

**Charles University
Faculty of Science**

Study program: Biology

Branch of study: Genetics, Molecular Biology and Virology



Bc. Tomáš Demeter

Induction of endogenous RNAi in mammalian cells

Indukce endogenní RNAi v savčích buňkách

Diploma Thesis

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

Prague, 2017

Prohlášení

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

V Praze, 10. 8. 2017

Tomáš Demeter

Pod'akovanie

V prvom rade by som chcel poďakovať môjmu školiťovi Petrovi Svobodovi, za možnosť stať sa členom jeho tímu, za zverený projekt a za rady a trpezlivosť pri písaní tejto práce.

Chcel by som poďakovať všetkým členom Laboratória epigenetických modifikácií, obzvlášť Radkovi Malíkovi za jeho nesmiernu ochotu a neoceniteľné rady, za pomoc pri klonovaní a za prípravu a starostlivosť o bunky v mojej neprítomnosti.

Ďalej by som chcel poďakovať mojej rodine a priateľom za ich pomoc a podporu. Som nesmierne vďačný mojím rodičom za ich starostlivosť, obetavosť, za poskytnutie všetkých zdrojov potrebných k ukončeniu štúdia a za to že vo mňa vždy verili.

Špeciálne poďakovanie patrí mojej priateľke za jej ohľaduplnosť a rady počas písania tejto práce. Ďakujem, že ma dokážeš podporiť a motivovať v najťažších chvíľach.

TABLE OF CONTENTS

ABSTRACT	6
ABSTRAKT	7
LIST OF ABBREVIATIONS	8
1. INTRODUCTION	11
1.1. Double-stranded RNA	11
1.2. RNA silencing.....	12
1.2.1. RNAi.....	12
1.2.1.1. Roles of RNAi	13
1.2.2. miRNA pathway.....	14
1.2.3. Interplay between RNAi and miRNA pathway.....	15
1.2.4. Dicer	16
1.2.4.1. Roles of Dicer.....	17
1.2.4.2. Dicer ⁰	18
1.2.5. Argonaute proteins	19
1.2.6. TARBP2.....	21
1.3. IFN response.....	22
1.3.1. PKR	23
1.3.1.1. Activation of PKR	23
2. AIMS OF THE THESIS	25
3. METHODS	26
3.1. Construction of dsRNA and reporter RNA expressing plasmids.....	26
3.1.1. Ligation and chemical transformation.....	26
3.1.2. PCR screening	26
3.1.3. Cloning.....	27
3.1.3.1. Cloning <i>Mos</i> and <i>Lin28a</i> sequence into convergent huH1↔ U6 plasmid	27
3.1.3.2. Cloning <i>Mos</i> and <i>Lin28a</i> sequence in both orientations after U6 promoter	28
3.1.3.3. Cloning <i>Mos</i> and <i>Lin28a</i> sequence in both orientations after CMV promoter	29
3.1.3.4. Cloning <i>MosIR</i> and <i>Lin28IR</i> sequence downstream of U6 promoter.....	30
3.1.3.5. Cloning <i>MosIR</i> and <i>Lin28IR</i> sequence downstream of CMV promoter	31
3.1.3.6. Cloning <i>Lin28a-Mos</i> chimeric sequence downstream of U6 promoter	32
3.1.3.7. Cloning <i>Mos-Lin28a</i> chimeric sequence downstream of SV40 promoter	33
3.2. Cell culture	33
3.3. Dual-Luciferase assay	34

3.4.	Quantitative PCR.....	34
4.	RESULTS	35
4.1.	Production of dsRNA.....	35
4.2.	Detection a reporter degradation using dual-luciferase assays	37
4.2.1.	RNAi in Dicer ^S expressing cells.....	41
4.2.2.	RNAi in Dicer ^O expressing cells	42
4.2.3.	Induction of RNAi by dsRNA substrates in PKR deficient cells	45
4.2.4.	Induction of RNAi by dsRNA substrates in TARBP2 deficient cells	47
4.2.5.	U6-driven hairpins do not trigger sequence non-specific effect.....	49
4.3.	Detection of RNAi-like effect by qPCR.....	51
4.3.1.	CAG-driven <i>Renilla</i> reporter	52
4.3.2.	U6-driven chimeric reporter	52
4.3.2.1.	CMV produced hairpins do not induce RNAi-like effect	54
4.3.3.	CMV-driven chimeric reporter	55
4.3.4.	CAG-driven Firefly reporter	57
5.	DISCUSSION.....	58
5.2.	PKR inhibition does not increase the RNAi induction	61
5.3.	Inhibition of RNAi by the deletion TARBP2.....	61
5.4.	U6-driven hairpins are immune to sequences non-specific degradation	62
5.5.	Detection of RNAi-like effect is influenced by the type of the reporter	62
6.	CONCLUSIONS.....	64
7.	REFERENCES	65
8.	SUPPLEMENTARY DATA	79

ABSTRACT

Double-stranded RNA (dsRNA), a double helix formed by two antiparallel complementary RNA strands, is a unique structure with a variety of biological effects. dsRNA can be introduced into the cell from exogenous sources or it can be produced endogenously. There are four basic mechanisms producing dsRNA: inverted repeat transcription, convergent transcription, pairing of sense and antisense RNAs produced *in trans*, and RNA dependent RNA polymerase-mediated synthesis dsRNA. Different mechanisms of production determine additional structural features of dsRNA, such as dsRNA termini, mismatches etc. These features may affect cellular response to dsRNA. Recognition of dsRNA can trigger several responses that act in sequence-specific or sequence-independent manners. The main sequence-specific response triggered by dsRNA is RNA interference (RNAi) is. Our laboratory has been studying mechanism of induction of RNAi in mammalian cells using one specific type of long dsRNA expression system. The dsRNA used in these experiments formed hairpin structure with long 5' and 3' single-strand RNA overhangs. We hypothesized that other dsRNA substrates might be more efficient than the one used in mammalian RNAi experiments since 2002.

Accordingly, the main aim of my thesis was to compare efficiency of different dsRNA substrates in induction of RNAi-like effects. To address this point, I produced various dsRNA substrates representing different mechanisms of dsRNA formation. Our experiments included two types of intramolecular duplexes: one with a blunt end and another one carrying longer overhang at the 3' terminus. In addition, I produced dsRNA by base-pairing of sense and antisense RNA strands transcribed either by convergent transcription or at two separate loci (plasmids). These strategies for dsRNA production mimic dsRNA derived from base-pairing of complementary transcripts expressed in the nucleus. As a measure of sequence-specific and sequence-independent effects, I used luciferase reporters where a dual-luciferase assay was used to monitor reporter expression and qPCR to specifically quantify reporter transcripts. Our results suggest that hairpin substrates with blunt ends can induce robust RNAi. Furthermore, such hairpins do not activate sequence-independent effects involving protein kinase R and interferon activation while they can induce RNAi also in the absence of TARBP2.

Key words: double-stranded RNA, Interferon response, RNA interference, Dicer

ABSTRAKT

Dvojvláknová RNA, je unikátna štruktúra s mnohými biologickými účinkami. Je tvorená z dvoch protichodných komplementárnych RNA vlákien, ktoré môžu byť produkované samotnými bunkami alebo sa do nich môžu dostať z vonkajších zdrojov. Existujú štyri hlavné mechanizmy jej vzniku: transkripcia z obrátených repetícií, konvergentná transkripcia, komplementárne párovanie (*in trans*) a syntéza sprostredkovaná RNA dependetnou RNA polymerázou. Rôzne mechanizmy produkcie určujú ďalšie štruktúrne vlastnosti dvojvláknových RNA, napríklad ich konce, chybné zaradenie nukleotidových báz a iné. Štruktúrne vlastnosti môžu ovplyvňovať to, akým spôsobom bunka odpovedá na prítomnosť dvojvláknových RNA. Táto odpoveď môže byť sekvenčne-špecifická alebo sekvenčne-nešpecifická. Hlavnou sekvenčne-špecifickou odpoveďou na prítomnosť dvojvláknovej RNA v bunke je RNA interferencia. Naša skupina študovala mechanizmus indukcie RNA interferencie v cicavčích bunkách pomocou špecifického typu dvojvláknovej RNA. Táto dvojvláknová RNA vytvára vlásenku s dlhými jednovláknovými presahmi na oboch koncoch. Predpokladali sme, že dvojvláknové RNA substráty s inými štruktúrami môžu byť efektívnejšie ako ten, ktorý sme požívali v našich experimentoch od roku 2002.

Hlavným cieľom mojej diplomovej práce bolo porovnať účinnosť rôznych dvojvláknových RNA substrátov na indukciu RNA interferencie v cicavčích bunkách. Preto som skonštruoval niekoľko plazmidov produkujúcich dvojvláknové RNA substráty, ktoré reprezentovali rôzne mechanizmy produkcie dvojvláknovej RNA. Naše experimenty obsahovali dva typy vlásienok: jedna bola ukončená tupými koncami a druhá dlhým presahom na 3' - konci. Okrem nich som vytvoril plazmidy produkujúce dvojvláknové RNA pomocou párovania dvoch protichodných vlákien. A to buď pomocou konvergentnej transkripcie, alebo transkripcie jednotlivých vlákien z rôznych plazmidov. Tieto stratégie produkcie dvojvláknovej RNA kopírujú jej vznik pomocou komplementárnych transkriptov v jadre. Na monitorovanie RNA interferencie som využil duálny luciferázový reportérový systém a kvantitatívnu PCR. Naše výsledky naznačujú, že vlásenkový substrát s tupými koncami dokáže indukovať silnú RNA interferenciu. Navyše sme zistili že tieto substráty neaktivujú sekvenčne-nezávislú odpoveď sprostredkovanú proteín kinázou R a dokážu indukovať RNA interferenciu aj v neprítomnosti TARBP2.

Kľúčové slová: dvojvláknová RNA, interferónová odpoveď, RNA interferencia, Dicer

LIST OF ABBREVIATIONS

A	anti-sense
DGCR8	DiGeorge syndrome critical region 8
Dicer ^O	oocyte-specific Dicer isoform in <i>Mus musculus</i>
Dicer ^S	somatic Dicer isoform in <i>Mus musculus</i>
DMSO	Dimethyl sulfoxide
dsRBD	dsRNA-binding domain
dsRBP	double-stranded RNA binding protein
dsRNA	double-stranded RNA
DUF283	domain of unknown function 283
eIF2	eukaryotic translational initiation factor 2
FL	firefly luciferase
HELICc	helicase conserved carboxy-terminal
HEV71	human enterovirus 71
IFN	interferon
IFG	interferon-induced genes
LB	lysogeny-broth medium
LINE	long interspersed nuclear element
mESC	embryonic stem cells
MID	middle domain
NoV	Nodamura virus
nt	nucleotide
PACT	protein activator of PKR
PAZ	Piwi Argonaute Zwillle

Piwi	P-element induced wimpy testis
PKR	protein kinase R
PolII	polymerase II
PolIII	polymerase III
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
P- bodies	processing bodies
rasiRNA	repeat-associated siRNA
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RL	<i>Renilla</i> luciferase
RLC	RISC loading complex
RL-Lin28ab	<i>Renilla</i> luciferase reporter with <i>Lin28ab</i> cognate sequence
RL-Mos	<i>Renilla</i> luciferase reporter with <i>Mos</i> cognate sequence
shRNA	short hairpin RNA
S	sense
SAP	Shrimp Alkaline Phosphatase
SINE	short nuclear interspersed element
siRNA	small interfering RNA
TAR	trans-activation response
TARBP2	TAR binding protein
tRFs	tRNA related fragments
tRNAs	transfer RNAs
VSR	viral suppressors of RNA

WAGO worm-specific Argonaute

1. INTRODUCTION

1.1. Double-stranded RNA

Double-stranded RNA (dsRNA) is a unique biologically important structure shaped as a right handed A-form double-helix. It can be formed by base-pairing of two complementary RNA strands from two RNA molecules or as an intermolecular duplex within one molecule.

dsRNA is specifically recognised by various dsRNA binding proteins (dsRBPs). The association with different dsRBPs can determine how a cell responds to the dsRNA accumulation. Recognition of dsRNA can trigger several responses that can act in sequence-specific or sequence-independent manners. However, the mechanism by which the cell directs this processing is not entirely understood. One of the underlying causes might stem from the fact that dsRNA can have additional structural features (namely loops and single-strand RNA overhangs) depending on the mechanism of its production. There are four basic mechanisms by which a dsRNA can be formed: convergent transcription, inverted repeat transcription, pairing *in trans* and RNA dependent RNA polymerase (RdRP) mediated amplification of dsRNA (Fig. 1.) The aforementioned structural features associated with different mechanisms of production might be important for the differentiation of various types of dsRNA and their efficient processing.

Biologically, dsRNA can be produced by the cell itself as an endogenous molecule or can originate from exogenous sources (including viral infection).

Endogenous dsRNA can be generated by all four aforementioned mechanisms, however RdRP-mediated production of endogenous dsRNA has been observed only in plants and invertebrates, whose genomes encode RdRPs. The exact roles of endogenous dsRNA are not entirely understood, however, several different models have been proposed to explain dsRNA function such as RNA masking, transcriptional and post-transcriptional interference and RNA editing (Lavorgna *et al.*, 2004).

Typically, the formation of dsRNA in mammalian cells was considered a by-product of viral replication. Almost all viruses produce dsRNA structures at some point of their life cycle. The genome of dsRNA viruses is a source of dsRNA by itself. Single-stranded RNA viruses produce dsRNA intermediates during their replication. Additionally, dsDNA viruses can produce dsRNA molecules as the result of bidirectional transcription. This might suggest that

the ability to sense the presence of dsRNA has evolved as a defence mechanism against viral infections (summarised in Kumar & Carmichael, 1998).

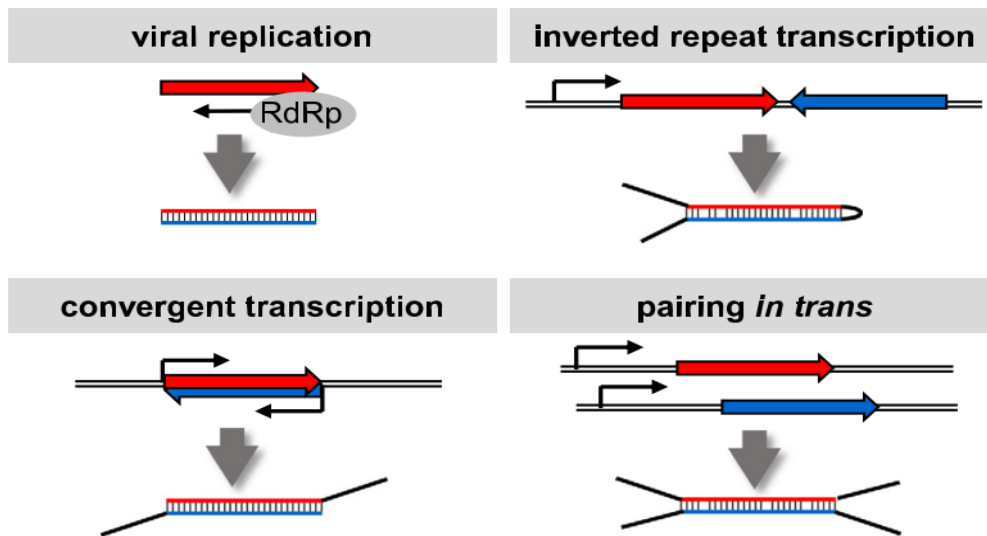


Fig. 1 RNAi substrates. A schematic representation of different mechanisms of dsRNA production. The structural features are determined by the mechanism of transcription. Adapted from Svoboda, 2014.

1.2. RNA silencing

RNA silencing is one of the important cellular responses that can be triggered by dsRNA. While the components of RNA silencing pathways possibly evolved as an defence response against genomic parasites, other fundamental roles emerged during the course of evolution as adaptive innovations allowing further control of the regulation of gene expression (Obbard, Gordon, Buck, & Jiggins, 2009; Pratt & Macrae, 2010).

1.2.1. RNAi

RNA silencing was first described almost 30 years ago when Napoli and co-workers observed transgene-induced silencing causing unexpected loss of colouring on *Petunia* flowers (Napoli, Lemieux, & Jorgensen, 1990). However, it was the work of Fire and Mello in *Caenorhabditis elegans*, who reported that dsRNA triggers sequence-specific degradation of complementary mRNAs and that dsRNA is inducing the silencing effect, significantly more efficiently than the previously employed antisense RNAs (Fire *et al.*, 1998). Since then, RNAi-like effects were observed in cells of many eukaryotic organisms including some mouse and human cell types (summarised in Agrawal *et al.*, 2003). Although, there are several definitions

of RNAi, I will use this term as it was used in the original sense in which dsRNA triggers sequence-specific degradation of complementary RNA.

During RNAi, endogenous or exogenous dsRNA is recognised by Dicer and processed into so-called small interfering RNAs (siRNAs). Animals, siRNAs are approximately 22 nt long and are characterised by 5' monophosphate and a hydroxyl group at the 3' termini (Bernstein, Caudy, Hammond, & Hannon, 2001).

Dicer and a siRNA duplex associate with dsRBPs and Argonaute protein, AGO2 in a complex, which loads a small RNA on AGO2 and yields the effector complex of RNA, the so-called RNA induced silencing complex (RISC) (MacRae, Ma, Zhou, Robinson, & Doudna, 2008). The small RNA duplex is then unwound, and strand selection takes place. The mechanism by which RISC recognise a proper guide strand (i.e. the strand guiding the RNAi effect) from a so-called passenger strand involves sensing the thermodynamic asymmetry along small RNA duplex, which apparently determines the strand used for RISC loading and the strand, which will be degraded (Schwarz *et al.*, 2003). Studies in *Drosophila melanogaster* showed that dsRBP partner of Dicer acts as asymmetry sensor, binding to the more stable siRNA duplex end and orienting Dicer to the strand that is easier to unwind (Tomari, Matranga, Haley, Martinez, & Zamore, 2004).

Once loaded, the single siRNA strand guides RISC to the complementary mRNA. The efficiency of a small RNA binding to its target is asymmetrical, the 3'- end of the guide strand binds to mRNA less effectively than the 5'- end (Stefan Ludwig Ameres, Martinez, & Schroeder, 2007; Wee, Flores-Jasso, Salomon, & Zamore, 2012). The key part of the guide RNA involved in the target recognition are bases 2-7, the so-called "seed region" (Lewis, Burge, & Bartel, 2005; Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003). Upon recognition and binding of a perfectly or nearly perfectly complementary mRNA, the PIWI domain of AGO2 catalyses mRNA slicing. This endonucleolytic cleavage occurs between mRNA nucleotides that pair with siRNA nucleotides 10 and 11 and leaves 5'-phosphate on one end and a 3'- hydroxyl group on the other end (Yuan *et al.*, 2005). The cleaved mRNA fragment is degraded by XRN1 while the 5' fragment would be degraded by the exosome.

1.2.1.1. Roles of RNAi

RNAi plays various roles in different organisms. As stated before it is debated that this pathways originally evolved as defence against viruses and mobile elements. This feature is

still utilised by many organisms including plants, yeasts and invertebrates (summarised in Ding and Voinnet, 2007). In the case of mammals, the role of RNAi in the antiviral response is controversial (De Veer, Sledz, & Williams, 2005; Gantier & Williams, 2007). The failure to experimentally detect RNAi in mammalian cells could be partially explained by the suppression of RNAi by viral suppressors of RNA silencing (VSR) encoded by viruses. Some of the tested viruses likely encoded VSRs that prevented the production of siRNAs, whose absence was interpreted as dysfunctional RNAi (Ding & Voinnet, 2007; Haasnoot, Westerhout, & Berkhout, 2007; Wu, Wang, & Ding, 2011). Some recent reports, however indicate that under specific circumstances, including inactivation of VSRs, RNAi function as an antiviral response (Li, Lu, Han, Fan, & Ding, 2013; P V Maillard *et al.*, 2013; Pierre V Maillard, Veen, Deddouche-grass, & Rogers, 2016).

For example, B2 protein of Nodamura virus (NoV) inhibits Dicer and subsequently biogenesis of small RNAs (Sullivan, Ganem, Sullivan, & Ganem, 2005). Knock-out of B2 gene resulted in production of small 21- to 23- nt long virus-derived small RNAs and lower accumulation of NoV viral particles (P V Maillard *et al.*, 2013). A recent study identified a new VSR 3A in the human enterovirus 71 (HEV71), whose deletion yielded viral derived siRNAs in Dicer dependent and interferon (IFN) independent manner. These viral siRNAs had properties of canonical siRNAs, were loaded on RISC, and specifically silenced cognate viral RNA resulting in decreased replication and pathogenicity of HEV71 (Qiu *et al.*, 2017). Although several earlier studies showed similar results in non-physiological conditions, Qiu and co-workers were the first to report antiviral RNAi under physiological conditions in mammalian cells and mice (Qiu *et al.*, 2017).

Taken together, the amount of evidence suggesting that mammalian RNAi may play a role in the response to viral infections is growing. The presence of active RNAi effectors in mammalian cells is consistent with the notion that RNAi may be one of the layers of cell defence, possibly more pronounced in specific cell types, where other defence mechanisms are not developed. At the same time, it is also possible that these proteins have a different primary role, such as function in gene regulation through the miRNA pathway.

1.2.2. miRNA pathway

The miRNA pathway is evolutionarily conserved RNA silencing mechanism regulating gene expression at a post-transcriptional level.

The miRNA pathway has been extensively described in a number of reviews (Stefan L Ameres & Zamore, 2013; Ha & Kim, 2014). Briefly, the canonical mammalian miRNA pathway is initiated by transcription of endogenous primary miRNAs (pri-miRNAs) by polymerase II. Pri-miRNAs contain one or several hairpin structures, which are recognised and processed into precursor miRNAs (pre-miRNAs). This processing is performed by the microprocessor complex consisting of RNaseIII protein Drosha and DiGeorge syndrome critical region 8 (DGCR8), an RNA binding protein. A pre-miRNA is 60-70 nt long small hairpin with a 2 nt overhang at the 3' - end and 5' phosphate. These characteristics are important for export from the nucleus, which is mediated by Exportin 5, and also for binding to Dicer. Dicer cleave pre-miRNA into ~22 nt miRNAs, which are subsequently loaded onto RISC.

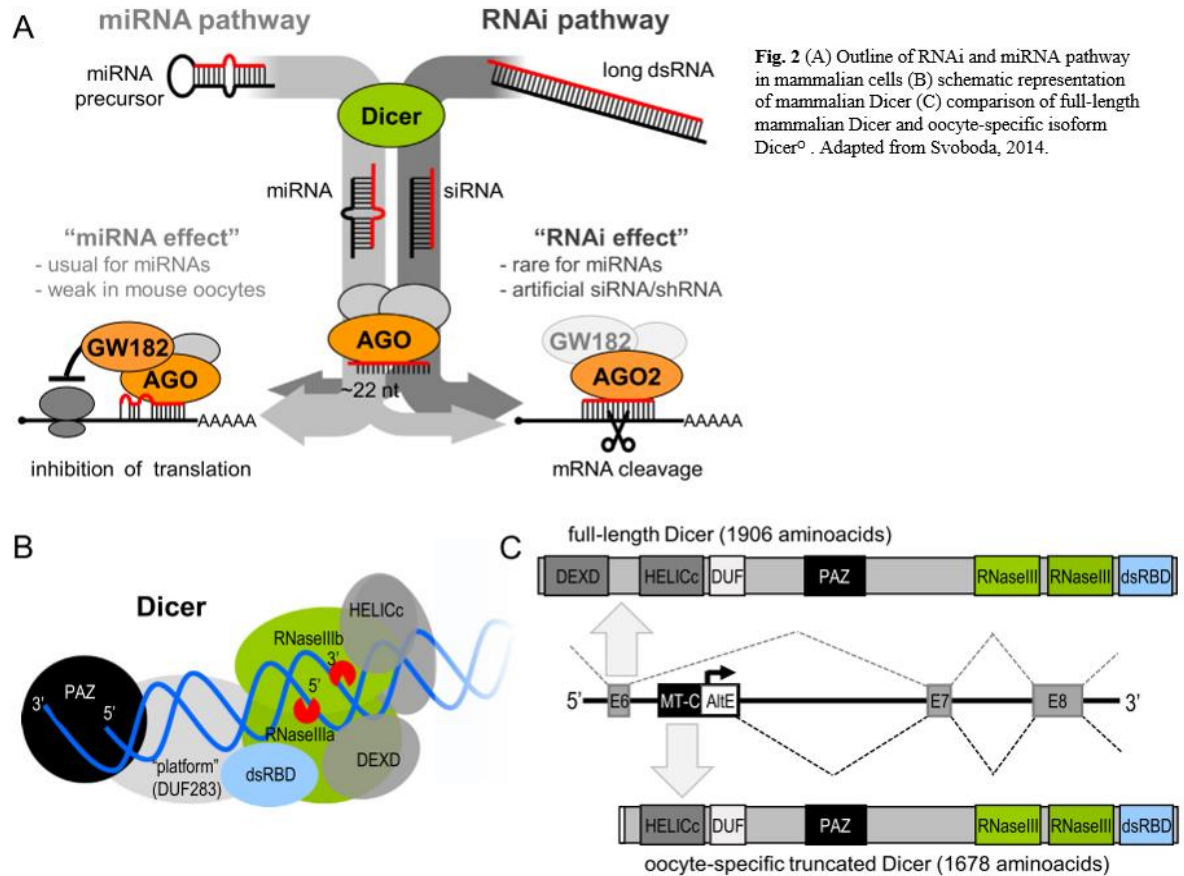
Typically, miRNAs have imperfect complementarity and induce silencing by translational repression rather than endonucleolytic mRNA cleavage. It is estimated that miRNAs regulate expression of up to 60% mammalian mRNAs (Friedman, Farh, Burge, & Bartel, 2009). However, miRNAs with perfect complementarity to mRNA target were shown to be able to mediate the endonucleolytic cleavage (Meister *et al.*, 2004). The key distinction between mammalian RNAi and miRNA pathways is thus the substrate processed by Dicer rather than the silencing mechanism itself.

