universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Evaluation of Candidate Factors for the
Post-transcriptional Regulation of microRNA Expression

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1. Introduction

The great time of deoxyribonucleic acid, commonly known as DNA, has passed. DNA was a celebrity decades ago, when Watson and Crick were the first to predict the double-helical nature of DNA (1953) or when Meselson and Stahl discovered the mechanism of DNA replication (1958). Even the first knock-out mouse was born nearly twenty years ago. DNA is old-fashioned. Today, ribonucleic acid (RNA) is everything. RNA is thought to have played around on Earth before everything else existed. It is still there and full of surprises: the discovery of a very short and special type of RNA in the late 1990ies has led to a paradigm shift in biology about the knowledge on how gene expression is regulated. The Central Dogma of molecular biology again had to be revised.

1.1 The Central Dogma

Originally, the Central Dogma was formulated by F. Crick[1]. In his paper from 1958 "On Protein Synthesis", he discussed the direction of information flow in the cell.

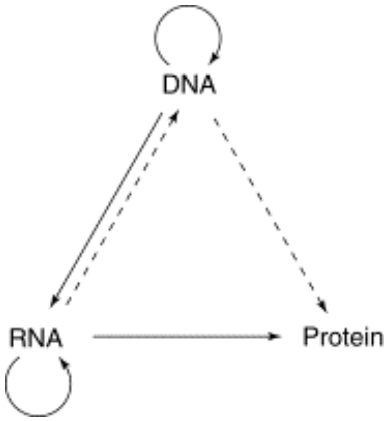


Figure 1: Crick wrote about this scheme: "Solid arrows represent probable transfers, dotted arrows possible transfers. The absent arrows represent the impossible transfers postulated by the central dogma. They are the three possible arrows starting from proteins." Adapted from Thieffry, 1998[1].

With DNA, RNA, and protein being the three corners of a triangle he explained three types of transfers existing between them (Figure 1): "unlikely" transfers from proteins to nucleic acids, "possible" transfers from RNA to DNA or DNA to proteins, and "known" transfers from DNA to RNA to proteins.

Although the Central Dogma as proclaimed by Crick neither excluded a reverse flow of information from RNA to DNA nor stated that the flow of information always stops at protein level it was interpreted in a more restrictive way: the conventional Central Dogma of molecular biology defines the information flow from storage (DNA) to effectors (proteins), i.e. that DNA makes RNA and RNA makes protein, and most importantly that this flow is not reversible. In this scheme, RNA was considered only as an intermediate in the flow of information.

The conventional view of the dogma was later challenged by the findings that RNAs can act as enzymes or "ribozymes", by the discovery of retroviruses, alternative splicing, mRNA editing, and prions[1]. All these phenomena did not fit into the conventional idea of DNA being the only source of hereditary information and the announcement of a pre-historic "RNA World" provided further support to the view that RNA is more important than previously thought.

1.2 RNA World: The Molecular Biologists' Dream

When two independent research groups discovered a new role of RNA in the 1980s[2], showing that this molecule is not only an intermediate in the flow of genetic information, but also able to take over enzymatic functions (ribozyme), the idea of self-replicating systems at the origin of life consisting only of RNA evolved. W. Gilbert was the first to propose the existence of an "RNA World" in 1989, picturing a hypothetical stage in the origin of life preceding the DNA/RNA/protein world[3]. The idea of an RNA World would resolve the "chicken and egg problem" of which came first, nucleic acids or proteins, as RNA alone combines informational and catalytic roles previously splitted between RNA and proteins.

The "Molecular Biologists' Dream" was a term coined by Joyce & Orgel in 1999 to describe the origin of the RNA World based on extrapolations of the findings of prebiotic chemists[4]. They proposed that nucleotides were formed by prebiotic reactions on Earth from nucleosides and sugars and combined to polynucleotides with the help of a mineral catalyst. Then, after double-strand RNAs were generated by template-directed synthesis, at least one of these RNAs melted and gave rise to a ribozyme, an enzymatically active RNA, which started copying itself and its complement. The "Molecular Biologists' Dream" suggests that life descends from only one or a few RNA molecules that started replication of polynucleotides four billion years ago. However, this dream is mere speculation and other hypotheses on the origin of life exist as well, which try to circumvent the problems of an RNA World, namely that the issue of RNA stability and synthesis remain implausible. In the "pre-RNA World", an RNA-like polymer is proposed which possesses the catalytic and templating functions of RNA and is easily replicated[5]. Nevertheless, current opinion in biology holds that RNA did not only play a major role at the origin of life (either at the beginning or after the pre-RNA World) but is also one of the key players in complex organisms of today[6]. For the journal "Science" latest

discoveries of RNA function were nominated as the "2002' Breakthrough of the Year", for the Nobel Foundation it was even more: in 2006 two researchers were awarded the Nobel Prize in medicine for their findings in gene regulation by small RNAs.

1.3 Small RNAs

1.3.1 Beginnings of RNA Silencing: when flowers turned pale

First hints that a novel layer of genetic regulation was waiting to be deciphered emerged in 1990, when Napoli and colleagues generated transgenic petunia flowers by introducing an additional gene copy for the pigment synthesis enzyme chalcone synthase[7, 8]. Aiming at an enhanced coloration of the petals, they unexpectedly yielded flowers with pigmentation patterns or even completely white petals (Figure 2).

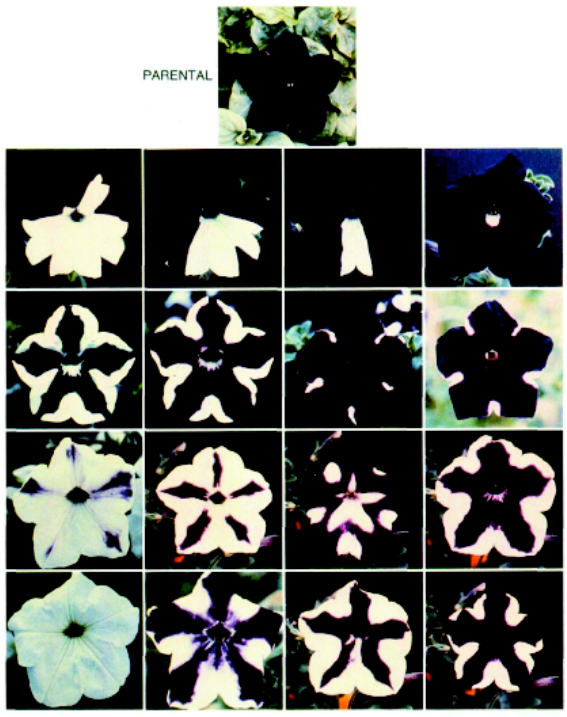


Figure 2: Phenotypes of transgenic petunia flowers (Napoli 1999). A normal phenotype of petunia flowers is depicted on top. Each row shows four flowers from a single transgenic plant displaying the variety of petal patterns found on an individual petunia plant.

This phenomenon was termed "co-suppression" or "post-transcriptional gene silencing" (PTGS), as the introduced gene copy did not enhance, but blocked the expression of the endogenous gene, leading to the loss of coloration due to the lack of the pigmentation enzyme in plant cells transfected with the gene copy.

