typically partially

complementary to 3' untranslated regions (3'UTRs) of targeted mRNA. The lack of complementarity between miRNA and target mRNA results in inhibition of translation of target mRNA and removal of polyA tail, which leads to mRNA degradation. In case, that miRNA is perfect complementary to mRNA, the cleavage by AGO2 occurs.

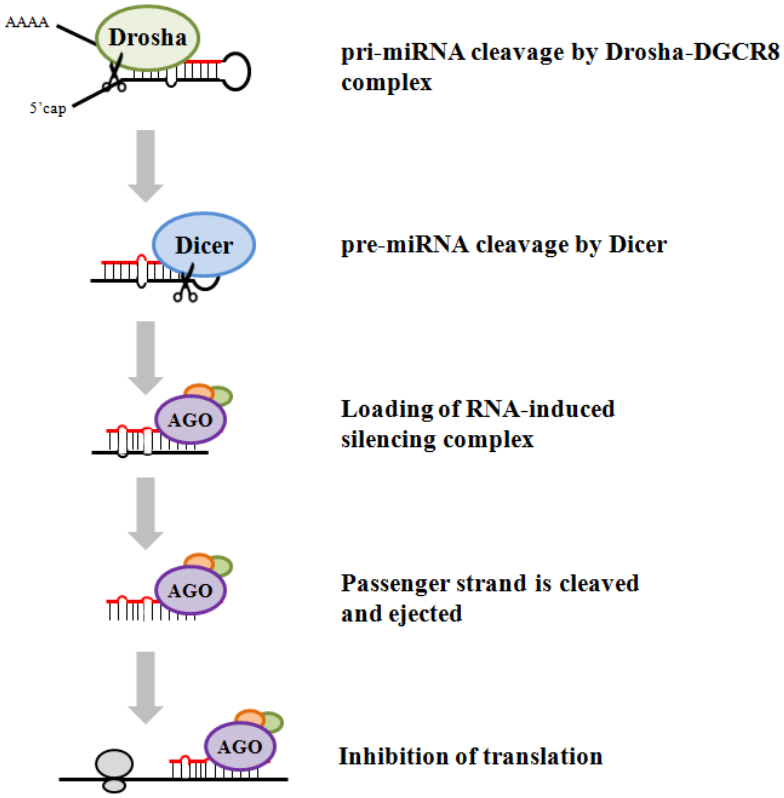

Figure 4 - microRNA pathway. Primary miRNA (pri-miRNA) is cleaved in nucleus by Drosha-DGCR8 complex. Generated pre-miRNA translocated into the cytoplasm, where is cleaved by Dicer into miRNA duplexes. One of the miRNA strands is incorporated in the RNA-induced silencing complex (RISC), where it serves as guide for miRNA-mediated silencing of partially complementary mRNAs, by inhibiting translation and removing of polyA tail.

1.5.2. RNA interference

The canonical RNAi is one of the evolutionary conserved effects of dsRNA. RNAi denotes for sequence-specific mRNA degradation induced by long dsRNA and the molecular mechanisms of canonical RNAi was summarized in number of reviews (Filipowicz et al., 2005; Macrae et al., 2006b; Carthew and Sontheimer, 2009; Jinek and Doudna, 2009; Liu and Paroo, 2010). Briefly, RNAi is initiated by the RNase III enzyme Dicer (Figure 5), which is cleaving long dsRNA at ~ 21-25 nt intervals, generating short interfering RNAs (siRNAs) with 5' phosphate group and 3' dinucleotide overhangs at their termini. After Dicer-mediated cleavage, one of the siRNA strands is incorporated into the RISC. The passenger strand is cleaved by Argonaut protein and it is removed from complex. siRNA strand loaded in RISC complex then serves as guide for cleaving perfectly complementary mRNAs by AGO2. The cleaved mRNA is released and rapidly degraded. After cleavage, the RISC is recycled and is

able to cleave another mRNA molecule. RNAi is sequence-specific, thus it causes cleavage of perfectly complementary or nearly perfect complementary target mRNAs. In presence of mismatches or bulges, the resulting effect is translation inhibition, rather than cleavage.

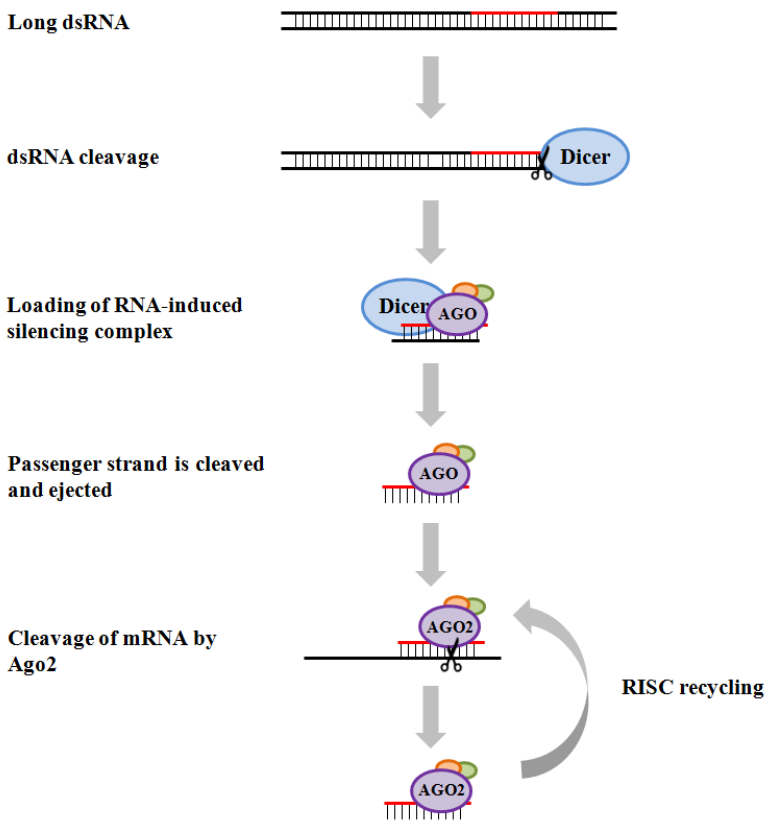

Figure 5 - Canonical RNAi pathway. RNAi is initiated by the RNase III enzyme Dicer, which is cleaving dsRNA at 22 nt intervals, generating short interfering RNA duplexes. One of the siRNA strands is incorporated into RISC, where it serves as guide for cleaving perfectly complementary mRNAs. Cleaved mRNA is released and degraded; RISC is recycled and prepared to bind another mRNA.

1.5.3. Dicer

Dicer, the key component of RNA silencing machinery, recognizes and cleaves dsRNA substrates into functional small RNAs (Bernstein et al., 2001). Dicer is a large multi-domain protein (~ 220 kDa) present in almost all eukaryotic organisms. Dicer consists of two tandem catalytic RNase III domains, dsRBD, DUF283 domain, PAZ domain and DExD helicase domain (Figure 6a) (summarized in Jinek and Doudna, 2009). The PAZ domain recognizes 5'phosphate and 2 nt 3'overhang of dsRNA substrates, while two RNase III domains form a pseudodimer, which facilitate the cleavage (Macrae et al., 2006b; Park et al., 2011). The distance between PAZ and RNase III domains determines the length of small RNAs (Macrae et al., 2006b). dsRBD of Dicer facilitates the interaction with dsRNA and it is also able to interact with other DRBPs; TRBP2 and PACT are known interacting partners of Dicer (Haase et al., 2005; Lee et al., 2006). Moreover, it was shown that dsRBD of Dicer functions as a nuclear localization signal (Doyle et al., 2013). DExD helicase domain recognizes

dsRNA termini and terminal loops of hairpin precursors (Welker et al., 2011; Ma et al., 2012) and it was shown that this domain has autoinhibitory function (Ma et al., 2008).

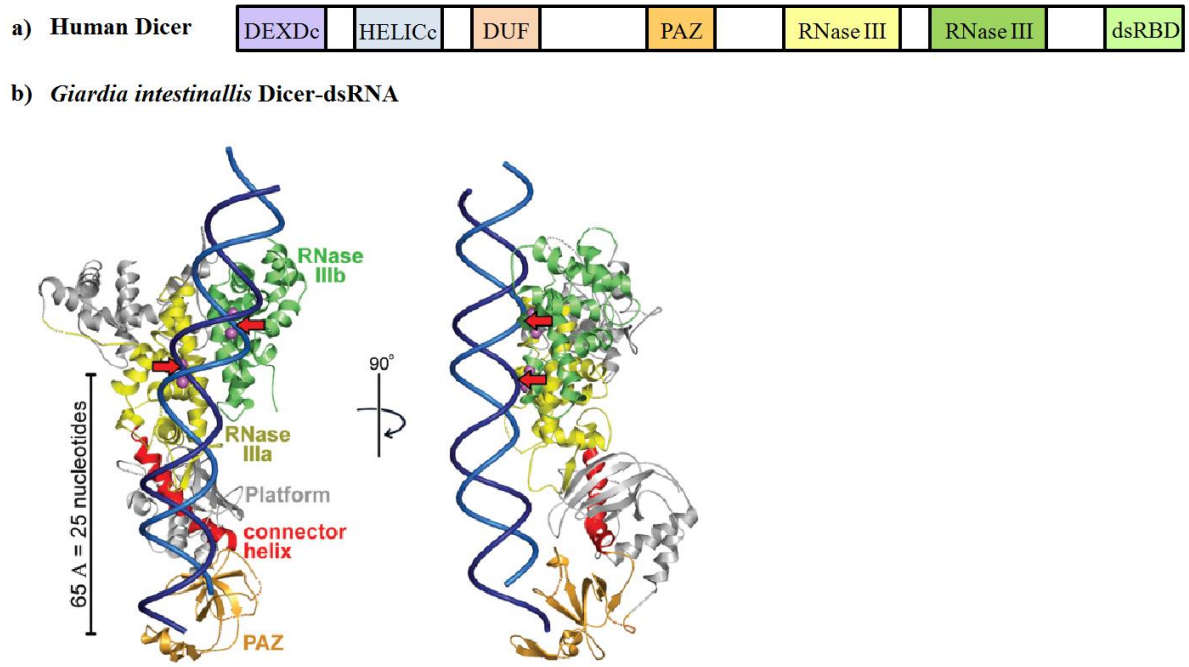

Figure 6 – Structure of Dicer protein. (a) Schematic structure of human Dicer, indicating DEXDc helicase domain, HELICc domain, DUF283, PAZ, RNase III and dsRBD. (b) Front and side views of crystal structure of *Giardia intestinalis* Dicer with modelled dsRNA. Red arrows indicate cleavage points. (Adapted from Macrae et al., 2006a).

Some organisms contain several Dicer homologues with distinct functions (reviewed in Murphy et al., 2008). Ciliates, such as *Tetrahymena thermophila*, and fungi contain a single copy of Dicer. Plants have four homologues of Dicer (DCL1-4), each with specialized function in small RNA biogenesis and related pathways (Xie et al., 2005). Nematodes, such as *Caenorhabditis elegans*, and vertebrates contain a single Dicer gene, while *Drosophila* species contain two Dicer genes, Dcr1 and Dcr2; Dicer 1 functions in the miRNA biogenesis, while Dicer 2 is required for the RNAi pathway.

As mentioned above, one single mammalian gene encoding Dicer is involved in biogenesis of siRNA and miRNA (Murphy et al., 2008). But interesting situation appears in mice; in somatic cell, the processing of dsRNA into siRNA is inefficient, but high amounts of endo-siRNAs are present in oocytes (Nejepinska et al., 2012). A recent study reveals that divergence of small RNA pathways in somatic cells and oocytes arises from presence of oocyte-specific isoforms ($Dicer^{O}$) (Figure 7a), which lack the autoinhibitory DExH/D domain and shows high efficiency of processing dsRNA into siRNAs in mouse oocytes (Flemr et al., 2013). This oocyte-specific isoform resulted from insertion of retrotransposon,

which occur relatively recently in the *Muridae* family (Flemr et al., 2013). Dicer^{O} is dominant isoforms of Dicer in mouse oocytes; protein level of full-length Dicer (Dicer^{S}) (Figure 7b) in oocytes is minimal. It was also confirmed that expression of Dicer^{O} isoforms is essential for proper oocyte function (Flemr et al., 2013).

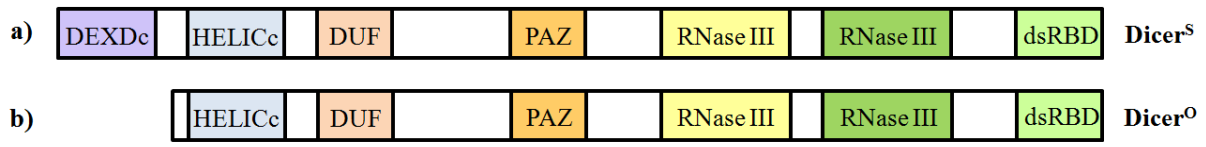

Figure 7 – Schematic structure of Dicer protein. (a) Full-length Dicer – Dicer^{S}. (b) N-terminally truncated Dicer^{O}.

1.6. Roles of RNAi

The canonical RNAi was firstly described in C.elegans in 1998 (Fire et al., 1998), since then RNAi effects were observed in wide range of animal taxa, including mammals. The molecular mechanism, key factors and physiological functions were studied using biochemical and genetic approaches. The RNAi is often considered as form of innate immunity, because dsRNA can originate during virus replication or transcription of repetitive elements. However, the evidence of antiviral function of RNAi in mammals was not proven, even though the experimentally induced RNAi is able to inhibit replication of viruses (van Rij and Andino, 2006).

Moreover, studies on RNAi mutants suggested that besides antiviral role and role in suppression of mobile elements, RNAi plays also roles in regulation endogenous genes and development (reviewed in Filipowicz et al., 2005). It is likely, that RNAi in animals originated from an ancient innate immunity response, but in mammals, the defensive role of RNAi was probably replaced by more sophisticated form of immune system and the original role of RNAi in suppression of mobile element was retained only in the female germline (summarized in Cullen, 2006). Moreover, mouse oocytes adopted RNAi for regulation of endogenous genes (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000).

1.7. Interplay between different pathways

Three different pathways, IFN response, ADAR editing and RNAi, recognize dsRNA and mediate different cellular responses. Targeting of particular dsRNA into different pathways is influence by presence of specific DRBPs, but also the type and structure of dsRNA, length, subcellular localization, origin of dsRNA can affect response to dsRNA. Moreover, response

to dsRNA depends on tissue or organisms, in which dsRNA is present. For example, in mammals, dsRNA found in cytoplasm can trigger IFN response or RNAi; on the other hand, ADAR editing occurs prevalently in nucleus (reviewed in Wang and Carmichael, 2004). Moreover, ADAR editing is absent in protozoa, yeast and plants (Jin et al., 2009), while IFN response originated in early vertebrates (Schultz et al., 2004).

1.7.1. RNAi and IFN response

Several mechanistical connections between IFN response and RNAi were found. The first connection is presence of two DRBPs – TRBP2 and PACT in these two pathways. It was shown that both these proteins are able to bind Dicer and PKR, but they have different functions (reviewed in Saunders and Barber, 2003). TRBP2 is known subunit of mammalian RISC complex (Haase et al., 2005). On the other hand, PACT is activator of PKR, while TRBP2 cause PKR inhibition (Benkirane et al., 1997; Patel and Sen, 1998).

The second connection is the fact, that helicases involved in RNAi in *C.elegans* are significant homologous to helicases acting in IFN response in mammals (RIG-1, MDA5, LPG2, mentioned in section 1.2.1.3) (summarized in Gantier and Williams, 2007). These observations indicate that IFN response, which evolved later than RNAi, adopted several components from RNAi pathway.

1.7.2. RNAi and ADAR editing

It is possible that effects of ADAR editing can antagonize effects of RNAi, because these two pathways may compete for the same dsRNA substrate (Nishikura, 2006). Extensive studies confirmed that extensive edited dsRNA became resistant to cleaving by Dicer (Scadden and Smith, 2001a), thus the increasing level of ADAR editing can reduce production of small interfering RNAs (summarized in Nishikura, 2006). On the contrary, hypoedited dsRNA can be processed *in vitro* by Dicer (Zamore et al., 2000; Scadden and Smith, 2001a). In this case, the resulting siRNA can contain up to one I:U pair, which can reduce the ability of siRNA recognize mRNA and thus reduce the efficiency of RNAi (reviewed in Nishikura, 2010).