1.2.3. Interplay between RNAi and miRNA pathway

RNAi and miRNA pathways are interconnected in mammals. Upon binding to Dicer, both of these pathways employ the same machinery. Essential role of these proteins in both pathways complicate the research of RNAi in mammalian cells. Whereas plants and worms express several Dicer and active AGO proteins (Grishok *et al.*, 2001; Jannot, Boisvert, Banville, & Simard, 2008; Mallory & Vaucheret, 2010), each producing small RNAs from different sources, mammals poses only one Dicer involved in both siRNA and miRNA pathways (J. Liu *et al.*, 2004; Meister *et al.*, 2004). Therefore, any intrusion influences not only RNAi but the production of miRNAs as well, complicating the evaluation of these experiments.

This entanglement between RNAi and miRNA pathways indicate common evolutionary origins. Phylogenetic studies suggest that the components of RNA silencing machinery have been functional in the last common ancestor of all eukaryotic organisms. It is assumed, that this machinery consisted of one Dicer, one Argonaute- like polypeptide, one Piwi-like protein and one RdRP. (Cerutti & Casas-Mollano, 2006). RdRP is involved in the amplification of RNAi by the production of more dsRNA from siRNA substrates. However, as

RdRP has not been observed in mammalian cells, I will not be mentioning it in details. Although, it is worth mentioning that ping-pong cycle implemented in the protection of mammalian germ line have a similar function (Brennecke *et al.*, 2007).



1.2.4. Dicer

Dicer or Dicer-like enzymes are one of the key members in the biogenesis of small RNAs in all eukaryotic organisms. However, the number of genes encoding specific Dicer proteins varies between different groups. There are four Dicer homologues in plants, called DCL1-4 (Baulcombe, 2004). It is assumed, that each of these enzymes is specialised for processing of a specific group of small RNAs (Xie, Allen, Wilken, & Carrington, 2005). The phylogenetic tree of animals indicates, that there was a duplication, which gave rise to two Dicer genes early in the evolution of metazoans. However, not all of their descendants retained them (Mukherjee, Campos, & Kolaczowski, 2013). *Drosophila melanogaster* carry both Dicer1 and Dicer2 genes, each of them being specialised for a specific small RNA pathway (Gao, Wang, Blair, Zheng, & Dou, 2014; Young Sik Lee *et al.*, 2004). On the other hand, all vertebrates and some invertebrates rely on a single Dicer protein (Kim *et al.*, 2005; Zhang,

Kolb, Jaskiewicz, Westhof, & Filipowicz, 2004). Moreover, Dicer has been lost from some fungi and from some parasitic protists (Drinnenberg *et al.*, 2009; Ullu, Tschudi, & Chakraborty, 2004).

Full-length mammalian Dicer has not been crystallised yet. However, several studies have tried to determine its structure (Lau *et al.*, 2012; Taylor *et al.*, 2013; Tian *et al.*, 2014; Wilson *et al.*, 2015). Cryo-electron microscopy revealed that Dicer is L-shaped protein with seven main functional domains. From N- terminus they are: helicase domain containing DExD/H, helicase conserved carboxy-terminal (HELICc) domain, the domain of unknown function 283 (DUF283), Piwi Argonaute Zwillie (PAZ) domain, RNaseIIIa and RNaseIIIb domains and finally dsRNA-binding domain (dsRBD). The helicase domain with DExD/H box creates a clamp-like structure at the base of the L shape, which is supposedly wrapping around dsRNA (Lau *et al.*, 2012). It has been reported that this domain plays a role in auto-inhibition of Dicer (Flemr *et al.*, 2013) (see 1.2.4.2). DUF283 domain has a particular role in the binding of Dicer and his dsRBP partners (Ota *et al.*, 2013). According to the recent *in vitro* study, it has been suggested that DUF283 domain can also bind single-stranded nucleic acids and accelerates binding between short RNAs and their targets (Kurzynska-kokorniak *et al.*, 2016). At the top of the L-shape is the PAZ domain that binds the 3'- end of a small RNA in a sequence-independent manner (Lingel, Simon, Izaurralde, & Sattler, 2004). The binding is facilitated by the recognition of a 2 nt overhang on the 3' terminus (Lingel *et al.*, 2004). Interestingly, one study on *Caenorhabditis elegans* and *Drosophila melanogaster* suggest that helicase domain can promote the processing of small RNA substrates that lack this characteristic (Welker *et al.*, 2012). The length of a dsRNA product is determined by the distance between the PAZ domain and the active catalytic site consisting of both RNase IIIa and RNaseIIIb domains. Both RNase domains are centred on the lower half of Dicer. It is assumed, that each RNase cleaves one strand of a dsRNA substrate (Zhang *et al.*, 2004).

1.2.4.1. Roles of Dicer

One of the endonucleolytic functions of Dicer involves cleavage of transfer RNAs (tRNAs). Processing of tRNAs results in the production of a heterogeneous class of small RNAs called tRNA related fragments (tRFs). It has been shown that tRFs map to the DNA sequences of tRNA genes and co-immunoprecipitate with Argonaute proteins (Cole *et al.*, 2009; Yong Sun Lee, Shibata, Malhotra, & Dutta, 2009). Although, their function is not yet understood, a role in RNA silencing mechanisms has been proposed.

Dicer was also shown to cleave distinct groups of small nucleolar RNAs into snoRNA-derived RNAs. These molecules apparently associate with Argonaute proteins and show potential role in the regulation of translation and mRNA processing (Ender *et al.*, 2008).

Dicer is also involved in regulation of retrotransposons. It has been demonstrated that both long interspersed nuclear elements (LINEs) and short nuclear interspersed elements (SINEs) are negatively regulated by Dicer (Heras *et al.*, 2013; Kaneko *et al.*, 2011; Yang & Kazazian, 2006). LINEs produce dsRNA as a result of convergent transcription. Subsequently, dsRNA is processed by Dicer into repeat-associated siRNAs (rasiRNAs). rasiRNAs are loaded onto Argonaute proteins and used to silence LINE-1 by RNA-directed DNA methylation (Faulkner, 2013). *Alu* elements are the most abundant members of SINE group of retrotransposons in the human genome. *Alu* elements can produce Dicer substrates in two ways: (i) transcript of a single *Alu element* can fold back creating a hairpin RNA or (ii) two distinct *Alu* transcripts base-pair producing a stable dsRNA. These dsRNAs are apparently cleaved by Dicer into repeat-induced RNAs which can provoke a stem cell to leave proliferative stem cell stage (Tarallo *et al.*, 2012).

Nuclear RNA silencing in mammalian cells was for some time considered a controversial topic. However, recent studies suggest that RNA silencing plays a significant role in the nucleus. It has been showed that Dicer associates with a nuclear pore complex and is transported to the nucleus (Ando *et al.*, 2011). Some studies propose a role of Dicer in the sequence-specific regulation of rDNA transcription through heterochromatin formation (Volpe *et al.*, 2002). In mammals, this siRNA-mediated silencing is executed by histone deacetylases and DNA methyltransferases (Morris, Chan, & Jacobsen, 2004). Nevertheless, the nuclear localisation and function of mammalian Dicer are probably the least understood aspect of Dicer biology.

1.2.4.2. Dicer^O

As mentioned before, both miRNA and siRNA pathways utilise the same Dicer protein in mammals. However, the efficiency of generation of these small RNAs varies in different cell types. For example, in mammalian somatic cells, miRNA is readily produced from their precursor molecules. However, the cleavage of a dsRNA substrate into siRNAs is not very efficient (Chakravarthy, Sternberg, Kellenberger, & Doudna, 2010).

In this context, mouse oocytes represent a valuable exception in mammalian cells. These cells show a high efficiency in the production of siRNAs from a dsRNA substrate, (P Svoboda, Stein, Hayashi, & Schultz, 2000; Wianny & Zernicka-Goetz, 2000) while both miRNAs and piRNAs seems to be inessential, as oocytes can tolerate their loss in the early development (Carmell *et al.*, 2007; Deng & Lin, 2002; J. Ma *et al.*, 2010). Moreover, sequence-independent degradation is not induced by a dsRNA in mouse oocytes (Stein, Zeng, Pan, & Schultz, 2005), further facilitating their usefulness in RNAi research.

Indeed, research of RNAi in mouse oocytes lead to the discovery of highly active oocyte-specific Dicer isoform (Dicer^O) (Flemer *et al.*, 2013). This isoform evolved in *Muridae* family of rodents as a consequence of an MT-C retrotransposon insertion into the exon 6 of Dicer gene. This resulted in the expression of the N-terminally truncated isoform, lacking DExD helicase domain (Flemer *et al.*, 2013). Rodent-specific MT retrotransposons are known to spread through the murine genome, where they can serve as oocyte-specific alternative promoters modifying gene expression (Flemer *et al.*, 2013; Peaston *et al.*, 2004).

Although, somatic Dicer (Dicer^S) is also expressed in oocytes, Dicer^O represents the major Dicer isoform. The amount of Dicer^O increase during the growth of an oocyte; however, the levels of transcription drop after fertilisation (Flemer *et al.*, 2013). Loss of alternative promoter of Dicer^O results in the deregulation of endogenous siRNA targets and female sterility, possibly caused by spindle defects. This implies that RNAi regulation is essential for the development of mice oocytes. Interestingly, the loss of Dicer^O did not affect levels of miRNAs or male fertility (Flemer *et al.*, 2013).

The efficiency of Dicer^O in the processing of dsRNA into siRNAs was further characterised in mouse embryonic stem cells (mESC) expressing Dicer^O isoform. These experiments showed increased processing of ectopically expressed dsRNA compared to Dicer^S (Flemer *et al.*, 2013). This was in agreement with a previous study showing that inhibition of helicase domain by protease treatment enhances dsRNA processing in vitro (Zhang, Kolb, Brondani, Billy, & Filipowicz, 2002). Furthermore, similar results were obtained with mutant human Dicer lacking helicase domain (Kennedy *et al.*, 2016). These results indicate an auto-inhibitory role of Dicer's helicase domain. However, further studies with a wider range of dsRNA substrates are necessary for the characterization of increased processivity of Dicer^O.

1.2.5. Argonaute proteins

Another key components of RNA silencing machinery are Argonaute proteins. Members of Argonaute family consist of three main types of proteins: i) AGO proteins are named after *Arabidopsis thaliana* protein AGO1, ii) PIWI proteins, which are homologous to *Drosophila melanogaster* P-element induced wimpy testis (Piwi) protein and iii) worm-specific Argonaute (WAGO) proteins, which were characterised in *Caenorhabditis elegans*. WAGO proteins serve as secondary Argonaute proteins acquiring their substrates from primary Argonautes or through small RNA amplification processes mediated by RdRP (Yigit *et al.*, 2006).

As outlined above, it is assumed that the last common ancestor of eukaryotes contained one Piwi-like and one AGO-like protein (Cerutti & Casas-Mollano, 2006). It was suggested that the Piwi-like protein would localise to the nucleus and would play a role in transcriptional silencing, whereas the AGO-like protein was responsible for the regulation on mRNA level (Cerutti & Casas-Mollano, 2006).

Argonaute genes have undergone a significant degree of duplication, and the number of the genes found in different organisms varies from one in *Saccharomyces pombe* to twenty-seven in *Caenorhabditis elegans* (Mallory & Vaucheret, 2010). In vertebrates, four AGO proteins can associate with small RNAs; however, only AGO2 was shown as catalytically active (J. Liu *et al.*, 2004). Nonetheless, some parasitic organisms have lost AGO proteins completely (Ullu *et al.*, 2004).

Argonaute proteins can be functionally redundant in some organisms, like in the case of *Caenorhabditis elegans* (Grishok *et al.*, 2001). However, in several other organisms AGO proteins have specialised and have non-overlapping functions (Mallory & Vaucheret, 2010). For example, in *Arabidopsis thaliana*, different AGO proteins are responsible for processing distinct species of small RNAs (Dunoyer, Himber, Ruiz-ferrer, Alioua, & Voinnet, 2007).

AGO proteins consist of four main domains: N-terminal domain, PAZ domain, middle (MID) domain and PIWI domain. PAZ domain of AGO proteins plays a similar role as in Dicer. It supposedly binds the 2-nt overhang of the 3' - end of a small RNA (Lingel *et al.*, 2004). The MID domain anchors the 5' end of a small RNA by the interactions between the 5' terminal base and conserved tyrosine (Jinek & Doudna, 2009). It was also reported that the MID domain contains MC motif which can bind cap structure and is necessary for regulation of translation (Kiriakidou *et al.*, 2007). The Piwi domain shares some similarities to RNase-H enzymes (Jinek & Doudna, 2009). These enzymes catalyse the cleavage of RNA in RNA/DNA complexes. The

Piwi domain contains degenerate RNase-H-like fold composed of DEDX tetrad in the catalytic centre. This allows for endonucleolytic cleavage of a target RNA (Nakanishi, Weinberg, Bartel, Patel, & Program, 2013; Yuan *et al.*, 2005). The cleavage products contain 5'-phosphate and a 3'-OH, which is also similar to the products of RNase-H like proteins (Martinez & Tuschl, 2006). The N-terminal domain contains a loop that plays a particular role during unwinding of small RNAs and removing of the passenger strand (Hauptmann *et al.*, 2013; Kwak & Tomari, 2012). Despite this, the exact mechanisms and whether additional factors are necessary for this process is still unclear.

1.2.6. TARBP2

TAR binding protein (TARBP2) was originally discovered as a protein stimulating HIV-1 promoter in human cells upon binding TAR (trans-activation response) RNA (Gatignol, Buckler-White, Berkhout, & Jeang, 1991). TARBP2 is also involved in the blocking of the activation of protein kinase R (PKR). Several mechanisms of PKR inhibition has been observed. First, TARBP2 can sequester dsRNA substrates which activate PKR (Blair *et al.*, 1995). Second, TARBP2 can bind PKR directly through dsRBDs of both proteins (Park *et al.*, 1994). Third, TARBP2 can interact with protein activator of PKR (PACT) (Daher *et al.*, 2009). In addition to its involvement in HIV replication and the regulation of PKR, TARBP2 is one of the three core components of RISC loading complex (RLC).

Generally, RLC consist of one AGO protein, one Dicer and one dsRBP. The exact dsRBP associated with Dicer and AGO differs from one organism to another. In *Caenorhabditis elegans* it is a protein called RDE-4, in *Drosophila melanogaster* it is R2D2 or Loqs, and in humans and mice, it is TARBP2 (Chendrimada *et al.*, 2005; Förstemann *et al.*, 2005; Q. Liu, 2003; Tabara, Yigit, Siomi, & Mello, 2002). Even though several studies reported that these proteins form a complex with AGO2, it still remains unclear whether their binding is direct or mediated through Dicer. However, it has been showed that TARBP2 binds to Dicer through small subdomain between DExH/D and helicase domains (Daniels *et al.*, 2009) and that deletion of this subdomain, as well as binding of TARBP2 to it, enhances the rate of cleavage of RNA precursors by Dicer (Soifer *et al.*, 2008). Additionally, Gredell *et al.* showed that when RLC does not contain TARBP2, Dicer-AGO2 interaction is decreased, suggesting that TARBP2 stabilises RLC. This study also proposes that TARBP2 helps in the orientation of AGO2 towards the specific end of siRNA and contributes to the guide strand selection (Gredell, Dittmer, Wu, Chan, & Walton, 2010).

What also remains unsolved is whether the RLC remains intact during the cleavage of small RNAs or dissociate after miRNA/siRNA is loaded on AGO2. The lack of evidence for co-localisation of Dicer and TARBP2 into P-bodies indicates that while they are involved in a precursor cleavage and loading of small RNAs onto AGO2, they are not reacquired for the slicing of mRNA itself. (Pare *et al.*, 2009).

To sum up, the role of TARBP2 in the RNA silencing is very poorly understood. One study has showed that depletion of TARBP2 affected pre-miRNA processing *in vitro*, but it had no effect in *in vivo* experiments (Haase *et al.*, 2005). Another study in human cells reported that knockout of TARBP2 resulted in a decrease of miRNA levels (Chendrimada *et al.*, 2005). The effect of TARBP2 on RNA machinery is apparently also affected by other binding partners, and even by post-translational modifications of TARBP2 (Chen *et al.*, 2015; Kok, Ng, Ching, & Jin, 2007). Unfortunately, studies investigating the effects of TARBP2 on the processing of dsRNAs are few and far between.

Taken together, the exact role of natural RNAi and its components in mammalian cells is still uncertain. RNAi techniques are frequently used for the silencing of genes in mammalian cells; however, they are usually accomplished by injection of artificially produced siRNAs into a cell. The injected siRNAs are then adapted by natural RNA silencing machinery leading to the sequence-specific silencing of targeted genes. Nonetheless, this technique omits the dsRNA processing of canonical RNAi. There are two key reasons: First, the processing of dsRNA by somatic Dicer is inefficient. Second, the presence of long dsRNAs in cytoplasm often triggers sequence non-specific degradation of RNA known as IFN response (Goubau, Deddouche, & Reis, 2013).

1.3. IFN response

IFN are members of inducible cytokines produced in a cell as a reaction to the foreign molecules, activating an antiviral state of a cell and non-specifically inhibiting gene expression. It is estimated that IFNs stimulates transcription of around 2000 genes in the complicated cascade of receptors, signalling molecules and transcription factors (summarised in Fensterl & Sen 2009). Transcripts of the majority of IFN-induced genes (IFGs) participate on the stimulation of the cellular response and propagation of the signals from the origin of the infection. Relatively few characterised ISG products are the effectors of the antiviral protection itself.

1.3.1. PKR

Probably the best-characterized ISG with a direct role in the antiviral defence is serine/threonine kinase PKR. Upon activation, PKR induces global inhibition of translation. This inhibition is stimulated by phosphorylation of alpha subunit of eukaryotic translational initiation factor 2 (eIF2) (Samuel, 1993). At the initiation of translation, eIF2 promotes delivery of Met-tRNA to ribosomal subunit 40S. This transfer is coupled with the hydrolysis of eIF2-bound GTP, which subsequently leads to the inactivation of eIF2. The initiation of the next round of translation is dependent on the reactivation of eIF2, which is catalysed by the exchange of GDP for GTP-mediated by eIF2-B (Hershey, 1991). However, phosphorylation of the alpha subunit of eIF2 increases its binding to eIF2B by 100-fold. This leads to competitive inhibition of eIF2 and global inhibition of translation (Sudhakar *et al.*, 2000; Thomis & Samuel, 1992).

1.3.1.1. Activation of PKR

At low levels, PKR is ubiquitously expressed in all differentiated cells. It is regulated by tumour suppressor protein p53, IFN-I stimulated response elements, transcription factors Sp-1 and Sp-3 and several other proteins (Das, Ward, Markle, & Samuel, 2004; Kuhen & Samuel, 1997; Ward & Samuel, 2002; Yoon, Lee, Lim, & Bae, 2009). Nonetheless, PKR normally exists in inactive, monomeric, un-phosphorylated form, and has to be activated before it can function as an antiviral factor (Robertson & Mathews, 1996). The activation of PKR can be achieved by a variety of stimuli like heparin, or proteolytic cleavage and removal of auto-inhibitory N-terminus of PKR by the apoptotic caspase -8, -3 and -7 (Saelens, Kalai, & Vandenabeele, 2001). However, the most important PKR activation mechanism for the context of this thesis is the activation upon dsRNA binding. However, I will focus on the activation by dsRNA binding as this is the most important PKR activation mechanism for my thesis.

Two distinct models of PKR activation upon dsRNA binding have been proposed. One of the models, the so-called auto-inhibition model, proposes that dsRBD of latent PKR is associated with its kinase domain. This supposedly blocks binding of eIF2 and other PKR targets. The inhibition is lifted upon binding of dsRNA by dsRBD, which releases kinase domain and allows for activation and phosphorylation of PKR substrates (Robertson & Mathews, 1996).

At present, the evidence suggests that dimerization rather than direct blocking of kinase domain plays the crucial role in the activation of PKR. (Carpick *et al.*, 1997; Robertson & Mathews, 1996). Current data propose that activation of PKR is induced by the binding of dsRNA, which leads to dimerization of PKR and subsequent auto-phosphorylation. Activated PKR then recognises and phosphorylates its targets (Clemens, 1997). PKR activity is characterised by a "bell-shaped" curve, meaning that low levels of dsRNA stimulate the activation whereas in higher concentrations dsRNA have inhibitory effects. These observations were explained by a hypothesis according to which low concentrations favour assembly of the proteins on a single molecule of dsRNA while higher dsRNA levels dilute PKR monomers (Kostura & Mathews, 1989).

The activation of PKR by dsRNA is also dependent on the length and possibly on the structure of the dsRNA substrates. It has been suggested that the ideal length of dsRNA for the activation of PKR is approximately 85 nt. However, PKR stimulation has been achieved even with dsRNA as short as 33 nucleotides (Manche, Green, Schmedt, & Mathews, 1992). Interestingly, short-hairpin RNAs (shRNAs) the size of 16 nt with a single-stranded tail of 11 nt at the 5'-, 3'- or both ends were a potent inductor of PKR (Zheng & Bevilacqua, 2004). Nallagatla *et al.* showed that shRNA induce PKR activity in a 5-triphosphate-dependent manner whereas the efficiency of induction of PKR by long dsRNA is not affected by the presence of hydroxyl, monophosphate or triphosphate termini. (Nallagatla *et al.*, 2007). One of the explanations for these findings might be their resemblance to the replicative intermediates of RNA viruses, which would also contain double-stranded regions flanked with single-stranded overhangs, and 5'- triphosphate (Richards, Martin, & Jense, 1984). These structures might play a key role in distinguishing between viral and native RNA, considering most cellular transcripts have 7-methyl guanosine cap or 5'- monophosphate.

While it is debated that this potent sequence non-specific reaction has replaced RNAi in the defence against viral infection (Gantier and Williams 2007; Svoboda 2014), it remains a complication for the investigation of the role of RNAi in mammalian somatic cells.

2. AIMS OF THE THESIS

The main aim of my thesis was to elucidate how different structural features of dsRNA substrates influence the induction of RNAi in mammalian cells.

Achieving this aim involved:

- Generation of plasmid construct expressing different dsRNA structures
- Testing the induction of RNAi by dsRNA substrates in mammalian cells using dual-luciferase assay and Quantitative PCR

3. METHODS

3.1. Construction of dsRNA and reporter RNA expressing plasmids

3.1.1. Ligation and chemical transformation

Typically, restriction digest was performed in 50 µl reaction, at 37°C overnight (5 -20 U of restriction enzyme, 5 µl of competent buffer and the maximum amount of DNA sample, when necessary the volume was brought up to 50 µl with H₂O). Before ligation, linearized plasmids or PCR fragments were run on 1% agarose gel in lithium bromide buffer with 0.1% ethidium bromide (Sigma), desired bands were cut out from the gel and fragments were extracted using QIAquick gel Extraction Kit (Qiagen). The vector backbones were incubated with Shrimp Alkaline Phosphatase (SAP) (New England Biolabs) for 1 hour at 37°C. SAP was inactivated by heat at 65°C for 10 minutes, and 250 ng of dephosphorylated plasmid was ligated with an insert in a vector: insert molar ratio 1:3. The ligation was conducted with 2 U of T4 DNA ligase (Thermo Scientific) at room temperature for 30 minutes.

4 µl of the ligation mix was added to 80 µl of chemically competent *Escherichia coli* TOP10 cells. Transformation mix was kept on ice for 30 minutes, incubated at 42°C for 60 seconds and then again put on ice for at least 2 minutes. Cells were transferred into 750 µl of Lysogeny Broth (LB) medium, incubated at 37°C, 250 RPM for 1 hour and variable amounts were spread on two LB agar plates with 100 µg/ml of ampicillin each. Agar plates were incubated overnight at 37°C.

3.1.2. PCR screening

Presence or absence of insert DNA in a plasmid construct was determined by colony PCR. Each colony was added to 0.2 ml tube containing 50 µl of LB medium with ampicillin (100 µg/ml). 1 µl of a colony containing medium was added to PCR reaction (0.5 of Taq polymerase, 2 µl of 10x Taq buffer, 1,5 µl of 25mM MgCl₂, 0.3 µl of forward primer 10 µM, 0.3 µl of reverse primer 10 µM, 0.4 µl 12.5 mM dNTPs, 14 µl of H₂O).

Typically, PCR programme was set up as follows: Initial denaturation for 3 minutes at 98°C. 30 cycles of: 30 seconds at 95°C, 40 seconds at various temperatures (see table S1), 40 seconds at 68°C. The final extension was performed for 5 minutes at 68°C.

Next, samples were loaded on 1 % agarose gel and run in lithium bromide buffer containing 0.1% ethidium bromide (Sigma). Positive colonies were inoculated into 6 ml of liquid LB medium with ampicillin (100 µg/ml), cultivated overnight at 37°C and plasmid were

isolated using QIAprep Spin Miniprep Kit. 750 ng of isolated plasmid and 20 pM of sequencing primer in a total volume of 10 μ l were sent for sequencing.

3.1.3. Cloning

I used previously constructed pol2-EGFP_MosIR (Nejepinska *et al.*, 2012), pol2-EGFP_Lin28abIR (Flemr, Moravec, Libova, Sedlacek, & Svoboda, 2014) and pJET_huH1 \leftrightarrow U6^{dsRNA} plasmids to generate my dsRNA expressing constructs. Also, pcDNA3.1 (-) (Invitrogen) was used for preparation CMV or SV40 promotor containing plasmids. The list of all the cloning and sequencing primers can be found in supplementary materials, tables S1 and S2 respectively.