In the following years more and more studies in different organisms from fungi to flies showed that the introduction of DNA or RNA sequences from a cellular gene into cells resulted in down-regulation of the corresponding mRNA[9]. However, insights into the mechanism did not arouse until 1998, when Craig Mello and Andrew Fire identified double-stranded RNA (dsRNA) to be the PTGS-inducing agent[10]. In their experiments they demonstrated that introduction of *unc-22* dsRNA into *C. elegans* resulted in a more effective down-regulation of *unc-22* mRNA than injecting only sense or antisense strands of the same RNA. Based on this and other observations, Fire and Mello could rule out the formerly

proposed antisense mechanism, which would suggest the formation of a hybrid RNA duplex between the introduced strand and the endogenous mRNA, leading to a block in translation. Shortly thereafter, Hamilton and Baulcombe provided additional evidence by their discovery of a correlation between PTGS and the presence of ~25-nucleotide (nt) antisense RNAs[11]. These small interfering RNAs (siRNAs) were complementary to target mRNAs and were never detected in the absence of PTGS, indicating a direct role in this mechanism. When Tuschl and colleagues demonstrated that chemically synthesized siRNAs were able to guide cleavage of their target mRNAs, a new era of gene silencing in mammalian cells was at the start[12, 13]. What followed next was a series of biochemistry work unveiling main actors in RNA silencing or RNA interference (RNAi): identification of Dicer as the dsRNA-siRNA converting enzyme[14], purification of RISC, the complex mediating cleavage of target mRNA[15], and identification of Ago2 as the protein factor with "Slicer" activity in RISC[16, 17]. Today it is known from both genetic and biochemical studies that the pathways underlying RNAi and factors involved exist in many eukaryotic organisms[18].

1.3.2 The RNAi pathway

RNAi is triggered by an RNase III-like enzyme termed Dicer, which cleaves long dsRNA into ~21-nt short interfering RNAs (siRNAs) with 2-nt 3' overhanging ends and phosphorylated 5' ends[19]. dsRNAs derive from exogenous sources like viruses or internal aberrant transcription of repetitive sequences. The siRNA duplexes are incorporated into a protein complex comprising Argonaute (Ago) proteins where one of the strands is removed ("passenger" strand). The Argonaute effector complex is guided by its remaining siRNA strand ("guide") to the target mRNA, where a single phosphodiester bond is cleaved between nucleotide position 10 and 11 counting from the 5' end of the siRNA guide[19, 20]. Figure 3 shows a diagram of the siRNA pathway.

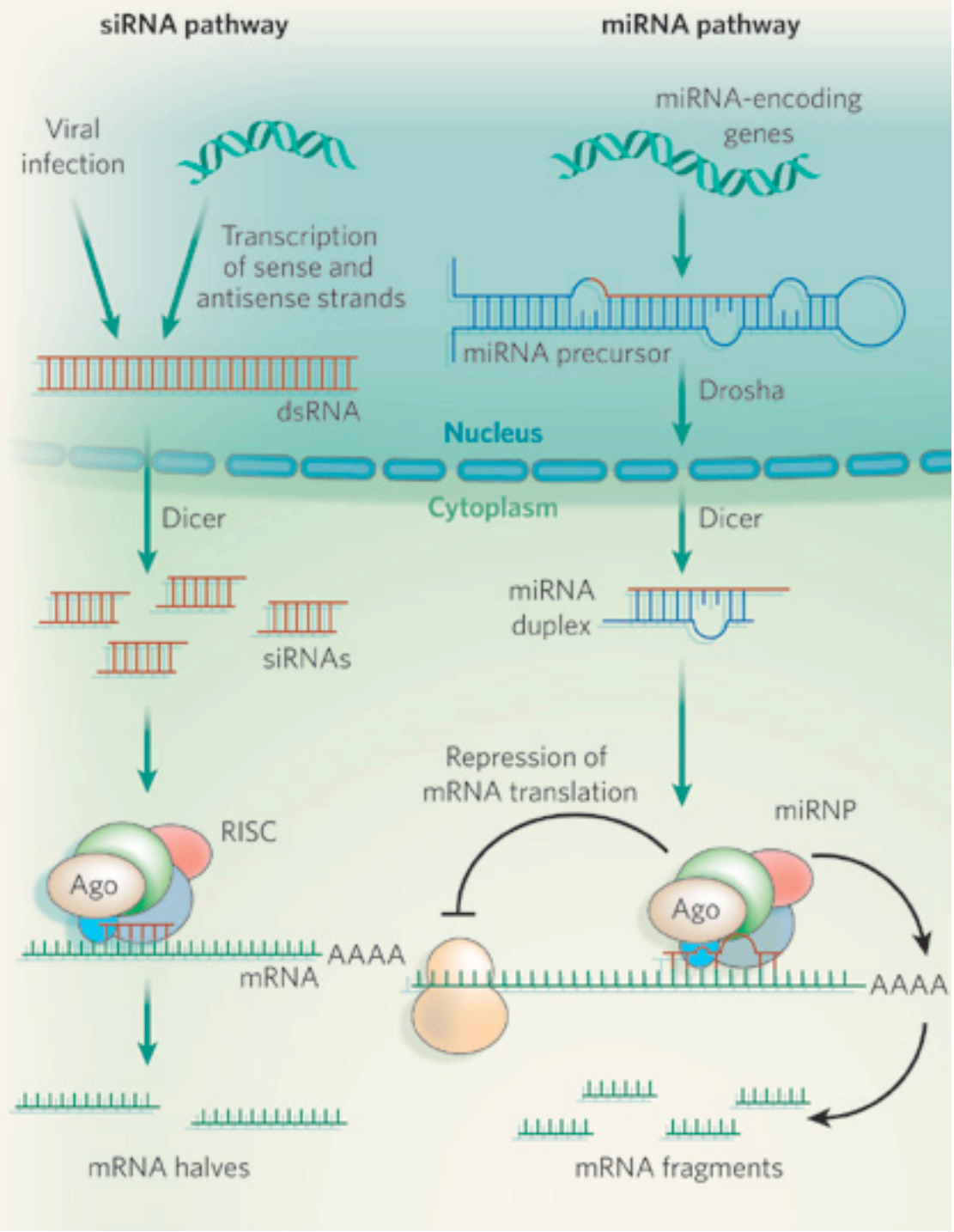


Figure 3: RNA silencing pathways. Left: siRNA pathway. siRNAs derive from dsRNA cleaved by Dicer and target RISC to the corresponding mRNA, resulting in cleavage and subsequent degradation of the mRNA. Right: miRNA pathway. miRNAs are transcribed from miRNA-encoding genes, cleaved by Drosha in the nucleus and exported into the cytoplasm, where they are procsessed by Dicer into functional and mature miRNAs. They guide RISC or miRNP to their target mRNA and induce repression of translation or mRNA decay. For details, see text. Figure adapted from Großhans, 2008.

1.3.3 Small RNAs now and then

siRNAs represented a completely though artificial new type of small RNAs. However, they were neither the first nor the last discovered. Members of the small RNA species were already found in the 1960ies in the nuclei and shortly after in the cytoplasm of mammalian cells[21, 22]. At that time, small RNAs were RNA molecules not directly involved in protein synthesis and sized only between 90-300 nt[23]. Later, small RNAs were also discovered in prokaryotes to be involved in regulatory and synthetic functions, e.g. control of gene expression, plasmid copy number, peptidoglycan synthesis, and phage DNA packing[24].

Today it is estimated that about 98% of transcribed genes in humans consists of small or non-coding RNAs[25]. Although proteins were always considered to be the cellular effectors and main regulators of transcription, the discovery of a new class of small RNAs in a seemingly small and insignificant organism, a worm, was influential in proposing that this type of RNA may account for the complexity and phenotypic variation of higher organisms[25].