Moreover, extensive ADAR editing can lead to degradation of targeted dsRNA, what results in reducing of expression of endo-siRNAs. Tudor-SN (Tudor staphylococcal nuclease), subunit of RNA-induced silencing complex, was identified as potential I-dsRNA specific ribonuclease (Scadden, 2005). Thus, the extensive ADAR editing can result in degradation of dsRNA by Tudor-SN, which causes inhibition of endo-siRNA expression (summarized in Nishikura, 2010).

In addition, in mammalian cells, a possibility emerges that ADAR1-L is able to sequester siRNAs. *In vitro* studies show that cytoplasmic ADAR1-L binds processed siRNA from dsRNA very tightly and without editing; what suggest that ADAR1-L inhibits siRNA function, by decreasing concentration of effective siRNAs (Yang et al., 2005). Interestingly, observation of increased expression of ADAR1 in mice injected with high siRNAs amount indicates that ADAR1 is important part of a cellular feedback in response to siRNA (Hong et al., 2005).

The importance of balance between RNAi and ADAR editing, is enhanced by facts that analyses of *C. elegans* strains lacking ADAR reveal connection between ADARs and regulation of transgenes expression (Knight and Bass, 2002). In *C. elegans*, it seems that ADAR editing of inverted repeats of transgenes prevents the silencing of the transgenes through RNAi (Knight and Bass, 2002). Moreover, transgene and viral silencing is very effective in plants and fungi, while these organisms lacks ADARs and ADAR editing system (Meister and Tuschl, 2004); therefore, the ADAR editing may evolved to regulate RNAi in animals (reviewed in Nishikura, 2010).

1.7.3. IFN response and ADAR editing

It was shown that interferons are able to induce expression of ADAR1-L (Patterson and Samuel, 1995) and these observations leads to question, if the ADAR editing is involved also in the host defence. There are several examples allocating connection between ADAR editing and IFN response and in all of them, the result is based on virus-host combination.

One of the examples is inhibition of replication of the hepatitis C virus by editing its RNA genome (Taylor et al., 2005). Another one is regulation of NF90 factors (nuclear factor 90), which are known to activate interferon-β, thus the suppression of NF90 by interacting with ADAR1 protects hematopoietic progenitor cells from apoptosis (Nie et al., 2005). Moreover, it was shown that ADAR1 is able to antagonize IFN response, by inhibiting PKR activation (Nie et al., 2007). Suppression of IFN response and apoptosis is documented in several viruses, for example human immunodeficiency virus or meales virus (Samuel, 2011). Thus, ADARs have both, proviral or antiviral role, but the particular response is dependent on a specific cell type, a particular virus, ADAR1 expression or other factors (reviewed in Samuel, 2012).

1.8. double-stranded RNA-binding proteins

DRBPs are a large family of proteins, which share evolutionary conserved dsRBD facilitating interaction with dsRNA (see section 1.1.1.) (Figure 8). These proteins can be divided into two groups: (1) DRBPs with enzymatic activity (e.g. PKR, Dicer or ADARs) and (2) proteins without enzymatic activity (e.g. TRBP2 or Staufen). DRBPs are involved in many different biological processes, including dsRNA signalling and host defence, RNA interference, mRNA elongation, editing, mRNA stability and splicing, transport, localization and regulation of translation (reviewed in Saunders and Barber, 2003).

In this thesis I choose four different DRBPs for further analyses in context of RNAi. Structures and main functions of these proteins are mention below.

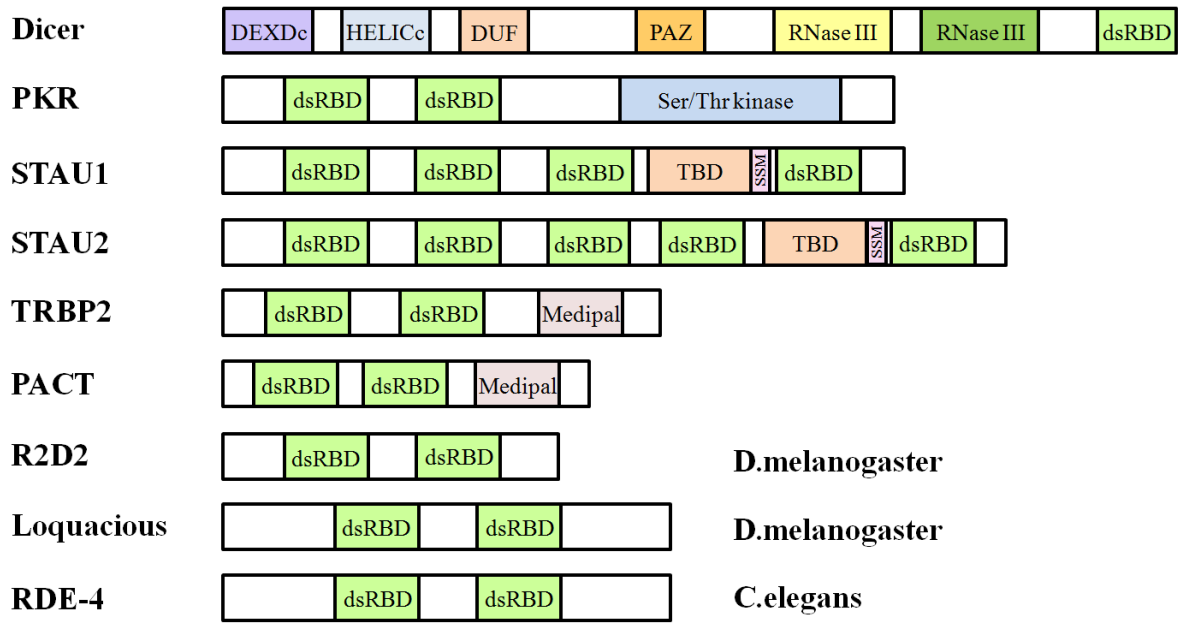

Figure 8 – Diagrams of double-stranded RNA-binding proteins. dsRBD – double-stranded RNA-binding domain, TBD – tubulin binding domain, SSM – Staufen-swapping motif.

1.8.1. STAU 1 and STAU 2

Mammalian cells produce two Staufen paralogs, STAU1 and STAU1, from two separate genes. Both, *Stau1* and *Stau2* genes produce several protein isoforms via alternative splicing. STAU1 and STAU2 consist of multiple dsRBD; dsRBD3 and dsRBD4 facilitate the interaction with dsRNA (reviewed in Saunders and Barber, 2003). Identity between STAU1 and STAU2 is ~50% (Furic et al., 2008), dsRBD3 and dsRBD4 are 78% and 81% identical, respectively (Wickham et al., 1999). In addition to dsRBD both Staufens contain tubulin-binding domain (TBD); and STAU2 contains additional dsRBD1 and only partial dsRBD5

compared to STAU1 (Allison et al., 2004). STAU1 and STAU2 can be found in cytoplasm in association with rough endoplasmic reticulum and are involved in several biological processes, including microtubule-dependent transport of RNAs to dendrites of polarized neurons and formation and maintenance of the dendritic spines of hippocampal neurons (reviewed in Park et al., 2013). Moreover, STAU1 and STAU2 function together in pathway called STAU1-mediated mRNA decay (SMD) (Park and Maquat, 2013).

1.8.2. TRBP2

TRBP2, TAR RNA binding protein or RISC-loading complex subunit TARBP2, was originally identified as cellular protein, which interact with HIV-1 trans-activating region and enhance HIV-1 translation and replication (Gatignol et al., 1991). Mouse homologue of TRBP2, PRBP (Prm-1 binding protein) is 93% identical with human TRBP2. TRBP2 consists of three dsRBD, with no other identifiable domains. TRBP2 is present in nucleus; in cytoplasm TRBP2 is associated with ribosomes and endoplasmatic reticulum. In addition to stimulation of HIV-1 expression, TRBP2 is involved in several other cellular processes (reviewed in Daniels and Gatignol, 2012). TRBP2 functions in IFN response, when it binds to PKR and inhibits PKR activation (Benkirane et al., 1997). TRBP2 also modulate the stress response by interacting with PACT (protein activator of PKR) (Daher et al., 2009). Moreover, TRBP2 associates with RNAi machinery and participate in production of small RNAs. TRBP2 in complex with Dicer facilitates the production of siRNAs (Kok et al., 2007) and ensures accurate biogenesis of miRNAs (Wilson et al., 2015).

1.8.3. PACT

PACT, protein activator of PKR, and its murine homologue (also known as RAX) are involved in modulation of PKR activation and IFN response. PACT is able to induce autophosphorylation of PKR leading to its activation (Peters et al., 2009). Moreover, like TRBP2, PACT interacts with Dicer and is integral part of RISC (Lee et al., 2006). Thus, both PACT and TRBP2 are important regulatory factors, which contribute to substrate and cleavage specificity during production of small RNAs (Lee et al., 2006). However, recent studies indicates, that PACT and TRBP2 have distinct effects on small RNA processing (Lee et al., 2013). In particular, PACT-Dicer complex inhibits the processing of siRNA, while TRBP2 in complex with Dicer stimulates both the miRNA and siRNA processing (Lee et al., 2013).

1.9. dsRNA expressing system

To express long dsRNA in cell cultures, I used dsRNA expressing system established in previous studies (Nejepinska et al., 2012). pCagEGFP MosIR (referred as MosIR) was produced by inserting fragment carrying *Mos* inverted repeat (IR) into the SspBI site downstream of the EGFP (enhanced green florescent protein) coding sequence in the pCagEGFP plasmid; promoter is composed of the CMV enhancer and β-actin core promoter (CAG) (Figure 9a) (Svoboda et al., 2001; Nejepinska et al., 2012). Resulting dsRNA forms long hairpin with perfect stem (520 bp) flanked by long single stranded overhangs (Figure 9b).

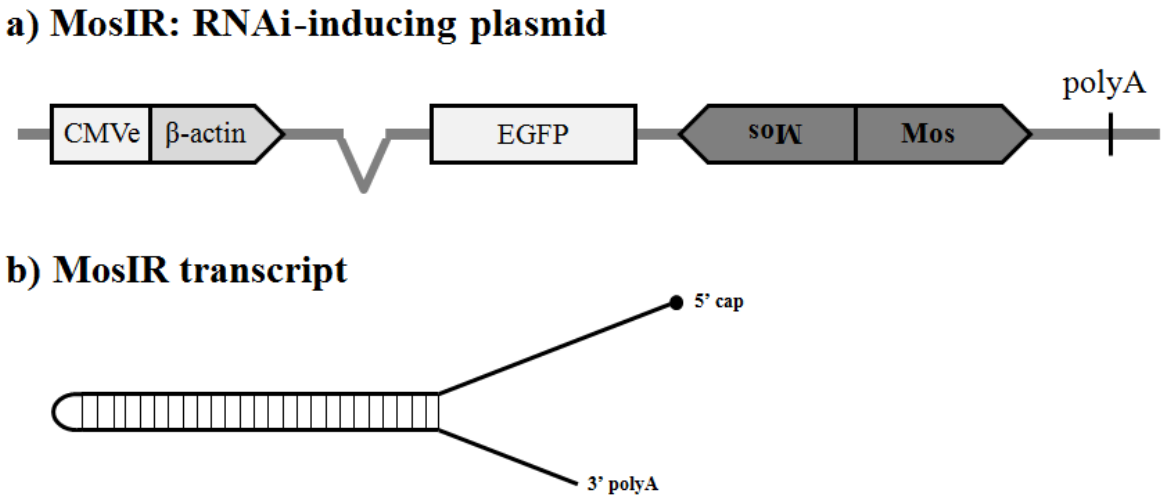

Figure 9 – dsRNA expressing vectors. (a) pCagEGFP MosIR – RNAi inducing plasmid. (b) MosIR transcript structure, long hairpin with perfect stem is flanked by single-stranded overhangs. (Adapted from Nejepinska et al., 2012).

There are several advantages of using this approach (reviewed in Nejepinska et al., 2012). (1) *Mos* is a dormant maternal mRNA stored in oocytes and *Mos* mRNA level in somatic cells is negligible. Moreover, the loss of *Mos* in somatic cells has no phenotype. (2) Expressed RNA efficiently folds into dsRNA upon transcription in vitro and induces specific RNAi effect in oocytes. (3) The amount of green fluorescence generated from EGFP coding sequence upstream of *Mos* hairpin is sufficient for monitoring expression of plasmids.

2. AIMS OF THE THESIS

The main objective of this Master's thesis was to elucidate how specific DRBPs influence entry of dsRNA into different pathways.

I addressed the following specific aims:

- binding of ectopically expressed dsRNA by candidate DRBPs
- effects of candidate DRBPs on RNAi

3. METHODS

3.1. Cell culture

Mouse embryonic stem cells (ESCs) expressing $Dicer^{O}$ and $Dicer^{S}$ were cultured on 0.1 % gelatin-coated plastic in Dulbecco's modified Eagle's medium (Sigma) containing 10 % fetal calf serum (FCS, Sigma), 1x NEAA 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential AA, penicillin/streptomycin, 50 µM 2-mercaptoethanol, leukemia inhibitory factor (LIF), 3 µM CHIR99021, 1 µM PD0325901 at 37 °C in 5 % CO_2. For transfection, cells were plated on a 24-well plate at density 100,000 cells/well and transfected 24 hours later using Lipofectamine 2000 (Thermo Scientific) or Lipofectamine 3000 (Thermo Scientific) according to the manufacturer's instructions.

Mouse NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) with 10 % FCS, penicillin and streptomycin at 37 °C in 5 % CO_2. For transfection, cells were plated on a 24-well plate at density 30,000 cells/well and transfected 24 hours later using TurboFect transfection reagent (Thermo Scientific) or polyethylenimin (PEI).

3.2. Preparing cell lysates

Cells were collected into 2 ml of PBS, 48 hours after transfection. Samples were centrifuged at maximal speed for 1 min and supernatants were removed. Cells were lysed with 200 µl of lysis buffer, passed through a needle and stored at ice for 10-15 min. Samples were centrifuged (15 min, 4 °C, 12,000g) and supernatants were used for immunoprecipitation or western blotting.

Lysis buffer:

20 mM	HEPES (pH 7.8)
100 mM	NaCl
1 mM	EDTA (pH 8.0)
0.5 %	IGEPAL-25 %
1 mM	fresh DTT
0.5 mM	PMSF
1 mM	NaF
0.2 mM	Na_3VO_4
2x	protease inhibitor cocktail set (Millipore)

2x	phosphatase inhibitor cocktail set (Millipore)
	RiboLock RNase inhibitor (Thermo Scientific)

3.3. Protein immunoprecipitation

Supernatants were diluted with binding buffer to 0.1 % total concentration of IGEPAL-25. HA-probe antibody (F-7, Santa Cruz Biotech., sc-7392 AC) conjugated with agarose beads were used for the protein immunoprecipitation. Beads was added to each sample and incubated on rotator overnight at 4°C. Beads were washed with 1000 µl of binding buffer 5 times (centrifugation at 4 °C, 4000g, and 4 min). The last wash was placed into a new tube. Immunoprecipitates were used for western blot and real-time PCR.

Binding buffer:

20 mM	HEPES (pH 7.8)
100 mM	NaCl
1 mM	EDTA (pH 8.0)
1 mM	fresh DTT
0.5 mM	PMSF
1 mM	NaF
0.2 mM	Na_3VO_4
2x	protease inhibitor cocktail set (Millipore)
2x	phosphatase inhibitor cocktail set (Millipore)
	RiboLock RNase inhibitor (Thermo Scientific)

3.4. Western blot

Lysates were mixed with 5x loading buffer (0.2 M Tris, 20 % glycerol, 10 mM β-mercaptoethanol, 1 % w/v Bromphenol Blue, 10 % w/v SDS), eluates were mixed with 5x loading buffer without SDS, because SDS was already present in elution buffer. Samples were incubated 5-10 min at 95 °C, centrifuged, loaded and resolved on SDS-PAGE (1012 % separating gel, 5 % stacking gel) using 100-150 V in 1x SDS running buffer (0.25 mM Tris, 2 M Glycine, 1 % SDS, pH ~ 8.8). Polyvinylidine fluoride membranes were soaked in 100% methanol for 30 sec and equilibrated in 1x semi-dry transfer buffer (12.5 mM Tris, 96 mM Glycine). The blotting apparatus was assembled (2x thick filter papers, 2x thin filter papers, membrane, gel, 2x thin filter papers, and 2x thick filter papers) and samples were transferred using power supply for 50 min at 35 V. The blotting apparatus was de-assembled

after blotting and membranes were soaked in 100 % for 30 sec, air dried and cut as needed. Membranes were then soaked again in methanol and equilibrate in 1xTTBS buffer (150 mM NaCl, 10 mM Tris, 0.05 % Tween 20, pH ~ 7.5). Membranes were blocked in blocking buffer (5% non-fat dry milk in 1xTTBS buffer) for 1 hour in room temperature. Membranes were stained overnight at 4°C with primary antibodies diluted in blocking buffer on a rocking platform. Membranes were washed 3x for 5 min with blocking buffer and incubated with secondary antibody diluted in blocking buffer for 1 hour at room temperature and subsequently washed 3x for 5 min in 1xTTBS buffer. Membranes were developed using Super signal West Femto Maximum Sensitivity substrate (Thermo Scientific) or Super signal West Pico Chemiluminiscent Substrate (Thermo Scientific) for 1 min, briefly dried, placed against film and exposed for 30 sec to 15 min. Specific modifications of each experiment are described on relevant places.