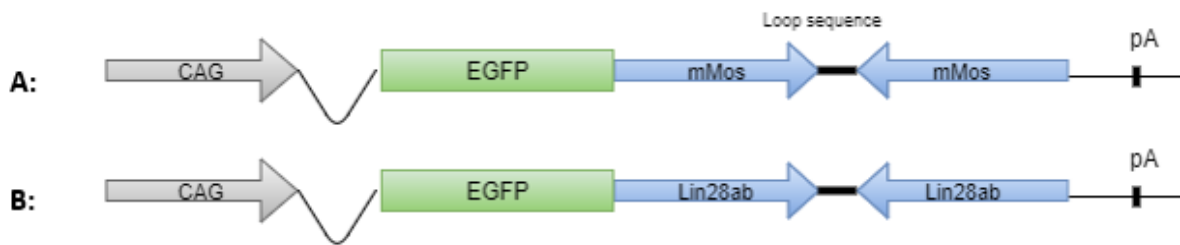


Fig. 3 A schematic representation of A: pol2-EGFP_MosIR plasmid and B: pol2-EGFP_Lin28abIR plasmid. Only the part relevant to this thesis is shown.

3.1.3.1. Cloning *Mos* and *Lin28a* sequence into convergent huH1 \leftrightarrow U6 plasmid

MosIR sequence of pol2-EGFP_MosIR plasmid and *Lin28a* sequence of pol2-EGFP_Lin28abIR plasmid were amplified by PCR using primer pair 1 and 2 respectively. PCR products were cloned separately into a pJET1.2 cloning vector (Thermo Scientific), and the presence of the inserts was controlled by PCR using plasmid pair 3. Positive samples were sequenced with pJET1.2 Forward Sequencing Primer (labelled as sequencing primer 1) (Thermo Scientific).

Both acquired plasmids plasmid were cut with HindIII, NotI^{HF} and ClaI restriction enzymes in NEB2.1 buffer (New England Biolabs) and pJET_huH1 \leftrightarrow U6^{dsRNA} was digested with HindIII and NotI^{HF} enzymes. Required fragments were separately cloned in between huH1 and U6 sequences of pJET_huH1 \leftrightarrow U6^{dsRNA} vector. Colonies were PCR screened by primer pair 4 and both pol3 \rightarrow Mos \leftarrow pol3 (Fig. 4A) and pol3 \rightarrow Lin28 \leftarrow pol3 (Fig. 4B) were sent for sequencing with sequencing primer 2.

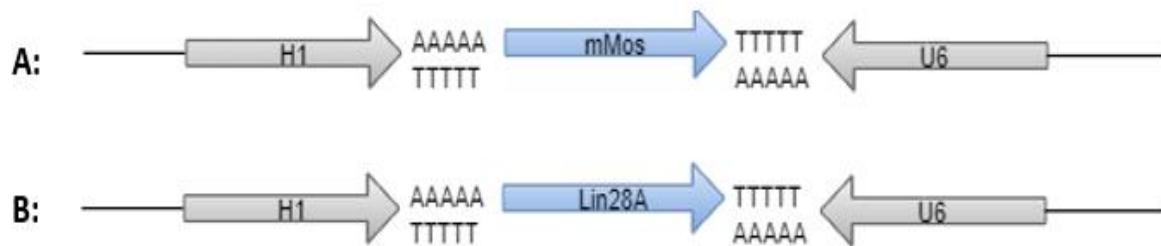


Fig. 4 A schematic representation of A: $\text{pol3} \rightarrow \text{Mos} \leftarrow \text{pol3}$ and B: $\text{pol3} \rightarrow \text{Lin28} \leftarrow \text{pol3}$ plasmids. dsRNA structures were generated by convergent transcription. Stretch of 5 Ts on both sides of a transcribed sequence terminates the transcription, generating dsRNA with blunt ends. Only the part relevant to this thesis is shown.

3.1.3.2. Cloning *Mos* and *Lin28a* sequence in both orientations after U6 promoter

The sequence of the U6 promoter of pJET_huH1 \leftrightarrow U6^{dsRNA} plasmid was amplified by primer pair 5 and PCR product was ligated into the pJET1.2 vector. Colony PCR was performed with primer pair 3, and positive samples were sent for sequencing with pJET1.2 Forward Sequencing Primer. The plasmid was then digested with MfeI and PstI restriction enzymes in NEB1.1 buffer (New England Biolabs) discarding both N- and C- terminal parts of the *eco47IR* lethal gene as well as their promoter. PCR screening was performed with primer pair 6 and positive plasmids were checked by sequencing with sequencing primer 3. This construct is referred to as pJET_U6^{NotI}.

MosIR and *Lin28aIR* sequences of *pol2*-EGFP_*MosIR* and *pol2*-EGFP_*Lin28aIR* plasmids were amplified using primer pairs 7 and 8 respectively. Products of the amplifications were cloned separately into four pJET1.2 plasmids, each product in both orientations, sense (S) and antisense (A). The colony PCR was performed using primer pair 3. Constructs pJET_*Mos*^{NotI} (S), pJET_*Mos*^{NotI} (A), pJET_*Lin28a*^{NotI} (S), and pJET_*Lin28a*^{NotI} (A) were sequenced using pJET1.2 Forward Sequencing Primer.

Plasmids generated by this cloning together with pJET_U6^{NotI} were digested by Not^{HF} restriction enzyme in CutSmart Buffer (New England Biolabs). Required fragments were cloned into pJET_U6^{NotI}. *pol3*_Mos (S) was screened with primer pair 9, *pol3*_Mos (A) with primer pair 10, *pol3*_Lin28a (S) with primer pair 11 and *pol3*_Lin28a (A) with pair 12. Positive constructs of were sequenced with sequencing primer 3. These constructs were used in pairs to generate individual strands of dsRNA from separate plasmids (Fig. 5).

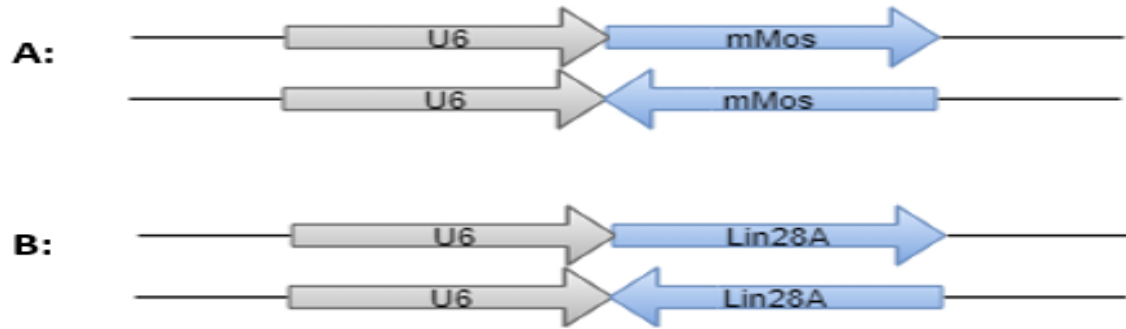


Fig. 5 A schematic representation of the two plasmid pairs containing polymerase III promoters. Each plasmid of an individual pair produced one strand of a dsRNA structure A: pol3_Mos (S) and pol3_Mos (A). B: pol3_Lin28a (S) and pol3_Lin28a (A). Only the part relevant to this thesis is shown.

3.1.3.3. Cloning *Mos* and *Lin28a* sequence in both orientations after CMV promoter

Plasmids pJET_Mos^{NotI} (S), pJET_Mos^{NotI} (A), pJET_Lin28a^{NotI} (S), pJET_Lin28a^{NotI} (A) and pcDNA3.1 (-) were digested by a NotI^{HF} enzyme in the CutSmart buffer. Necessary fragments (*Mos* and *Lin28a* sequences in both orientations) were ligated into four pcDNA3.1 (-) plasmids, and colony PCR was performed using primer pairs 13, 14, 15, 16 for pol2_Mos (S), pol2_Mos (A), pol2_Lin28a (S) and pol2_invLin28a (A) respectively. All construct generated by this cloning were sequenced using sequencing primer 4. These constructs were also used in pairs to generate individual strands of dsRNA from separate plasmids (Fig. 6).

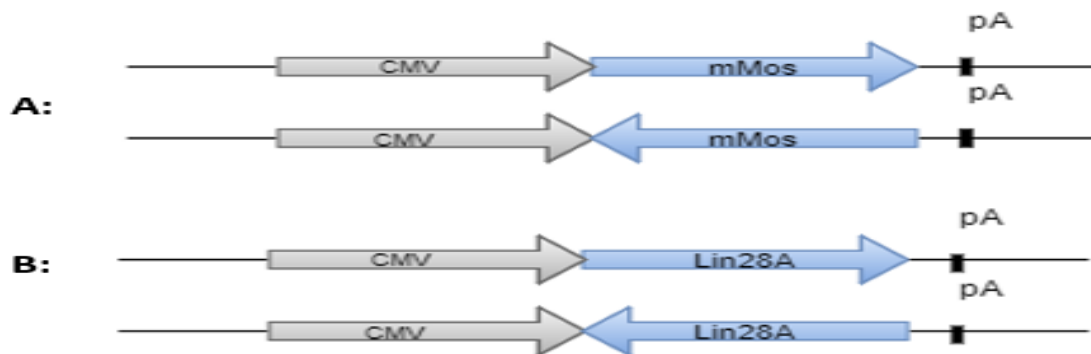


Fig. 6 A schematic representation of the two plasmid pairs containing polymerase II promoters. Each plasmid of an individual pair produced one strand of a dsRNA structure A: pol2_Mos (S) and pol2_Mos (A). B: pol2_Lin28a (S) and pol2_Lin28a (A). Only the part relevant to this thesis is shown.

3.1.3.4. Cloning *MosIR* and *Lin28IR* sequence downstream of U6 promoter

For the generation of pol3_MosIR plasmid (Fig. 7), the U6 sequence of huH1 \leftrightarrow U6^{dsRNA} construct was amplified by Q5 High-Fidelity DNA polymerase with primer pair 17. The reaction was performed in the volume of 50 μ l (10 μ l 5x Q5 Reaction Buffer, 1 μ l 10 mM dNTP, 2.5 μ l 10 μ M forward primer, 2.5 μ l 10 μ M reverse primer, 500 ng of template DNA, 12 μ l 5x Q5 High GC Enhancer, 0.5 μ l Q5 High-Fidelity DNA Polymerase, Nuclease-free water up to 50 μ l).

The thermocycler was set up as follows: Initial denaturation for 3 minutes at 98°C, 30 cycles (10 seconds at 98°C, 20 seconds at 70°C, 15 seconds at 72°C) and final extension for 2 minutes at 72°C. Samples were loaded on gel extracted and ligated into the pJET1.2 cloning vector. The presence of U6 insert was checked by colony PCR using primer pair 3 and by sequencing with pJET1.2 Forward Sequencing Primer.

Lethal *eco47IR* sequences of the pJET1.2 vector were cut out by MfeI and PstI restriction enzymes (New England Biolabs) in NEB 1.1 buffer. U6 promoter was ligated back into the vector; colony PCR was performed with primer pair 18 and plasmid was sent for sequencing with sequencing primer 3. This construct is referred to as pJET_U6^{KpnI}.

Generated construct and pol2-EGFP_MosIR plasmid were digested with KpnI restriction enzyme in KpnI buffer. For this digestion SAP was added into the restriction mix, and the reaction was incubated at 37°C for 24 hours. *MosIR* sequence was ligated into the vector in vector: insert molar ratio 1:6, colony PCR was performed using 5 % of dimethyl sulfoxide (DMSO) and primer pair 19. The screening by restriction digest was performed using the ApalI enzyme in the CutSmart buffer. Two reactions were prepared to sequence the inverted repeat from both ends. First sequencing was carried out with sequencing primer 4, second with sequencing primer 6 and both reactions contained 5 % DMSO.

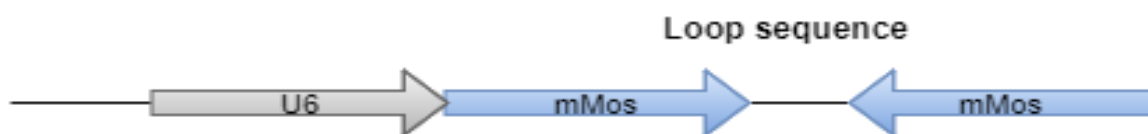


Fig. 7 A schematic representation of pol3_MosIR plasmid. mMos hairpin structure was transcribed by polymerase III from promoter U6. Only the part relevant to this thesis is shown.

Procedure for the generation of pol3_Lin28aIR plasmid (Fig. 8) was similar with following alterations: The U6 sequence of huH1 \leftrightarrow U6^{dsRNA} construct was amplified using primer pair 20. For the second digestion pol2-EGFP_Lin28abIR plasmid was used instead of pol2-EGFP_MosIR. pJET_U6^{HindIII} (analogous to pJET_U6^{KpnI}) was digested with HindIII in NEB2.1 buffer, while pol2-EGFP_Lin28abIR was digested with both HindIII and KpnI in NEB2.1 buffer. The *Lin28aIR* sequence was ligated into digested construct, and positive colonies were screened for by PCR with plasmid pair 21. Screening by restriction digest was performed by BamHI and PvuI in NEB 3.1 buffer (New England Biolabs). Positive samples were digested with BamHI to cut the inverted repeat improving sequencing results. The linearized plasmid was sent for sequencing with the same primers as for the pol3_MosIR.

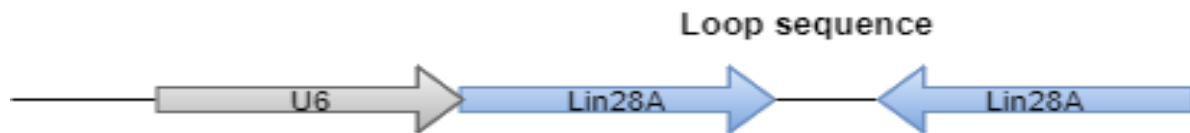


Fig. 8 A schematic representation of pol3_Lin28aIR plasmid. Lin28a hairpin structure was transcribed by polymerase III from promoter U6. Only the part relevant to this thesis is shown.

3.1.3.5. Cloning *MosIR* and *Lin28IR* sequence downstream of CMV promoter

For the preparation of pol2_MosIR plasmid (Fig. 9), pcDNA3.1 (-) and pol2-EGFP_MosIR were digested by KpnI enzyme with NEB1.1 buffer. *MosIR* sequence was ligated into a pcDNA plasmid, and PCR screening was performed in the presence of 5 % DMSO with primer pair 22. Samples were screened by restriction digest with ApaI in CutSmart buffer, and positive samples were sequenced in separate reactions with 5 % DMSO, with sequencing primers 4 and 6.



Fig. 9 A schematic representation of pol2_MosIR plasmid. mMos hairpin structure was transcribed from polymerase II promoter CMV. Only the part relevant to this thesis is shown.

Pol2_Lin28aIR (Fig. 10) was prepared by digestion of pol2-EGFP_Lin28aIR by HindIII and KpnI enzymes. Colony PCR was performed with 5 % DMSO and primer pair 23. Samples were further screened for by restriction digest with BamHI. Positive samples were linearized by BamHI digestion and sequenced with the same primers as for the pol2_MosIR.

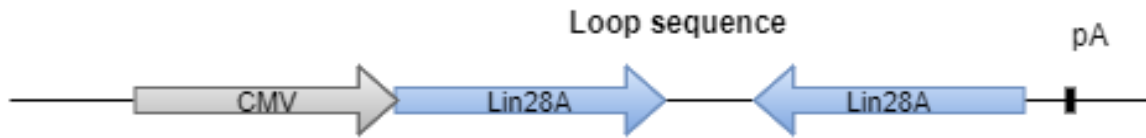


Fig. 10 A schematic representation of pol2_Lin28aIR plasmid. Lin28a hairpin structure was transcribed by polymerase II from promoter CMV. Only the part relevant to this thesis is shown.

We prepared two additional reporter expressing plasmids that were used for the detection of RNA degradation on RNA level using qPCR. These reporters carried chimeric *Mos-Lin28a* sequence that is controlled by U6 (Fig. 11) or SV40 (Fig. 12) promoter.

3.1.3.6. Cloning *Lin28a-Mos* chimeric sequence downstream of U6 promoter

For the production of U6 controlled reporter, *Mos* sequence of pol3_Mos (S) was amplified by primer pair 24 and *Lin28a* sequence of pol3_Lin28a (S) was amplified by primer pair 25. Both sequences were separately ligated into the pJET1.2 plasmid. The screening was performed by colony PCR using primer pairs 26 and 27 for *Mos* and *Lin28a* sequence containing constructs respectively. Then, both constructs were sequenced with pJET1.2 Forward Sequencing Primer. The plasmids generated in this cloning are referred to as pJET_Mos^{PstI-HindIII} and pJET_Lin28a^{NotI-HindIII-NotI}. These plasmids were produced by Radek Malík.

pJET_Lin28a^{NotI-HindIII-NotI} and pol3_Lin28a (S) plasmid were both digested with EagI^{HF} restriction enzyme (New England Biolabs) in CutSmart buffer. *Lin28a* sequence of pol3_Lin28a was replaced by *Lin28a* sequence from pJET_Lin28a^{NotI-HindIII-NotI} plasmid, gaining restriction sites for further cloning. This construct, pJET_Lin28a^{NotI-HindIII-PstI}, was screened for by primer pair 28. Positive samples were sequenced with sequencing primer 3.

In the next step pJET_Mos^{PstI-HindIII} and pJET_Lin28a^{NotI-HindIII-PstI} plasmids were digested with PstI, and HindIII restriction enzymes and *Mos* sequence was ligated upstream of

the *Lin28a* sequence. Colonies were screened by primer pair 29 and positives were sent for sequencing with sequencing primer 3.



Fig. 11 A schematic representation of *pol3_Lin28a-Mos* plasmid used as a reporter in qPCR experiments. Both target sequences on one reporter eliminated the variability caused by differences in reporter performance. Unique *Lin28a-Mos* chimeric sequence allowed for specific amplification and co-transfection with reporters that contained either *Lin28ab* or *Mos* sequence. Only the part relevant to this thesis is shown.

3.1.3.7. Cloning *Mos-Lin28a* chimeric sequence downstream of SV40 promoter

pol3_Mos-Lin28a and *pol2-EGFP_Lin28abIR* plasmids were digested by *EagI*^{HF} restriction enzymes, and *Mos-Lin28a* sequence was ligated downstream of the SV40 promoter, discarding EGFP sequence in the process. Colony PCR was performed with plasmid pair 30, and positive samples were sequenced with sequencing primer 5.

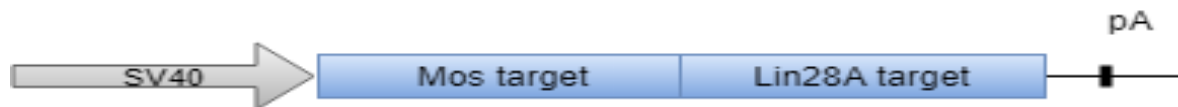


Fig. 12 A schematic representation of *pol2_Mos-Lin28a* plasmid used as a reporter in qPCR experiments. Similarly, chimeric cognate sequences on one reporter were generated in order to eliminate the variability caused by differences in reporter performance. Unique *Mos-Lin28a* sequence allowed for specific amplification and co-transfection with reporters that contained either *Lin28ab* or *Mos* sequence. Only the part relevant to this thesis is shown.

3.2. Cell culture

For the cultivation of mouse NIH 3T3 cells, I used Dulbecco's modified Eagle's medium (Sigma) with streptomycin, penicillin and 10 % fetal calf serum (Sigma). Cells were plated on 24 well plate at density 80,000 cells per well and cultivated for 24 hours at 37 °C and 5 % CO₂. Transfection was conducted in triplicates using Lipofectamine 3000 (Thermo Scientific) according to the manufacturer's instructions. The medium has been changed 24 hours after transfection and cells were collected 48 hours post transfection.

Mouse embryonic stem cells were plated on 0.1% gelatine-coated 24 well-plate at density 80,000 cell/well. Cells were cultivated in Dulbecco's modified Eagle medium (Sigma) with 10% fetal calf serum, 1x NEAA 1 mM sodium pyruvate, penicillin, 2mM L-glutamine,

non-essential AA, 50 μ M 2-mercaptoethanol, leukaemia inhibitory factor, 3 μ M CHIR99021, 1 μ M PD0325901 for 24 hours at 37°C and 5 % CO₂. Cells were transfected in triplicates 24 hours later using Lipofectamine 3000. The medium has been changed 24 hours after transfection, and the cells were collected 48 hours post transfection.

3.3. Dual-Luciferase assay

Luciferase activity was measured using Dual-Luciferase Reporter Assay (Promega) and Modulus Microplate reader (Turner Biosystems). 48 hours after transfection, were cells washed with 500 μ l of PBS and collected into 200 μ l of PPBT lysis buffer (0.2 % v/v Triton x-100, 100mM potassium phosphate buffer, pH7.8). Samples were centrifuged for 30 seconds at 15,000g, and 3.5 μ l of supernatants were used for luciferase assay. Additionally, 3.5 μ l of supernatants were also used for protein measurement by Bradford method using Bio-Rad Protein Assay (Bio-Rad). Dye Reagent Concentrate was diluted in 1:4 ratio with dH₂O and 200 of the mix was added to each sample. Samples were left at RT for 10 minutes, and total protein concentrations were measured at 595 nm on Thermo Multiskan EX (Thermo Scientific).

3.4. Quantitative PCR

Cells were washed by 500 μ l of PBS and RNA was isolated using RNeasy Plus Mini Kit (Quiagen) according to the manufacturer's instructions. Residual DNA contamination was removed by DNA-free DNA Removal Kit (Ambion). 1 μ l of DNase I was used per sample, and all the samples were incubated at 37°C for 30 minutes. Subsequently, 1 μ g of RNA, 1.5 μ l 100 μ M random hexamers and 1.5 μ l 12.5 mM dNTPs were incubated together at 65°C for 10 minutes. Reverse transcription was conducted using RevertAid Reverse Transcriptase (Thermo Scientific). cDNA was diluted in 2:1 ration and 2 μ l of each sample were used for qPCR. Measurements were performed on LC480 (Roche) and LC480II (Roche) using 2x maxima SYBR Green qPCR Master Mix (Fermentas) and 4 μ M of forward and reverse primers. The list of primers used for qPCR can be found in supplementary materials, table S3.

4. RESULTS

The aim of this project was to compare efficiency of different dsRNA molecules in the induction of RNAi-like effects. dsRNA were produced ectopically from transfected plasmid constructs. The measurements were conducted in various mouse cell lines in order to determine the influence of PKR, TARBP2 and different Dicer isoforms. I analysed these effects on protein levels using dual-luciferase assay as well as on the mRNA levels by qPCR.

4.1. Production of dsRNA

One of the questions we wanted to answer was whether bluntly ended dsRNA structures are better for the induction of RNAi-like effect in mammalian cells. As mentioned before, pre-miRNAs, which are common Dicer substrate in mammalian somatic cells, contain 2-nt overhang created by Drosha processing. Therefore we expected the dsRNA substrates with blunt ends to be more efficient than their counterparts with long overhangs. We have utilised differences in the expression between RNA polymerase II (polII) and RNA polymerase III (polIII) to produce dsRNA with overhangs and blunt ends respectively. Transcripts produced from PolII promoter, CMV, should contain cap at the 5' end and polyA-tail at the 3' - end. As mentioned above, it has been proposed that long overhangs have an inhibitory effect on Dicer processing . Therefore, dsRNA produced upon binding of anti-parallel strands produced by polII should contain overhangs at its termini. On the other hand, transcripts produced from PolIII promoter, U6, have triphosphate at the 5' end and more defined 3' end that is not polyadenylated. Thus, we assumed that dsRNA produced from this promoter would have blunt ends.

We used these promoters in different orientations with respect to the transcribed regions to mimic different mechanism of the production of endogenous dsRNA (Fig. 13).

Two sets of dsRNA expressing plasmids were prepared. The first set expressed dsRNA with partial *Mos* gene sequence. This gene encodes dormant maternal mRNA, which is stored in oocyte until the resumption of meiosis (Gebauer *et al.*, 1997). Its elimination manifests as parthenogenetic activation of ovulated eggs, however, *Mos*^{-/-} animals appear normal otherwise (Hashimoto *et al.*, 1994; Stein, Svoboda, & Schultz, 2003).

The second set contained *Lin28a* sequence. *Lin28a* and its homologue *Lin28b* encode RNA binding protein interfering with Let-7 maturation during the oocyte-to-zygote transition (Viswanathan *et. al.*, 2008). Lin28 proteins also have roles in pluripotency, growth,

development and metabolism summarised in Shyh-Chang and Daley, 2013. However, during early development, no effect on gene expression has been shown upon depletion of Lin28 proteins (Flemr *et al.*, 2014).

The first plasmids I used in my experiments are pol2-EGFP_MosIR and pol2-EGFP_Lin28aIR. Substrates from these constructs are transcribed by PolII from CAG promoter. They contain a synthetic intron followed by EGFP coding sequence upstream of an inverted repeat. Therefore, these transcripts should generate a hairpin structure with the 5' cap and overhangs on both ends.

The second hairpin structure I used in these experiments was produced by pol2_MosIR and pol2_Lin28aIR plasmids. These constructs were produced from CMV promoter by PolII. They do not contain EGFP coding sequence nor the synthetic intron. The substrates generated from these plasmids should have 5' - cap and 3' - end overhang.