1.4 microRNAs - A new class of small RNAs

Lin-4 was a small RNA identified in 1993 as a temporal regulator in the development of the worm *Caenorhabditis elegans*[26, 27], followed by let-7 in 2000[28]. In the same year, let-7 was found to be conserved in a wide range of animal species, leading to the introduction of the term "small temporal RNAs" (stRNA) for the newly discovered short, non-coding RNAs[29]. The existence of additional stRNAs involved in developmental control was proposed at the same time[29]. Only one year later, several groups announced the discovery of many more stRNAs in various organisms, which were from now on termed microRNAs[30-32].

Today, the term microRNA (miRNA) describes a class of small non-coding, single-stranded RNA molecules possessing a crucial regulatory function in diverse biological processes[18, 20, 33]. They are encoded by either specific genes (exons), in intergenic regions or in introns of protein-coding genes[34]. After transcription and several processing steps a 19-24 nt single-stranded RNA stretch is formed which is incorporated into an effector complex. Nowadays, hundreds of miRNAs in plants, worms, flies, mammals and even viruses in various tissues and organs are known. Their function is not restricted to

development, but they are involved in many regulatory pathways. In humans, more than 600 miRNAs have been sequenced according to the miRBase Sequence Database (http://miRNA.sanger.ac.uk), predicted to regulate the expression of more than 30% of the genes in the human genome[35]. In the last few years, miRNAs have been shown to play pivotal roles in controlling cell differentiation, proliferation and apoptosis both in native and disease conditions[33, 36]. Although the pace of progress in elaborating the nature of RNA silencing is overwhelming, many details on biology, mechanism and targets of miRNAs remain to be uncovered[18, 33].

1.5 miRNA Biogenesis

Like any other family of cellular RNAs, the origin of miRNAs resides in the genome. From here, they are transcribed by RNA polymerase II or III in mono- or polycistronic fashion, often being several kilobases long[33]. These transcripts, named primary miRNAs (pri-miRNAs) display features of common mRNAs like a m^7G-cap and a poly-A-tail to prevent immediate degradation. Still in the nucleus, an RNase III enzyme called Drosha assisted by the RNA-binding protein Pasha/DGCR8 cleaves the pri-miRNAs. The cleavage product, a ~ 70 nt long miRNA precursor (pre-miRNA) with a 2-nt 3' overhang, is transported into the cytoplasm through the nuclear pores with the aid of exportin-5. The export of pre-miRNAs into the cytoplasm allows the second processing step to take place, which resembles in many ways the siRNA pathway. The second step, also termed maturation step, is catalysed by Dicer in complex with Loquacious (TRBP in mammals) and results in a 19-24 nt long duplex with again a 2-nt 3' overhang. Similar to siRNAs, the miRNA duplex is incorporated into an effector complex termed RNA-induced silencing complex (RISC) or miRNP for miRNA-ribonucleoprotein. The main components of RISC are proteins of the Argonaute (Ago) family, which bind the mature miRNA via their PAZ domain. In humans, there are 4 Ago proteins (Ago1-4). Several assembly and regulatory proteins associated to the complex have also been identified.

Only one strand of the duplex, the "guide" strand, remains bound to RISC, while the other ("passenger" strand) is removed. The preference for one strand lies in the thermodynamic stability of the duplex ends: the strand with the thermodynamically least stable 5' end remains in the complex. The strand bound to RISC guides the effector complex to the target mRNA and thereby blocks translation or induces degradation.

1.6 Mode of (inter)action

miRNAs control gene expression by blocking translation or promoting mRNA degradation in a sequence-specific manner[18, 37]. In plants, they bind their target mRNA almost perfectly, thereby inducing endonucleolytic mRNA cleavage followed by degradation. Metazoan miRNAs require perfect complementarity to the miRNA target site only for the nucleotides at position 2-8, referred to as the "seed" region[35]. Precise binding of this heptamer to mRNA target sites in the 3'-untranslated region (3- UTR) is sufficient for miRNA functioning. However, additional features in the central or 3' region of the miRNA improve silencing efficacy[37]. An unpaired region, called "bulge", is characteristic for miRNAs in contrast to siRNAs.

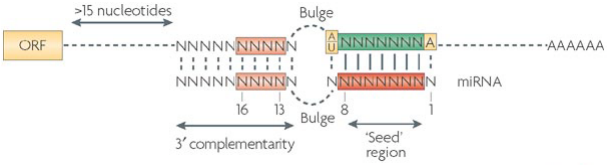


Figure 4: miRNA-mRNA interaction. Metazoan miRNAs basepair with their target sequence in the 3′ UTR of mRNAs imperfectly. Precise complementarity is only required for the "seed" region (depicted in dark red).

The regulatory potential of miRNAs lies in their ability to influence the pattern of gene expression. miRNAs have been shown to play a role in mRNA decay, inhibition and sometimes even stimulation of translation[18]. The most common mode of action employed by miRNAs is the repression of target mRNA translation. Nevertheless, controversy exists about the exact mechanism. Four different ways of repressing translation have been published until now: co-translational protein degradation, inhibition of translation elongation, premature termination of translation, and inhibition of translation initiation[38] (Figure 5).

It has remained elusive at which step miRNAs interfere with translation. Established methods like examining polysome-bound mRNAs in sucrose gradients have resulted in contradicting interpretations of the silencing mechanism[39-41]. Furthermore, experiments demonstrating a cap-dependent repression illustrate a convincing mechanism, nevertheless it was shown as well that silencing occurs in a cap-independent way[40-42]. Experimental evidence for each of the possibilities has been provided, resulting in an ongoing debate whether the dispute arises from different experimental approaches and interpretations thereof or reflects the diversity of miRNA silencing mechanisms.

The influence of miRNAs in mRNA decay is known in more detail. By imperfectly binding to the target site, miRNAs induce removal of the 3' poly(A) tail from the mRNA, resulting in 5' decapping and subsequent exonucleolytic digestion starting from the 5' end.

The individual contribution of mRNA destabilization and translational repression, respectively, differs from one mRNA to another[18]. Figure 5 gives an overview on the published mechanisms of miRNA action including different proposed mechanisms of translational repression.