Primary antibodies:

STAU1 antibody (Millipore, AB 5781, rabbit, 1:1500)
STAU2 antibody (Pierce, PA5-30721, rabbit, 1:2000)
TRBP2 antibody (Santa Cruz Biotech., sc-292550, rabbit, 1:1000)
PACT antibody (Santa Cruz Biotech., sc-18768, goat, 1:1000)
anti-HA high affinity (Roche, 11867423001, clone 3F10, rat, 1:2500)

Secondary antibodies:

goat anti-rabbit IgG (H+L), HRP conjugate (Pierce, 31460, 1:50,000)
goat anti-rat IgG (H+L), HRP conjugate (Pierce, 31470, 1:50,000)
rabbit anti-goat IgG (H+L), HRP conjugate (Thermo Scientific, A16142, 1:10,000)

3.5. Real-time PCR

Total RNA from samples was isolated using RNAzol according to the manufacturer's instructions. Expression of specific mRNAs was analyzed by real-time PCR. RNA was reverse transcribed using reverse transcriptase (RevertAid Premium Reverse Transcriptase, Fermentas) and random hexanucleotides (Fermentas). Reverse transcriptase was omitted in control (-RT) samples. Resulted cDNA was diluted 2:1 with water and 2.4 µl was used as a template for 8 µl real-time PCR reaction. Real-time PCR was performed on LC480 (Roche) machines using 4 µl of 2x Maxima SYBR Green qPCR Master Mix (Fermentas) and 1.6 µl of primer mix (forward and reverse primer, 4 µM each).

3.6. Luciferase-based reporter assay

3.6.1. RNAi monitoring system

For monitoring RNAi activity, I used a well-established luciferase-based reporter assay (Figure 10) (Ma et al., 2010). Briefly, plasmid carrying *Mos* inverted repeat is transcribed, newly-formed MosIR dsRNA is recognized by Dicer and is cleaved into siRNAs. The following step after siRNA production is selection and loading of one of its strand onto the RISC. The selected strand subsequently serves as sequence-specific guide for recognition of mRNA that will be degraded. In the RNAi monitoring system, the *Renilla* luciferase (RL) reporter contains the *Mos* sequence, which is targeted by siRNAs originated from MosIR dsRNA. Targeting of siRNAs to the *Mos* sequence present in RL reporter leads to specific cleavage of RL reporter mRNA (Figure 11a) and RNAi activity is measured as decrease of RL reporter activity. As a control reporter we used Firefly luciferase (FL) reporter (Figure 11b), which do not contain any target site for siRNAs originated from MosIR dsRNA.

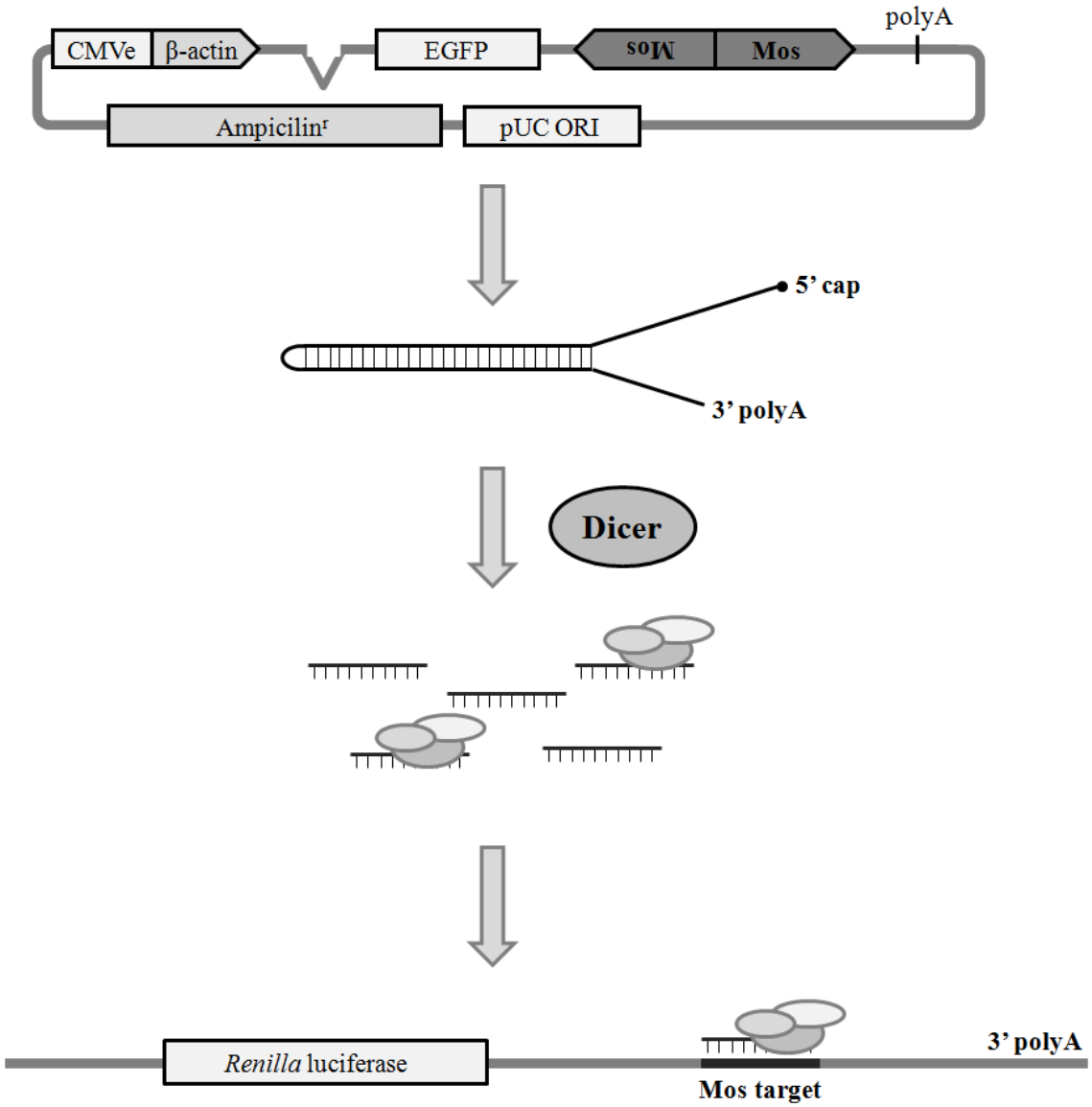

Figure 10 – Luciferase-based system for monitoring RNAi efficiency. MosIR plasmid is transcribed; mRNA forms long dsRNA stem and Dicer cleaves this dsRNA into siRNAs. One of the siRNAs strand is loaded onto RISC and serves as sequence specific guide for the degradation of targeted mRNA. siRNAs originated from MosIR dsRNA specifically bind the *Mos* sequence present in *Renilla* luciferase reporter. The targeting of siRNAs to the Mos sequence result in the specific degradation of RL reporter mRNA and the decrease of RL reporter activity.

Plasmids expressing dsRNA are shown in Figure 12. pCagEGFP MosIR plasmid expresses *Mos* inverted repeats forming dsRNA; as a control plasmid we used pCagEGFP Mos-Mos plasmid carrying *Mos* fragment, with the same sequence as MosIR, but into head to tail orientation, which expression results in a single stranded RNA. Elavl2IR plasmid expresses different dsRNA, which is used as control for specific RL down-regulation, because only in case of MosIR dsRNA, newly-formed siRNAs are complementary to *Mos* target sequence present in RL and cause its degradation.

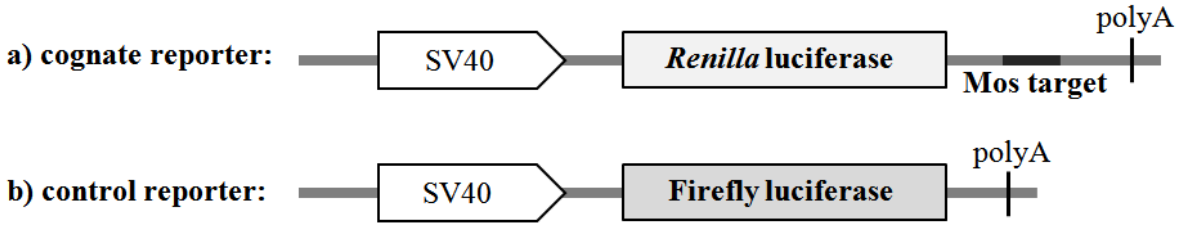

Figure 11 – Schematic composition of functional parts of Mos reporters. a) Renilla luciferase reporter contains Mos target, which is targeted by siRNAs originated from MosIR plasmid. b) Firefly luciferase reporter serves as control reporter for normalization.

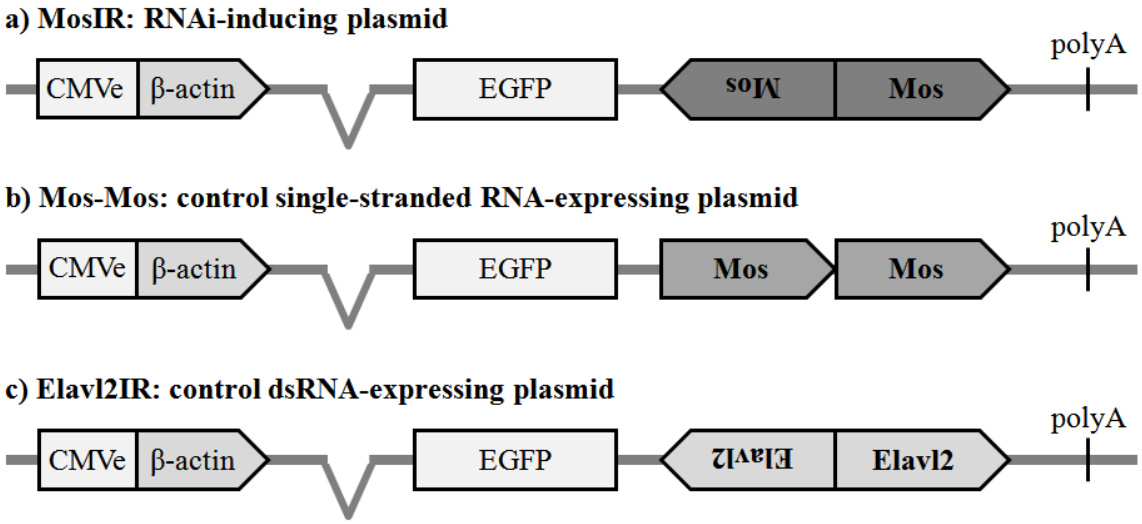

Figure 12 – Schematic composition of functional parts of plasmids producing double-stranded or single-stranded RNA in luciferase assay. a) MosIR: RNAi-inducing plasmid. b) Mos-Mos: control single-stranded RNA-expressing plasmid. c) Elavl2 IR: control dsRNA-expressing plasmid.

3.6.2. miRNA monitoring system

A similar strategy was used for monitoring the miRNA pathway. Three different reporters for monitoring miR-30 (Figure 13) were used (Ma et al., 2010): 1x perfect miR-30 (1xP miR-30) contains one perfect target site for miR-30 and mimics siRNA-like degradation of targeted reporter; 4x bulged miR-30 (4xB miR-30) contains four partially complementary sites for miR-30; the targeting miR-30 to these sites results in the translational inhibition of the targeted reporter and subsequent mRNA degradation (presence of multiple sites increase the efficiency of inhibition after targeting of miR-30); the last reporter 4x mutated miR-30 (4xM miR-30) serves as control reporter, because all four sites are mutated and no miR-30 are able to bind these sites and cause repression.

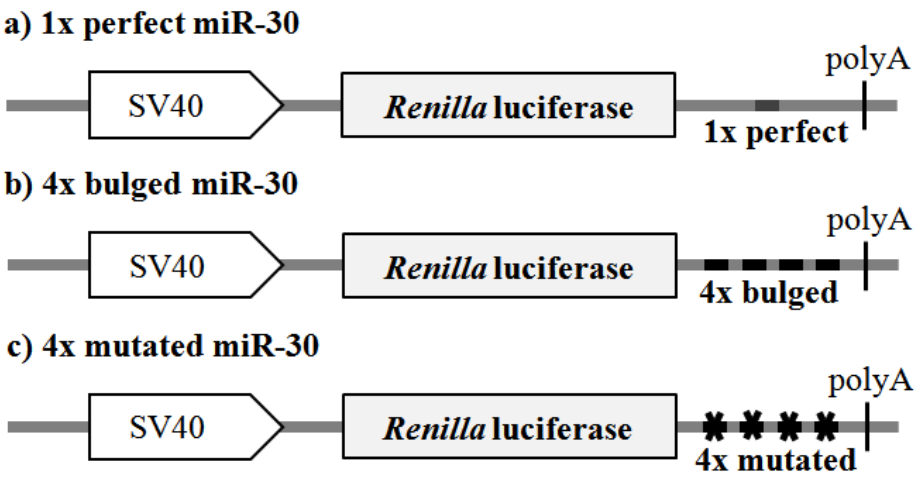

Figure 13 – Schematic composition of functional parts of reporters used for monitoring endogenous miRNA pathways. a) 1x perfect miR-30 reporter carries one perfect miR-30 target and serves for mimicking RNAi. b) 4x bulged miR-30 reporter carries 4 bulged sites and targeting miR-30 cause inhibition of translation of RL reporter. c) 4x mutated miR-30 reporter carries 4 mutated sites and serves as control reporter.

3.6.3. Experimental procedure

Transfected cells were washed in PBS and lysed in 200 µl of 1x PPBT lysis buffer (0.2 % v/v Triton x-100, 100 mM potassium phosphate buffer, pH 7.8). The lysates were cleared by a short centrifugation, and the luciferase activity was measured with Dual-Luciferase Reporter Assay system (Promega) on Modulus Microplate Reader (Turner Biosystems) luminometer. The obtained luminescence was corrected using the total protein amount in sample lysate measured by Bradford Protein Assay (Bio-Rad). Specific modifications of each experiment are described on corresponding places.

4. RESULTS

Main aims of this project were (1) to analyze binding of ectopically expressed dsRNA by candidate DRBPs and (2) to test their effects on RNAi.

4.1. Expression on DRBPs

For the DRBP overexpression, I used expression plasmids carrying an SV40 early enhancer/promoter, an HA-tag fused with a coding sequence of a particular DRBP cloned from mouse oocytes, an SV40 3'UTR polyA signal and a coding sequence of ampicillin resistance (Figure 14).