The last hairpin structure in my experiments was produced from pol3_MosIR and pol3_Lin28aIR plasmids. These substrates are expressed by PolIII and therefore are expected to have blunt ends with no 5' - cap.

Next, I used pol3 → Mos ← pol3 and pol3 → Lin28a ← pol3 plasmids generating dsRNA substrates by convergent transcription from PolIII promoters. These substrates are also supposed to have blunt ends and no 5' – cap. The rationale behind the production of these constructs was to test whether the induction of RNAi-like effect would be decreased when the dsRNA structure is generated from two molecules compared to the intermolecular hairpin.

I also used a combination of two plasmids for the production of dsRNA. In this system, each plasmid should produce one complementary RNA strand. The first plasmid pair used to generate dsRNA consist of pol3_Mos (S) and pol3_Mos (A). This system is denoted together as trans-SA pol3_Mos for the better orientation in the figures below. The second dsRNA produced this way is expressed from pol3_Lin28a (S) and pol3_invLin28a (A) plasmids, denoted together as trans-SA pol3_Lin28a. The dsRNA substrate produced by each plasmid pair should also have blunt ends. However, the RNAi induction should be even less efficient than of the substrates produced by convergent transcription. The reasoning for this is that the individual strands of a dsRNA will be generated in different loci, reducing the efficiency of dsRNA formation even further.

The last substrates used in my experiments also use a combination of two plasmids for dsRNA generation. The difference is that these substrates are transcribed from CMV promoter by PolIII. The first pair consist of pol2_Mos (S) and pol2_Mos (A) denoted together as trans-SA pol2_Mos. The second pair is made of pol2_Lin28a (S) and pol2_invLin28a (A) plasmids denoted together as trans-SA pol2_Lin28a. We expected these dsRNA substrates to be the least efficient because of (i) low dsRNA formation and (ii) long overhangs at the 3' - end.

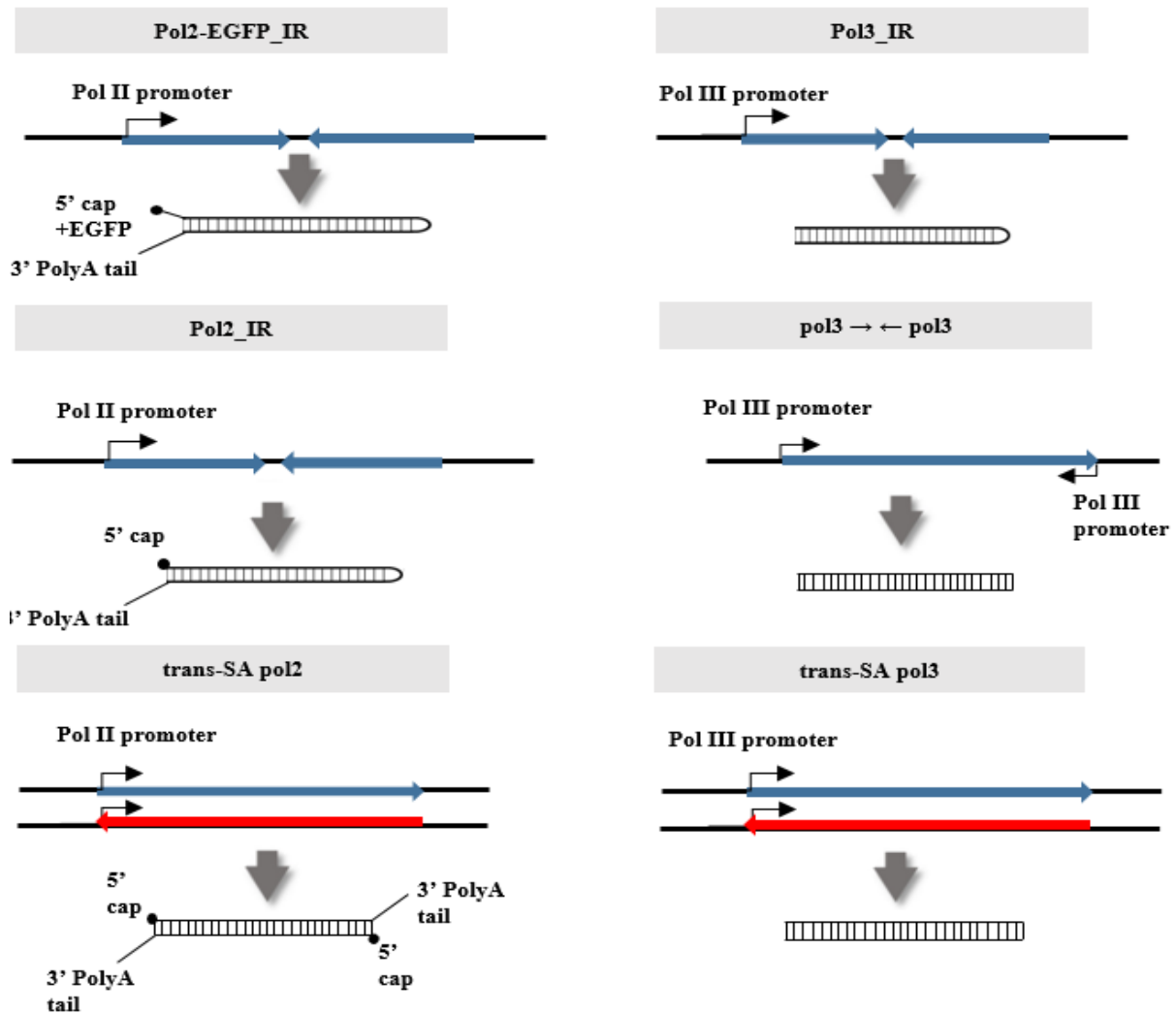


Fig. 13 Representation of different mechanisms of dsRNA production from plasmid constructs utilised in my experiments.

4.2. Detection a reporter degradation using dual-luciferase assays

The luciferase assay is commonly used as a tool for studying gene expression. It is based on measuring light generated by luciferases. These enzymes convert chemical energy released during luciferin oxidation into the luminescent signal. In theory, the strength of a

luciferase signal is proportional to the levels of the luciferase. Therefore, the fusion of a gene of interest with the luciferase allows for determining gene expression changes by measuring differences in the emitted light.

Dual-luciferase assay is a modification of this system, which implements two distinct luciferase enzymes, in our case, firefly and *Renilla*. This system allows for measurement of changes in the expression of each luciferase from a single sample. At first, cells expressing both firefly and *Renilla* luciferases are lysated, and the firefly luciferase reagent is added to the lysate. The luminescent signal produced by the firefly luciferase is measured and then quenched by adding the second reagent. Simultaneously, this second reagent initiates production of the *Renilla* luciferase signal.

In my experiments, I co-transfected cells with plasmids expressing different reporter luciferases. *Renilla* luciferase (RL) reporter contain a sequence that is complementary to either *Mos* or *Lin28* dsRNA derived siRNAs (Fig. 14). *Mos* reporters (RL-*Mos*) were transcribed from plasmid phRL_SV40-*Mos*. *Lin28ab* reporters (RL-*Lin28ab*) were expressed from phRL_SV40-*Lin28ab* plasmid (Fig. 14).

Firefly luciferase (FL) reporter was expressed from plasmid pGL4-SV40 (Fig. 14). This reporter had no cognate sequence and was used to measure sequence non-specific effects. All the measurement obtained from RL activity were normalised to FL.

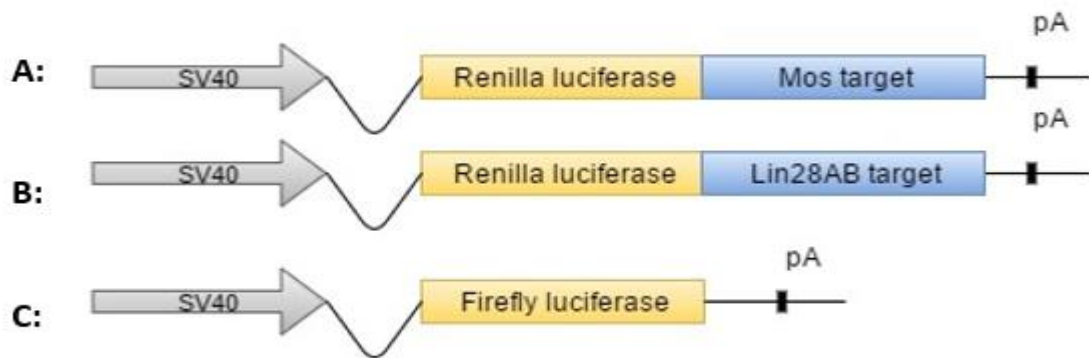


Fig. 14 Schematic representation of the plasmids expressing reporter mRNAs used in Dual-Luciferase Assays. A: phRL_SV40-*Mos* B: phRL_SV40-*Lin28ab* C: pGL4-SV40. Only the part relevant to this thesis is shown.

Each dual-luciferase assay experiment consisted of several samples. Every sample in an individual experiment was co-transfected with (i) two plasmids producing mRNA reporter pair (FL and one of the RL reporters) and (ii) different dsRNA expressing plasmid (or plasmid

pair) (Fig. 16A). Therefore if Dicer was to efficiently process dsRNA substrate into siRNA molecules, they should be targeted onto a cognate sequence of RL mRNA via RISC. This would lead to the cleavage of the RL reporter and should be observable as a downregulation of the RL reporter signal. Both sets of dsRNA expressing plasmids were in every experiment.

One sample was co-transfected with a Pol2_MosMos plasmid (Fig. 15). This plasmid transcribes the same sequence as pol2-EGFP_MosIR plasmid however in head-to-tail repetition. The sequence in this orientation is not capable of dsRNA formation, and therefore it is a good negative control for my experiments. The ration of RL/FL activity in the samples co-transfected with pol2_MosMos plasmid was set to one. The RL/FL ratios of the rest of the samples were normalised to this control.

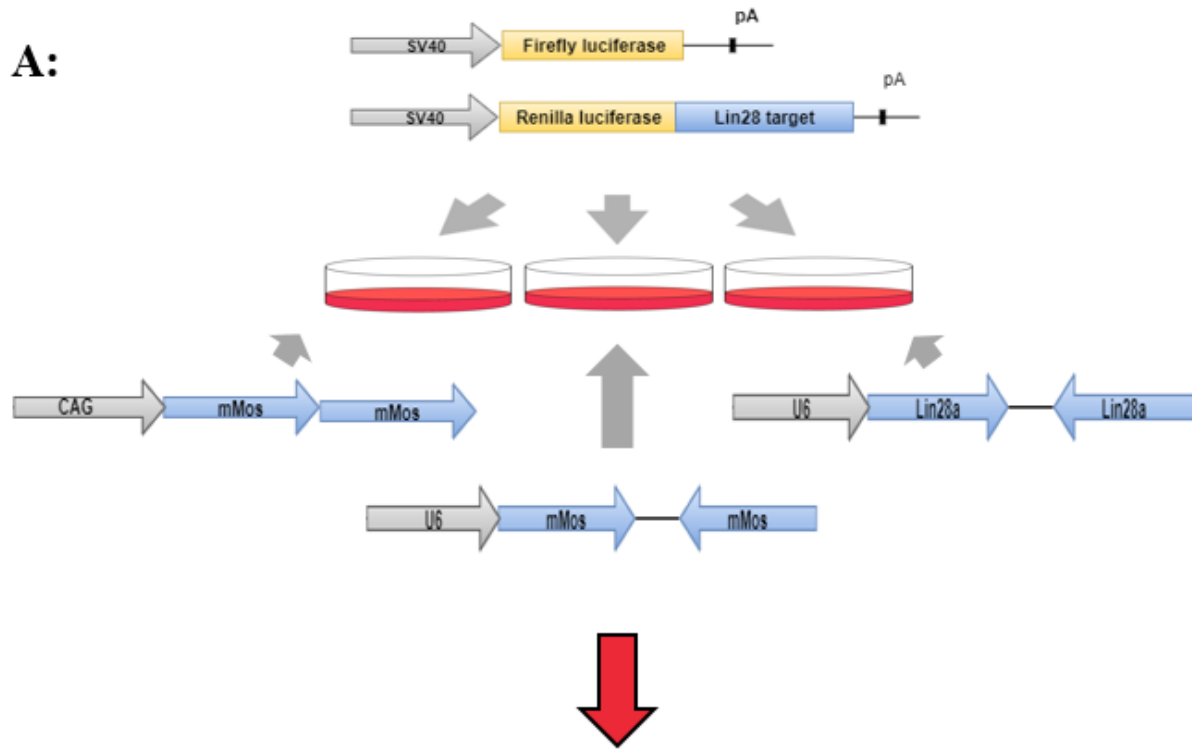


Fig. 15 Schematic representation of the Pol2_MosMos plasmid. The head-to-tail orientation of two Mos repetition will not allow for dsRNA formation. Only the part relevant to this thesis is shown.

This experimental design allowed to compare two types of information. We could determine which structure is the most effective in the induction of the mRNA degradation, by comparing different structures with the same sequence. Moreover, by comparing dsRNA with the same structure but different sequences (*Mos* or *Lin28a*) we could determine whether the effect was indeed sequence-specific or not (Fig. 16B).

Additionally, we could use both sets of dsRNA substrates (*Mos* and *Lin28a*) as samples or control samples depending on which RL reporter was expressed in the individual experiment. Meaning, that when RL reporter containing *Mos* cognate sequence in its mRNA was transcribed, we would expect to see a decrease of normalised RL luciferase signal in those samples that were co-transfected with plasmids encoding *Mos* dsRNA. Samples co-transfected with *Lin28* dsRNA should remain unaffected. On the other hand, when samples were co-transfected with RL reporter containing *Lin28ab* target sequence in its mRNA, the RNAi-like effect should be detectable only in samples co-transfected with *Lin28* dsRNA while *Mos* dsRNA samples should not be affected. Off note, all the measurements were corrected using total protein amount in sample lysate.

A:



B:

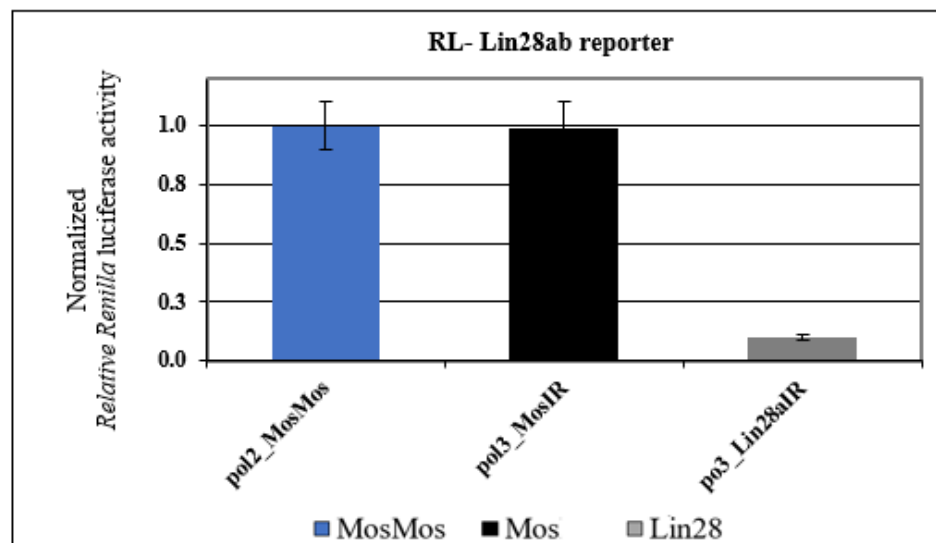


Fig. 16 The depiction of dual-luciferase assay experiment. A: Illustration of a co-transfection of three samples. Each sample was co-transfected with two reporter expressing plasmids and one substrate expressing plasmid. B: A fictional example of Normalised Relative *Renilla* activity. The ration of RL/FL activity in pol2_MosMos control sample was set to one. The rest of the samples were normalised to pol2_MosMos. The figure shows a substantial decrease in normalised relative *Renilla* luciferase activity in samples co-transfected with pol3_Lin28aIR compared to pol3_MosIR suggesting that its degradation was caused by RNAi-like effect. It should be noted that this system is not able to detect a decrease of the RL/FL signal under 20%. The main reason for this is unequal co-transfection. Separate experiments conducted in our lab suggest that around 20% of the cells used in these co-transfection experiments are not transfected with all the plasmids. The cells co-transfected only by a reporter expressing plasmid and no substrate expressing plasmid are responsible for the background.

4.2.1. RNAi in Dicer^S expressing cells

Initial experiments were performed in NIH 3T3 mouse fibroblast cells. This Dicer^S expressing cell line has been used in our laboratory in the past to determine the effect of various dsRBPs on RNAi in mammalian cells. However, the induction of RNAi-like degradation using pol2-EGFP_MosIR or pol2-EGFP_Lin28abIR dsRNA hairpins was unsuccessful. Therefore, we reasoned that this cell line was a good starting point in determining whether newly prepared dsRNA substrates are more efficient.

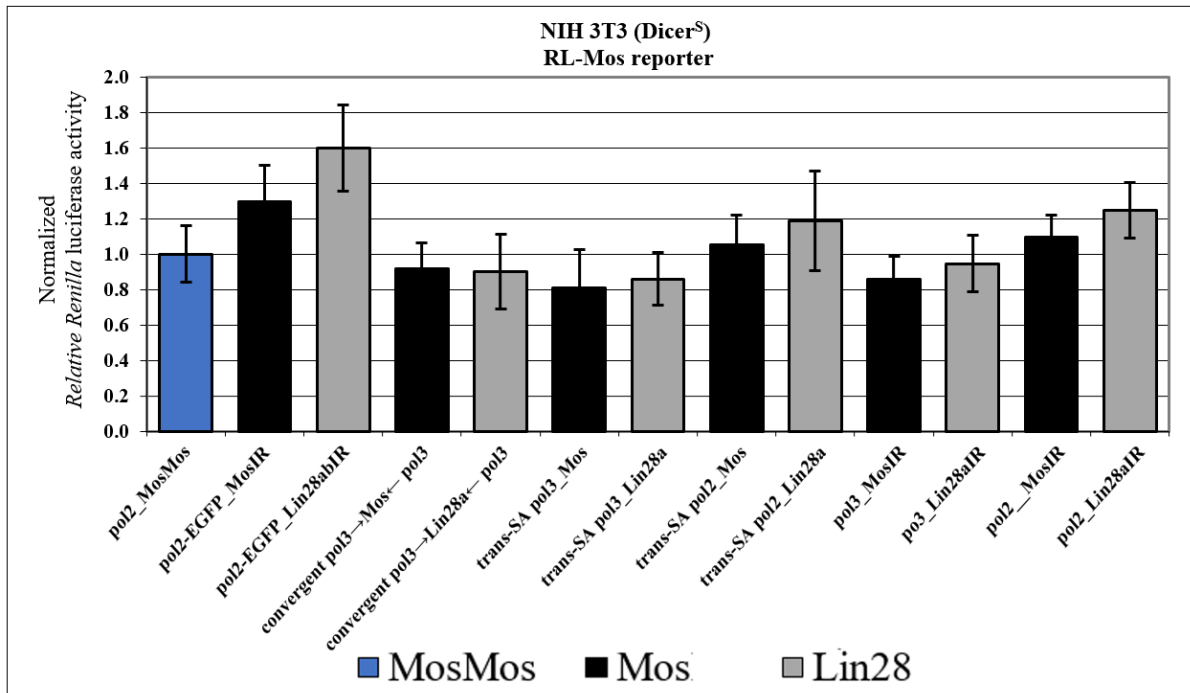


Fig. 17 Normalised Relative *Renilla* luciferase activity in NIH 3T3 cells using RL-Mos reporter.

The first experiment in NIH 3T3 cell line has been conducted with RL-Mos reporter. Sequence dependent degradation would be manifested as lower RL/FL activity in a *Mos* samples compared to Pol2_MosMos control as well as their Lin28 counterpart. However, normalised data did not show substantial difference between RL activity in samples containing *Mos* dsRNA and *Lin28* dsRNA.

Next experiment has been conducted in a similar manner. However, this time NIH 3T3 cell line was co-transfected with RL-Lin28ab reporter instead of RL-Mos reporter. Thus, any sequence-specific degradation of RL target reporter should be apparent by downregulation of RL-Lin28ab reporter activity in samples co-transfected with Lin28 dsRNA.

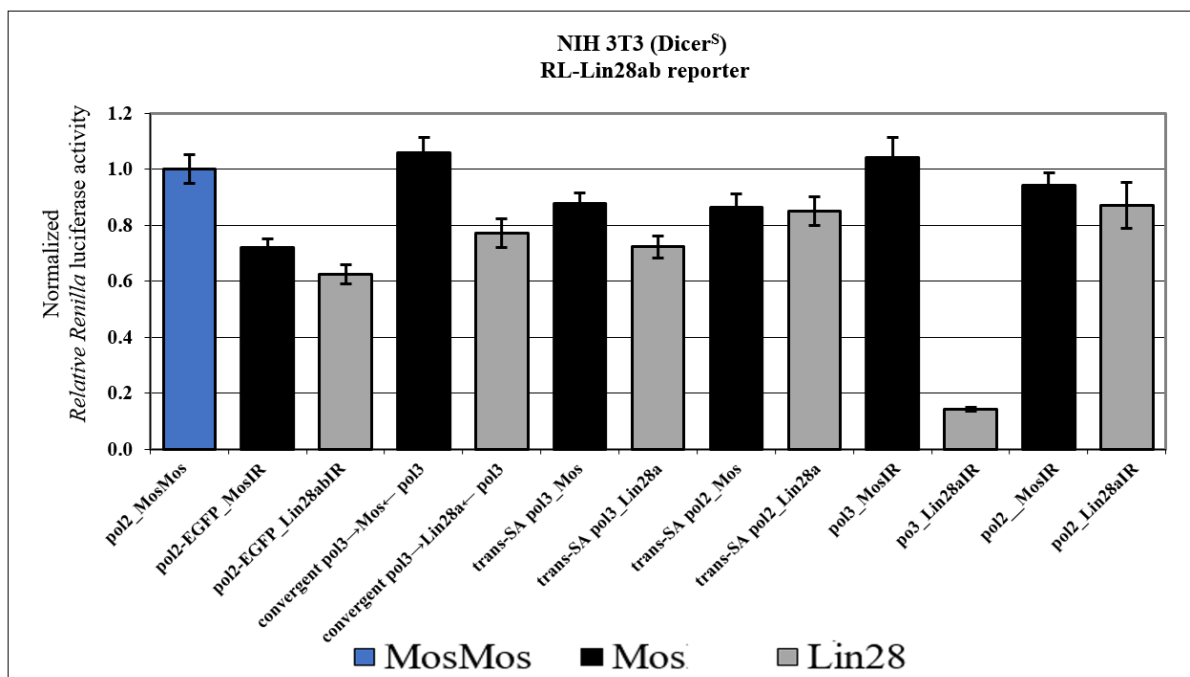


Fig. 18 Normalised Relative *Renilla* luciferase activity in NIH 3T3 cells using RL-Lin28ab reporter.

The results of this experiment, show a specific decrease of RL-Lin28ab reporter signal co-transfected with pol3_Lin28aIR plasmid. This indicates that *Lin28* hairpin substrate transfected by polymerase III from U6 promoter, induced robust sequence-specific degradation of RL-Lin28ab reporter. The outcome of this experiment was surprising because NIH 3T3 cells express Dicer^S isoform. This isoform is not very active, and as mentioned above, previous experiments could not detect any RNAi-like effect. However, as pol3_Lin28aIR substrate is produced from a single molecule, and polymerase III promoter is supposed to generate transcripts with blunt ends, this experiment indicated supported our initial hypothesis. The induction of RNAi-like effect is dependent on the probability of the formation of dsRNA and on the type of ends of a dsRNA molecule. The rest of the substrates were not able to induce sequence-specific degradation. Therefore different cell types have been tried.

4.2.2. RNAi in Dicer^O expressing cells

Out of all the candidates, only one substrate indicated the induction of RNAi-like effect in the experiments with NIH 3T3 cells. Therefore, further experiments were required to determine how RNAi is affected by substrate termini and the likelihood of a dsRNA formation. In the next step, we chose to conduct the experiments in a cell line that has been shown to induce RNAi-like effect by pol2-EGFP_MosIR construct (Flemr *et al.*, 2013). I refer to this

cell line as to JM7. This cell line has Dicer^S gene replaced by the gene encoding more efficient Dicer^O isoform in the endogenous region. As mentioned in the section 1.2.4.2., Dicer^O shows high efficiency in the processing of a dsRNA into siRNAs (Flemr *et al.*, 2013).