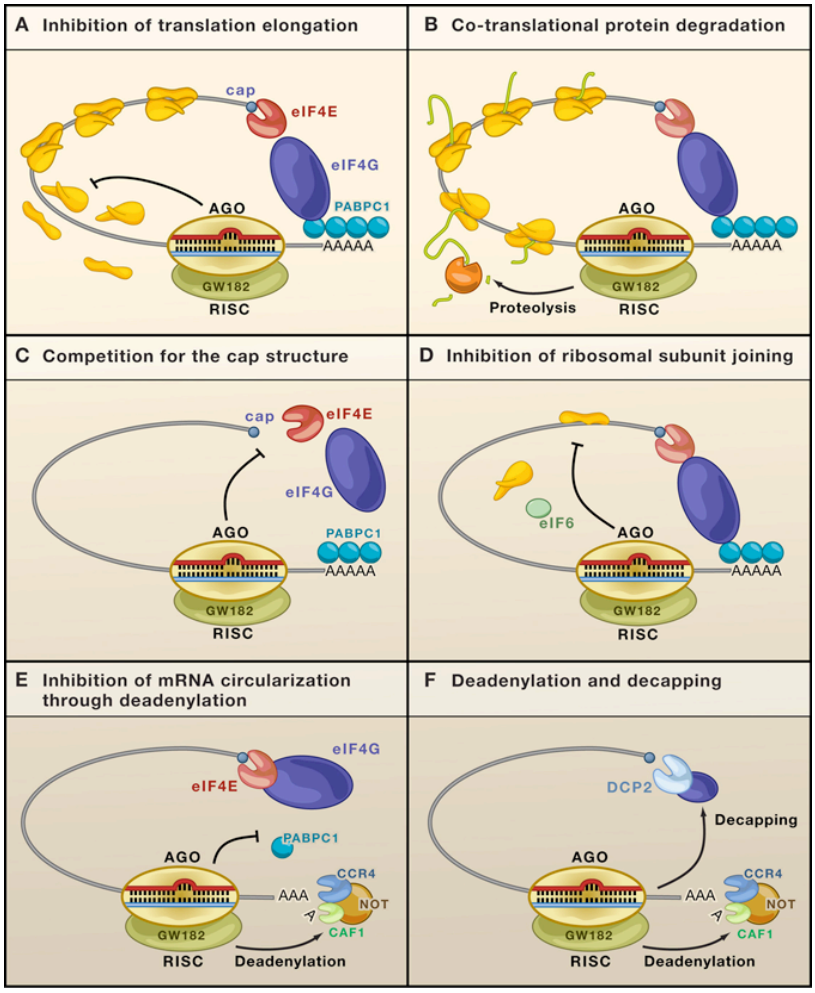


Figure 5: Mechanisms of miRNA action. (A) Postinitiation mechanism. miRNAs block translational elongation. (B) Co-translational protein degradation. In this model, translation is not inhibited, but the translated nascent polypaptide is degraded immediately. (C-E) Initiation mechanisms. miRNAs inhibit translation at an early step, so that elongation cannot take place. (F) mRNA decay. Bound miRNAs induce deadenylation and decapping of the target mRNA. Adapted from Eulalio, 2008.

1.7 Regulation of miRNA expression

When the first two miRNAs, lin-4 and let-7, were discovered, they were also found to be developmentally regulated[26, 28]. A loss of let-7 led to the recurrence of larval cell fates during adult stages, while increased let-7 levels induced the expression of adult fates during larval stages. Developmental timing of miRNA expression or tissue-specific

expression patterns was subsequently detected for many vertebrate miRNAs[43]. The majority of differentially regulated miRNAs may be due to regulation on transcriptional level as most miRNAs are transcribed in an RNA Polymerase II-dependent way[33]. However, several studies indicate that post-transcriptional regulation of miRNAsexpression might also contribute to the timing- or tissue-specific miRNA pattern. One example is miR-38 in *C. elegans*, where a post-embryonic down-regulation of the mature miRNA occurs, while the precursor miRNA is expressed throughout all stages from larval to adult worms[44]. This observation suggested an inhibition of miRNA maturation either by intervening in nuclear export or by directly blocking the accessibility of Dicer or the precursor[33].

Differential accumulation of precursor or mature miRNA was also found in rats engineered to overexpress individual miRNAs[45], indicating a post-transcriptional regulation of processing efficiency. Another study showed differential regulation in cancerous versus normal colorectal tissue of human samples[46], followed by a recent study in different tissues, tumors, and cancer cell lines revealing widespread discrepancy between the levels of precursor and mature miRNA[47]. Examining expression patterns of miRNAs in zebrafish brains, Kapsimali et al. also supported the idea of a post-transcriptional regulation, as they found that miRNAs transcribed from the same cluster nevertheless display differential expression[48].

In general, regulation of miRNA expression may occur at different levels: inhibition of Drosha processing, prevention of nuclear export, or blocking of Dicer processing. Thomson and colleagues showed that a large fraction of miRNAs in mouse is down-regulated post-transcriptionally at the Drosha processing step[49]. Last year, Guil and Cáceres were the first to identify a protein factor involved in regulation of miRNA biogenesis[50]. They could show that the RNA binding protein hnRNP A1 was essential during Drosha processing of pri-miR-18a. RNAi-based depletion of the auxiliary protein factor resulted in reduction of *in vitro* processing activity and decreased levels of pre-miR-18. Control experiments revealed that the effect of hnRNP A1 on processing was specific for pri-miR-18a, supporting the idea that individual miRNAs require specific cofactors during biogenesis. SMAD proteins were also found to play an auxiliary role in Drosha processing of pri-miR-21[51].

First preliminary insights into mechanistic details of regulation of Drosha processing were provided by two independent research groups: this year, the labs of Gregory and

Hammond demonstrated that lin-28 selectively blocks miRNA processing at the Drosha step by binding to conserved nucleotides in the loop region of pri-let-7[52-54]. More studies concerning let-7 processing by lin-28 or regulation of Drosha in general can be envisioned in the near future.

1.8 Background of this Thesis

To understand the complex regulatory networks of miRNAs which involves the regulation of one mRNA target by many miRNAs, and the control of several targets by a single miRNA, regulation of miRNA expression needs to be investigated[55]. Several research groups have demonstrated regulation of Drosha processing by various factors[49-51] (see section 1.7 for more details). Nevertheless, the exact mechanism of regulation remains largely mysterious.

However, control of miRNA biogenesis at other steps than Drosha processing is even less understood. Wulczyn showed that let-7 processing is inhibited at the precursor level in undifferentiated EC cells[56]. Some progress has been made recently, when Rybak and colleagues identified lin-28 to be an inhibitor of pre-let-7 Dicer processing, challenging previous work from Hammond and Gregory labs[57] (see section 1.7). However, Rybak explained the discrepancy in results by a multi-level post-transcriptional control of let-7 expression, involving inhibition at Drosha and Dicer processing in parallel.

Regulation of miRNA biogenesis at Dicer level is also subject of ongoing research in the Martinez Lab[58-60]. In 2006, Obernosterer *et al.* used Northern blots and *in situ* hybridizations to demonstrate that the precursor of the brain-specific miRNA miR-138 is ubiquitously found in mouse, whereas the mature transcript is restricted to neurons of the neo-cortex, the hippocampus, the cerebellum, and to cells of the fetal liver[60]. Thus, an additional layer of miRNA regulation besides the regulation on promoter level was proposed. Using *in vitro* processing assays, they provided evidence for the presence of a proteinaceous inhibitory factor in cells not expressing miR-138, ruling out the possibility of a Dicer activator.

In later studies, Martinez *et al.* used mutation analyses to reveal the importance of the secondary structure of pre-miR-138 for recognition by the inhibitor. The structure of pre-miR-138 includes a 2-nt overhang at the 5' end, two central loops, a one-nucleotide bulge and a terminal loop (Figure 6). Dicer cleaves 5' of or within the second loop, resulting in a

23 or 24 nt long mature miR-138. Closing loop 1 (Figure 6) by site-directed mutagenesis resulted in the recovery of Dicer processing activity in HeLa cell extracts indicating that the inhibitory effect is mediated via recognition of loop 1[58].

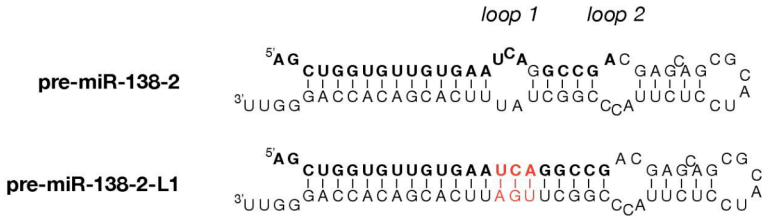


Figure 6: Secondary structure of pre-miR-138. Top: The 69 nt long precursor comprises two central and one terminal loop and a single mismatch close to the terminal loop. Down: structure of mutated pre-miR-138, where loop 1 was closed. Figure adapted from Leuschner, 2007.