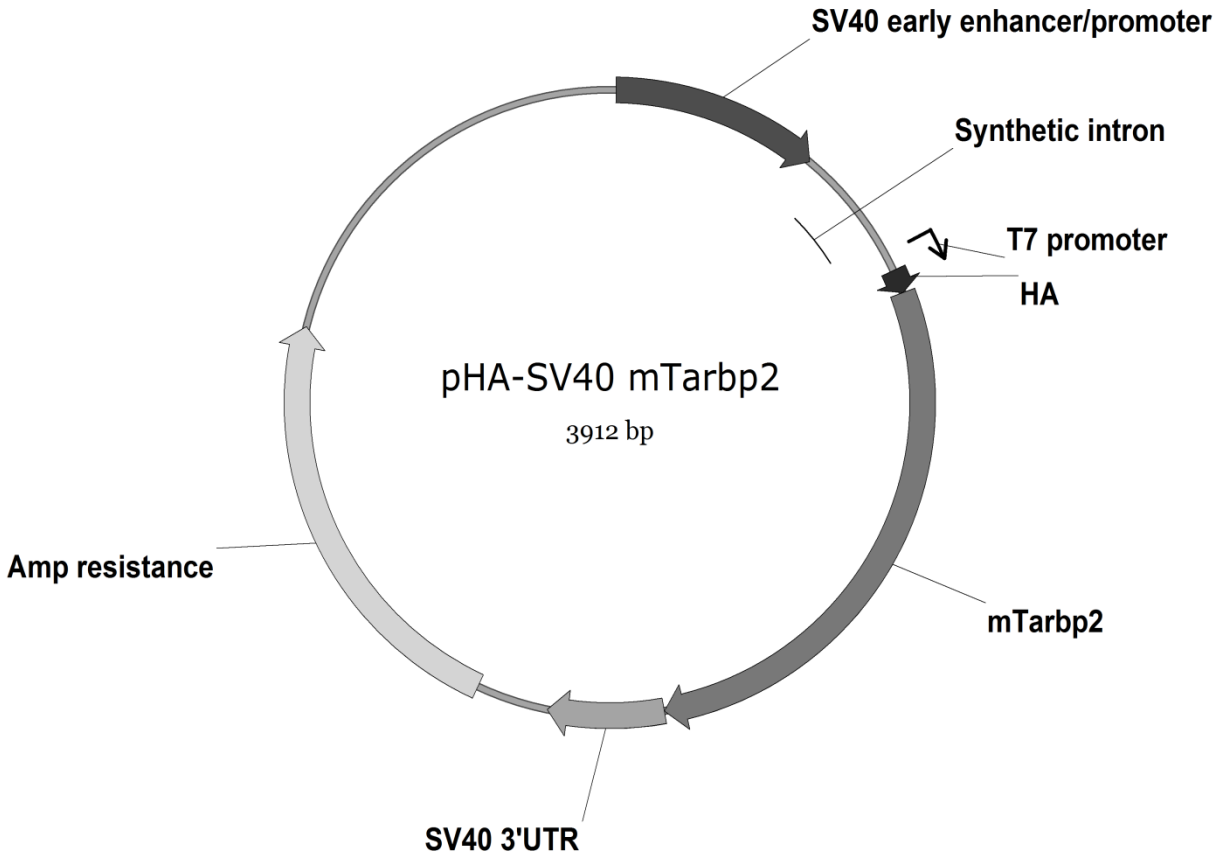

Figure 14 – Schematic representation of a DRBP (TRBP2) expression plasmid. The plasmid carries SV40 early enhancer/promoter, synthetic intron, T7 promoter; HA-tag fused with the DRBP coding sequence, SV40 3'UTR polyA signal and ampicillin resistance. Plasmids were produced and validated by sequencing.

Next, I verified that candidate proteins were expressed in transfected cells using specific antibodies or an antibody against the HA-tag. I transfected NIH 3T3 cells with constructs carrying DRBPs; untransfected cells were used as a control. Cells were collected and lysed 48 hours after transfection. 30 µg of protein from lysates were resolved on a polyacrylamide gel, which was then used for blotting.

In the first round of western blot staining, I used specific primary antibodies against DRBPs (Figure 15 a-d). Results indicated that the STAU1-specific primary antibody recognized the endogenous STAU1 (Figure 15a, Control) as well as endogenous STAU1 in cells transfected with STAU1 or in cells transfected with STAU2; however, the STAU1-specific primary antibody did not recognize overexpressed STAU1 in transfected cells. Moreover, STAU1-specific antibody did not recognized only STAU1, but it recognized other protein with a lower molecular weight of ~ 50 kDa, and also a different protein with higher molecular weight of ~ 100 kDa. The specific antibody against STAU2 did not specifically recognize the endogenous STAU2 protein, as no band of expected size was present in untransfected cells (Figure 15b, Control). Moreover, the STAU2-specific antibody did not recognize the overexpressed STAU2 (Figure 15b, second lane), while it apparently interacted with overexpressed STAU1 (Figure 15b, first lane). Taken together, these specific primary antibodies did not appear suitable for immunoprecipitation.

The TRBP2-specific primary antibody recognized the endogenous TRBP2 in untransfected cells (Figure 15c, Control) and we also observed strong signal in transfected cells corresponding to overexpressed TRBP2. In contrast, the specific antibody against PACT was not functional, as it did not recognize any specific protein (Figure 15d).

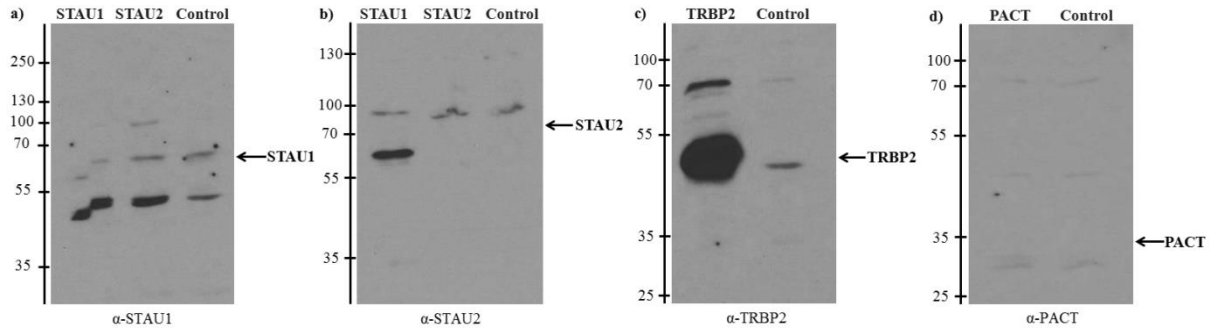

Figure 15 – Detection of DRBPs using specific antibodies. NIH 3T3 cells were transfected with 2 µg of construct carrying DRBPs. Cells were collected 48 hrs after transfection. 30 µg of protein from lysates were loaded on polyacrylamide gel and western blot was performed. a) STAU1 antibody (1:1500). b) STAU2 antibody (1:2000). c) TRBP2 antibody (1:1000). d) PACT antibody (1:1000). Arrows indicate expected size of DRBPs (kDa). Control = untransfected cells.

Taken together, since specific antibodies did not seem to be suitable for immunoprecipitation experiments and only TRBP2 specifically recognized endogenous TRBP2 as well as overexpressed TRBP2; I decided to take advantage of the N-terminal HA-tag and we detected overexpressed proteins using anti-HA high affinity antibody. In this case, much better results were obtained compared to results obtained with specific primary antibodies against DRBPs.

Using anti-HA high affinity antibody I specifically detected STAU1, TRBP2 and PACT (Figure 16 a-c).

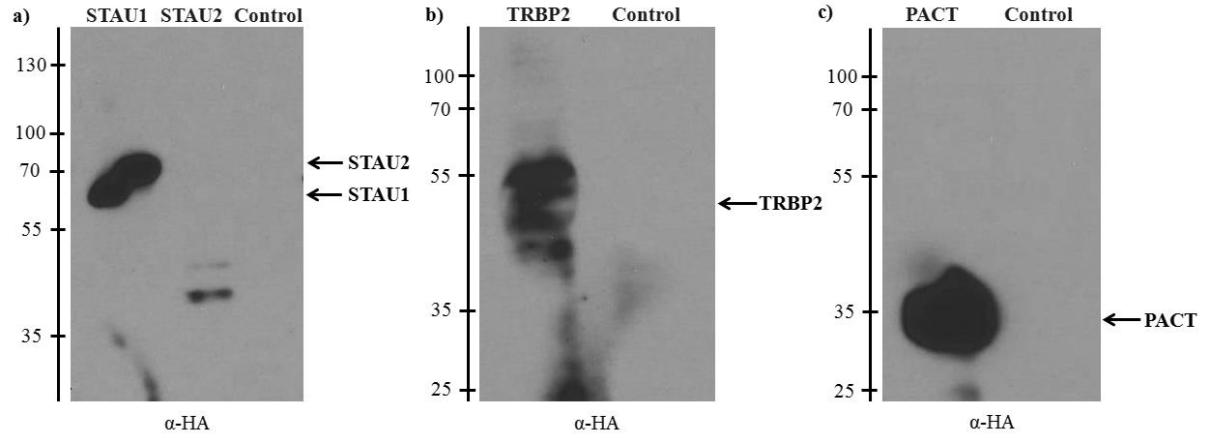

Figure 16 – Detection of DRBPs using HA-antibody. NIH 3T3 cells were transfected with 2 µg of construct carrying DRBPs. Cells were collected 48 hrs post-transfection. 30 µg of protein from lysates were loaded on polyacrylamide gel and western blot was performed. Anti-HA-high affinity antibody was used for detection of overexpressed DRBPs. Arrows indicate expected size of DRBPs (kDa). Control = untransfected cells.

Surprisingly, no detectable amount of STAU2 with expected size was present in cell lysates, while a weak band of ~ 45 kDa was detected; indicating that the full-length STAU2 was not present, but a truncated form of STAU2 protein was detected. To test whether this result was caused by expression plasmid, I validated the sequence of STAU2 by sequencing. I searched for point mutations or premature stop codons in the coding sequence of STAU2, which could cause a generation of truncated form of STAU2. However none of such alteration was present compared to reference sequence suggesting that this might be a post-transcriptional effect.

To rule out the effect of the expression plasmid backbone, I re-cloned STAU2 into different backbones. The EGFP Flag and the pCi neo backbones were used for the expression of STAU2 (Figure 17). The expected molecular weight of STAU2 was ~ 70 kDa from pSV40 and pCi neo plasmids; in case of EGFP Flag plasmid, STAU2 fused to EGFP should have ~ 100 kDa.

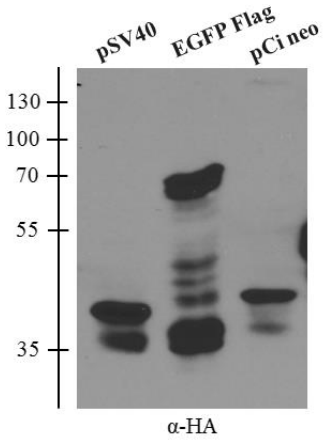

Figure 17 – Detection of STAU2 using HA-antibody in different backbones. NIH 3T3 cells were transfected with 2 µg of construct carrying STAU2. Three different backbones were used: pSV40, EGFP Flag and pCi neo. 48hrs after transfection we collected and lysed cells. 30 µg of protein from lysates were loaded on polyacrylamide gel and western blot was performed. Anti-HA high affinity antibody was used for detection of overexpressed STAU2. In all three cases, detected STAU2 has smaller size than predicted size, indicating truncation of STAU2 in cells.

However, the full-length form of STAU2 was not detected in any case. Only truncated versions were expressed from the three different backbones, indicating that the different backbones did not have any effect on the truncation of STAU2 protein.

Taken together, western blotting revealed that specific antibodies against DRBPs were not suitable for the protein immunoprecipitation, in contrast to western blotting using primary antibody against HA-tag. Therefore, I decided to take advantage of HA-tag present at N-terminus of DRBPs. Candidate DRBPs were expressed from plasmids containing SV40 early promoter in all following experiments.

4.2. Protein immunoprecipitation using HA-tag

To test the protein immunoprecipitation efficiency, I transfected NIH 3T3 cells with DRBP-expressing constructs and I performed immunoprecipitation using the HA-probe antibody conjugated with agarose. Approximately 500 µg of total protein was used for immunoprecipitation; 45 µg of total protein from the initial lysate was stored as input. Immunoprecipitated DRBPs were eluted from HA-probe antibody by elution buffer (125 mM Tris-HCl, 4 % w/v SDS, pH 6.8) and subjected to SDS PAGE electrophoresis followed by western blotting. The presence of DRBPs in eluates was analyzed by anti-HA high affinity antibody (Figure 18, eluates). Inputs (12.5 % of the initial amount used for immunoprecipitation) served as a positive control for the presence of DRBPs in transfected cells (Figure 18, inputs).

I readily detected HA-tagged DRBPs in eluates in case of all candidate DRBPs; there was a clear enrichment of STAU1 and truncated STAU2 protein in eluates compared to inputs indicating that immunoprecipitation was efficient. The efficiency of immunoprecipitation of TRBP2 and PACT was lower as shown by comparison of input with respective eluates.

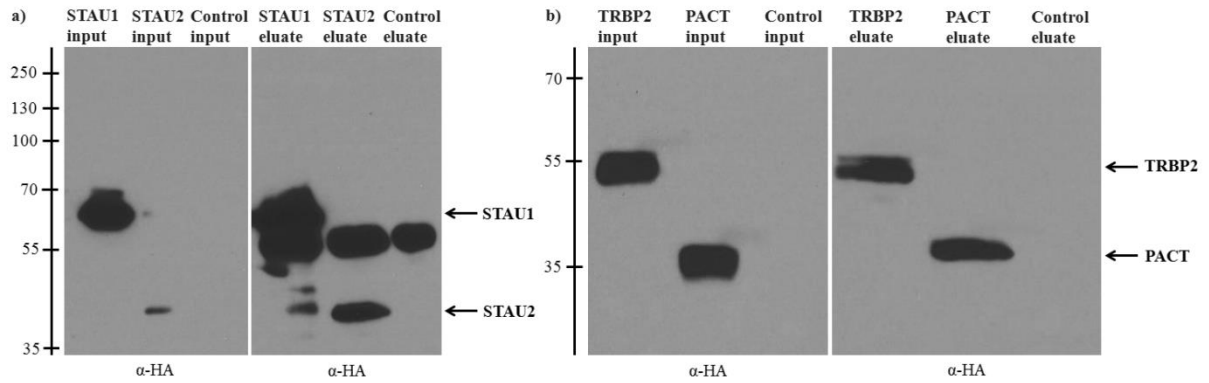

Figure 18 – Detection of immunoprecipitated DRBPs through HA-tag. NIH 3T3 cells were collected 48 hours after transfection. DRBPs were immunoprecipitated by the HA-probe antibody conjugated with agarose beads; the whole volume of eluates was resolved on a polyacrylamide gel. Inputs (40 µg of total protein concentration, 12.5 % of initial sample used for immunoprecipitation) served as positive control of the presence of HA-tagged DRBPs in transfected cells. Western blot was performed with anti-HA high affinity primary antibody. Arrows indicate expected size of DRBPs (kDa). Detected bands with molecular weight ~ 55 kDa probably originated from unspecific recognition of the heavy chain of the HA-probe antibody. Control = untransfected cells.

Results of the immunoprecipitation and western blot implied that the HA-probe antibody conjugated with agarose specifically recognized HA-tagged DRBPs and thus immunoprecipitation using HA-probe antibody was used in following experiments.

4.3. Binding of DRBPs to MosIR

Candidate DRBPs contain three to five dsRBD, which mediate interaction with dsRNA (Stefl et al., 2010), but do they bind ectopically expressed MosIR dsRNA? To test binding of MosIR by candidate DRBPs I co-transfected cells with DRBP-expressing plasmids and with MosIR or Mos-Mos plasmids. MosIR plasmid expresses dsRNA and Mos-Mos plasmid is control ssRNA expressing plasmid. DRBPs should bind to MosIR transcript, which forms dsRNA, and not to single-stranded Mos-Mos RNA transcript. The complex of DRBP with bound RNAs was immunoprecipitated using HA-probe antibody. The total RNA was isolated from samples and reverse transcribed with random hexamers as primers for reverse transcription. Subsequently, I performed the real-time PCR with primers specific to MosIR and Mos-Mos to amplify RNAs bound to DRBPs. As a control, I used plasmids carrying different HA-tagged proteins, which should not specifically interact with dsRNA (EFGP, RFP and LacZ).

During the real-time PCR, a DNA binding dye binds to newly amplified double-stranded DNA, which cause the fluorescence of the dye and the positive reaction is detected as accumulation of fluorescence signal. The number of cycles required for fluorescence signal to cross the fluorescence background is represented by the Ct value (cycle threshold). Ct values

are inversely proportional to the amount of DNA; the lower Ct value indicates the higher amount of DNA present in sample. Thus I analyzed Ct values of eluates by the normalization to corresponding Ct values of inputs, which eliminates the difference in the transfection efficiency between individual samples, and to Mos-Mos, which allows us to eliminate the presence of the MosIR unspecific binding.

The results after normalization should reveal if DRBPs interact with MosIR or not. The relative fold enrichment is shown in Figure 19.