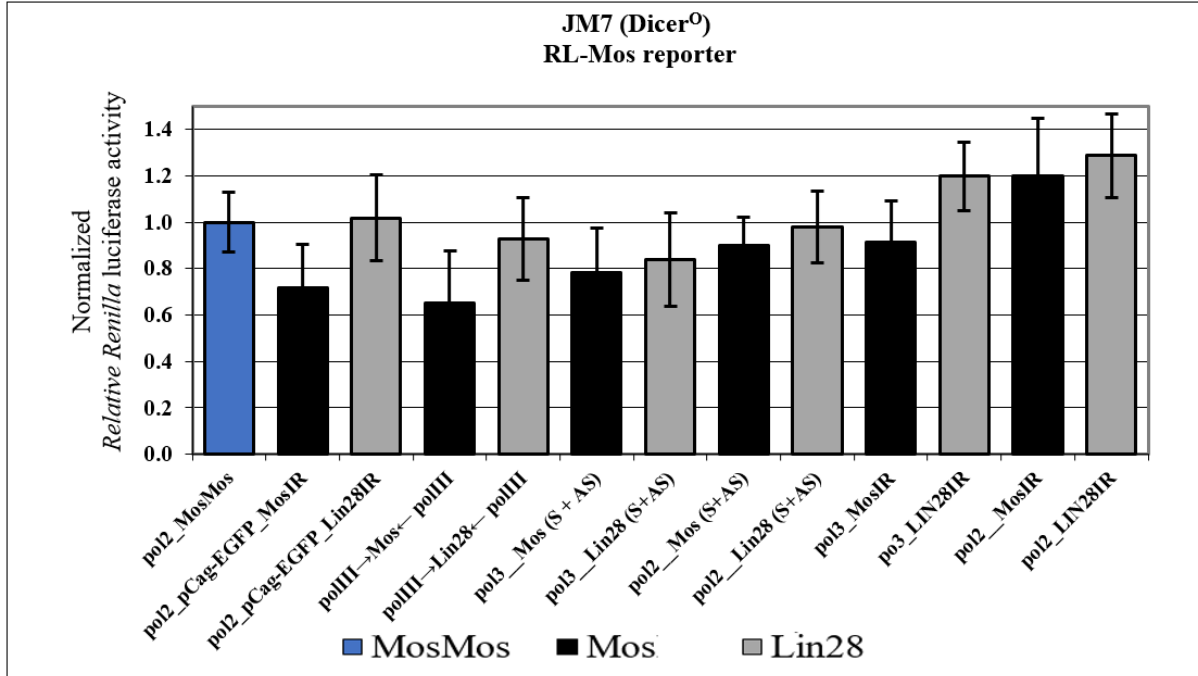


Fig. 19 Normalised Relative *Renilla* luciferase activity in mESC clone JM7 cells using RL-Mos reporter.

The experiments conducted with RL-Mos reporter did show minor improvement compared to what we could detect in NIH 3T3 cells using the same reporter. The levels of RL signal in the samples co-transfected with *Mos* dsRNA expressing plasmids is lower compared to their analogues with *Lin28a* sequence. Namely, pol2-EGFP_MosIR, pol3→Mos←pol3 and pol3_MosIR substrate were able to decrease normalised relative RL reporter signal by ~ 30%.

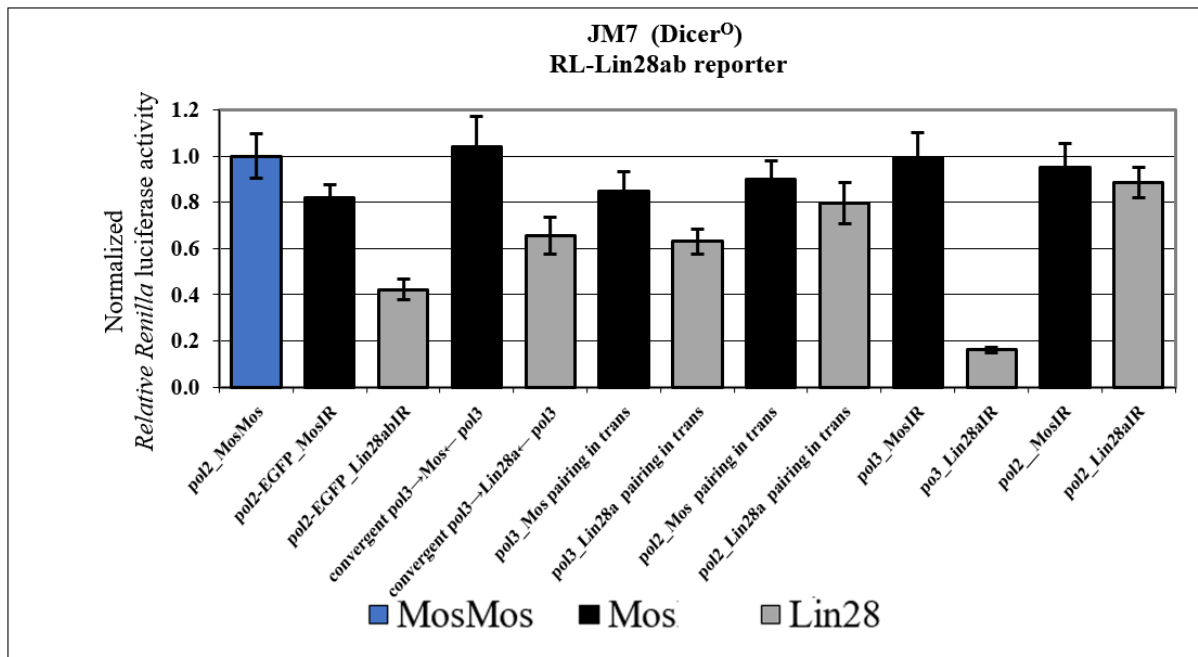


Fig. 20 Normalised Relative *Renilla* luciferase activity in mESC clone JM7 cells using RL-Lin28ab reporter.

Interestingly, the RNAi-like effect became much more apparent when we repeated the experiment with the same cell line using RL-Lin28ab reporter. The results indicate that the most effective substrate was again *Lin28* hairpin produced from U6 promotor by polIII. Samples co-transfected with this substrate showed a decrease of the normalised RL signal by ~ 80%. Pol2-EGFP_Lin28abIR also seemed more effective than its MosIR counterpart in the previous experiments, decreasing normalised RL signal by ~ 40%. In this experiments, we could also see that normalised RL signal decreased in samples co-transfected with pol3→Lin28←pol3 plasmid by ~ 35%. Moreover, samples where dsRNA was formed by *in trans* mechanism from U6 promotor also indicated low RNAi-like effect. Substrates produced from SV40 promotor, without intron or EGFP sequence showed little to no RL/FL signal decrease.

Taken together, these results support our initial hypothesis. The induction of sequence-specific degradation in mammalian cells seems to be dependent on the types of dsRNA ends, preferring blunt ends produced by polymerase III over the 3' - end overhangs from polymerase II. In addition, it also indicates the importance of the efficiency of dsRNA formation. Hairpins produced from a single molecule are the most efficient substrates. The substrates with blunt ends produced by convergent transcription and hairpins with long overhangs are comparably efficient. Third, are the bluntly ended substrates produced from two plasmids. The least

effective seem to be two molecular substrates with long overhangs produced from two plasmids.

4.2.3. Induction of RNAi by dsRNA substrates in PKR deficient cells

In the following experiments, I have tried to determine the effect of PKR on the efficiency of the induction of RNAi in mammalian cells. As mentioned above, it has been proposed that the RNAi pathway in mammalian somatic cells might be masked by more potent PKR induced IFN response (summarised in De Veer *et. al.*, 2005; Gantier and Williams, 2007). To test this hypothesis, I have performed dual-luciferase assays in NIH 3T3 Δ PKR and JM7 Δ PKR cell lines. These cells are modified NIH 3T3 and JM7 cells used in the previous experiments. The inhibition of PKR in these cells have been achieved by a deletion of exons 2-5 using CRISPR/Cas9 technology. Residual PKR could be produced from this locus; however, it would lack dsRNA binding domain. Therefore, it should not induce sequence non-specific response triggered by the presence of a dsRNA in the cytoplasm. Our working hypothesis was, that inhibition of PKR in these cell lines should increase the probability of directing dsRNA into a sequence-specific pathway, rather than initiating complete translation shut-down by interferon response.

Co-transfection experiments were performed as described above. I determined normalised *Renilla* luciferase activity in Δ PKR cell lines. Then, I compared these measurements to those obtained in a respective cell line without PKR deletion. The stimulation of RNAi-like effect should manifest itself as a decreased normalised *Renilla* luciferase signal in Δ PKR cells.

Initial experiments were performed in NIH 3T3 Δ PKR cell line expressing Dicer^S. Previous results in NIH 3T3 cells did not show detectable RNAi-like effect for most of the samples with the exception of those co-transfected with pol3_Lin28aIR. Therefore I decided to test whether performing the same experiments in NIH 3T3 Δ PKR cells would increase the ability of other substrates to induce sequence-specific degradation. However, the results of dual-luciferase assays did not show any substantial decrease of RL/FL signal compared to NIH 3T3 cell (Fig. 21). Moreover, experiments in which samples were co-transfected with plasmid producing RL-Mos reporter also failed to show induction of RNAi (data not shown). This indicates that the deletion of dsRBD of PKR does not stimulate RNAi-like effect in Dicer^S expressing NIH 3T3 cells.

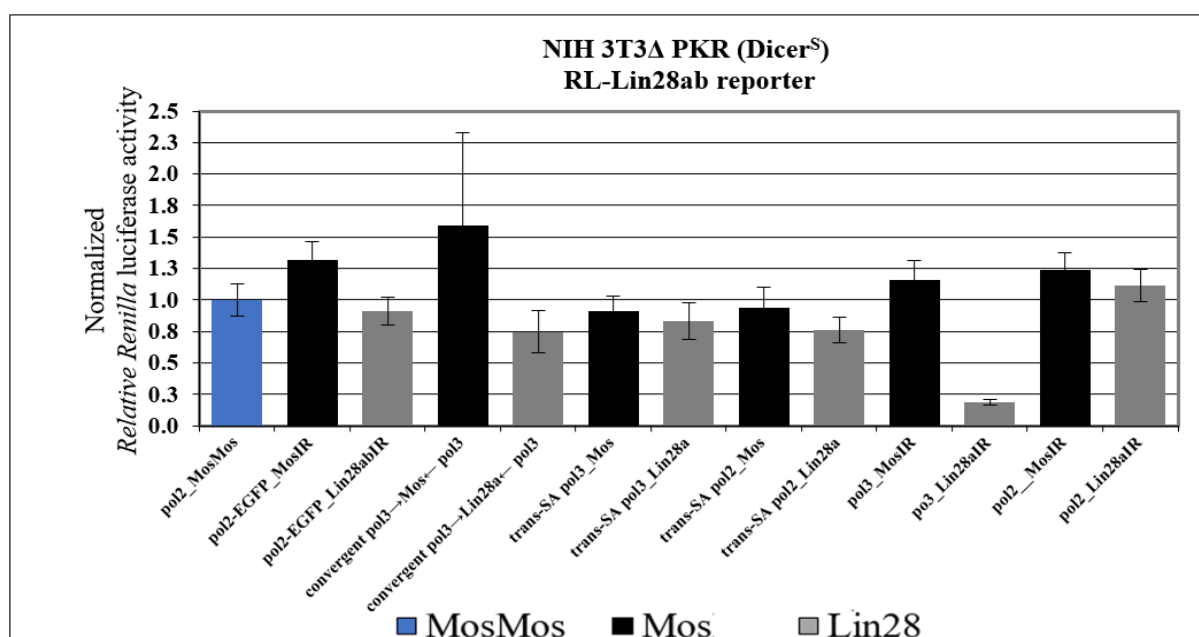


Fig. 21 Normalised Relative *Renilla* luciferase activity in NIH 3T3 ΔPKR cells using RL-Lin28ab reporter.

I performed similar experiments in JM7 ΔPKR cell line in order to investigate the effect of PKR on RNAi in Dicer^O expressing cells. Dual-luciferase assays conducted in JM7 cells indicated active RNAi-like effect in samples where RL-Lin28ab reporter was expressed. However, this observation could not be replicated with dsRNA substrates carrying *Mos* sequence in experiments with RL-Mos reporter. Therefore, we decided to test whether inhibition of PKR would increase the efficiency of *Mos* substrates in the induction of RNAi-like effect.

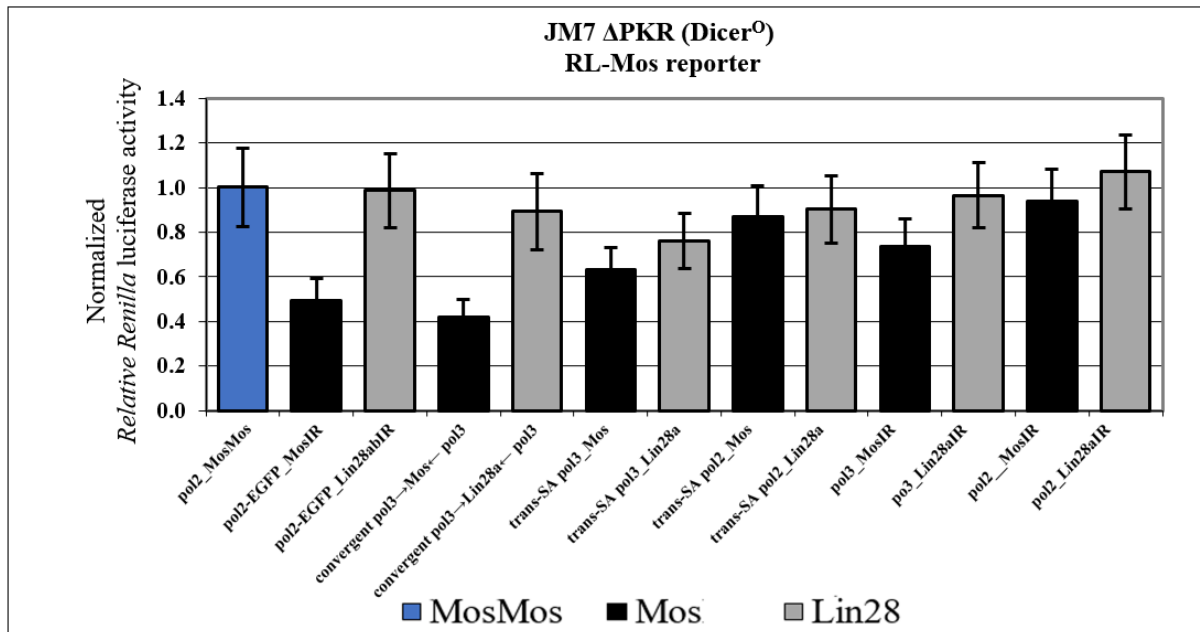


Fig. 22 Normalised Relative *Renilla* luciferase activity in JM7 3T3 Δ PKR cells using RL-Mos reporter.

Nonetheless, experiments carried out with RL-Lin28ab reporter did not generate conclusive results. Pol2-EGFP_Lin28abIR substrate indicated a slight increase in RNAi-like effect; however, these measurements are not very reliable. The reasons for this are the observations showing that inhibition of PKR in JM7 cells caused substantial variations in the generated RL/FL signal, both between triplicates and individual experiments (data not shown). Moreover, the rest of the substrates used in these experiments did not show any improvement in the induction of RNAi-like effect. In fact, samples co-transfected with pol3→Lin28←pol3 plasmid and pol3__Lin28 (S+A) plasmids, appeared to be even less efficient in this cell line compared to the original JM7 cells (data not shown).

Altogether these experiments indicate that inhibition of PKR in mESC expressing Dicer⁰ does not necessarily stimulate the induction of RNAi-like effect.

4.2.4. Induction of RNAi by dsRNA substrates in TARBP2 deficient cells

The second protein I focused on was RLC component, TARBP2. As described above this dsRBP can influence the cellular response to the presence of dsRNA in several ways. First, it has been reported that the association of TARBP2 with Dicer stimulates the cleavage of RNA precursors by Dicer (Soifer *et al.*, 2008). Second, TARBP2 was also showed to inhibit the activation of PKR by dsRNA (Blair *et al.*, 1995; Daher *et al.*, 2009; Park *et al.*, 1994). Therefore, we hypothesized that a deletion of TARBP2 would have opposite effect on RNAi

compared to PKR, causing the cells to lose their ability to direct dsRNA substrates into the RNAi machinery. In my experiments, I have used JM7 Δ TARBP2 cell line. Again, these cells were prepared from JM7 cell line with CRISPR/Cas9 technology. However, in this case, the entire TARBP2 gene was deleted. I conducted dual-luciferase assays and compared results obtained in this cell line with the data acquired in experiments on JM7 cells.

First, I performed experiments using plasmid expressing RL-Mos reporter. This showed that the signal of normalised *Renilla* luciferase obtained in JM7 Δ TARBP2 cells has increased upon TARBP2 deletion (Fig. 23). Samples co-transfected with pol2-EGFP_MosIR, pol3→Mos← pol3 and pol3_MosIR plasmids indicated low RNAi-like effect in the experiments performed on JM7 cells. However, this effect could not be observed in the absence of TARBP2. This suggests that RNAi-like effect was inhibited as we expected. Anyhow, experiments in JM7 cells using RL-Mos reporter did not indicate potent sequence-specific degradation. Therefore, we co-transfected JM7 Δ TARBP2 cells with plasmid producing RL-Lin28ab reporter to fully visualise the effect of TARBP2 deletion.

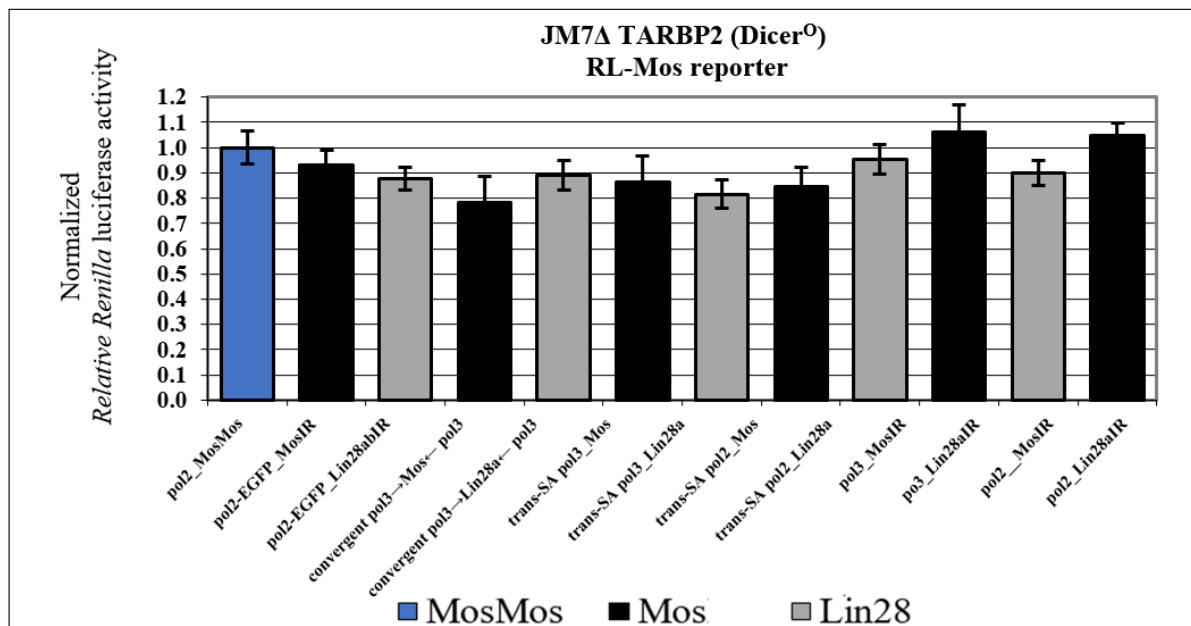


Fig. 23 Normalised Relative *Renilla* luciferase activity in JM 7 Δ TARBP2 cells using RL-Mos reporter.

Experiments conducted with RL-Lin28ab reporter in JM7 cells indicated RNAi-like effect in samples co-transfected with Pol2-EGFP_Lin28abIR plasmid, pol3→Lin28←pol3 plasmid or pol3__Lin28 (S+A) plasmids. As expected, this effect disappeared upon the deletion of TARBP2 in these samples (Fig. 24). The strongest RNAi-like effect we observed in JM7

cells was in a sample co-transfected with pol3_Lin28aIR plasmid, where we could detect robust sequence-specific decrease of RL-Lin28ab reporter signal (~ 80%). Surprisingly, dual-luciferase assays performed in JM7 Δ TARBP2 cell line showed similar outcome in this sample (Fig. 24).

Dual-luciferase assays conducted in these cells showed that RNAi-like effect induced by most of the dsRNA substrates disappears after TARBP2 deletion. However, it is not clear why samples co-transfected with pol3_Lin28aIR plasmid indicated TARBP2 independent RNAi-like effect.

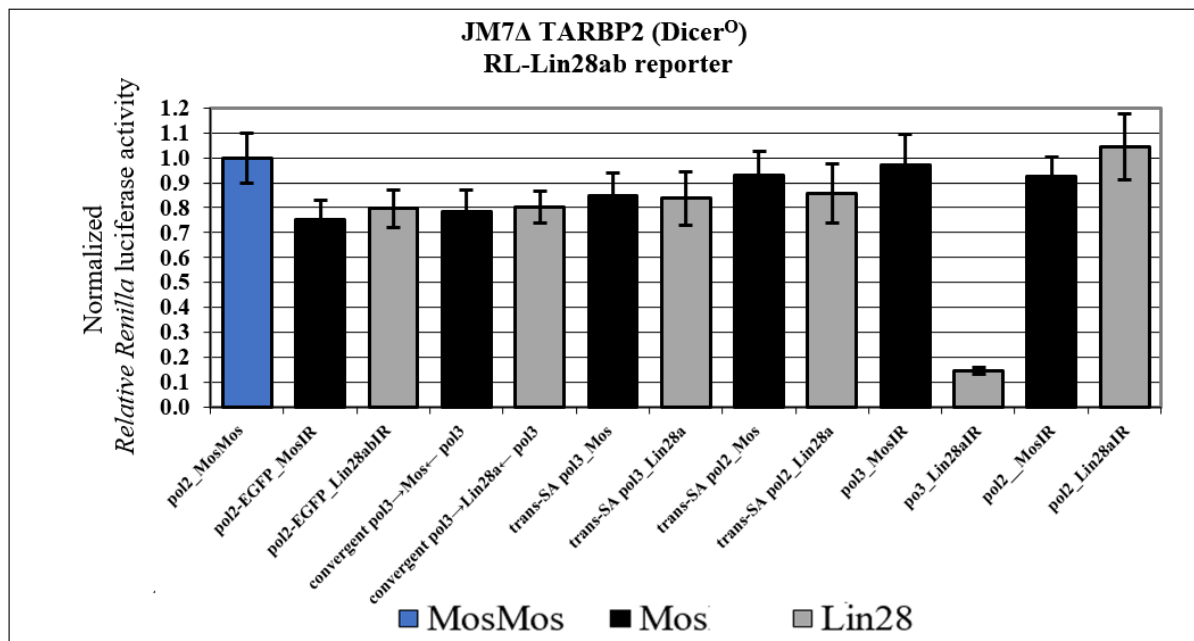


Fig. 24 Normalised Relative *Renilla* luciferase activity in JM7 Δ TARBP2 cells using RL-Lin28ab reporter.

4.2.5. U6-driven hairpins do not trigger sequence non-specific effect

During the evaluation of raw data from dual-luciferase assays conducted in the JM7 Δ TARBP2 cells, we discovered interesting phenomenon. We realised that the signal of FL reporter in the samples co-transfected with pol3_MosIR and pol3_Lin28aIR plasmids is much stronger compared to the samples co-transfected with plasmids producing hairpins from polII promoters (data not shown). We thought that one possible explanation for this might be, that hairpins produced from U6 promoters are not inducing sequence non-specific effect.

Therefore, we conducted another round of experiments in order to investigate this phenomenon. We used cell line termed as RS10. These cells are similar to the JM7 cell line,

meaning that they are also homologous for Dicer^O, with the insertion in the same loci. But apart from JM7 cells, RS10 cell line is not RNAi proficient. It also shows higher amounts of PKR (data not shown) and displays strong sequence non-specific degradation. Therefore, if hairpins transcribed from U6 promoter are resistant against non-specific degradation, the effect should be fairly visible in this cell line.

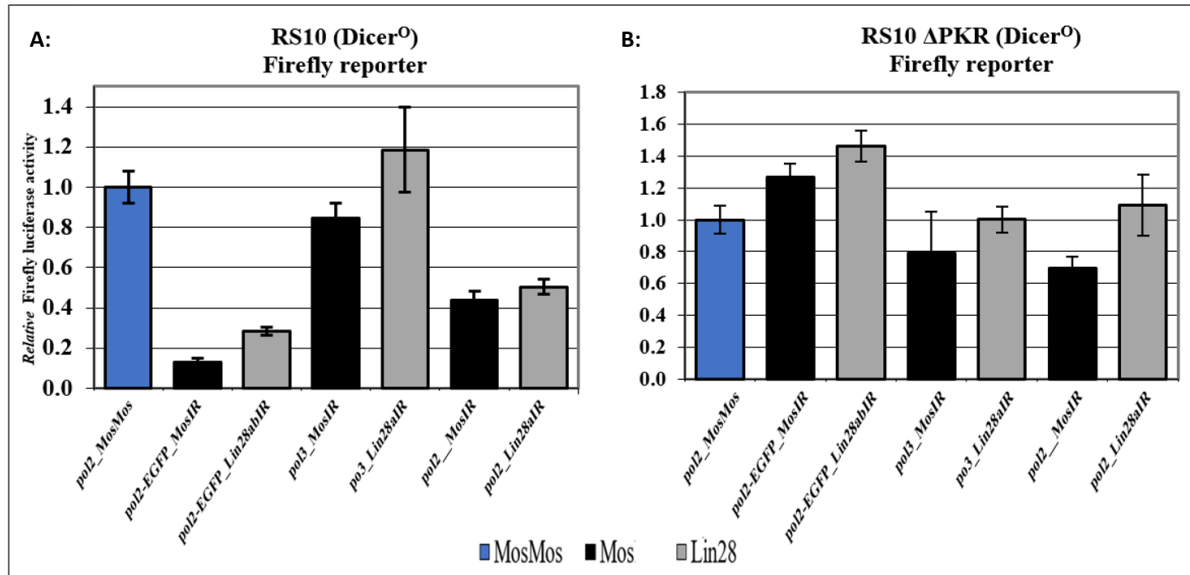


Fig. 25 Relative Firefly luciferase activity in: A RS10 cells B RS10 ΔPKR cells.