1.9 Aim of this Thesis

In an effort to purify and identify the unknown inhibitor, a combination of classical chromatography and affinity purification had been employed[58]. The classical chromatography approach involved seven purification steps using various resins and elution conditions, yielding large numbers of putative inhibitors after mass-spectrometry analysis. A second, independent affinity purification approach using StreptoTag technology[61] was employed in parallel to minimize the amount of candidate proteins co-eluting with the inhibitory activity.

Comparing mass-spectrometry and size analysis results from the classical purification series with data from the affinity chromatography approach revealed that a single protein of approximately the correct size as determined by gel filtration (15-20 kDa) appeared in both independent data sets. The protein found in both mass-spectrometry analyses was signal recognition particle 14 (SRP14).

SRP14 is a highly conserved cytoplasmic ribonucleoprotein with a function in sorting secretory and membrane proteins to the endoplasmic reticulum as part of the SRP (signal recognition particle) complex[62]. SRP recognizes signal sequences on nascent polypeptide chains, resulting in an arrest of protein synthesis. The ribosome-SRP complex is then directed to the endoplasmic reticulum to the SRP receptor, where SRP dissociates from the ribosome and protein synthesis is continued with the simultaneous translocation of the emerging polypeptide into the endoplasmic reticulum.

SRP consists of six protein cofactors and a single 300-nt RNA molecule[63] (Figure 7). Two of these polypeptides, SRP9 and SRP14, form a stable heterodimer *in vitro* and only then bind to a specific 87-nt long RNA sequence called *Alu* region[64].

As SRP9/14 can exist as a heterodimer without SRP, the possibility exists that it might play additional roles by forming complexes with *Alu*-like RNAs or even different small RNAs[65]. Furthermore, SRP9 and SRP14 accumulate in 20-fold excess over SRP in primate cells as RNA-bound complex or in free cytoplasmic form. Due to the RNA binding capability, the proposed additional roles of SRP9/14 heterodimer, and its excess over SRP, it constituted an interesting candidate to test for the putative inhibitory function in miRNA biogenesis. To examine a possible inhibitory role of the heterodimer SRP9/14 in Dicer processing, recombinant expression of this candidate protein together with its binding partner SRP9 as well as RNAi-mediated knock-down in HeLa cells was performed, followed by testing of the depleted extract and the recombinant protein, respectively, on Dicer in *in vitro* processing assays.

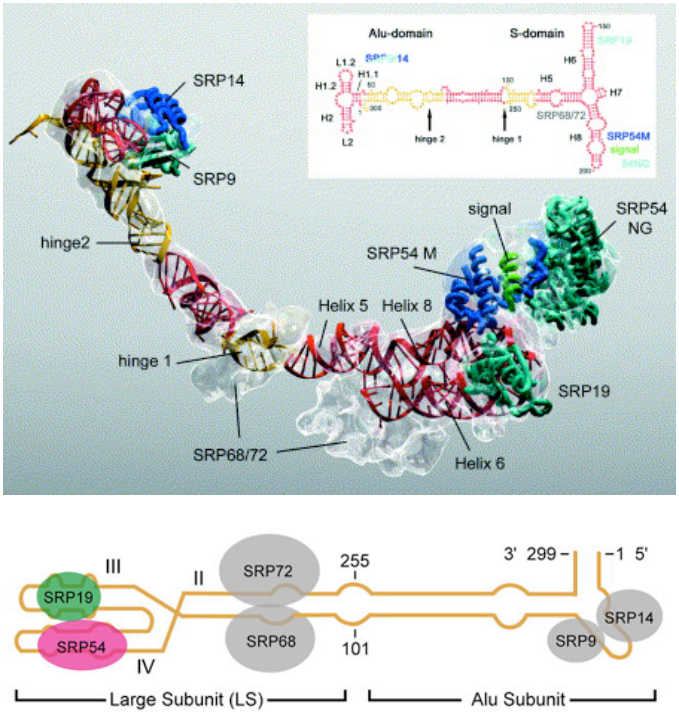


Figure 7: Structure of SRP. (Top) Molecular model of SRP. Cyan, blue and grey: SRP proteins; red and yellow: 7S RNA; green: signal sequence. Adapted from Halic, 2005. (Bottom) Schematic representation of SRP. Proteins are shown in colored and grey ovals, RNA is depicted in orange. SRP9 and SRP14 form a heterodimer and bind to the *Alu* Subunit of SRP. Adapted from Maity, 2007.

2. Results

Two approaches were employed in order to test the role of SRP9/14 in miRNA processing as a putative candidate protein for the inhibition of miRNA maturation.

a) Recombinant expression of SRP9 and SRP14 in bacteria,
b) Knockdown of SRP9 and SRP14 in HeLa cells by means of RNA interference.

First, recombinant expression allowed the addition of SRP9 and SRP14 to the already established *in vitro* processing assays of pre-miR-138. Inhibition of *in vitro* pre-miRNA processing using recombinant protein would provide evidence for SRP9/14 being a valid candidate.

In the second part, the aim of investigation was whether SRP9/14-depleted HeLa cell extracts would be able to process pre-miR-138. Recovery of processing activity would indicate that SRP14 alone or in complex with SRP9 is indeed involved in inhibition of Dicer.