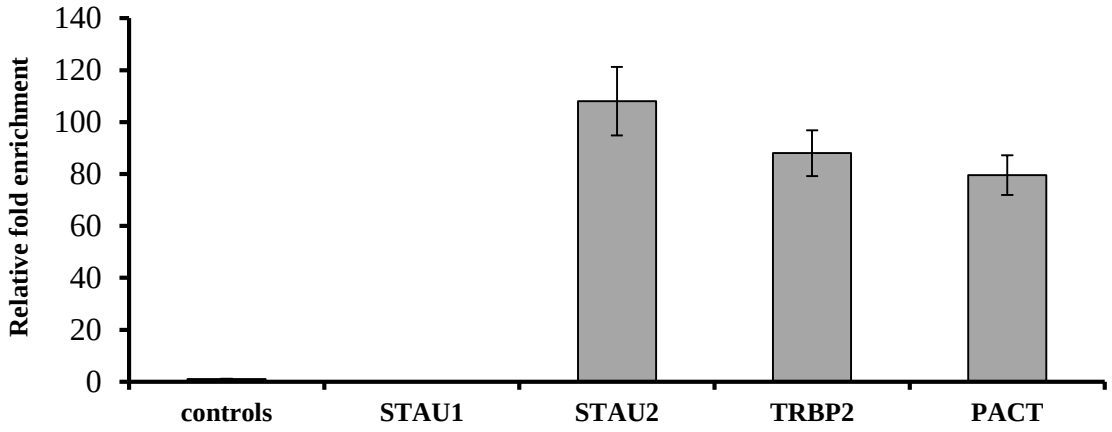

Figure 19 – Binding of ectopically expressed MosIR to DRBPs. The Ct values of eluates were normalized to corresponding Ct values of inputs; furthermore MosIR samples were normalized to Mos-Mos samples. Relative fold enrichment shows that STAU2, TRBP2 and PACT bind to MosIR, but STAU1 does not. Graph shows results from two independent measurements. Controls = average relative fold enrichment in cells expressing EGFP, RFP and LacZ. Controls are set to one.

Increased relative fold enrichment in cells expressing HA-tagged TRBP2 protein and HA-tagged PACT protein indicated that DRBPs were bound to MosIR dsRNA in comparison to mean of relative fold enrichment in cells expressing control proteins. The relative fold enrichment of STAU1 expressing cells was similar to relative fold enrichment in cells expressing control proteins, suggesting that STAU1 protein did not bind MosIR dsRNA.

Surprisingly, the increased relative enrichment was observed in cells expressing STAU2, which indicated that the truncated STAU2 protein bound MosIR dsRNA. Thus results from immunoprecipitation and real-time PCR suggest that the truncated STAU2 protein still contains a functional dsRBD and has an ability of binding to MosIR dsRNA.

4.4. Effects of DRBPs on RNAi

Despite problematic STAU2 expression and STAU1 binding to MosIR dsRNA, I decided to examine effects of all DRBPs on RNAi using a luciferase-based RNAi assay (section 3.6.).

To test effects of DRBPs on RNAi, I co-transfected cells with (i) a set of plasmids expressing dsRNA or ssRNA, which serve as RNAi inducers, (ii) a set of reporters, and (iii) a DRBP (Figure 20).

NIH 3T3 mouse fibroblasts were transfected with two plasmids, which served as RNAi sensor. *Renilla* luciferase (RL) reporter mRNA contains specific target sequence for siRNAs and the targeting of siRNAs causes down-regulation of RL reporter activity. Firefly luciferase (FL) reporter mRNA does not contain target sequence and serves for the elimination of effects, which can affect the activity of both luciferase reporters non-specifically. Together with luciferase reporter plasmids, cells were co-transfected with one of three different RNAi inducers. MosIR plasmid carriers an inverted repeat (IR) of *Mos* sequence, and its expression results in the formation of dsRNA. MosIR transcript can be subsequently cleaved by Dicer into siRNAs. siRNAs originating from MosIR would then repress a RL reporter carrying complementary sequence to the *Mos* sequence. The specific targeting by siRNAs thus results in reduced RL reporter activity, while the FL reporter activity remains unaffected, since FL reporter has no complementary sequence to *Mos* sequence. To test the sequence specific effects of the suppression of RL reporter I transfected cells with Elavl2IR plasmid instead of the MosIR plasmid. The Elavl2IR transcript forms dsRNA and yields siRNAs, but they are not complementary to the *Mos* target, thus the RL reporter activity is not affected. In addition to dsRNA expressing plasmids, I also used a control Mos-Mos plasmid, which has the same size and sequence MosIR plasmid, but *Mos* sequences are in a tandem head-to-tail arrangement. The head-to-tail orientation of *Mos* sequences yields single-stranded RNA, which is not processed into siRNAs. Therefore, Mos-Mos plasmid is a good negative control for estimating the RNAi activity.

Together with dsRNA or ssRNA expressing plasmids and set of RL and FL reporters, cells were co-transfected with a plasmid expressing DRBP. To distinguish specific effects of each DRBP on RNAi, I also co-transfected cells with plasmids expressing proteins (control proteins), which do not specifically bind dsRNA. I used LacZ, EGFP and RFP, because of their availability, different molecular weights and absence of effects on RNA silencing (Nejepinska et al., 2014).

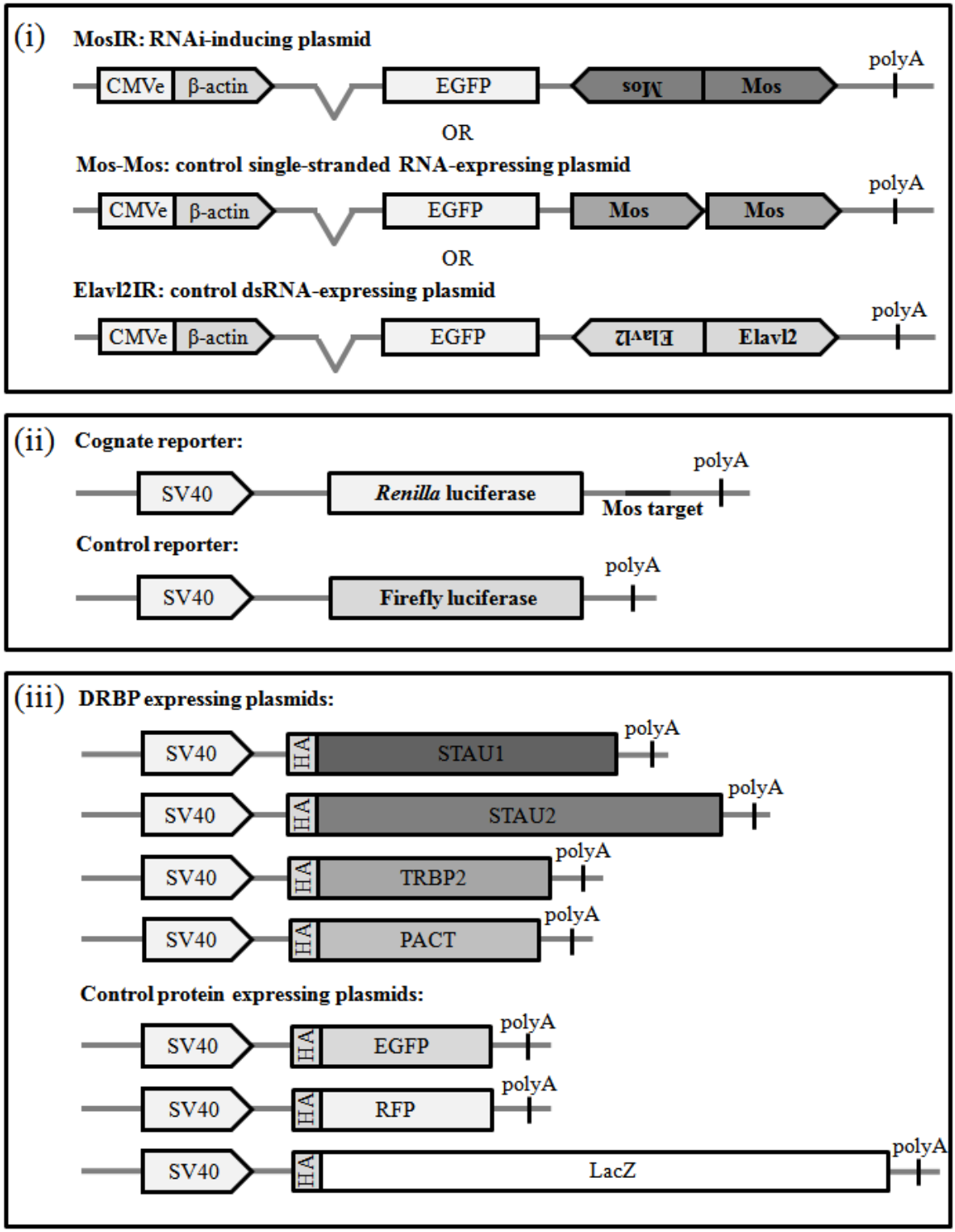

Figure 20 – Scheme of the luciferase-based RNAi assay, which can reveal the specific effect of each DRBP. Boxes represent relevant parts of plasmids producing double-strand s or single-strand RNA (i), the set of reporters (ii) and candidate DRBPs (iii).Overexpression of DRBPs is not sufficient to induce RNAi

Initial experiments were performed in NIH 3T3 cells mouse fibroblasts. Measured values of RL reporter activity were normalized to values of FL reporter activity; this eliminates unspecific effects, which influences both luciferase reporters, such as differences in

transfection efficiency. The ratio of RL reporter activity and FL reporter activity (relative RL activity) in Mos-Mos expressing cells was set to one.

The basic activity of RNAi in 3T3 cells would be manifested as lower relative RL activity in cells, which expressed control proteins and MosIR, compared to relative RL activity in cells, which expressed control proteins and Mos-Mos (Figure 21, control proteins). However, normalized data showed that relative RL activity in cells expressing MosIR and control proteins was not lower compared to relative luciferase activity in cells expressing Mos-Mos and control proteins. In fact, it appeared slightly higher. This suggests that intrinsic RNAi activity in NIH 3T3 cells is minimal if any.

The lack of detectable RNAi activity in NIH 3T3 cells, however, was not a critical issue for the original goal, which was whether the overexpression of DRBPs would stimulate RNAi in somatic cells. When I overexpressed STAU2 and TRBP2, the RNAi activity was comparable to activity of RNAi observed in cells expressing control proteins, suggesting that DRBPs do not stimulate RNAi in NIH 3T3 cells. In case of the overexpression STAU1, a minor decrease in the relative RL activity was observed in cells expressing STAU1 and MosIR relative to cells expressing control proteins (Figure 21). This reduction was statistically significant ($p = 0.027$). The difference between relative RL activity in cells expressing MosIR and PACT and the mean of relative RL activity of cells expressing MosIR and control proteins was also statistically significant ($p = 0.001$) suggesting a possible role PACT in RNAi. However, similar decreases of relative RL activity were observed in cells expressing Elavl2. This suggested that this decrease might be sequence-independent and thus not caused by RNAi.

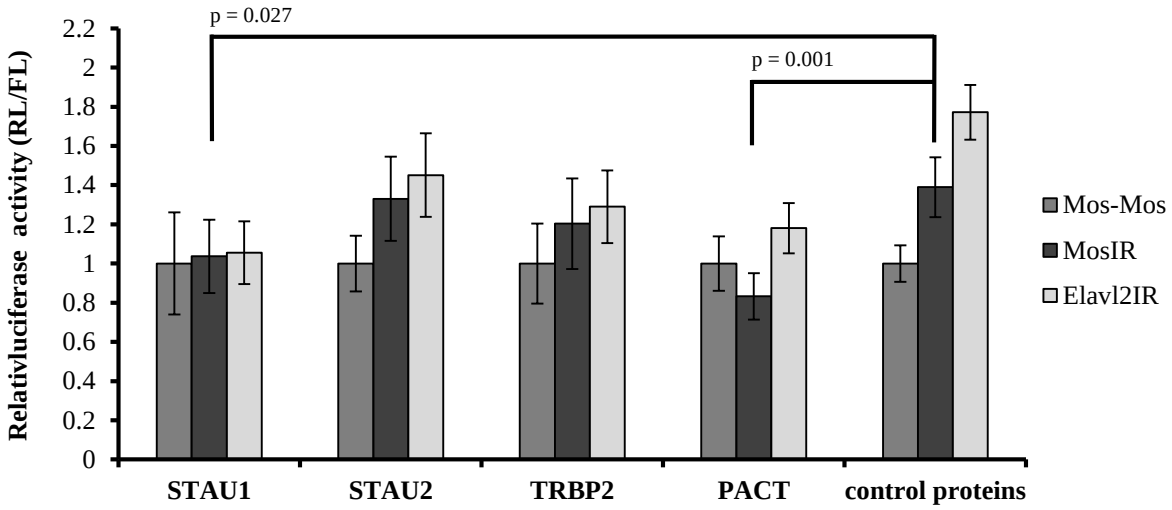

Figure 21 – Relative luciferase activity (RL/FL) in NIH 3T3 cells. NIH 3T3 cells were grown in a 24-well plate and co-transfected with 50 ng of each luciferase reporter; 250 ng of either MosIR or Mos-Mos or Elavl2IR; and 250 ng of candidate DRBPs or control proteins. The luciferase assay was performed 48 h post-transfection. RL reporter activity was normalized to FL reporter activity and the relative RL activity in cells expressing Mos-Mos was set to one. Results are shown as means +/- SD of four independent experiments performed in triplicates. Statistical significance was evaluated according to unpaired two-tailed t-test.

Off note is that the relative RL activity was higher in cells expressing Elavl2IR and controls compared to cells expressing MosIR or Mos-Mos (Figure 21); while the cause of this effect is unclear, this difference is not important for the main goal of this experiment, which was to test effects of DRBPs on RL activity in cells expressing MosIR.

Taken together, these results suggested that overexpression of DRBPs is not sufficient to improve naturally inefficient RNAi.

4.4.1. dsRNA-mediated suppression is inhibited by DRBPs

When evaluating luciferase results, it is important to examine raw data, which can provide additional information that is lost after normalization. This was especially revealing in the case examining experiment in NIH 3T3 cells.

When examining raw values of RL reporter activity, I noticed in controls that RL reporter activity in cells expressing MosIR or Elavl2IR was down-regulated by ~ 50 % relative to cells expressing Mos-Mos (Figure 22, control proteins). This effect seemed to be the same as dsRNA effect, which was earlier described by Nejepinska et al (Nejepinska et al., 2014), it was shown that dsRNA expressing plasmids inhibit co-transfected reporters in a sequence independent manner and the inhibition is also independent on co-transfected reporter type (Nejepinska et al., 2014). Consistent with this, a similar effect was observed in controls on

the non-targeted FL reporter (Figure 23). FL reporter activity in cells expressing MosIR and Elavl2IR was also down-regulated by ~ 50 % compared to cells expressing Mos-Mos; implying that this effect is sequence-independent and dsRNA-mediated (Figure 23).

dsRNA-mediated suppression of co-transfected reporters was also observed in cells expressing PKR. The RL reporter activity as well as FL reporter activity in cells expressing MosIR and Elavl2IR was down-regulated by ~ 50 % compared to cells expressing Mos-Mos. Moreover, the down-regulation of both, the RL and FL reporter activities were increased in cells expressing Mos-Mos and PKR, this presumably reflects generally reduced plasmid expression in the presence of high levels of PKR.