The design of the experiments did not change; however, we focused on the FL reporter signal obtained in samples co-transfected with plasmids producing hairpin dsRNA. The results of this experiment showed that FL reporter do not seem to be affected by sequence non-specific degradation in the samples co-transfected with plasmids producing hairpins from U6 promoters (Fig. 25A). On the other hand, substrates produced from promoters for polII displayed substantial non-specific degradation (Fig. 25A).

In the next step, I co-transfected another RS10 cell line. These cells are referred to as RS10 ΔPKR. They contain the same PKR exon 2-5 deletion as JM7 cell line. The goal of this experiment was to examine, how a deficiency of PKR would affect the FL signal in samples with hairpins produced from polII promoters. We speculated, that if the difference in the FL reporter signal is caused by PKR, then the deletion of PKR should cause an increase of FL signal in these samples. Moreover, if the bluntly ended hairpins are not affected by the presence of PKR, the relative levels of FL reporter signal should remain unchanged in samples containing U6 transcribed hairpins.

The results of this experiment show the effect we expected (Fig. 25B). The FL reporter signal in the samples co-transfected with plasmids producing hairpins from polII promoters has increased upon PKR deletion. This effect is best visible with hairpins produced from pol2-EGFP_MosIR and pol2-EGFP_Lin28abIR plasmids. Samples co-transfected with pol2_MosIR and pol2_Lin28aIR seem to be affected as well. However, the increase of the FL signal is not as dramatic. On the other hand, samples co-transfected with pol3_MosIR and pol3_Lin28aIR show very similar levels of relative FL-reporter signal in both cell lines.

Taken together, these data suggest that hairpin substrates produced from U6 promoter are not affected by sequence non-specific degradation mediated by PKR.

4.3. Detection of RNAi-like effect by qPCR

The results obtained by dual-luciferase assays indicated that substrates with *Mos* sequence were less effective in the induction of RNAi-like effect compared to the analogous substrates with *Lin28* sequence. We have prepared new reporter expressing plasmids, to rule out the possibility that this difference is caused by ineffective RL-Mos reporter. The newly prepared plasmids expressed reporters with a chimeric sequence consisting of *Lin28a* and *Mos* cognate sequences. We used either U6 or SV40 promoters for their expression (Fig. 11 and Fig. 12 respectively). The reporters were designed without synthetic intron and more importantly without *Renilla* luciferase coding sequence. Therefore, we utilised qPCR for the detection of RNAi-like effect on mRNA levels. The mRNA levels of each reporter were measured separately by specific primers. Thus, we could co-transfect each sample with three reporter expressing plasmids and one dsRNA expressing plasmid. We used Beta-2-Microglobulin and POU Class 5 Homeobox 1 as housekeeping genes. All measurements were normalised to non-transfected cells. Reporters carrying cognate sequences were normalised to the mRNA levels of FL reporter to determine the sequence-specific effect.

We focused our attention on hairpins because dual-luciferase assays indicated only limited induction of RNAi-like effect by other dsRNA substrates. Furthermore, I have used two additional hairpins in these experiments. First was transcribed from pol2-EGFP_RlucIR plasmid. This plasmid expresses hairpin substrate with a sequence complementary to the *Renilla* luciferase sequence of RL reporter. Second substrate was transcribed from pol2-EGFP_AmpIR plasmid. This transcript forms a hairpin structure however, it does not contain a sequence complementary to any of our reporters. Therefore, we implemented it in our experiments as a negative control.

4.3.1. CAG-driven *Renilla* reporter

The first qPCR experiment was conducted in JM7 cell line, using chimeric reporter expressed from pol3_Lin28a-Mos plasmid as well as RL-Lin28ab and FL reporters. The reason for using JM7 cell line was that dual-luciferase assays conducted in these cells indicated robust RNAi-like effect when using RL-Lin28ab reporter. Therefore, we wanted to determine whether we can observe a similar effect on mRNA level.

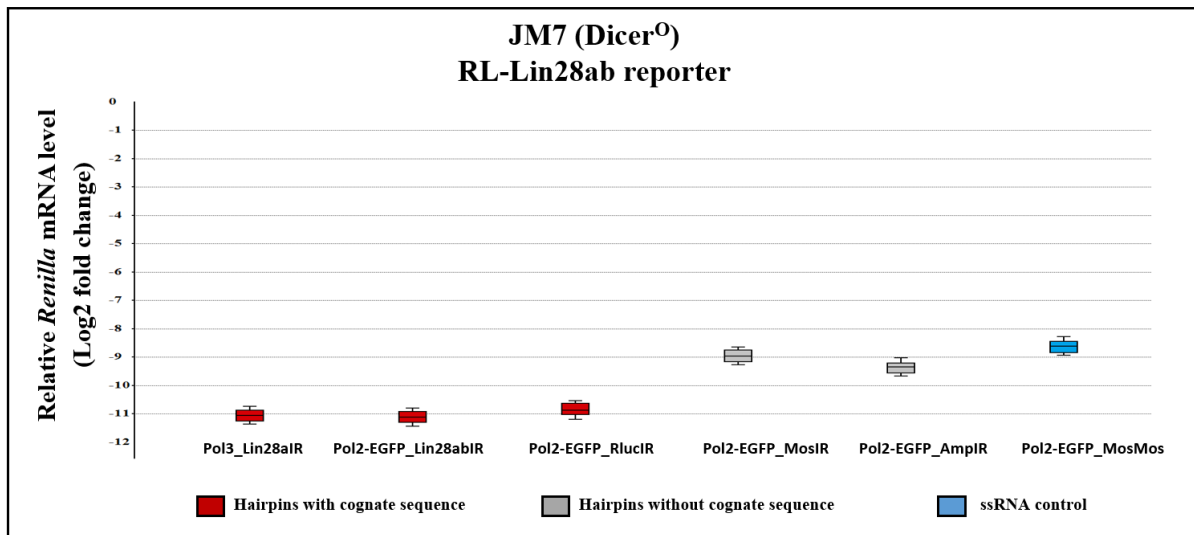


Fig. 26 Normalised Relative *Renilla* luciferase activity in JM7 cells using RL-Lin28ab reporter.

The results (Fig. 26) showed reduced levels of RL-Lin28ab reporter in samples co-transfected with Pol3_Lin28aIR, Pol2-EGFP_Lin28abIR and Pol2-EGFP_RlucIR plasmids. All of these hairpins carry a sequence that is complementary to the RL-Lin28ab reporter. On the other hand, samples co-transfected with pol2-EGFP_MosIR and Pol2-EGFP_AmpIR plasmids showed comparable RL/FL levels to those observed with Pol2-EGFP_MosMos negative control. These data supported our earlier observations from dual-luciferase assays. Both Pol3_Lin28aIR and Pol2-EGFP_Lin28abIR seem to induce RNAi-like effect. Moreover, this experiment showed that we can detect this effect on mRNA level.

4.3.2. U6-driven chimeric reporter

Next, I wanted to examine whether, we can see the RNAi-like effect with the newly prepared, chimeric reporter. Hairpins transcribed from pol2_AmpIR and pol2-EGFP_RlucIR served as negative control.

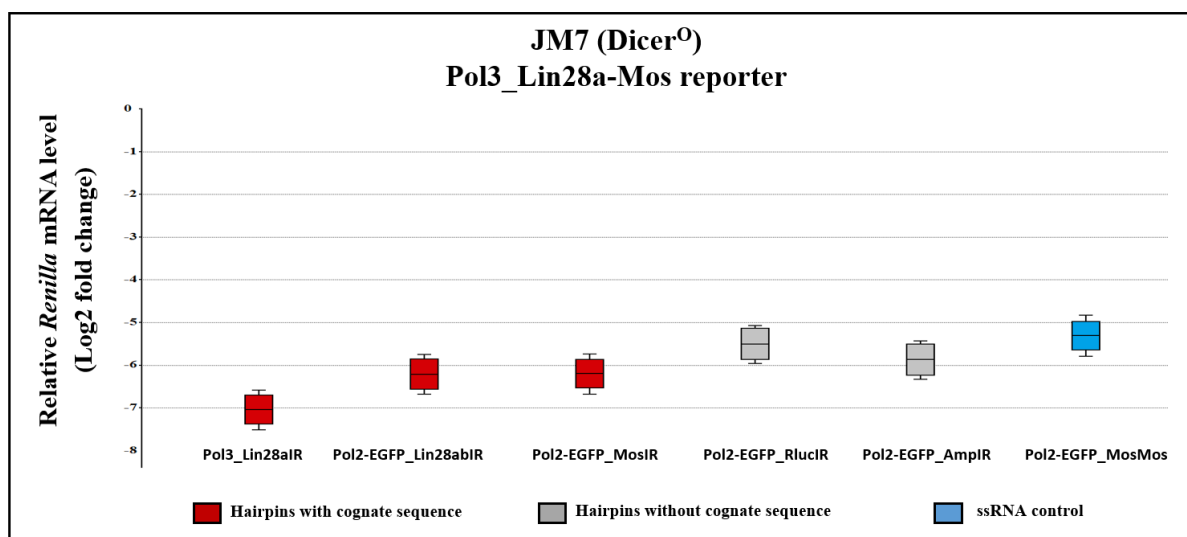


Fig. 27 Normalised Relative *Renilla* luciferase activity in JM7 cells using reporter transcribed from Pol3_lin28a-Mos plasmid.

Surprisingly, the results indicated that pol3_Lin28a-Mos reporter is specifically degraded only by polIII transcribed hairpin (Fig. 27). Samples co-transfected with plasmids pol2-EGFP_MosIR and pol2-EGFP_Lin28abIR showed only small indication of the induction of RNAi-like effect. These observations were in a conflict with the previous qPCR experiment (Fig. 26), which indicated similar RNAi induction by hairpins produced from pol3_Lin28a and pol2-EGFP_Lin28abIR.

I tried to further analyse the induction of RNAi-like effect by hairpins produced from U6 promoter. Dual-luciferase assays showed low efficiency of dsRNA substrates with *Mos* sequence when used with RL-Mos reporter. Therefore, I tried to examine its effectiveness in the induction of RNAi with the chimeric reporter transcribed from U6 promoter. We also wanted to investigate the inconsistency of the results obtained in the last two experiments. It seemed that co-transfection of pol2-EGFP_Lin28abIR plasmid with plasmid generating RL-Lin28ab reporter resulted in the induction of RNAi-like effect. However, when we produced the same substrate with the chimeric U6 transcribed reporter, we could not see RNAi-like effect clearly.

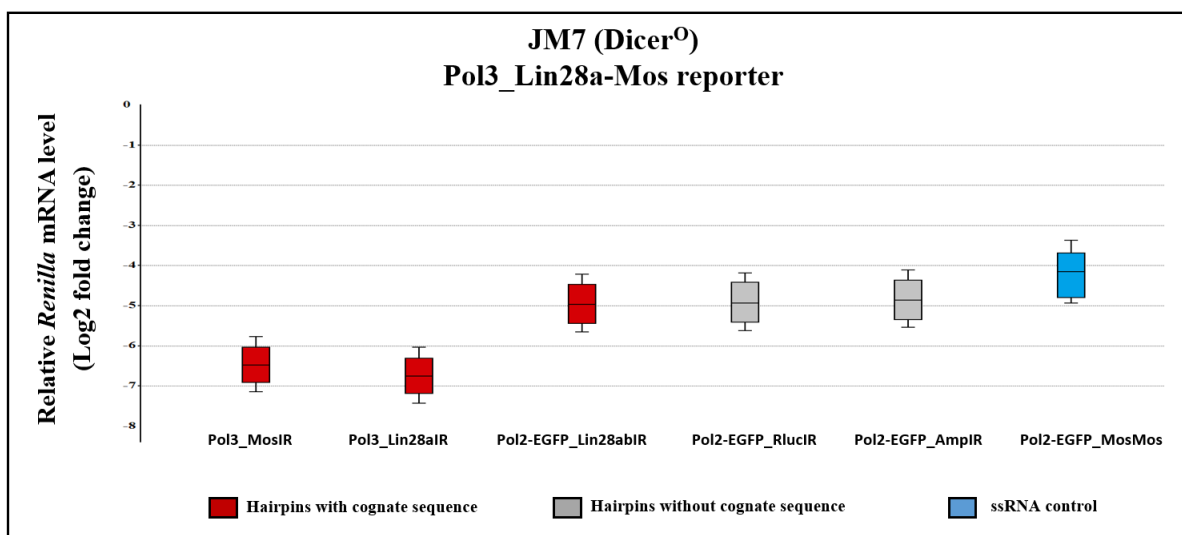


Fig. 28 Normalised Relative *Renilla* luciferase activity in JM7 cells using reporter transcribed from Pol3_Lin28a-Mos plasmid.

The results of this experiment showed similar amounts of pol3_Lin28a-Mos transcribed reporter in samples co-transfected with pol3_MosIR and pol3_Lin28aIR. This imply that bluntly ended hairpin with *Mos* sequence can induce RNAi-like effect on the levels comparable to those of its *Lin28a* analogue. These observations suggest that the variability of the data obtained from RL-Mos and RL-Lin28ab reporters in dual-luciferase assays was probably caused by faulty reporter.

This experiment also indicated that we can observe RNAi-like effect when we co-transfect plasmids producing both hairpins and reporter from U6 promoter (Fig. 28). However, we could not see indications of sequence-specific degradation when we co-transfected plasmids producing hairpins by polII and reporters by polIII. Taken together, the results of these qPCR experiments and dual-luciferase assays imply that U6 transcribed hairpins can cause sequence-specific degradation of reporters transcribed from both U6 and SV40 promoters. On the other hand, pol2-EGFP_Lin28abIR hairpin induces RNAi-like effect only on reporter transcribed by polymerase II.

4.3.2.1. CMV produced hairpins do not induce RNAi-like effect

Dual-luciferase assays did not show any indication of RNAi-like effect induced in samples co-transfected with pol2_MosIR and pol2_Lin28aIR plasmids. Therefore, I sought to determine whether we could observe any effect using chimeric reporters. Because pol3_MosIR

and pol3_Lin28aIR were able to induce strong RNAi-like effect I used them as positive controls for this experiments.

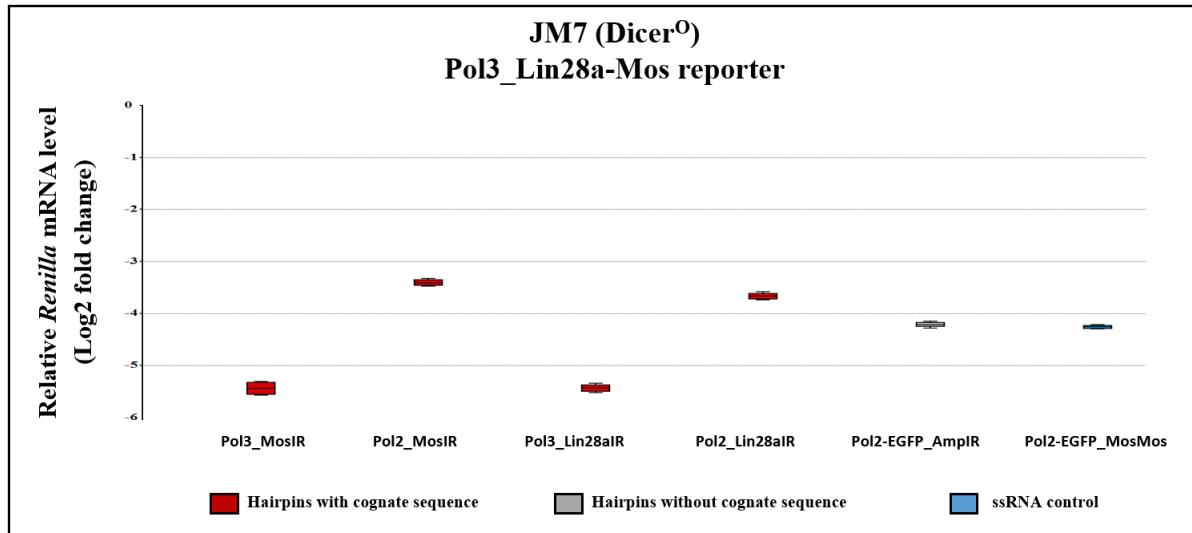


Fig. 29 Normalised Relative *Renilla* luciferase activity in JM7 cells using reporter transcribed from Pol3_lin28a-Mos plasmid.

This qPCR experiment supported our observations from dual-luciferase assays. Samples co-transfected with plasmids producing hairpins with a long overhang at the 3' – end failed to show any signs of RNAi on U6 produced chimeric reporter (Fig. 29). Furthermore, analysis of normalised relative levels of RL-Lin28ab reporter also supported these results (Fig. 30). Compared to hairpins produced from pol2-EGFP_Lin28abIR and Pol2-EGFP_MosIR plasmids, these hairpins are transcribed from CMV promoter. They also lack a synthetic intron and the EGFP sequence. However, whether these are the reasons for their inefficiency in the induction of RNAi with SV40 and U6 driven reporters is not clear.

4.3.3. CMV-driven chimeric reporter

Next, I tried to determine whether hairpins produced from pol2_MosIR and pol2_Lin28aIR plasmids can induce sequence-specific degradation of the second chimeric reporter we produced. This reporter is transcribed from pol2_Mos-Lin28a plasmid. In this experiment, both hairpins and reporters are produced from SV40 promoter, and they do not carry any coding sequence or synthetic intron. Again, both substrates failed to show indications of RNAi. Taken together, substrates without intron and coding region transcribed by

polymerase II from SV40 promoter did not show induction of RNAi regardless of which reporter we used.

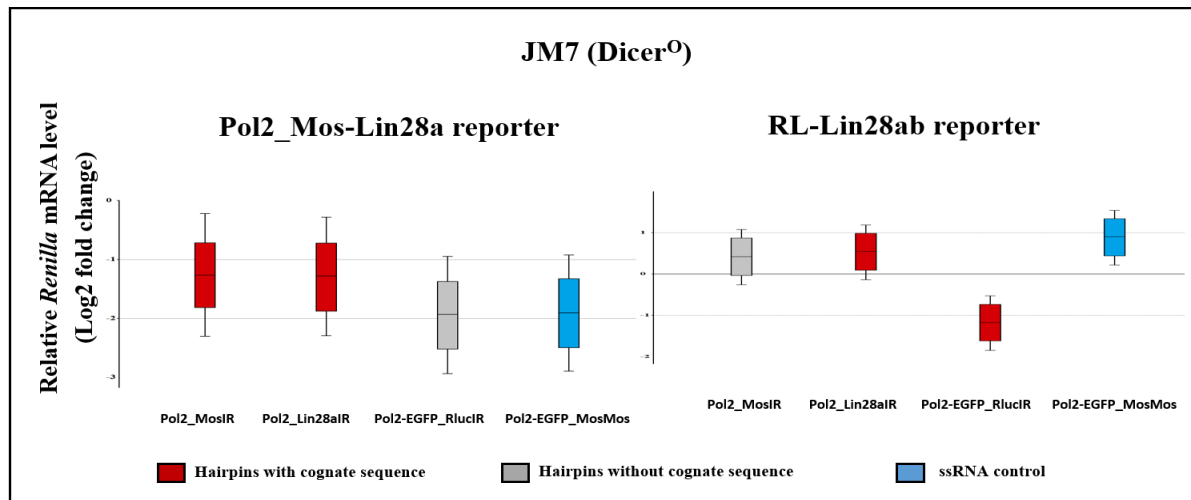


Fig. 30 Normalised Relative *Renilla* luciferase activity in JM7 cells using reporter transcribed from Pol3_lin28a-Mos plasmid and RL-Lin28ab reporter.

I also tried to investigate whether remaining hairpin substrates can induce sequence-specific degradation of the reporter transcribed from pol2_Mos-Lin28a plasmid. I chose the substrates generated from Pol3_MosIR, Pol3_Lin28aIR and Pol2-EGFP_Lin28abIR plasmids because samples co-transfected with these plasmids indicated RNAi-like effect in previous experiments. Interestingly, the normalised relative levels of pol2_Mos-Lin28a reporter in all samples showed similar levels to the negative control (Fig. 31). This indicates that neither of the hairpin substrates induced RNAi.

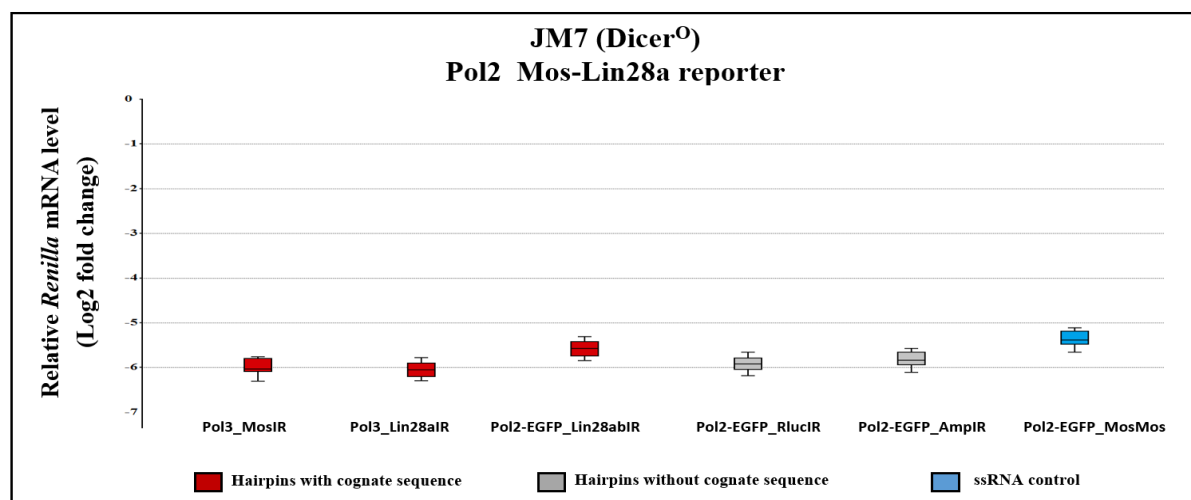


Fig. 31 Normalised Relative *Renilla* luciferase activity in JM7 cells using reporter transcribed from Pol3_Lin28a-Mos plasmid.

4.3.4. CAG-driven Firefly reporter

Previous experiments conducted using dual-luciferase assays indicated that the substrates transcribed by polymerase III from U6 promoters were immune to sequence non-specific degradation. I carried out qPCR experiments using RS10 cell line to further analyse this effect on mRNA level. As mentioned above these cells are characterised by robust sequence non-specific degradation of RNA. I analysed the levels of firefly reporter, as this reporter does not have a cognate sequence to any of our substrates. The results were again normalised to non-transfected cells.

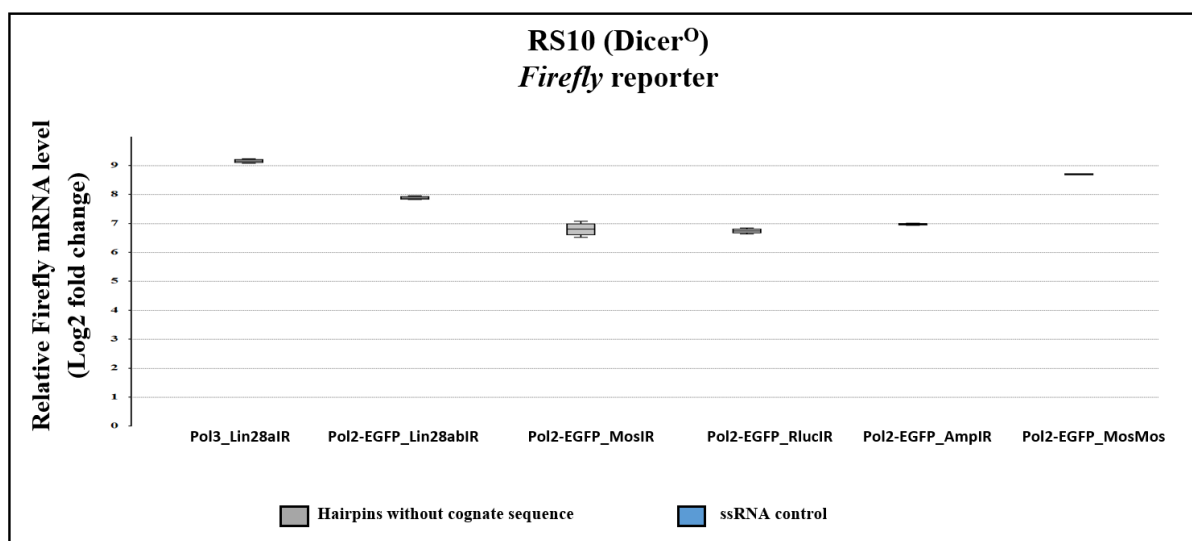


Fig. 32 Relative Firefly luciferase activity in RS10 cells.

Results of this experiment support the data observed by dual-luciferase assay. The substrate produced from U6 promotor show higher levels of relative FL reporter compared to the rest of the samples. Moreover, these levels are comparable to those measured in the sample co-transfected with negative control. Additionally, a similar effect could be observed in samples co-transfected with pol3_MosIR plasmid (data not shown).

5. DISCUSSION

Somatic mammalian cells have several mechanisms responding to the dsRNA. These include sequence-specific RNAi degradation and sequence-independent IFN response.

The induction of canonical RNAi-like effect using substrate produced by pol2-EGFP_MosIR plasmid was shown to be inefficient in mouse somatic cells (Nejepinska *et al.*, 2012). In contrast, expression of MosIR in mouse oocytes triggers RNAi effect (Svoboda *et al.*, 2001; Stein *et al.*, 2003; Nejepinska *et al.*, 2012). This was attributed to the presence of truncated Dicer^O isoform in mouse oocytes. (Flemr *et al.*, 2013). Dicer^O was shown to be more effective in siRNA generation both *in vitro* and *in vivo* (Flemr *et al.*, 2013). Moreover, it was shown that Dicer^O is capable of the induction of RNAi in mESCs (Flemr *et al.*, 2013).