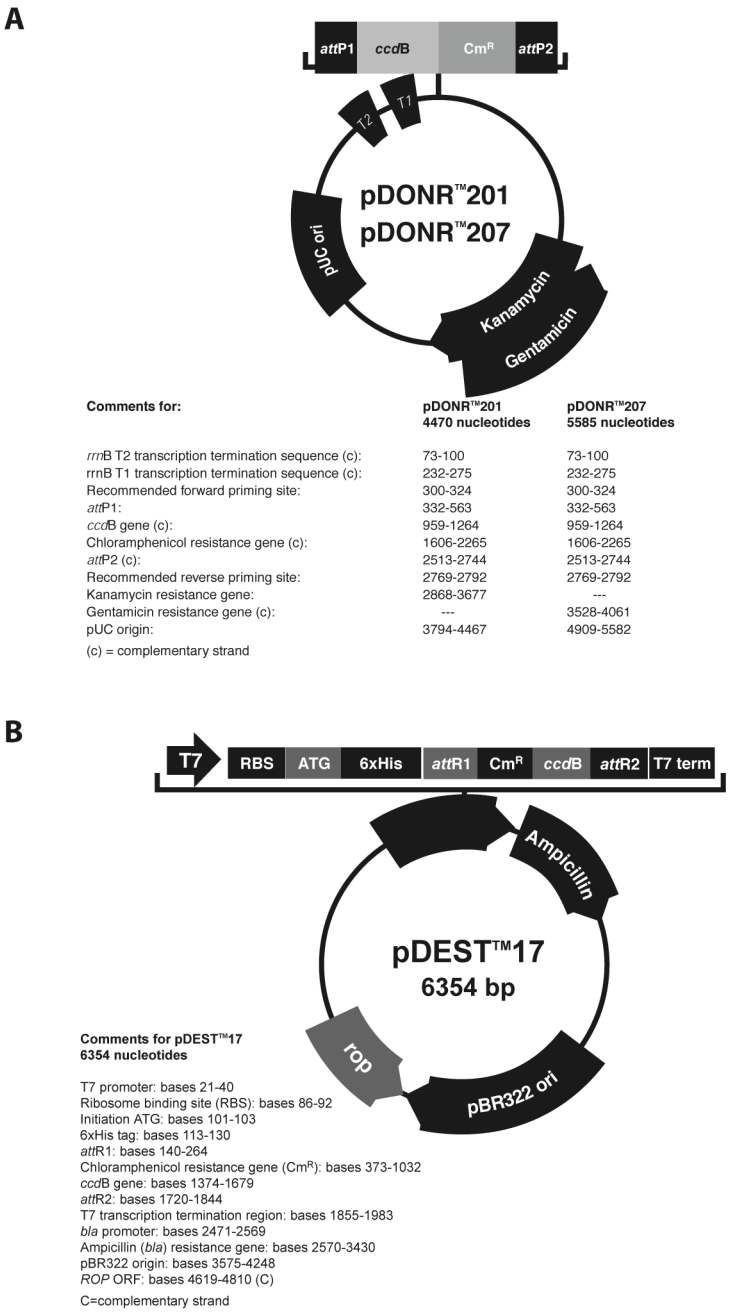
2.1 Recombinant Expression of SRP14

2.1.1 Cloning of SRP9 and SRP14

One approach to test a potential candidate for its putative function is its recombinant expression and subsequent addition to the functional test. To express a recombinant protein, the gene of interest needs to be inserted into a vector specialized for expression, like pDEST17 which contains a T7 promoter for IPTG-inducible transcription. Thus, the first aim was to insert human SRP9 and SRP14 genes into the bacterial expression vector. For cloning, the Gateway® Technology developed by Invitrogen was chosen (see Methods section 5.8.1).

SRP9 and SRP14 were amplified from the RIKEN clone 9530033C02 (261 bp) and F830102009 (333 bp), respectively. Using Gateway® Technology, universal attB-sites were added to the 5' and 3' ends of the cloned gene. attB sites serve as recombination sites to insert the attached gene into a pDONR vector (pDONR201), which again contains recombination sites to shuttle the gene of interest into an optional destination vector. For the purpose of this thesis, pDEST17 was used as destination vector (Figure 8). Final

sequencing analysis revealed that the SRP9 and SRP14, respectively, were successfully cloned into pDEST17.



Comments for:	pDONR™201 4470 nucleotides	pDONR™207 5585 nucleotides
*rrn*B T2 transcription termination sequence (c):	73-100	73-100
rrnB T1 transcription termination sequence (c):	232-275	232-275
Recommended forward priming site:	300-324	300-324
*att*P1:	332-563	332-563
*ccd*B gene (c):	959-1264	959-1264
Chloramphenicol resistance gene (c):	1606-2265	1606-2265
*att*P2 (c):	2513-2744	2513-2744
Recommended reverse priming site:	2769-2792	2769-2792
Kanamycin resistance gene:	2868-3677	---
Gentamicin resistance gene (c):	---	3528-4061
pUC origin:	3794-4467	4909-5582

(c) = complementary strand

Comments for pDEST™17
6354 nucleotides

T7 promoter: bases 21-40
Ribosome binding site (RBS): bases 86-92
Initiation ATG: bases 101-103
6xHis tag: bases 113-130
*att*R1: bases 140-264
Chloramphenicol resistance gene (Cm^R): bases 373-1032
*ccd*B gene: bases 1374-1679
*att*R2: bases 1720-1844
T7 transcription termination region: bases 1855-1983
bla promoter: bases 2471-2569
Ampicillin (*bla*) resistance gene: bases 2570-3430
pBR322 origin: bases 3575-4248
ROP ORF: bases 4619-4810 (C)

C=complementary strand

Figure 8: (A) pDONR201 vector map. (B) pDEST17 vector map. pDEST17 contains an ampicillin resistance gene and a coding sequence for a N-terminal His-tag. SRP9 and SRP14 were inserted after the His-tag via recombination, replacing the chloramphenicol resistance and the ccdB gene, the latter one preventing cells from growing on culture. Adapted from Invitrogen.

pDEST17-SRP9 and pDEST17-SRP14 were then used for bacterial expression of His-tagged SRP9 and SRP14.

2.1.2 Expression and purification of SRP9 and SRP14

pDEST17-SRP9 and pDEST17-SRP14 were transformed in chemically competent *E. coli* (Rosetta™) and expressed overnight upon IPTG induction (see "Methods", section 5.6.1, for details). After bacterial lysis, His-tagged SRP9 and SRP14 were purified using Ni-column affinity chromatography. During the purification, aliquots of each step were taken and loaded on a polyacrylamide gel to check for enrichment of the recombinant protein.

Figure 9A shows a Coomassie stain with aliquots from SRP9 purification. Surprisingly, the band emerging after induction of protein expression was detected between 10 kDa and 15 kDa., exceeding the expected 9 kDa for SRP9 slightly. Pellet and input both comprised similar levels of SRP9, indicating that the bacterial lysate contained only half of total protein. The flow-through was devoid of SRP9, implying that the column was not overloaded. During the washing steps, some of the protein was eluted probably due to increasing concentrations of imidazole in all of the wash buffers (25-100 mM). Nevertheless, the majority of protein was eluted with 300 mM in elutions 1 and 2.

Figure 9B shows a Coomassie-stained gel from SRP14 purification. A band at 15 kDa existing in the induced aliquot but not in the uninduced sample indicated that SRP14 expression was successfully initiated upon adding IPTG to the medium. Due to overload of the third lane (pellet), it cannot be clarified, whether the majority of the recombinant protein remained in the pellet or in the supernatant after bacterial lysis. The flow-through still contained a large amount of SRP14, indicating that the Ni-column was oversaturated. The concentration of imidazole was increased during the four washing steps (25-100 mM), which might be responsible for partially elution of bound SRP14 with washing buffer. Similar to purification of SRP9, the majority of the protein was eluted with 300 mM imidazole in elution 1 and 2.