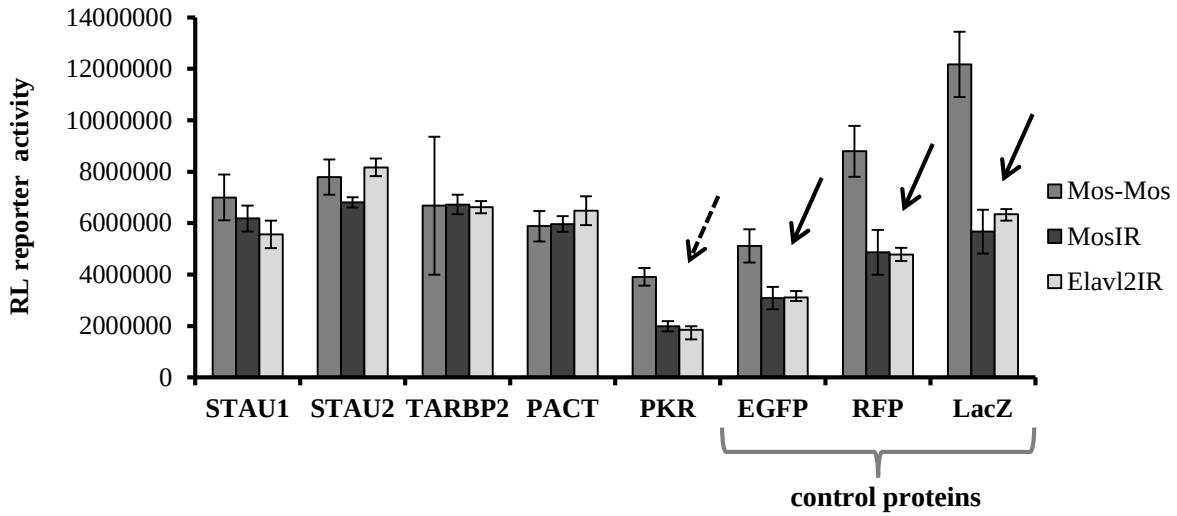

Figure 22 – *Renilla* luciferase reporter activity in 3T3 NIH cells. EGFP, RFP and LacZ were used as negative controls for DRBPs. Black arrows indicate dsRNA-dependent non-specific effect, which is inhibited in cells expressing DRBPs. Dashed arrow indicates the general reduction of co-transfected reporters expression in the presence of high levels of PKR.

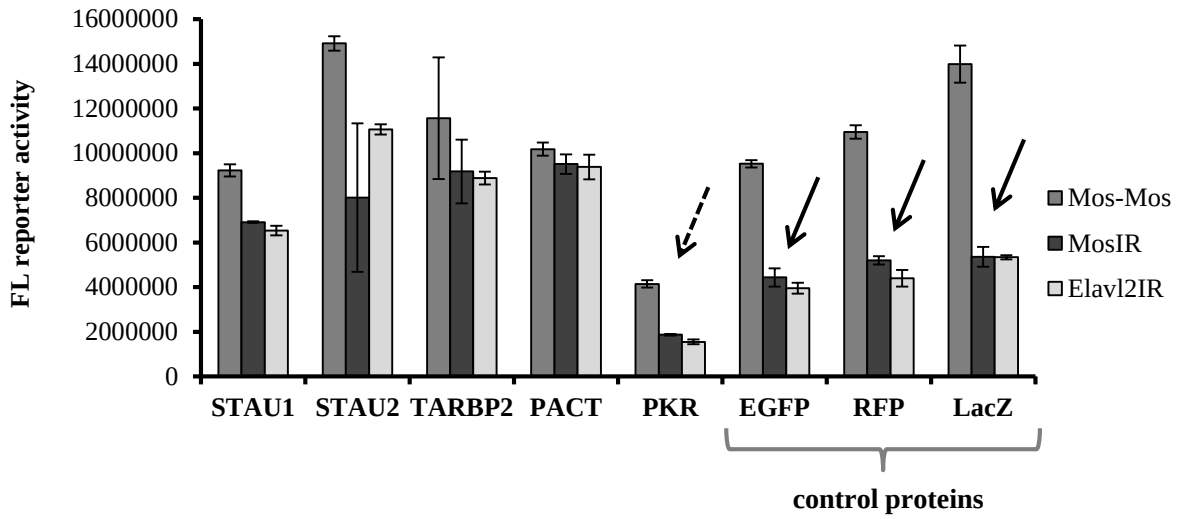

Figure 23 –Firefly luciferase reporter activity in 3T3 cells. EGFP, RFP and LacZ were used as negative controls for DRBPs. Black arrows indicate dsRNA-dependent non-specific effect, which is inhibited in cells expressing DRBPs. Dashed arrow indicates the general reduction of co-transfected reporters expression in the presence of high levels of PKR.

Interestingly, dsRNA-dependent down-regulation of RL reporter activity did not occur in cells expressing candidate DRBPs, which suggested that dsRNA-mediated sequence-independent suppression of was blocked by DRBPs (Figure 22). The similar result was observed in FL reporter activity; however, this relief was partial in cells expressing STAU1, STAU2, TRBP2, and complete only in cells expressing PACT. This difference could be caused by increased expression of PACT in cells and unequal sensitivity of both luciferases to dsRNA effects. Importantly, this observation can explain apparently lower relative RL activity in cells expressing MosIR and PACT when compared to cells expressing MosIR and controls (Figure 21). Since PACT expression caused higher FL reporter expression, the relative RL activity would appear lower (Figure 21).

There are several possibilities how DRBPs could affect dsRNA-mediated sequence-independent repression of reporters. Our working hypothesis is that DRBPs directly bind dsRNA and mask its recognition by PKR; alternatively DRBPs could bind PKR and inhibit its activity itself.

In any case, our data revealed that all four expressed DRBPs inhibit dsRNA-mediated sequence-independent repression of reporters in NIH 3T3 cells.

4.5. Testing of DRBPs effects on functional RNAi in ESCs

Previous results showed that overexpression of candidate DRBPs did not reactivate RNAi in somatic cells. To rule out that this effect was a consequence by lacking RNAi, I tested whether the DRBP overexpression can affect the RNAi activity in cells where RNAi is active.

The best model of cell culture with active RNAi is Dicer^{O}-expressing embryonic stem cell line (ESCs) (Flemr et al., 2013). As mentioned in section 1.5.3., the mouse oocyte-specific truncated Dicer isoform, Dicer^{O}, shows highly efficient processing of dsRNA into siRNAs (Flemr et al., 2013). ESCs expressing the full-length Dicer, Dicer^{S}, were used as a control, as RNAi activity is very low in these cells (Ma et al., 2008, Flemr, 2013 #9).

4.5.1. ESCs behave similarly to NIH 3T3 cells

Dicer^{S}-expressing ESCs were reported to have a low level of endogenous RNAi (Ma et al., 2008; Flemr et al., 2013). Thus, I took the advantage of this fact and I used ESCs expressing Dicer^{S} as a control to ESCs expressing Dicer^{O}.

The luciferase-based RNAi assay was performed in Dicer^{S}-expressing ESCs similarly as in NIH 3T3 cells. I observed a slight down-regulation (~ 20 %) of relative RL activity in cells expressing MosIR and control proteins compared to cells expressing Mos-Mos and control proteins (Figure 24). This down-regulation, which was not statistically significant, did not seem to be caused by RNAi, since it was observed when for Elavl2IR was expressed in cells. The measurement error was higher in ESCs than in NIH 3T3 cells; this was probably caused by lower transfection efficiency resulting in higher heterogeneity of samples among biological replicates.

The relative RL activity in cells expressing MosIR and DRBPs was not down-regulated compared to relative RL activity in cells expressing MosIR and control proteins, suggesting that overexpression of candidate DRBPs is not sufficient to stimulate RNAi in ESCs expressing Dicer^{S} (Figure 24).

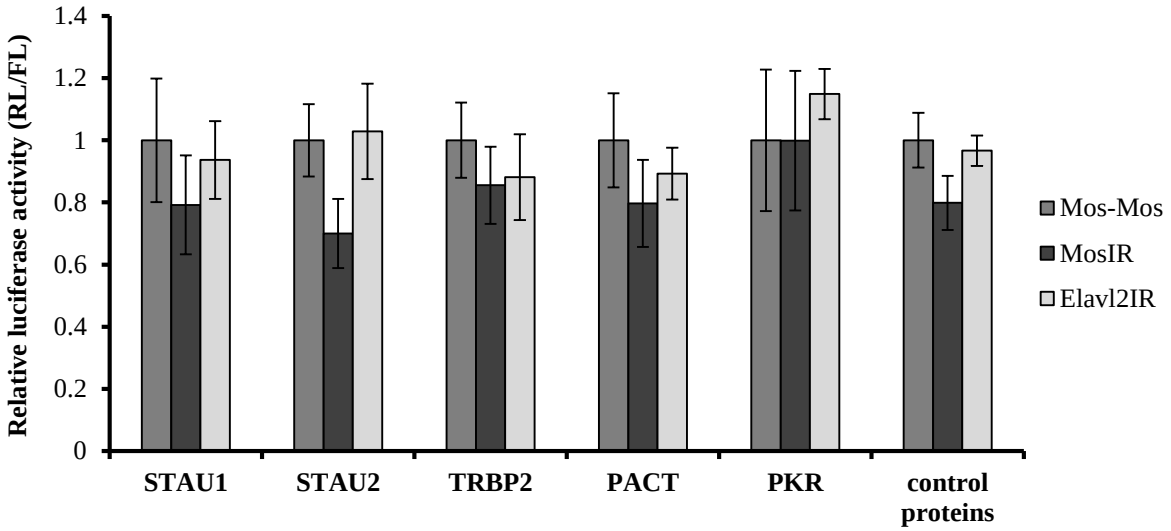

Figure 24 – Relative luciferase activity (RL/FL) in Dicer^S-expressing ESCs. ESCs cells were grown in a 24-well plate and co-transfected with 50 ng of each reporter; 250 ng of either MosIR or Mos-Mos or Elavl2IR; and 250 ng of candidate DRBPs or control proteins. The luciferase assay was performed 48 h post-transfection. RL reporter activity was normalized to FL reporter activity and the relative luciferase activity in cells expressing Mos-Mos was set to one. Results are shown as means +/- SD of up to three independent experiments performed in triplicates.

Down-regulation of raw RL and FL reporter activities in cells expressing MosIR or Elavl2IR and control proteins compared to cells expressing Mos-Mos and control proteins suggests that dsRNA-mediated effect suppressing RL and FL reporters in the presence of MosIR and Elavl2IR, is present also in ESCs expressing Dicer^S (Figure 25 and 26). Similarly to NIH 3T3 cells, overexpression of PKR increased dsRNA-mediated down-regulation of co-transfected reporters in cells expressing MosIR, Elavl2IR as well as Mos-Mos. This suggests that the PKR overexpression causes the suppression of plasmid regardless of whether dsRNA expressing plasmid is present or not.

Importantly, suppression of co-transfected reporters in cells expressing MosIR or Elavl2IR was partially inhibited in cells with overexpressed DRBPs implying that DRBPs might mask dsRNA from its recognition by PKR. These results essentially reproduce previous results obtained in NIH 3T3 cells.

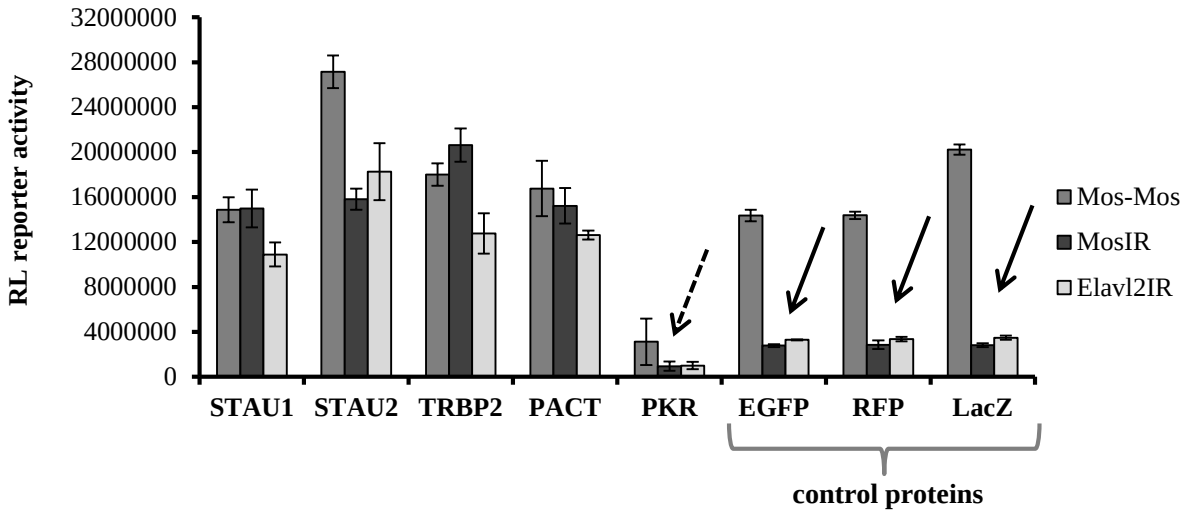

Figure 25 – *Renilla* luciferase reporter activity in Dicer^S-expressing cells. EGFP, RFP and LacZ were used as negative controls for DRBPs. Black arrows indicate dsRNA-dependent non-specific effect, which is inhibited in cells expressing DRBPs. Dashed arrow indicates the general reduction of co-transfected reporters expression in the presence of high levels of PKR.

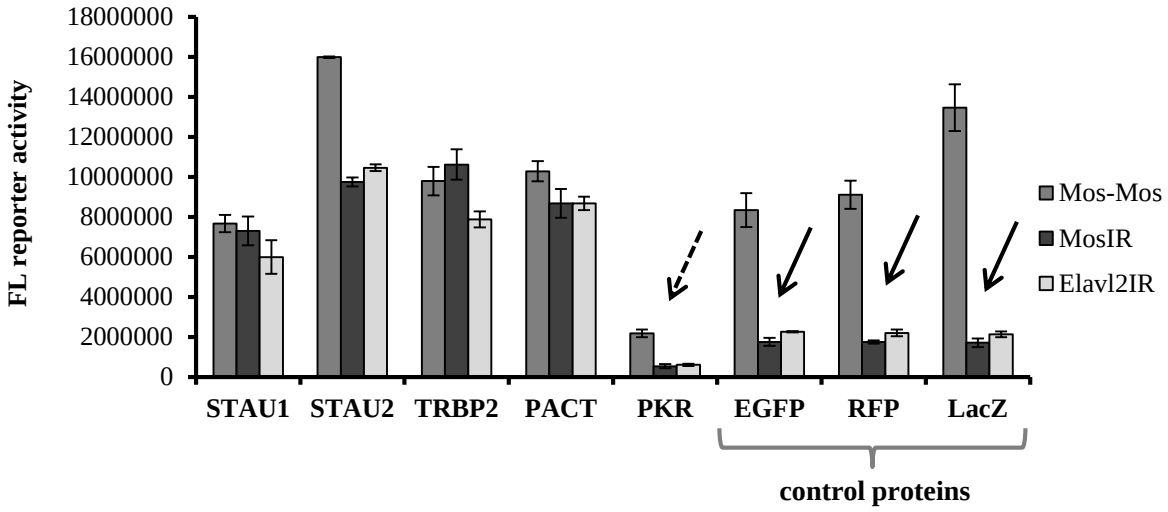

Figure 26 – Firefly luciferase reporter activity in Dicer^S-expressing cells. EGFP, RFP and LacZ were used as negative controls for DRBPs. Black arrows indicate dsRNA-dependent non-specific effect, which is inhibited in cells expressing DRBPs. Dashed arrow indicates the general reduction of co-transfected reporters expression in the presence of high levels of PKR.

Taken together, overexpression of DRBPs was not sufficient to stimulate RNAi also in Dicer^S-expressing ESCs and dsRNA-mediated sequence-independent suppression of co-transfected reporters was again inhibited by overexpressed DRBPs.

4.5.2. RNAi enhanced by Dicer^{O} is inhibited by TRBP2 and PACT

The previous results showed that overexpression of DRBPs did not activate RNAi in NIH 3T3 cells nor in Dicer^{S}-expressing ESCs. To exclude the possibility that this effect was a consequence of inactive RNAi, I performed luciferase-based RNAi assay in Dicer^{O}-expressing ESCs similarly. Highly active RNAi in Dicer^{O}-expressing ESCs were reporter by Flemr et.al. (Flemr et al., 2013). Consistently, robust down-regulation (~ 60 %) of relative luciferase activity was observed in cells expressing MosIR and control proteins compared to cells expressing Mos-Mos and control proteins suggesting that RNAi is highly efficient in cells expressing Dicer^{O} (Figure 27). At the same time, no down-regulation of relative RL activity was observed in cells expressing Elavl2IR and control proteins compared to cells expressing Mos-Mos and control proteins.