In my thesis, I tried to determine how other factors could stimulate canonical RNAi in mammalian cells. Emphasis was placed predominantly on the structural features of various dsRNA substrates. Our laboratory has been using system generating hairpin substrate with single-stranded overhangs for the study of RNAi in mammalian cells. However, it has been reported that long overhangs have an inhibitory effect on the Dicer processing (Vermeulen *et al.*, 2005). Therefore, we hypothesized that other dsRNA substrates might be more suitable for the induction of RNAi in mammalian cells.

I have constructed several plasmids producing dsRNAs with various structural features, to determine the most efficient substrate for the induction of canonical RNAi in mammalian cells. Our experiments included two types of intramolecular duplexes. First was produced with a blunt end, while the second carried overhang at the 3' termini. In addition, we produced dsRNA formed by base-pairing of distinct sense and antisense RNA strands. These strands were transcribed either in one locus by convergent transcription or at two separate loci.

Afterwards, I determined the efficiency of these substrates in both Dicer^S and Dicer^O expressing cells. Finally, I tested the effects of the PKR and TARBP2 on the RNAi induction by these dsRNA structures. I employed dual-luciferase assays and qPCR to observe the degradation of ectopically produced reporters on protein and mRNA levels respectively.

5.1. Structural of dsRNA influence the induction of RNAi in mammalian cells

First, we tested dsRNA substrates with various structural features carrying *Mos* sequence from the original pol2-EGFP_MosIR plasmid. These substrates did not show any substantial improvement in comparison to the original dsRNA. Repeating the experiment using substrates with the same structural features but *Lin28a* sequence showed an apparent increase in RNAi-like effect. Corresponding with our hypothesis, the efficiency of RNAi induction using *Lin28a* substrates seemed to be (i) influenced by the type of dsRNA termini, (ii) proportional to the probability of a dsRNA formation.

The data obtained from these experiments indicated that the most effective substrate for RNAi induction in mammalian cells is a hairpin molecule with blunt ends produced by polIII from pol3_Lin28aIR plasmid. Samples co-transfected by this plasmid showed the most noticeable decrease in normalized *Renilla* luciferase signal. Furthermore, qPCR experiments using different reporter also indicated sequence-specific degradation in the samples co-transfected with this plasmid.

Interestingly, our experiments in NIH 3T3 cell line indicate that the dsRNA substrate generated from pol3_Lin28aIR plasmid induced RNAi in Dicer^S expressing cells. These findings were unexpected as Dicer^S has been considered very inefficient in a dsRNA processing. At this point, it is unclear why this samples showed RNAi-like effect and further studies are necessary to resolve this finding.

The second most effective structures for the induction of RNAi seemed to be hairpins produced from pol2-EGFP_MosIR and pol2-EGFP_Lin28aIR plasmids. Apart from the rest of the pol2 driven substrates used in my experiments, these hairpins are transcribed from CAG promoter. Moreover, these transcripts contain overhangs at both termini as well as EGFP coding sequence and synthetic intron. We observed a substantial difference in the induction of RNAi-like effect between these hairpins and the CMV driven hairpins which are also transcribed by polII. However, whether the cause of this inequality was the different promoter or some other factor is not clear. The RNAi induction by hairpins produced from polII promoters was not detected in Dicer^S expressing cells and was much less visible in the cells expressing Dicer^O.

These observations are in agreement with a previous study conducted in HEK 393T cells with PKR knockout and N-truncated human Dicer isoform (Kennedy *et al.*, 2016). Authors of the study compared the induction of RNAi by dsRNA substrates produced by different polymerases. They reported that both polII and polIII transcripts were processed by Dicer and induced RNAi-like effect. However, the induction of RNAi by dsRNA substrates produced by polIII was more effective.

dsRNA structures produced by convergent transcription also indicated RNAi-like effect in the Dicer^O expressing JM7 cell line. However, the observed effect was weaker compared to the U6 and CAG driven hairpin substrates. We speculated that the reduced efficiency is caused by the mechanism of the formation of this dsRNA. Although, there might be another reason. The sense strand of this dsRNA was produced from H1 promoter. We utilized this promoter because it is commonly used for the expression of shRNA during RNAi silencing experiments. However, one study reported that transcription from H1 promoter might have multiple start sight, which could result in a variability at the 5'termini (H. Ma *et al.*, 2014). Thus, low efficiency in the RNAi induction might not be caused only by the reduced dsRNA formation but also by the unstructured overhangs of these structures.

dsRNA substrate produced from two plasmids were not expected to be very efficient. Moreover, we assumed that the pol2 transcribed variant would be the least effective of all the dsRNA structures because of the overhangs generated by the specific mechanism of transcription. These predictions were supported by the obtained data as we could not observe convincing indications of RNAi-like effect induced by these substrates.

Results of the dual-luciferase assays indicated that *Mos* and *Lin28* substrates with analogous structures have different efficiencies in the degradation of their cognate reporter. At first, we were not sure why we could not replicate some of the results obtained using RL-Lin28ab reporter. I thought there might be three possible reasons: (i) the data obtained in experiments with RL-Lin28ab reporter do not show canonical RNAi (ii) dsRNA substrates containing *Mos* sequence are not capable of the potent induction of RNAi (iii) dsRNA substrates containing *Mos* sequence are capable of the induction of RNAi but we are not able to detect it due to faulty RL-Mos reporter. To resolve this issue, we prepared a new plasmid; expressing reporter with the chimeric Lin28a-Mos sequence. We utilized qPCR to detect the degradation of this reporter on mRNA level. These experiments showed that co-transfection of

the U6-driven chimeric reporter with both pol3_MosIR and pol3_Lin28aIR plasmids resulted in the comparable decrease of normalised reporter mRNA levels. This indicates that the discrepancies in RNAi induction between *Mos* and *Lin28a* substrates observed in dual-luciferase assays were probably caused by faulty reporter rather than by biological intrinsic cause.

5.2. PKR inhibition does not increase the RNAi induction

In the next round of experiments, we performed dual-luciferase assays in NIH 3T3 Δ PKR and JM7 Δ PKR cells. We speculated that RNAi and sequence non-specific degradation mediated by PKR might compete for the dsRNA substrates. Therefore, we supposed that the inactivation of PKR could have a stimulating effect on the RNAi. However, comparing the normalised *Renilla* luciferase signal obtained in NIH 3T3 cells and NIH 3T3 Δ PKR cells did not show any substantial difference. Similarly, comparison of JM7 and JM7 Δ PKR cells showed only minor effect induced by dsRNA substrates produced from pol2-EGFP_MosIR or pol3→Mos←pol3 plasmids. Nevertheless, these observations could not be reliably replicated in experiments with RL-Lin28ab reporter. On the contrary, some samples showed increased normalised *Renilla* luciferase signal in Δ PKR cell lines.

Taken together, inactivation of PKR did not stimulate RNAi in my experiments. However, our Δ PKR cell lines are prepared by the deletion of PKR exons 2-5, which might results in the production of PKR lacking dsRBD. Although in theory, this deletion should be sufficient for PKR inactivation, it might be possible that truncated PKR disrupts other cellular pathways or dsRBPs involved in the RNAi.

5.3. Inhibition of RNAi by the deletion TARBP2

As mentioned before TARBP2 is a component of RLC. The exact role of this dsRBP in the RNAi is not very well understood. However, it has been proposed that TARBP2 assist during siRNA selection and loading on AGO2 (Gredell *et al.*, 2010). Moreover, TARBP2 is supposedly an inhibitor of PKR (Blair *et al.*, 1995; Daher *et al.*, 2009; Park *et al.*, 1994). Therefore, we expected that the loss of TARBP2 would result in the stimulation of sequence-independent degradation and inhibition of RNAi-like effect. To test this hypothesis, we performed dual-luciferase assays in Dicer⁰ expressing mESCs with a TARBP2 knockout. The results showed that all but one sample lost the ability to reduce normalised *Renilla* luciferase signal. The samples co-transfected with pol3_Lin28aIR plasmid did not show any apparent

difference compared to the results obtained in the TARBP2 viable cell line. At this point, it is not clear why substrates produced by this plasmid appeared to cause RNAi-like effect even in the absence of TARBP2.

The fact that these results were not replicated in samples co-transfected with pol3_MosIR plasmid might indicate that this effect is caused by factors other than canonical RNAi. However, it should be noted that underperforming RL-Mos reporter might have affected detection of the effect. To resolve this issue, qPCR experiments using chimeric pol3_Lin28A-Mos reporter should be performed in the near future.

5.4. U6-driven hairpins are immune to sequences non-specific degradation

As described before FL reporter do not contain cognate sequence to any of the substrates I used. In our experiments, it is implemented as an indicator of sequence non-specific inhibition of expression. However, analysing raw data revealed that samples co-transfected with pol3_MosIR and pol3_Lin28aIR plasmids generated stronger FL signal compared to the samples co-transfected with plasmids expressing hairpins from pol2 promoters. This led us to speculation that hairpins transcribed by polIII might be immune to the sequence-independent degradation.

To address this, we conducted dual-luciferase assays in RS10 and RS10 Δ PKR cell lines. RS10 cells co-transfected with plasmids producing hairpins from U6 promoter showed 40 - 60% stronger FL signal compared to the samples co-transfected with plasmids producing hairpins from polII promoters. However, when we repeated the experiment in RS10 Δ PKR cells, the relative FL signal from the samples with polIII-driven hairpins increased, while it did not change in samples with U6-driven hairpins. Additionally, our experiment conducted using qPCR delivered similar results.

Taken together, these results suggest polIII-driven hairpins induce sequence non-specific degradation mediated by PKR. On the other hand, hairpins transcribed from U6 promoter seem not to activate this response.

5.5. Detection of RNAi-like effect is influenced by the type of the reporter

The main reason for the conduction of the qPCR experiments was to determine whether the discrepancies between the performances of dsRNA substrates with *Mos* and *Lin28* sequence is caused by the under-performing RL-Mos reporter. However, these experiments revealed more about the type of reporter needed for the detection of RNAi-like effect.

- Sequence specific degradation of the chimeric reporter produced from pol2_Mos-Lin28a plasmid could not be detected by any of the hairpin substrates we tried.
- Chimeric reporter transcribed from U6 promoter indicated RNAi-like effect only when co-transfected with plasmids producing hairpins from U6 promoter.
- RL-Lin28ab reporter showed RNAi-like effect when co-transfected with both U6 and CAG transcribed hairpins.

6. CONCLUSIONS

The main aim of my thesis was to determine how different dsRNA structures can influence the induction of canonical RNAi in mammalian cells. I have found that:

- The most effective substrates for the induction of RNAi-like effect were hairpin substrates with blunt ends, presumably because of their better Dicer accessibility.
- Samples co-transfected with pol3_Lin28aIR plasmid showed robust RNAi in Dicer^O as well as in Dicer^S expressing cells demonstrating that Dicer^S can provide sufficient support for siRNA biogenesis.
- RNAi-like effect was not stimulated by the deletion of PKR 2-5 exons, perhaps because of inefficient inhibition or PKR's off-targeting.
- Hairpin produced from pol3_Lin28aIR seemingly induced RNAi-like effect in the absence of TARBP2.
- Levels of the Firefly reporter were not affected by the U6-driven hairpins, possibly because these substrates do not induce sequence non-specific effect mediated by PKR.

7. REFERENCES

- Agrawal, N., Dasaradhi, P. V. N., Mohmmmed, A., Malhotra, P., Bhatnagar, R. K., & Mukherjee, S. K. (2003). RNA interference: biology, mechanism, and applications. *Microbiology and Molecular Biology Reviews* : *MMBR*, 67(4), 657–85. <https://doi.org/10.1128/MMBR.67.4.657>
- Ameres, S. L., Martinez, J., & Schroeder, R. (2007). Molecular Basis for Target RNA Recognition and Cleavage by Human RISC. *Cell*, 130(1), 101–112. <https://doi.org/10.1016/j.cell.2007.04.037>
- Ameres, S. L., & Zamore, P. D. (2013). Diversifying microRNA sequence and function. *Nature Reviews. Molecular Cell Biology*, 14(8), 475–88. <https://doi.org/10.1038/nrm3611>
- Ando, Y., Tomaru, Y., Morinaga, A., Burroughs, A. M., Kawaji, H., Kubosaki, A., ... Hayashizaki, Y. (2011). Nuclear pore complex protein mediated nuclear localization of dicer protein in human cells. *PLoS ONE*, 6(8). <https://doi.org/10.1371/journal.pone.0023385>
- Baulcombe, D. (2004). RNA silencing in plants. *Nature*, 431(7006), 356–363. <https://doi.org/10.1038/nature02874>
- Bernstein, E., Caudy, A. A., Hammond, S. M., & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, 409(6818), 363–366. <https://doi.org/10.1038/35053110>
- Blair, E., Roberts, C., Snowden, W., Gatignol, A., Benkirane, M., & Jeang, K.-T. (1995). Expression of TAR RNA-Binding Protein in Baculovirus and Co-Immunoprecipitation with Insect Cell Protein Kinase. *J Biomed Sci*, 2, 322–329. Retrieved from <https://link.springer.com/content/pdf/10.1007%2F02255219.pdf>
- Brennecke, J., Aravin, A. A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., & Hannon, G. J. (2007). Discrete Small RNA-Generating Loci as Master Regulators of Transposon Activity in *Drosophila*. *Cell*, 128(6), 1089–1103. <https://doi.org/10.1016/j.cell.2007.01.043>
- Carmell, M. A., Girard, A., van de Kant, H. J. G., Bourc'his, D., Bestor, T. H., de Rooij, D. G., & Hannon, G. J. (2007). MIWI2 Is Essential for Spermatogenesis and Repression of Transposons in the Mouse Male Germline. *Developmental Cell*, 12(4), 503–514.

<https://doi.org/10.1016/j.devcel.2007.03.001>

- Carpick, B. W., Graziano, V., Schneider, D., Maitra, R. K., Lee, X., & Williams, B. R. G. (1997). Characterization of the Solution Complex between the Interferon- induced , Double-stranded RNA-activated Protein Kinase and HIV-I Trans-activating Region RNA *, 272(14), 9510–9516.
- Cerutti, H., & Casas-Mollano, J. A. (2006). On the origin and functions of RNA-mediated silencing: from protists to man, 50(2), 81–99. <https://doi.org/10.1007/s00294-006-0078-x>.On
- Chakravarthy, S., Sternberg, S. H., Kellenberger, C. A., & Doudna, J. A. (2010). Substrate-specific kinetics of dicer-catalyzed RNA processing. *Journal of Molecular Biology*, 404(3), 392–402. <https://doi.org/10.1016/j.jmb.2010.09.030>
- Chen, C., Zhu, C., Huang, J., Zhao, X., Deng, R., Zhang, H., ... Yu, J. (2015). SUMOylation of TARBP2 regulates miRNA/siRNA efficiency. *Nature Communications*, 6, 8899. <https://doi.org/10.1038/ncomms9899>
- Chendrimada, T. P., Gregory, R. I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., & Shiekhattar, R. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature*, 436(7051), 740–744. <https://doi.org/10.1038/nature03868>
- Clemens, M. J. (1997). PKR--a protein kinase regulated by double-stranded RNA. *The International Journal of Biochemistry & Cell Biology*, 29(7), 945–949. [https://doi.org/10.1016/S1357-2725\(96\)00169-0](https://doi.org/10.1016/S1357-2725(96)00169-0)
- Cole, C., Sobala, A., Lu, C., Thatcher, S. R., Bowman, A., Brown, J. W. S., ... Hutvagner, G. (2009). Filtering of deep sequencing data reveals the existence of abundant Dicer-dependent small RNAs derived from tRNAs. *RNA (New York, N.Y.)*, 15(12), 2147–60. <https://doi.org/10.1261/rna.1738409>
- Daher, A., Laraki, G., Singh, M., Melendez-Peña, C. E., Bannwarth, S., Peters, A. H. F. M., ... Gatignol, A. (2009). TRBP control of PACT-induced phosphorylation of protein kinase R is reversed by stress. *Molecular and Cellular Biology*, 29(1), 254–65. <https://doi.org/10.1128/MCB.01030-08>
- Daniels, S. M., Melendez-Peña, C. E., Scarborough, R. J., Daher, A., Christensen, H. S., El

- Far, M., ... Gatignol, A. (2009). Characterization of the TRBP domain required for dicer interaction and function in RNA interference. *BMC Molecular Biology*, 10, 38. <https://doi.org/10.1186/1471-2199-10-38>
- Das, S., Ward, S. V., Markle, D., & Samuel, C. E. (2004). DNA Damage-binding Proteins and Heterogeneous Nuclear Ribonucleoprotein A1 Function as Constitutive KCS Element Components of the Interferon-inducible RNA-dependent Protein Kinase Promoter *, 279(8), 7313–7321. <https://doi.org/10.1074/jbc.M312585200>
- De Veer, M. J., Sledz, C. A., & Williams, B. R. G. (2005). Detection of foreign RNA: Implications for RNAi. *Immunology and Cell Biology*. <https://doi.org/10.1111/j.1440-1711.2005.01337.x>
- Deng, W., & Lin, H. (2002). miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. *Developmental Cell*, 2(6), 819–830. [https://doi.org/10.1016/S1534-5807\(02\)00165-X](https://doi.org/10.1016/S1534-5807(02)00165-X)
- Ding, S., & Voinnet, O. (2007). Review Antiviral Immunity Directed by Small RNAs, 413–426. <https://doi.org/10.1016/j.cell.2007.07.039>
- Drinnenberg, I. A., Weinberg, D. E., Xie, K. T., Mower, J. P., Wolfe, K. H., Fink, G. R., & Bartel, D. P. (2009). RNAi in budding yeast, 326(5952), 544–550. <https://doi.org/10.1126/science.1176945.RNAi>
- Dunoyer, P., Himber, C., Ruiz-ferrer, V., Alioua, A., & Voinnet, O. (2007). Intra- and intercellular RNA interference in *Arabidopsis thaliana* requires components of the microRNA and heterochromatic silencing pathways, 39(7). <https://doi.org/10.1038/ng2081>
- Ender, C., Krek, A., Friedländer, M. R., Beitzinger, M., Weinmann, L., Chen, W., ... Meister, G. (2008). A Human snoRNA with MicroRNA-Like Functions. *Molecular Cell*, 32(4), 519–528. <https://doi.org/10.1016/j.molcel.2008.10.017>
- Faulkner, G. J. (2013). Retrotransposon Silencing During Embryogenesis: Dicer Cuts in LINE. *PLoS Genetics*, 9(11). <https://doi.org/10.1371/journal.pgen.1003944>
- Fensterl, V., & Sen, G. C. (2009). Interferons and viral infections. <https://doi.org/10.1002/biof.6>
- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent

- and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391(6669), 806–811. <https://doi.org/10.1038/35888>
- Flemr, M., Malik, R., Franke, V., Nejepinska, J., Sedlacek, R., Vlahoviček, K., & Svoboda, P. (2013). A retrotransposon-driven Dicer isoform directs endogenous siRNA production in mouse oocytes. *Cell*, in press.
- Flemr, M., Moravec, M., Libova, V., Sedlacek, R., & Svoboda, P. (2014). Lin28a Is Dormant , Functional , and Dispensable During Mouse Oocyte-to-Embryo, 90(May), 1–9. <https://doi.org/10.1095/biolreprod.114.118703>
- Förstemann, K., Tomari, Y., Du, T., Vagin, V. V., Denli, A. M., Bratu, D. P., ... Zamore, P. D. (2005). Normal microRNA maturation and germ-line stem cell maintenance requires loquacious, a double-stranded RNA-binding domain protein. *PLoS Biology*, 3(7), 1187–1201. <https://doi.org/10.1371/journal.pbio.0030236>
- Friedman, R. C., Farh, K. K. H., Burge, C. B., & Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research*, 19(1), 92–105. <https://doi.org/10.1101/gr.082701.108>
- Gantier, M. P., & Williams, B. R. G. (2007). The response of mammalian cells to double-stranded RNA. *October*, 454(1), 42–54. <https://doi.org/10.1097/OPX.0b013e3182540562>.The
- Gao, Z., Wang, M., Blair, D., Zheng, Y., & Dou, Y. (2014). Phylogenetic Analysis of the Endoribonuclease Dicer Family, 9(4), 1–7. <https://doi.org/10.1371/journal.pone.0095350>
- Gatignol, A., Buckler-White, A., Berkhout, B., & Jeang, K. T. (1991). Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. *Science (New York, N.Y.)*, 251(5001), 1597–600. <https://doi.org/10.1126/science.2011739>
- Goubau, D., Deddouche, S., & Reis, C. (2013). Cytosolic Sensing of Viruses. *Immunity*, 38(5), 855–869. <https://doi.org/10.1016/j.immuni.2013.05.007>
- Gredell, J. A., Dittmer, M. J., Wu, M., Chan, C., & Walton, S. P. (2010). Recognition of siRNA asymmetry by TAR RNA binding protein. *Biochemistry*, 49(14), 3148–3155. <https://doi.org/10.1021/bi902189s>
- Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., ... Mello, C. C. (2001). Genes and Mechanisms Related to RNA Interference Regulate Expression of the Small

- Temporal RNAs that Control *C. elegans* Developmental Timing, *106*, 23–34.
- Ha, M., & Kim, V. N. (2014). Regulation of microRNA biogenesis. *Nature Reviews. Molecular Cell Biology*, *15*(8), 509–524. <https://doi.org/10.1038/nrm3838>
- Haase, A. D., Jaskiewicz, L., Zhang, H., Lainé, S., Sack, R., Gatignol, A., & Filipowicz, W. (2005). TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. *EMBO Reports*, *6*(10), 961–7. <https://doi.org/10.1038/sj.embor.7400509>
- Haasnoot, J., Westerhout, E. M., & Berkhout, B. (2007). RNA interference against viruses : strike and counterstrike, *25*(12). <https://doi.org/10.1038/nbt1369>
- Hashimoto, N., Watanabe, N., Furuta, Y., Tamemoto, H., Sagata, N., Yokoyama, M., ... Ikawa, Y. (1994). Parthenogenetic activation of oocytes in c-mos-deficient mice. *Nature*, *370*(6484), 68–71. <https://doi.org/10.1038/370068a0>
- Hauptmann, J., Dueck, A., Harlander, S., Pfaff, J., Merkl, R., & Meister, G. (2013). Turning catalytically inactive human Argonaute proteins into active slicer enzymes. *Nature Publishing Group*, *20*(7), 814–817. <https://doi.org/10.1038/nsmb.2577>
- Heras, S. R., Macias, S., Plass, M., Fernandez, N., Cano, D., Eyra, E., ... Cáceres, J. F. (2013). The Microprocessor controls the activity of mammalian retrotransposons. *Nature Publishing Group*, *20*(10), 1–7. <https://doi.org/10.1038/nsmb.2658>
- Hershey, J. W. B. (1991). Translational Control in Mammalian Cells. *Annu. Rev. Biochem*, *60*:717-55.
- Jannot, G., Boisvert, M.-E. L., Banville, I. H., & Simard, M. J. (2008). Two molecular features contribute to the Argonaute specificity for the microRNA and RNAi pathways in *C. elegans*. *RNA (New York, N.Y.)*, *14*(5), 829–35. <https://doi.org/10.1261/rna.901908>
- Jinek, M., & Doudna, J. A. (2009). A three-dimensional view of the molecular machinery of RNA interference. *Nature*, *457*(January), 405–412. <https://doi.org/10.1038/nature07755>
- Kaneko, H., Dridi, S., Tarallo, V., Gelfand, B. D., Fowler, B. J., Cho, W. G., ... Ambati, J. (2011). DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. <https://doi.org/10.1038/nature09830>
- Kennedy, E. M., Whisnant, A. W., Kornepati, A. V. R., Marshall, J. B., Bogerd, H. P., &

- Cullen, B. R. (2016). Production of functional small interfering RNAs by an amino-terminal deletion mutant of human Dicer. *Proceedings of the National Academy of Sciences*, 113(42), E6547–E6547. <https://doi.org/10.1073/pnas.1615446113>
- Kim, J. K., Gabel, H. W., Kamath, R. S., Kennedy, S., Dybbs, M., & Bertin, N. (2005). Functional Genomic Analysis of RNA Interference in *C. elegans*, 308(May), 1164–1168.
- Kiriakidou, M., Tan, G. S., Lamprinak, S., Planell-saguer, M. De, Nelson, P. T., & Mourelatos, Z. (2007). An mRNA m 7 G Cap Binding-like Motif within Human Ago2 Represses Translation, 1141–1151. <https://doi.org/10.1016/j.cell.2007.05.016>
- Kok, K. H., Ng, M. H. J., Ching, Y. P., & Jin, D. Y. (2007). Human TRBP and PACT directly interact with each other and associate with dicer to facilitate the production of small interfering RNA. *Journal of Biological Chemistry*, 282(24), 17649–17657. <https://doi.org/10.1074/jbc.M611768200>
- Kostura, M., & Mathews, M. B. (1989). Purification and Activation of the Double-Stranded RNA-dependent eIF-2 kinase DAI. *Molecular and Cellular Biology*, 9(4), 1576–1586. Retrieved from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC362574/pdf/molcellb00052-0204.pdf>
- Kuhen, K. L., & Samuel, C. E. (1997). Isolation of the Interferon-Inducible RNA-Dependent Protein Kinase Pkr Promoter and Identification of a Novel DNA Element within the 5' Flanking Region of Human and Mouse Pkr Genes, 130(227), 119–130.
- Kumar, M., & Carmichael, G. G. (1998). Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. *Microbiology and Molecular Biology Reviews : MMBR*, 62(4), 1415–34. Retrieved from <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=98951&tool=pmcentrez&rendertype=abstract>
- Kurzynska-kokorniak, A., Pokornowska, M., Koralewska, N., Hoffmann, W., Bienkowska-Szewczyk, K., & Figlerowicz, M. (2016). Revealing a new activity of the human Dicer DUF283 domain in vitro, (April), 1–13. <https://doi.org/10.1038/srep23989>
- Kwak, P. B., & Tomari, Y. (2012). The N domain of Argonaute drives duplex unwinding during RISC assembly, 19(2), 145–152. <https://doi.org/10.1038/nsmb.2232>
- Lau, P., Guiley, K. Z., De, N., Potter, C. S., Carragher, B., & Macrae, I. J. (2012). The

- molecular architecture of human Dicer. *Nature Publishing Group*, 19(4), 436–440.
<https://doi.org/10.1038/nsmb.2268>
- Lavorgna, G., Dahary, D., Lehner, B., Sorek, R., Sanderson, C. M., & Casari, G. (2004). In search of antisense, 29(2). <https://doi.org/10.1016/j.tibs.2003.12.002>
- Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J., & Carthew, R. W. (2004). Distinct Roles for Drosophila Dicer-1 and Dicer-2 in the siRNA / miRNA Silencing Pathways, 117, 69–81.
- Lee, Y. S., Shibata, Y., Malhotra, A., & Dutta, A. (2009). A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). *Genes & Development*, 23(22), 2639–49.
<https://doi.org/10.1101/gad.1837609>
- Lewis, B. P., Burge, C. B., & Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, 120(1), 15–20. <https://doi.org/10.1016/j.cell.2004.12.035>
- Lewis, B. P., Shih, I.-H., Jones-Rhoades, M. W., Bartel, D. P., & Burge, C. B. (2003). Prediction of Mammalian MicroRNA Targets. *Cell*, 115(7), 787–798.
[https://doi.org/10.1016/S0092-8674\(03\)01018-3](https://doi.org/10.1016/S0092-8674(03)01018-3)
- Li, Y., Lu, J., Han, Y., Fan, X., & Ding, S. (2013). RNA Interference Functions as an Antiviral Immunity Mechanism in Mammals.
- Lingel, A., Simon, B., Izaurralde, E., & Sattler, M. (2004). Nucleic acid 3' -end recognition by the Argonaute2 PAZ domain, 11(6), 576–577. <https://doi.org/10.1038/nsmb777>
- Liu, J., Carmell, M. A., Rivas, F. V., Hammond, S. M., Joshua-tor, L., & Hannon, G. J. (2004). Argonaute2 Is the Catalytic Engine of Mammalian RNAi, 305(September), 1437–1442.
- Liu, Q. (2003). R2D2, a Bridge Between the Initiation and Effector Steps of the Drosophila RNAi Pathway. *Science*, 301(5641), 1921–1925.
<https://doi.org/10.1126/science.1088710>
- Ma, H., Wu, Y., Dang, Y., Choi, J., Zhang, J., & Wu, H. (2014). Pol III Promoters to Express Small RNAs: Delineation of Transcription Initiation, (March), 1–11.
<https://doi.org/10.1038/mtna.2014.12>
- Ma, J., Flemr, M., Stein, P., Berninger, P., Malik, R., Zavolan, M., ... Schultz, R. M. (2010).