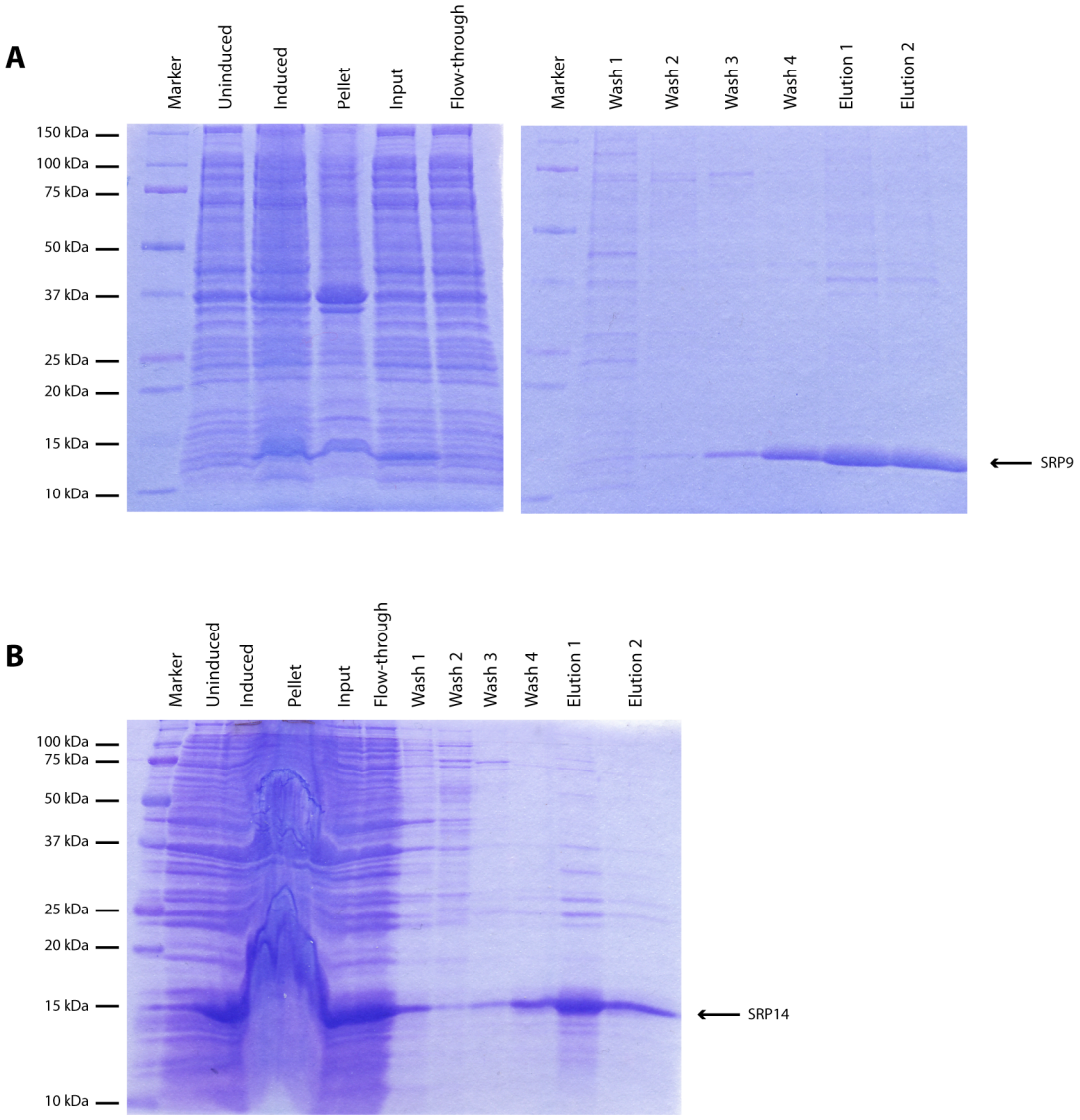


Figure 9: (A) Purification of SRP9 on Ni-column. 45 µg of cell lysate or protein was loaded per sample well. The Coomassie stain shows that upon IPTG induction, SRP9 was expressed (lane "Uninduced" vs. lane "Induced"). Enrichment of SRP14 was successful in eluate 1 and 2 (300 mM imidazole), although the samples were contaminated with various proteins. (B) Purification of SRP14 on Ni-column. 45 µg of cell extract or protein was loaded per sample well. Expression of SRP14 was induced with IPTG. His-tagged SRP14 was purified on Ni-column and eluted at 300 mM imidazole in elution 1 and 2.

2.1.3 Analysis of recombinant SRP9/14 in processing assay

The processing assay was performed using radiolabeled pre-miR-138, recombinant Dicer enzyme (rDcr) and dialyzed recombinant SRP9/14. Results indicated that only the addition of undiluted SRP9/14 resulted in inhibition of processing in the *in vitro* assay, but not diluted SRP9/14 (1:5, 1:10, 1:50, 1:100). pre-miR-19 and pre-miR-138L1, a mutated variant of pre-miR-138 (see also Figure 6) served as control substrates. Contrary to pre-miR-138, pre-miR-138L1 displays no inhibition of processing by HeLa cell extracts due to the closing of loop 1. The inhibitory effect of undiluted SRP9/14 was observed with

both pre-miR-138 and pre-miR-138L1, indicating that this effect is not specific for pre-miR-138 but due to unspecific binding of SRP9/14 to RNA (Figure 10). Inhibition of Dicer processing after addition of recombinant protein might be due to an unspecific RNA binding by SRP9/14, blocking the access of Dicer to the precursors.

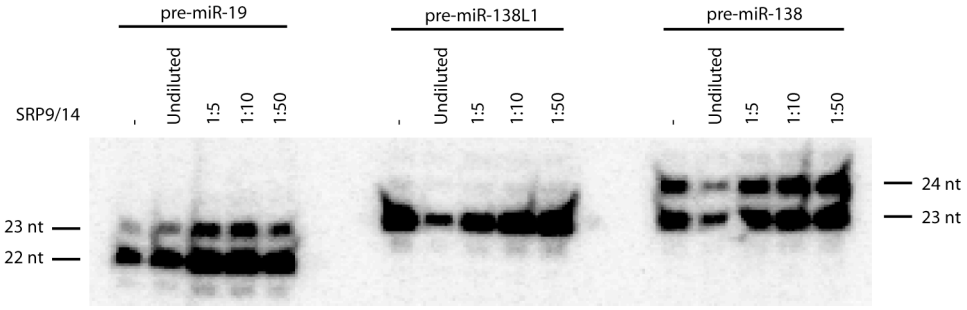


Figure 10: Dicer *in vitro* processing assay with SRP9/14. 5 µl of the reaction mix were loaded per sample well. Incubation of pre-miR-138 with SRP9/14 and Dicer resulted in a reduction of mature miRNA in the undiluted SRP9/14 sample. The same effect was detected when incubating with control pre-miR-138L1, indicating that an unspecific RNA binding effect of SRP9/14 resulted to inhibition of processing by Dicer. The usually included control pre-miR-19 did not work in all assays involving SRP9/14, as can be seen here in the left-most set of bands.

2.1.4 Cloning of the fusion protein SRP9-myc-SRP14

One explanation why SRP9 and SRP14 did not show the expected effect of inhibition was the possibility that SRP9 and SRP14 could not form the heterodimer required to bind RNA properly due to unequal molarities of both proteins. For example, SRP9 and SRP14 do not bind to *Alu* sequences as monomers[66]. As it seemed to be difficult to simulate the native conditions under which formation of the SRP9/14 complex was facilitated, a different strategy was employed. A fusion protein of SRP9 and SRP14 linked by a myc epitope has been shown to fulfill the biological function of the heterodimer, i.e. binding to *Alu* RNA[66]. Generation of this fusion protein would resolve problems of unequal protein molarity.

The fusion protein SRP9-myc-14 consisted of the SRP9 protein fused at its C-terminus to a myc-tag (-EQKLISEED), which was linked to the N-terminus of SRP14 (Figure 11). SRP14 followed by myc epitope and SRP9 has been shown to be functional as well[66], but generation of this permutation was not pursued here.

Preparing the constructs for the fusion protein was more complicated than cloning the individual recombinant proteins. A short linker sequence comprising 51 nucleotides (myc-

tag) had to be inserted between the 3' end of the SRP9 gene (*srp9*, 261 bp) and the 5' end of SRP14 gene (*srp14,* 333 bp*)*. The first cloning strategy resulted in the generation of a fusion protein consisting only of *srp9* linked to *srp14* at its 3' end, omitting inclusion of the linker. Therefore, a second improved cloning strategy was developed, which proved to be successful (for exact method of cloning see section 5.8.2). The resulting gene fragment for the fusion protein was successfully inserted into pDEST17. Sequencing analysis confirmed correct pDEST9-myc-14 clones, of which one was selected for protein expression in bacteria.