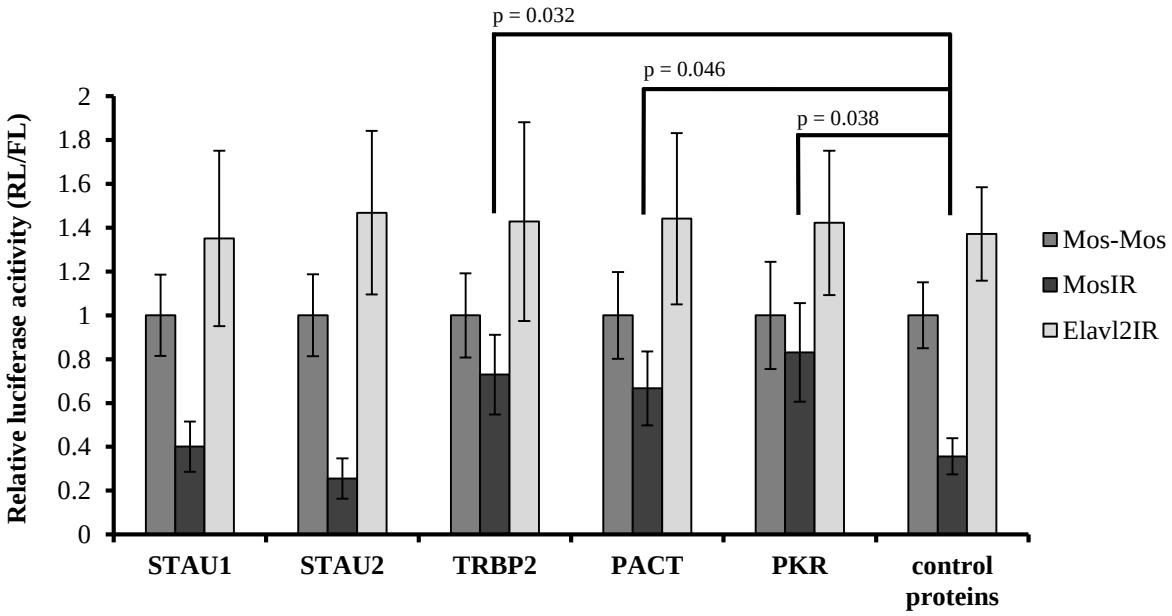

Figure 27 – Relative luciferase activity (RL/FL) in Dicer^{O}-expressing ESCs. ESCs were grown in a 24-well plate and co-transfected with 50 ng of each reporter; 250 ng of either MosIR or Mos-Mos or Elavl2IR; and 250 ng of candidate DRBPs or control proteins. The luciferase assay was performed 48 h post-transfection. RL reporter activity was normalized to FL reporter activity and the relative luciferase activity in cells expressing Mos-Mos was set to one. Results are shown as means +/- SD of up to three independent experiments performed in triplicates. Statistical significance was evaluated according to unpaired two-tailed t-test.

The relative RL activity observed in cells expressing MosIR and STAU1 or STAU2 was comparable to the relative RL activity in cells expressing MosIR and control proteins indicating that STAU1 and STAU2 overexpression did not have any significant effect on RNAi activity.

Interestingly, down-regulation of the relative RL activity in cells expressing MosIR and TRBP2 was reduced when compared to cells expressing MosIR and control proteins. A similar inhibition was also observed in cells expressing MosIR and PACT as well as in cells expressing MosIR and PKR.

Taken together, these results suggest that the overexpression of TRBP2, PACT or PKR protein results in significant inhibition of RNAi activity in ESCs expressing Dicer^{O}.

4.5.3. dsRNA-mediated suppression of co-transfected reporters is relieved in Dicer^{O}-expressing cells

dsRNA-mediated suppression of co-transfected reporters was observed in every cell type examined. As mentioned in section 4.4.2., this suppression is PKR-dependent and overexpression of PKR increases the suppression of co-transfected reporters in cell expressing MosIR, Elavl2IR and Mos-Mos. However, in Dicer^{O}-expressing ESCs, the down-regulation of the FL reporter activity in cells expressing MosIR or Elavl2IR was partially relieved compared to the down-regulation of the FL reporter activity observed in Dicer^{S}-expressing cells (Figure 28). Interestingly, the down-regulation of FL reporter activity in cells expressing MosIR or Elavl2IR and PKR was comparable in Dicer^{O}-expressing ESCs and in Dicer^{S}-expressing ESCs, indicating that PKR can antagonize Dicer^{O} attenuation of dsRNA-mediated sequence-independent effect.

In conclusion, dsRNA-mediated sequence-independent suppression was partially relieved in cells with efficient RNAi suggesting that RNAi may antagonize PKR pathway.

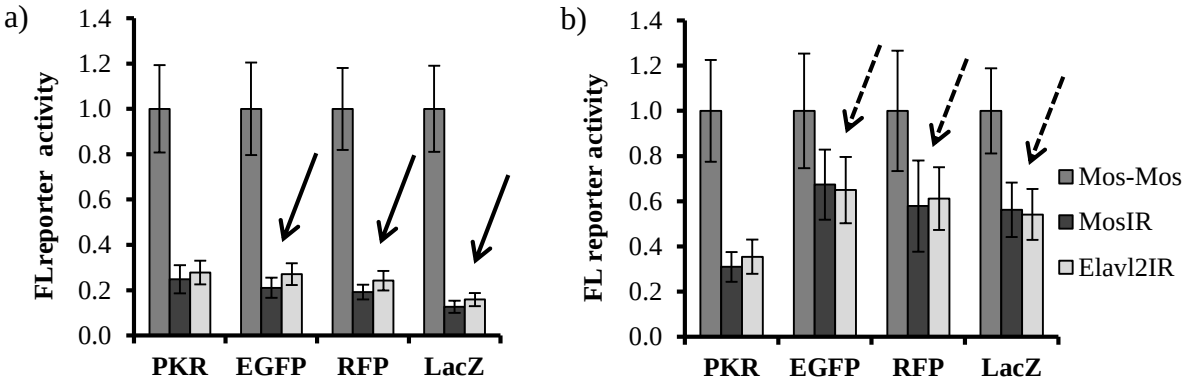

Figure 28 – Firefly luciferase reporter activity in Dicer^{S} (a) and Dicer^{O} (b) -expressing cells. FL reporter activity of Mos-Mos was set to one. In Dicer^{S}-expressing ESCs, the FL reporter activity was down-regulated in cells expressing MosIR and Elavl2IR compared to cells expressing Mos-Mos, indicating the presence of dsRNA-mediated sequence-independent suppression (black arrows). dsRNA-mediated suppression was relieved in ESCs expressing Dicer^{O} (dashed arrows).

4.6. miRNA pathway remains unaffected after DRBPs overexpression

Altogether, results from Dicer^{O} and Dicer^{S}-expressing ESCs indicated that Dicer^{O} is important and sufficient for efficient RNAi in mouse cell lines, as we did not observe efficient RNAi in ESCs expressing Dicer^{S}. Moreover, the overexpression of TRBP2 or PACT inhibited the RNAi activity in Dicer^{O}-expressing ESCs, which raised another question: What is the mechanism by which TRBP2 and PACT affect RNAi?

There are two main models, how TRBP2 or PACT can inhibit RNAi. First, TRBP2 and PACT proteins could bind dsRNA and prevent access of Dicer and cleavage of dsRNA. Second, TRBP2 and PACT could inhibit long dsRNA processing by Dicer by direct binding to it. To distinguish between these two scenarios, one could take advantage of the miRNA pathway. As mentioned above, Dicer plays an essential role in both RNAi and miRNA pathways. If overexpression of TRBP2 or PACT has an impact on miRNA pathway, it would indicate that TRBP2 and PACT proteins bind to Dicer and inhibit its activity directly. When the miRNA pathway would remain intact, it would suggest that the inhibition of RNAi by overexpression of TRBP2 and PACT is at the level of dsRNA recognition and processing.

To investigate the effect of TRBP2 or PACT on the miRNA pathway, I used different sets of luciferase reporters, which allow us for monitoring the endogenous miRNA activity. We opted for reporters carrying binding sites for the endogenous miR-30c, which is abundantly expressed in ESCs and NIH 3T3 cells. The schematic representation of miRNA reporters is shown in the section 3.6.2.

I used three different reporters for monitoring miR-30c: 1x perfect miR-30 (1xP miR-30) carries one perfect target site for miR-30c and mimics siRNA-like degradation of targeted reporter; 4x bulged miR-30 (4xB miR-30) corries four partially complementary sites for miR-30 and targeting miR-30 to these sites results in the translational inhibition of the targeted reporter, the last reporter 4x mutated miR-30 (4xM miR-30) carries four mutated miR-30 sites and serves as control reporter (Ma et al., 2010).

The luciferase assay was performed as described previously. Results from ESCs expressing Dicer^{O} are shown in Figure 29. The decrease of relative RL activity was observed in cells expressing four bulged 4xB miR-30 reporter and control proteins and also in cells expressing 1xP miR-30 reporter and control proteins compared to the relative luciferase activity observed in cells expressing 4xM miR-30. From this result we can assume, that both, the miRNA as well as siRNA-like pathways were efficient in ESCs expressing Dicer^{O}.

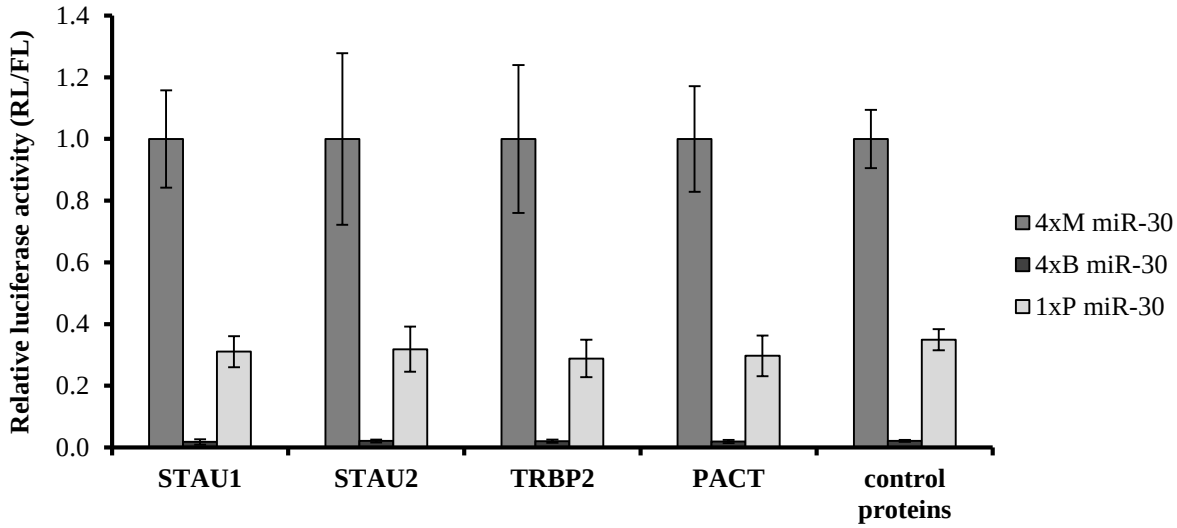

Figure 29 – Relative luciferase activity (RL/FL) in Dicer^{O}-expressing ESCs. ESCs were grown in a 24-well plate and co-transfected with 50 ng of each reporter, and 250 ng of candidate DRBPs or control proteins. The luciferase assay was performed 48 h post-transfection. RL reporter activity was normalized to FL reporter activity and the relative luciferase activity in cells expressing 4xM miR-30 reporter was set to one. Results are shown as means +/- SD of three independent experiments performed in triplicates.

Down-regulation of the relative RL activity in cells expressing 4xB miR-30 and 1xP miR-30 reporters was not significantly affected by any of overexpressed DRBPs in Dicer^{O}-expressing ESCs (Figure 29). Similar results were observed in Dicer^{S}-expressing ESCs (Figure 30) indicating that selected DRBPs do not influence miRNA pathway in ESCs.

A different level of 1xP miR-30 and 4xB miR-30 reporters repression was observed in NIH 3T3 cells compared to ESC expressing Dicer^{O} or Dicer^{S}. These differences in the down-regulation of relative RL activity presumably reflect differenced in miR-30 activities in different cell types (e.g. different miR-30c expression levels or different Argonautes expression levels).

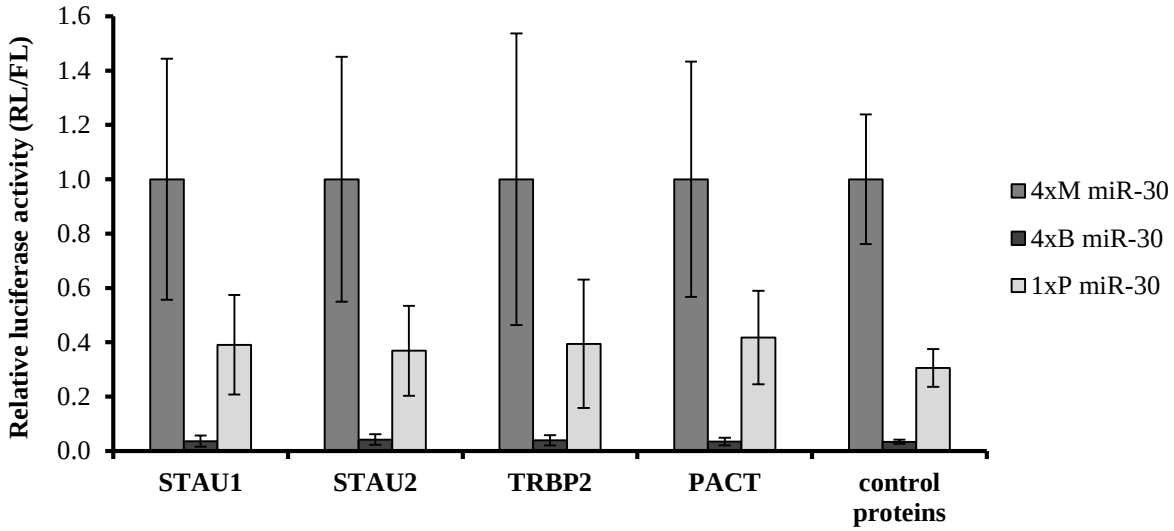

Figure 30 – Relative luciferase activity (RL/FL) in Dicer^{S}-expressing ESCs. ESCs were grown in a 24-well plate and co-transfected with 50 ng of each reporter and 250 ng of candidate DRBPs or control proteins. The luciferase assay was performed 48 h post-transfection. RL reporter activity was normalized to FL reporter activity and the relative luciferase activity in cells expressing 4xM miR-30 reporter was set to one. Results are shown as +/- SD of one independent experiment performed in triplicates.

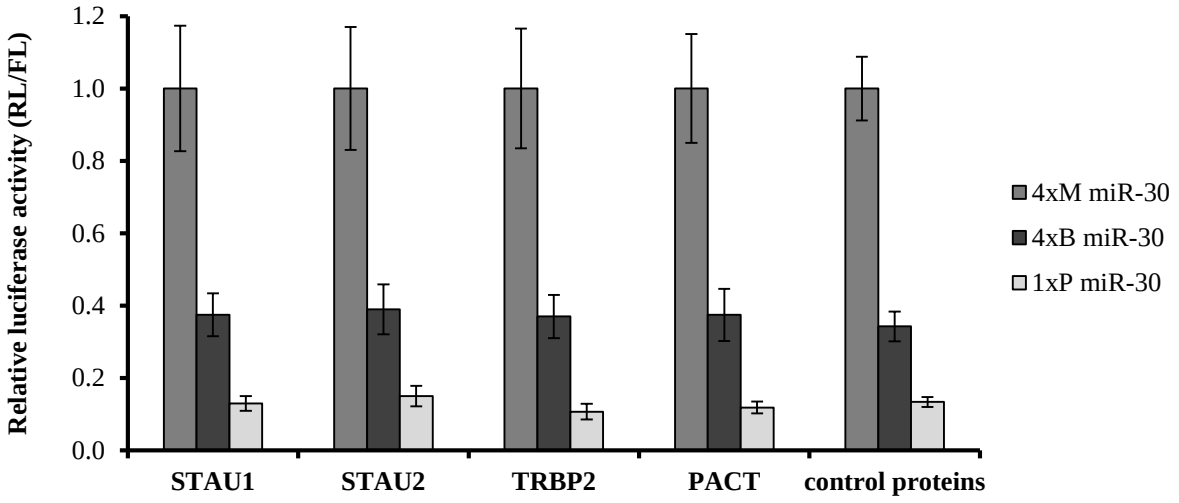

Figure 31 – Relative luciferase activity (RL/FL) in NIH 3T3 cells. NIH 3T3 cells were grown in a 24-well plate and co-transfected cells with 50 ng of each reporter and 250 ng of candidate DRBPs or controls. The luciferase assay was performed 48 h post-transfection. RL reporter activity was normalized to FL reporter activity and the relative luciferase activity in cells expressing 4xM miR-30 reporter was set to one. Results are shown as means +/- SD of two independent experiments performed in triplicates.