- MicroRNA Activity Is Suppressed in Mouse Oocytes. *Current Biology*, 20(3), 265–270.
<https://doi.org/10.1016/j.cub.2009.12.042>
- MacRae, I. J., Ma, E., Zhou, M., Robinson, C. V., & Doudna, J. A. (2008). In vitro reconstitution of the human RISC-loading complex. *Proceedings of the National Academy of Sciences*, 105(2), 512–517. <https://doi.org/10.1073/pnas.0710869105>
- Maillard, P. V, Ciaudo, C., Marchais, A., Li, Y., Jay, F., Ding, S. W., & Voinnet, O. (2013). Antiviral RNA interference in mammalian cells. *Science*, 342(6155), 235–238. <https://doi.org/10.1126/science.1241930>
- Maillard, P. V, Veen, A. G. Van Der, Deddouche-grass, S., & Rogers, N. C. (2016). Inactivation of the type I interferon pathway reveals long double-stranded RNA-mediated RNA interference in mammalian cells, 1–14.
- Mallory, A., & Vaucheret, H. (2010). Form , Function , and Regulation of ARGONAUTE Proteins, 22(December), 3879–3889. <https://doi.org/10.1105/tpc.110.080671>
- Manche, L., Green, S. R., Schmedt, C., & Mathews, M. B. (1992). Interactions between double-stranded RNA regulators and the protein kinase DAI. *Molecular and Cellular Biology*, 12(11), 5238–48. <https://doi.org/10.1128/MCB.12.11.5238>
- Martinez, J., & Tuschl, T. (2006). RISC is a 5' phosphomonoester-producing RNA endonuclease, 975–980. <https://doi.org/10.1101/gad.1187904.al>.
- Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., & Tuschl, T. (2004). Human Argonaute2 Mediates RNA Cleavage Targeted by miRNAs and siRNAs, 15, 185–197.
- Morris, K. V, Chan, S. W., & Jacobsen, S. E. (2004). Small Interfering RNA – Induced Transcriptional Gene Silencing in Human Cells, 305(August), 1289–1293.
- Mukherjee, K., Campos, H., & Kolaczowski, B. (2013). Evolution of animal and plant dicers: Early parallel duplications and recurrent adaptation of antiviral RNA binding in plants. *Molecular Biology and Evolution*, 30(3), 627–641. <https://doi.org/10.1093/molbev/mss263>
- Nakanishi, K., Weinberg, D. E., Bartel, D. P., Patel, D. J., & Program, B. (2013). Structure of yeast Argonaute with guide RNA, 486(7403), 368–374. <https://doi.org/10.1038/nature11211.Structure>

- Nallagatla, S. R., Hwang, J., Toroney, R., Zheng, X., Cameron, C. E., & Bevilacqua, P. C. (2007). 5'-triphosphate-dependent activation of PKR by RNAs with short stem-loops. *Science (New York, N.Y.)*, 318(5855), 1455–1458. <https://doi.org/10.1126/science.1147347>
- Napoli, C., Lemieux, C., & Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *The Plant Cell*, 2(4), 279–289. <https://doi.org/10.1105/tpc.2.4.279>
- Nejepinska, J., Malik, R., Filkowski, J., Flemr, M., Filipowicz, W., & Svoboda, P. (2012). DsRNA expression in the mouse elicits RNAi in oocytes and low adenosine deamination in somatic cells. *Nucleic Acids Research*, 40(1), 399–413. <https://doi.org/10.1093/nar/gkr702>
- Obbard, D. J., Gordon, K. H. J., Buck, A. H., & Jiggins, F. M. (2009). The evolution of RNAi as a defence against viruses and transposable elements. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 364(1513), 99–115. <https://doi.org/10.1098/rstb.2008.0168>
- Ota, H., Sakurai, M., Gupta, R., Valente, L., Wulff, B. E., Ariyoshi, K., ... Nishikura, K. (2013). ADAR1 forms a complex with dicer to promote MicroRNA processing and RNA-induced gene silencing. *Cell*, 153(3), 575–589. <https://doi.org/10.1016/j.cell.2013.03.024>
- Pare, J. M., Tahbaz, N., Lopez-Orozco, J., LaPointe, P., Lasko, P., & Hobman, T. C. (2009). Hsp90 Regulates the Function of Argonaute 2 and Its Recruitment to Stress Granules and P-Bodies. *Molecular Biology of the Cell*, 20, 3273–3284. <https://doi.org/10.1091/mbc.E09>
- Park, H., Davies, M. V, Langland, J. O., Chang, H. W., Nam, Y. S., Tartaglia, J., ... Venkatesan, S. (1994). TAR RNA-binding protein is an inhibitor of the interferon-induced protein kinase PKR. *Proceedings of the National Academy of Sciences of the United States of America*, 91(11), 4713–4717. <https://doi.org/10.1073/pnas.91.11.4713>
- Peaston, A. E., Evsikov, A. V, Graber, J. H., de Vries, W. N., Holbrook, A. E., Solter, D., & Knowles, B. B. (2004). Retrotransposons regulate host genes in mouse oocytes and preimplantation embryos. *Dev Cell*, 7(4), 597–606. <https://doi.org/10.1016/j.devcel.2004.09.004>

- Pratt, A. J., & Macrae, I. J. (2010). The RNA-induced Silencing Complex : A Versatile Gene-silencing Machine *, 284(27), 17897–17901. <https://doi.org/10.1074/jbc.R900012200>
- Qiu, Y., Xu, Y., Zhang, Y., Zhou, H., Deng, Y., Li, X., ... Zhang, Q. (2017). Human Virus-Derived Small RNAs Can Confer Article Human Virus-Derived Small RNAs Can Confer Antiviral Immunity in Mammals. *Immunity*, 46(6), 992–1004.e5. <https://doi.org/10.1016/j.immuni.2017.05.006>
- Richards, O. C., Martin, S. C., & Jense, H. G. (1984). Structure of Poliovirus Replicative Intermediate RNA Electron Microscope Analysis of RNA Cross-linked in Viva with Psoralen Derivative, 83, 325–340.
- Robertson, H. D., & Mathews, M. B. (1996). The regulation of the protein kinase PKR by RNA. *Biochimie*, 78, 909–14.
- Saelens, X., Kalai, M., & Vandenabeele, P. (2001). Translation Inhibition in Apoptosis, 276(45), 41620–41628. <https://doi.org/10.1074/jbc.M103674200>
- Samuel, C. E. (1993). The eIF-2cr Protein Kinases, Regulators of Translation in Eukaryotes from Yeasts to Humans, 268(11), 7603–7606.
- Schwarz, D. S., Hutvágner, G., Du, T., Xu, Z., Aronin, N., & Zamore, P. D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell*, 115(2), 199–208. [https://doi.org/10.1016/S0092-8674\(03\)00759-1](https://doi.org/10.1016/S0092-8674(03)00759-1)
- Shyh-Chang, N., & Daley, G. Q. (2013). Lin28: Primal regulator of growth and metabolism in stem cells. *Cell Stem Cell*. <https://doi.org/10.1016/j.stem.2013.03.005>
- Soifer, H. S., Sano, M., Sakurai, K., Chomchan, P., Sætrom, P., Sherman, M. A., ... Rossi, J. J. (2008). A role for the Dicer helicase domain in the processing of thermodynamically unstable hairpin RNAs. *Nucleic Acids Research*, 36(20), 6511–6522. <https://doi.org/10.1093/nar/gkn687>
- Stein, P., Svoboda, P., & Schultz, R. M. (2003). Transgenic RNAi in mouse oocytes: A simple and fast approach to study gene function. *Developmental Biology*, 256(1), 187–193. [https://doi.org/10.1016/S0012-1606\(02\)00122-7](https://doi.org/10.1016/S0012-1606(02)00122-7)
- Stein, P., Zeng, F., Pan, H., & Schultz, R. M. (2005). Absence of non-specific effects of RNA interference triggered by long double-stranded RNA in mouse oocytes. *Developmental Biology*, 286(2), 464–471. <https://doi.org/10.1016/j.ydbio.2005.08.015>

- Sudhakar, A., Ramachandran, A., Ghosh, S., Hasnain, S. E., Kaufman, R. J., & Ramaiah, K. V. A. (2000). Phosphorylation of Serine 51 in Initiation Factor 2 R (eIF2 R) Promotes Complex Formation between eIF2 R (P) and eIF2B and Causes Inhibition in the Guanine Nucleotide Exchange Activity of eIF2B †, 12929–12938.
- Sullivan, C. S., Ganem, D., Sullivan, C. S., & Ganem, D. (2005). A Virus-Encoded Inhibitor That Blocks RNA Interference in Mammalian Cells A Virus-Encoded Inhibitor That Blocks RNA Interference in Mammalian Cells, 79(12). <https://doi.org/10.1128/JVI.79.12.7371>
- Svoboda, P. (2014). Renaissance of mammalian endogenous RNAi. *FEBS Letters*. Federation of European Biochemical Societies. <https://doi.org/10.1016/j.febslet.2014.05.030>
- Svoboda, P., Stein, P., Hayashi, H., & Schultz, R. M. (2000). Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development*, 127(19), 4147–4156. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10976047
- Svoboda, P., Stein, P., & Schultz, R. M. (2001). RNAi in mouse oocytes and preimplantation embryos: effectiveness of hairpin dsRNA. *Biochem Biophys Res Commun*, 287(5), 1099–1104. <https://doi.org/10.1006/bbrc.2001.5707>
- Tabara, H., Yigit, E., Siomi, H., & Mello, C. C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-Box helicase to direct RNAi in *C. elegans*. *Cell*, 109(7), 861–871. [https://doi.org/10.1016/S0092-8674\(02\)00793-6](https://doi.org/10.1016/S0092-8674(02)00793-6)
- Tarallo, V., Hirano, Y., Gelfand, B. D., Dridi, S., Kerur, N., Kim, Y., ... Ambati, J. (2012). DICER1 loss and Alu RNA induce age-related macular degeneration via the NLRP3 inflammasome and MyD88. *Cell*, 149(4), 847–859. <https://doi.org/10.1016/j.cell.2012.03.036>
- Taylor, D. W., Shigematsu, H., Cianfrocco, M. A., Noland, C. L., Nagayama, K., Nogales, E., ... Berkeley, L. (2013). Substrate-specific structural rearrangements of human Dicer, 20(6), 662–670. <https://doi.org/10.1038/nsmb.2564.Substrate-specific>
- Thomis, D. C., & Samuel, C. E. (1992). Mechanism of interferon action : Autoregulation of RNA-dependent P1 / eIF-2a protein kinase (PKR) expression in transfected mammalian

- cells, 89(November), 10837–10841.
- Tian, Y., Simanshu, D. K., Ma, J., Park, J., Heo, I., & Kim, V. N. (2014). A Phosphate-Binding Pocket within the Platform-PAZ-Connector Helix Cassette of Human Dicer. *Molecular Cell*, 53(4), 606–616. <https://doi.org/10.1016/j.molcel.2014.01.003>
- Tomari, Y., Matranga, C., Haley, B., Martinez, N., & Zamore, P. D. (2004). A protein sensor for siRNA asymmetry. *Science (New York, N.Y.)*, 306(5700), 1377–80. <https://doi.org/10.1126/science.1102755>
- Ullu, E., Tschudi, C., & Chakraborty, T. (2004). Microreview RNA interference in protozoan parasites, 6, 509–519. <https://doi.org/10.1111/j.1462-5822.2004.00399.x>
- Vermeulen, A., Behlen, L., Reynolds, A., Wolfson, A., Marshall, W. S., Karpilow, J., & Khvorova, A. (2005). The contributions of dsRNA structure to Dicer specificity and efficiency. *RNA (New York, N.Y.)*, 11(5), 674–82. <https://doi.org/10.1261/rna.7272305>
- Viswanathan, S. R., Daley, G. Q., & Gregory, R. I. (2008). Selective blockade of microRNA processing by Lin-28. *Science (New York, N.Y.)*, 320(5872), 97–100. <https://doi.org/10.1126/science.1154040>
- Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I. S., & Martienssen, R. A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science (New York, N.Y.)*, 297(5588), 1833–1837. <https://doi.org/10.1126/science.1074973>
- Ward, S. V., & Samuel, C. E. (2002). Regulation of the Interferon-Inducible PKR Kinase Gene : The KCS Element Is a Constitutive Promoter Element That Functions in Concert with the Interferon-Stimulated Response Element, 146, 136–146. <https://doi.org/10.1006/viro.2002.1356>
- Wee, L. M., Flores-Jasso, C. F., Salomon, W. E., & Zamore, P. D. (2012). Argonaute divides Its RNA guide into domains with distinct functions and RNA-binding properties. *Cell*, 151(5), 1055–1067. <https://doi.org/10.1016/j.cell.2012.10.036>
- Welker, N. C., Maity, T. S., Ye, X., Aruscavage, P. J., Ammie, A., Liu, Q., & Bass, B. L. (2012). Dicer's helicase domain discriminates dsRNA termini to promote an altered reaction mode, 41(5), 589–599. <https://doi.org/10.1016/j.molcel.2011.02.005>.Dicer
- Wianny, F., & Zernicka-Goetz, M. (2000). Specific interference with gene function by double-

- stranded RNA in early mouse development. *Nat Cell Biol*, 2(2), 70–75. <https://doi.org/10.1038/35000016>
- Wilson, R. C., Tambe, A., Kidwell, M. A., Noland, C. L., Schneider, C. P., & Doudna, J. A. (2015). Dicer-TRBP complex formation ensures accurate mammalian MicroRNA biogenesis. *Molecular Cell*, 57(3), 397–408. <https://doi.org/10.1016/j.molcel.2014.11.030>
- Wu, Q., Wang, X., & Ding, S.-W. (2011). Viral suppressors of RNA-based viral immunity: Host targets, 8(1), 12–15. <https://doi.org/10.1016/j.chom.2010.06.009>.Viral
- Xie, Z., Allen, E., Wilken, A., & Carrington, J. C. (2005). DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*.
- Yang, N., & Kazazian, H. H. (2006). L1 retrotransposition is suppressed by endogenously encoded small interfering RNAs in human cultured cells. *Nature Structural & Molecular Biology*, 13(9), 763–771. <https://doi.org/10.1038/nsmb1141>
- Yigit, E., Batista, P. J., Bei, Y., Pang, K. M., Chen, C. G., Tolia, N. H., ... Mello, C. C. (2006). Analysis of the *C. elegans* Argonaute Family Reveals that Distinct Argonautes Act Sequentially during RNAi, 747–757. <https://doi.org/10.1016/j.cell.2006.09.033>
- Yoon, C., Lee, E., Lim, D., & Bae, Y. (2009). PKR , a p53 target gene , plays a crucial role in the tumor-suppressor function of p53.
- Yuan, Y.-R., Pei, Y., Ma, J.-B., Kuryaviy, V., Zhadina, M., Meister, G., ... Patel, D. J. (2005). Crystal Structure of *A. aeolicus* Argonaute, a Site-Specific DNA-Guided Endoribonuclease, Provides Insights into RISC-Mediated mRNA Cleavage, 19(3), 405–419. <https://doi.org/10.1016/j.molcel.2005.07.011>.Crystal
- Zhang, H., Kolb, F. A., Brondani, V., Billy, E., & Filipowicz, W. (2002). Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *EMBO Journal*, 21(21), 5875–5885. <https://doi.org/10.1093/emboj/cdf582>
- Zhang, H., Kolb, F. A., Jaskiewicz, L., Westhof, E., & Filipowicz, W. (2004). Single Processing Center Models for Human Dicer and Bacterial RNase III, 118, 57–68.
- Zheng, X., & Bevilacqua, P. C. (2004). Activation of the protein kinase PKR by short double-stranded RNAs with single-stranded tails. *RNA (New York, N.Y.)*, 10(12), 1934–45. <https://doi.org/10.1261/rna.7150804>

8. SUPPLEMENTARY DATA

S1: List of primers used for the cloning

Primer pair number	Sequences	Annealing temperature
1	FWD: 5'- CAAGCGGCCGCCATCAAGCAAGTAAACAAGTGC -'3 REV: 5'- TGAAGCTTGCACCAAGGACCTACGTGCATCCC -'3	66°C
2	FWD: 5'- TCAAGCTTGGAGTTTAAGGAAAGAGGCATCAATC -'3 REV: 5'- TAGCGGCCGCGGTTTGACACTTGTTTCGCTTCCC -'3	58°C
3	FWD: 5'- CGACTCACTATAGGGAGAGCGGC -'3 REV: 5'- CGGTTCTGATGAGGTGGTTAGC -'3	57°C
4	FWD: 5'- CAGGAAGATGGCTGTGAGGGAC -'3 REV: 5'- CGGTTCTTGGCCTTTTGCTGG -'3	57°C
5	FWD: 5'- CACAATTGAGGGCCTATTTCCCATGATTCC -'3 REV: 5'- GTCTGCAGAAAAAAGCGGCCGCGGTGTTTCGTCCTTTCCACAAG -'3	58°C
6	FWD: 5'- GGAATAAGGGCGACACGGAAATG -'3 REV: 5'- CGGTTCTTGGCCTTTTGCTGG -'3	58°C
7	FWD: 5'- CAAGCGGCCGCCATCAAGCAAGTAAACAAGTGC -'3 REV: 5'- TAAGCGGCCGCACTTCCCCAACTCAGTTG -'3	65°C
8	FWD: 5'- TCGCGGCCGCGGAGTTTAAGGAAAGAGGCATCAATC -'3 REV: 5'- TAGCGGCCGCGGTTTGACACTTGTTTCGCTTCCC -'3	67°C
9	FWD: 5'- GACTTGGTGTAGAGTCACGTTGCC -'3 REV: 5'- CGGTTCTTGGCCTTTTGCTGG -'3	57°C
10	FWD: 5'- GGAATAAGGGCGACACGGAAATG -'3 REV: 5'- GACTTGGTGTAGAGTCACGTTGCC -'3	57°C
11	FWD: 5'- CAAGGGAAATATCACACAGCCTTC -'3 REV: 5'- CGGTTCTTGGCCTTTTGCTGG -'3	53°C
12	FWD: 5'- GGAATAAGGGCGACACGGAAATG -'3 REV: 5'- CAAGGGAAATATCACACAGCCTTC -'3	53°C
13	FWD: 5'- GACTTGGTGTAGAGTCACGTTGCC -'3 REV: 5'- TAGAAGGCACAGTCGAGG -'3	52°C
14	FWD: 5'- CGTAACAACTCCGCCCCATTGAC -'3 REV: 5'- GACTTGGTGTAGAGTCACGTTGCC -'3	57°C
15	FWD: 5'- CAAGGGAAATATCACACAGCCTTC -'3 REV: 5'- TAGAAGGCACAGTCGAGG -'3	51°C
16	FWD: 5'- CGTAACAACTCCGCCCCATTGAC -'3 REV: 5'- CAAGGGAAATATCACACAGCCTTC -'3	53°C
17	FWD: 5'- CACAATTGAGGGCCTATTTCCCATGATTCC -'3 REV: 5'- GTCTGCAGAAAAAAGGTACCGGTGTTTCGTCCTTTCCACAAG -'3	70°C

18	FWD: 5'- GGAATAAGGGCGACACGAAATG -'3 REV: 5'- CGGTCCTGGCCTTTTGCTGG -'3	58°C
19	FWD: 5'- GGAATAAGGGCGACACGAAATG -'3 REV: 5'- GTGCATCCCAGCGGAGTTTCTG -'3	58°C
20	FWD: 5'- CACAATTGAGGGCCTATTTCCTATGATTCC -'3 REV: 5'- GTCTGCAGAAAAAAGCTTGGTGTTCGTCCTTTCCACAAG -'3	70°C
21	FWD: 5'- GGAATAAGGGCGACACGAAATG -'3 REV: 5'- GTTGTAGCACCTGTCTCCTT -'3	52°C
22	FWD: 5'- CGTAACAACCTCCGCCCCATTGAC -'3 REV: 5'- GTGCATCCCAGCGGAGTTTCTG -'3	58°C
23	FWD: 5'- CGTAACAACCTCCGCCCCATTGAC -'3 REV: 5'- GTTGTAGCACCTGTCTCCTT -'3	52°C
24	FWD: 5'- ATGCGGCCGCTAACAACATCTAGGGAATACTTG -'3 REV: 5'- TGAAGCTTGCACCAAGGACCTACGTGCATCCC -'3	62°C
25	FWD: 5'- TCAAGCTTGGAGTTTAAGGAAAGAGGCATCAATC -'3 REV: 5'- TAGCGGCCGCGGTTTGACACTTGTTCGCTTCCC -'3	58°C
26	FWD: 5'- CAAGGGAAATATCACACAGCCTTC -'3 REV: 5'- CGGTCCTGATGAGGTGGTTAGC -'3	55°C
27	FWD: 5'- GACTTGGTGTAGAGTCAGTTGCC -'3 REV: 5'- CGGTCCTGATGAGGTGGTTAGC -'3	57°C
28	FWD: 5'- GGAATAAGGGCGACACGAAATG -'3 REV: 5'- GGAACCTCTACCACACTCTATCTG -'3	53°C
29	FWD: 5'- GGAATAAGGGCGACACGAAATG -'3 REV: 5'- GGAACCTCTACCACACTCTATCTG -'3	53°C
30	FWD: 5'- CGGTCCTGGCCTTTTGCTGG -'3 REV: 5'- GGAACCTCTACCACACTCTATCTG -'3	53°C

S2: List of primers used for sequencing

Primer number	Sequence
1	5'- CGACTCACTATAGGGAGAGCGGC-'3
2	5'- CAGGAAGATGGCTGTGAGGGAC-'3
3	5'- GGAATAAGGGCGACACGAAATG-'3
4	5'- CGTAACAACCTCCGCCCCATTGAC-'3
5	5'- AGCAATAGCATCACAAATTTTAC-'3
6	5'- CGGTCCTGGCCTTTTGCTGG-'3

S3: List of primers used for qPCR

Primer pair number	Sequence
Primers for <i>Renilla</i> reporter:	FWD: 5'- CAGATTGTCCGCAACTACAACGCC -'3 REV: 5'- CTTACCCATTTCATCTGGAGCGTC -'3
Primers for <i>Firefly</i> reporter:	FWD: 5'- GCTACAAACGCTCTCATCGACAAG -'3 REV: 5'- GTATTTGATCAGGCTCTTCAGCCG -'3
Primers for U6_Lin28a-Mos and SV40_Lin28a-Mos reporters:	FWD: 5'- CAGAAACTCCGCTGGGATGCAC -'3 REV: 5'- GAAGGCTGTGTGATATTTCCCTTG -'3