Taken together, while miRNA pathway is active in Dicer^{O}-expressing ESCs, Dicer^{S}-expressing ESCs and NIH 3T3 cells, the overexpression of DRBPs did not affect miRNA activity. This demonstrates that the inhibition of RNAi activity in Dicer^{O}-expressing ESCs is not caused by modification of Dicer activity itself but the inhibition is rather at the level of recognition and processing of dsRNA.

5. DISCUSSION

The main objective of this Master's thesis was to examine how specific candidate DRBPs influence the entry of long dsRNA into different pathways. Mammalian cells have several mechanisms responding to dsRNA. These include sequence-independent interferon (IFN) response, RNA editing by adenosine deaminases and RNAi, sequence-specific mRNA degradation induced by long dsRNA.

To study dsRNA processing, we employed a dsRNA-expression model based on a hairpin RNA carrying *Mos* gene sequence (MosIR) (Nejepinska et al., 2012). Using this system in somatic cells, it was shown that expressed MosIR dsRNA does not induce the canonical IFN response, RNA editing was barely detectable, and processing of MosIR dsRNA by Dicer was inefficient (Nejepinska et al., 2012). In contrast to somatic cells, expression of MosIR dsRNA in mouse oocytes triggers a robust RNAi effect (Svoboda et al., 2001, Stein et al., 2003, Nejepinska et al., 2012). The high RNAi activity in mouse oocytes was attributed to the presence of a truncated version of Dicer ($Dicer^{O}$) (Flemr et al., 2013). $Dicer^{O}$ showed higher cleavage activity *in vitro* and was also able to efficiently produce siRNAs from long dsRNA in comparison to $Dicer^{S}$ *in vivo* (Flemr et al., 2013). It was also shown that $Dicer^{O}$ is sufficient to boost RNAi when expressed in ESCs (Flemr et al., 2013). In this thesis, I studied whether other factors could stimulate RNAi in somatic cells. Double-stranded RNA-binding proteins consisting of multiple dsRBDs appeared as good candidates, because they are involved in recognition and processing of dsRNA into different pathways, including RNAi, in different organisms.

For our study, we selected four different DRBPs: STAU1, STAU2, TRBP2 and PACT. While different roles of candidate DRBPs were studied in mammalian cells (summarized in Saunders and Barber, 2003), their function has not been examined in the context of the canonical RNAi pathway.

5.1. Effect of DRBPs in cells not displaying RNAi

As mentioned above, $Dicer^{O}$ is sufficient to augment RNAi when expressed in ESCs. I tested whether DRBPs could be sufficient to boost RNAi in cells, which do not display RNAi. First, I analyzed binding of ectopically expressed dsRNA by DRBPs and then I tested whether overexpression of selected DRBPs can stimulate RNAi activity in 3T3 cells and ESCs expressing $Dicer^{S}$.

I verified the expression of DRBPs and analysed dsRNA-binding by protein immunoprecipitation followed by real-time PCR. Effects of DRBPs on RNAi were examined using luciferase-based reporter RNAi assay and results revealed that DRBPs overexpression did not have any effect on RNAi activity in NIH 3T3 cells or in ESCs expressing Dicer^{S}. Below, I will discuss separately results concerning individual DRBPs.

5.1.1. STAU1

I did not observe STAU1 binding to MosIR dsRNA. This absence of binding was an unexpected result as ectopically expressed full-length STAU1 was readily detected in NIH 3T3 cells.

There are several possible explanations why I did not detect STAU1 binding to MosIR dsRNA. The first explanation that STAU1 protein does not bind MosIR dsRNA is less likely as we also observed inhibition of dsRNA-mediated sequence-independent suppression of co-transfected reporters in cells overexpressing STAU1 (Figure 22 and 23). This suggests that STAU1 indeed interacts with MosIR dsRNA to some extent, although I cannot formally rule out that STAU1 binds and affects other factors, like PKR. Alternative possibilities are that STU1 binding to MosIR dsRNA is weak or that STAU1 recognizes MosIR dsRNA and triggers the, so called, Staufen1-mediated mRNA decay (SMD). SMD is a translation-dependent regulatory mechanism, which occurs when STAU1 homodimer together with ATP-dependent RNA helicase Up-frameshift 1 (UPF1) bind to a STAU1-binding site downstream of a stop codon on mRNA (Kim et al., 2005). Subsequent enhancement of UPF1 activity, presumably by conformational change triggered by STAU1, leads to mRNA cleavage and degradation (reviewed in (Park and Maquat, 2013). STAU1-binding sites can be formed by intermolecular or intramolecular base-pairing within the 3'UTR of mRNA, mostly derived from Alu elements (Kim et al., 2007; Gong and Maquat, 2011). Therefore, it is possible that the dsRNA structure in the 3'UTR of MosIR transcript mimics STAU1-binding site and triggers SMD in cells overexpressing STAU1. The immunoprecipitated MosIR dsRNA is detected by real-time PCR using primers located in the 5' end of MosIR mRNA and such detection is superior in the terms of specificity (primers are directed to intron-containing region) over primers localized directly in dsRNA region (large contamination by plasmid-derived DNA). As I did not detect immunoprecipitated dsRNA in cells overexpressing STAU1, it might indicate that MosIR mRNA is degraded, which would support the SMD hypothesis. I have obtained preliminary data supporting this hypothesis, but it needs to be further tested, for example by inhibition of SMD pathway.

5.1.2. STAU2

The ectopic expression of HA-tagged STAU2 in NIH 3T3 cells revealed the presence of a C-terminally truncated version of STAU2 instead of the full-length STAU2. To exclude the role of expression vector, STAU2 sequence was validated by sequencing and no alterations compared to the reference sequence were identified. STAU2 coding sequence was re-cloned into different plasmid backbones; however, different plasmid backbones had no effect on the STAU2 truncation. This implies that truncation of overexpressed STAU2 is mediated by a post-transcriptional or a post-translational effect. This effect was not specific to NIH 3T3 cells, because similar C-terminal truncation was also observed in ESCs (data not shown).

Protein immunoprecipitation and subsequent real-time PCR revealed that the expressed STAU2 binds MosIR dsRNA despite of the C-terminal truncation, suggesting that at least one RBDs remains intact in the truncated STAU2 and is sufficient to bind dsRNA. Similar to the STAU1, truncated STAU2 inhibits the dsRNA-mediated sequence-independent suppression of co-transfected reporters. However, as for all tested DRBPs we did not observe any effect on RNAi.

Recently, it was shown that STAU2 protein can also trigger SMD (Park et al., 2013). Interestingly, we did not observe any defect in STAU2 binding to MosIR dsRNA, as we did for STAU1. This observation can be explained by the presence of the truncated version of STAU2 protein after ectopic expression in cell culture. It was shown that the C-terminal dsRBD together with Staufen-swapping motif, which resides in the region between Tubulin binding domain (TBD) and C-terminal dsRBD (Figure 8), are essential for homo- and hetero-dimerization and alterations of these motifs suppressed SMD activity (Gleghorn et al., 2013). Thus, a possible explanation why STAU2 would bind MosIR dsRNA and would not cause its degradation is that the C-terminally truncated STAU2 cannot form dimers and SMD activity is not present.

5.1.3. TRBP2 and PACT

Ectopically expressed TRBP2 and PACT were readily detected in cells. Protein immunoprecipitation and subsequent real-time PCR revealed that both TRBP2 and PACT bind MosIR dsRNA. However, the overexpression of TRBP2 did not have any effect on RNAi. I observed a slightly higher apparent RNAi activity in cells overexpressing PACT. However, decrease of normalized relative RL activity may be attributed to higher expression of FL reporter in the MosIR expressing cells, which may be caused by different sensitivity of

FL to sequence-independent effect in the presence of PACT and not by stimulated RNAi. Taken together, we did not observe any significant effect of overexpressed TRBP2 or PACT on RNAi activity in NIH 3T3 cells.

5.1.4. dsRNA-mediated sequence-independent suppression is inhibited by DRBPs

Interestingly, I observed that dsRNA-mediated suppression of co-transfected reporters, which is sequence-independent and involves PKR (Nejepinska et al., 2014), is inhibited by overexpression of all candidate DRBPs. There are several possible explanations of this inhibition.

Direct binding of DRBPs to MosIR dsRNA can result in masking of MosIR dsRNA from PKR and prevent PKR-dependent dsRNA-mediated suppression of co-transfected reporters. However, we still cannot exclude the possibility that some DRBPs directly bind to PKR and inhibit its activity. This could be indeed the case for TRBP2 or PACT.

TRBP2-mediated inhibition of PKR activity was initially attributed to the sequestration of dsRNA by TRBP2 (Park et al., 1994); however, further studies showed that TRBP2 directly binds to PKR and inhibit its activity (Benkirane et al., 1997; Gupta et al., 2003). Therefore, it is possible that TRBP2 inhibits PKR activity both directly and by sequestration of dsRNA (Daniels and Gatignol, 2012).

PACT is known activator of PKR and its overexpression enhances PKR phosphorylation, which is required for PKR activation (Daniels and Gatignol, 2012). However, I observed the inhibition of dsRNA-mediated suppression after PACT overexpression, which may suggest that the balance between PKR and PACT is tight and high concentration of PACT might cause modification of PKR activity or dsRNA sequestration.

In the case of STAU1, there is possible explanation that MosIR dsRNA is degraded through SMD and thus is not available for PKR activation and dsRNA-mediated suppression is thus inhibited. This assumption is supported by similar inhibition observed in $Dicer^{O}$-expressing ESCs (Flemr et al., 2013) where dsRNA pool available for PKR activation is depleted by $Dicer^{O}$-mediated cleavage.

Another possible explanation is that dsRBD could function as nuclear localization signal, similarly as in human Dicer (Doyle et al., 2013). Nuclear localization of DRBPs could result in sequestration of MosIR dsRNA in the nucleus, which would prevent dsRNA-mediated PKR-dependent suppression of co-transfected reports, because PKR is present exclusively in the cytoplasm. The nuclear localization would not affect the RNAi activity, because it was shown that Dicer is able to process dsRNA also in nucleus (Flemr et al., 2013). This hypothesis is consistent with my result that RNAi activity is not affected after overexpression of STAU1 in ESCs expressing Dicer^{O} (section 4.5.2). However, the exact localization of ectopically expressed HA-tagged STAU1 needs to be tested further.

5.2. Effects of DRBPs in cells with active RNAi

As the overexpression of DRBPs was not sufficient to activate RNAi in NIH 3T3 cells or ESCs expressing Dicer^{S}, I tested whether the overexpression of DRBPs can have effect in cells where RNAi is readily detectable. Our luciferase-based RNAi assay showed that RNAi is active in cells expressing Dicer^{O}, which is consistent with previous observations (Flemr et al., 2013). Thus, recognition and processing of MosIR dsRNA into functional siRNAs is active in Dicer^{O}-expressing cells. Moreover, we observed that dsRNA-mediated non-specific suppression of co-transfected reporters is inhibited in ESCs expressing Dicer^{O}, presumably by depletion of dsRNA pool available for PKR activation by Dicer^{O}-mediated cleavage. This suggests that RNAi antagonizes the PKR pathway.

Overexpression of STAU1 or C-terminally truncated STAU2 did not have any significant effect on the RNAi activity. Interestingly, the overexpression of TRBP2 and PACT inhibits the RNAi activity in Dicer^{O}-expressing cells.

It was shown that TRBP2 interacts with Dicer and also functions as a PKR inhibitor. It has been reported that the Dicer-TRBP2 interaction stabilizes Dicer and knockdown of TRBP2 results in suppression of miRNA biogenesis (Chendrimada et al., 2005). The role of TRBP2 in the Dicer stabilization and pre-miRNA processing remains controversial, as other studies did not verify these results (Haase et al., 2005; Lee et al., 2006). Later on, it had been demonstrated that depletion of TRBP2 has no significant effect on miRNA abundance; however it can affect Dicer processing of a subset of pre-miRNAs, which leads to generation of different-sized miRNAs (isomiRs) (Lee et al., 2013; Kim et al., 2014). TRBP2 and PACT were also suggested to have roles in RISC loading and guide strand selection (Tomari et al., 2004); however, the other studies suggested that TRBP2 or PACT may not be

necessary for this step (Betancur and Tomari, 2012; Noland and Doudna, 2013). Moreover, differential roles of TRBP2 and PACT in small RNA processing were elucidated *in vitro* (Lee et al., 2013). It was shown, that Dicer-PACT complex inhibits the pre-siRNA substrate processing compared to Dicer-TRBP2 complex or Dicer and that Dicer-TRBP2 complex stimulates pre-miRNA and pre-siRNA substrate processing (Lee et al., 2013). Taken together, roles of TRBP2 and PACT in RNA silencing are still poorly understood and their role in recognition and processing of dsRNA in canonical RNAi pathway *in vivo* was not examined until now.

Our working hypothesis was that overexpression of DRBPs might function similarly as RDE-4 in *C. elegans*. RDE-4 is double-stranded RNA-binding proteins, which domain structure is very similar to TRBP2 or PACT (Parker et al., 2008). It was shown, that RDE-4 is required for recognition and processing of dsRNA into siRNA and is not required for subsequent steps (Parrish and Fire, 2001; Tabara et al., 2002; Parker et al., 2006). Moreover, this assumption was emphasized as the RNAi was inefficient in rde-4 mutant *C. elegans* after injection of long dsRNA, but not after injection of siRNAs (Parrish and Fire, 2001).

However, my results obtained by luciferase-based RNAi assay in ESCs expressing Dicer^{O} revealed that RNAi activity is inhibited after ectopic expression of TRBP2 and PACT. This suggests that overexpression of TRBP2 or PACT did not facilitate the recognition and processing of dsRNA and our initial hypothesis based on RDE-4 functions was not confirmed.

As TRBP2 and PACT overexpression reduced the RNAi activity, I focused on examining the mechanism of the inhibition by TRBP2 and PACT. There are two different mechanisms how overexpression of TRBP2 or PACT can inhibit the RNAi activity: (1) TRBP2 and PACT can compete with Dicer for binding site on dsRNA and thus, the Dicer cannot cleave dsRNA into siRNA, or (2) TRBP2 and PACT can bind to Dicer and inhibit its activity *per se*. To test direct inhibition of Dicer activity by TRBP2 and PACT, I took the advantage of the fact, that Dicer operates in both RNAi and miRNA pathways and we used luciferase based assay for monitoring activity of endogenous miRNA pathway (section 3.6.2). However, results observed in Dicer^{O}-expressing ESCs revealed that activity of miRNA pathway is not affected by overexpressed DRBPs. This indicates that the inhibition of RNAi activity is not caused by modifying Dicer activity itself, but the inhibition occurs at the level of recognition and processing of dsRNA.

There is still an open possibility that TRBP2 or PACT contributes to RNAi, but they have an inhibitory role at high levels. Therefore, a similar luciferase-based RNAi assay should be tested in cells expressing different levels of DRBPs.

In any case, inhibition of RNAi activity by overexpression of TRBP2 and PACT in cells expressing Dicer^{O} can be further explored as a strategy to repress RNAi. This would allow for inhibiting RNAi pathway, without affecting miRNA pathway. The separation of these two pathways could be then used for studying specific factors involved in these two pathways and their interplay.

6. CONCLUSIONS

The main objective of this Master's thesis was to analyze binding of ectopically expressed dsRNA by candidate DRBPs and to test effects of candidate DRBPs on RNAi.

We found out:

- Full-length STAU1 was clearly detectable in transfected NIH 3T3 cells; however, I did not detect the binding of STAU1 to MosIR dsRNA and this inability may be caused by STAU1-mediated dsRNA decay.
- STAU2 was expressed in NIH 3T3 cells in a C-terminally truncated form; however, the truncated STAU2 retained the ability to bind MosIR dsRNA.
- TRBP2 and PACT were clearly detectable in transfected NIH 3T3 cells and both, TRBP2 and PACT, bind MosIR dsRNA.
- Overexpression of DRBPs did not stimulate inefficient RNAi in NIH 3T3 cells and ESCs expressing Dicer^{S}.
- Overexpression of DRBPs inhibited dsRNA-mediated sequence-independent suppression of co-transfected reporters, presumably by masking dsRNA from PKR.
- Ectopic expression of TRBP2 and PACT proteins inhibited RNAi activity at the level of recognition and processing of dsRNA by Dicer, not Dicer activity *per se*.

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