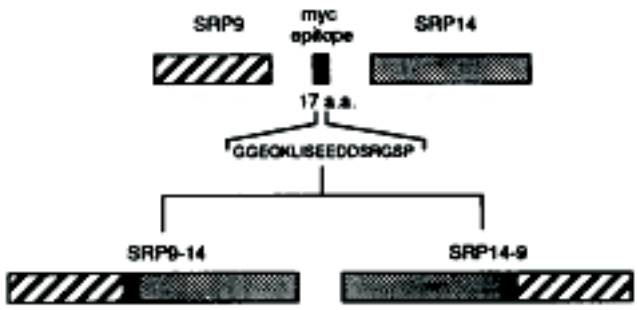


Figure 11: Permutations of SRP9-myc-14 fusion protein. In this study, only the permutation depicted on the lower left-hand side was generated and expressed. Here, SRP9 was C-terminally linked to myc, which was linked to the N-terminus of SRP14. Modified from Bovia, 1994.

2.1.5 Expression and purification of SRP9-myc-14

pDEST17-SRP9-myc-14 was transformed in chemically competent *E. coli* (Rosetta™) and expressed overnight upon IPTG induction (see Methods section 5.6.1 for details). After bacterial lysis, His-tagged SRP9-myc-14 was purified using Ni-column affinity chromatography. During the purification, aliquots of each step were taken and loaded on a polyacrylamide gel to check for enrichment of recombinant protein.

Figure 12 shows a Coomassie stain with aliquots from SRP9-myc-14 purification. A very strong band emerged after induction with IPTG at approximately 27 kDa, fitting to the data of the published fusion protein[66]. The apparent difference to the calculated molecular weight (24.5 kDa) was according to Bovia and colleagues due to the very basic C-terminal domain of SRP14. Lane 3 shows that the fusion protein was highly expressed upon induction, resulting in loss of half protein amount due to the low binding capacity of the resin. The fusion protein was eluted in an elution buffer containing 300 mM imidazole.

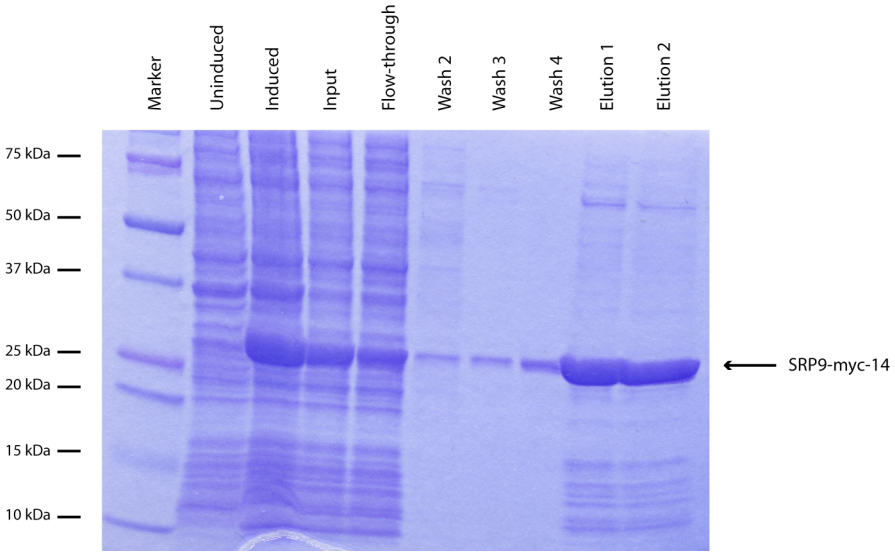


Figure 12: Purification of SRP9-myc-14 on Ni-column. 45 µg of cell lysate or protein was loaded per sample well. The Coomassie stain shows that upon IPTG induction, SRP9-myc-14 was expressed (lane "Uninduced" vs. lane "Induced"). Enrichment of SRP9-myc-14 was successful in eluate 1 and 2 (300 mM imidazole), although the samples were contaminated with various proteins. Elution 1 and 2 precipitated, there fore Wash 4 was used for *in vitro* processing assays (c = 1.5 µg/µl).

2.1.6 Analysis of SRP9-myc-14 in processing assay

For the processing assay, dialysed SRP9-myc-14 was added to radiolabeled pre-miR-138 and rDcr in several dilutions. Figure 13 shows processing of pre-miR-138 and two control precursors, pre-miR-19, and pre-miR-138L1.

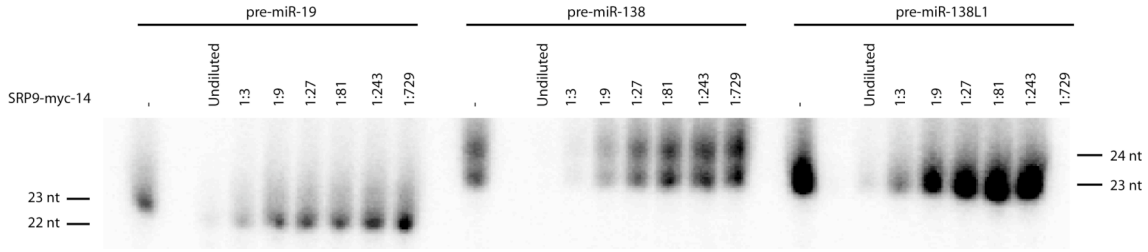


Figure 13: Dicer *in vitro* processing assay with SRP9-myc-14 fusion protein. Incubation of varying concentrations of the fusion protein with pre-miR-138 and two control precursors resulted in inhibition of all three precursors by high concentrations of the fusion protein and relieve of inhibition in dilutions of SRP9-myc-14. As both controls are processed normally by HeLa cell extracts in contrast to pre-miR-138, this result indicated that SRP-9-myc-14 did not possess inhibitory function in Dicer processing.

The single lane represents the positive control with RNAi buffer, showing active Dicer processing of the precursor. Comparison of pre-miR-138 with the two controls revealed that all three precursors were blocked in processing when adding undiluted or 1:3 diluted

fusion protein. Higher dilutions resulted in normal processing activity of Dicer. This indicates that the fusion protein binds RNA unspecifically, inhibiting Dicer access to all three precursors.

2.1.7 Analysis of effect of BSA on processing assay

The processing assay shown in Figure 13 reveals that unspecific binding sites for proteins exist on the precursor miRNAs, which might conceal a specific interaction of SRP9/14 with pre-miR-138. To solve this problem, saturation of these binding sites with bovine serum albumin (BSA) was performed. Radiolabeled pre-miR-138 and pre-miR-138L1 were incubated with rDcr and varying concentrations of BSA. Concentration of undiluted BSA solution was 0.8 µg/µl. 2 µl of undiluted or diluted BSA solutions were added to the reaction mix. Figure 14 shows that addition of BSA did not block access of Dicer to the precursor miRNAs, visualized by unaltered processing activity of Dicer.

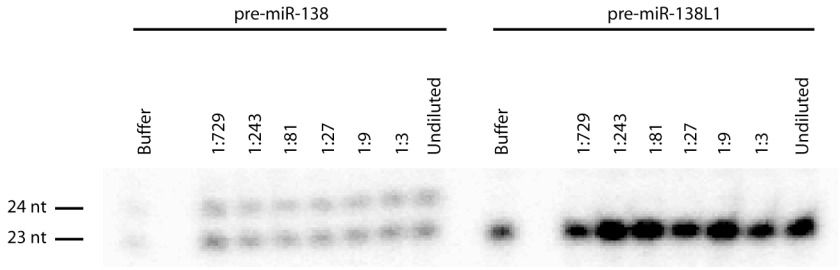


Figure 14: Dicer *in vitro* processing assay with BSA. 2 µl of a 0.8 µg/µl BSA solution and dilutions thereof were added to radiolabeled substrate and rDcr. BSA did not influence Dicer processing in pre-miR-138 or control precursor.

2.2 RNAi-mediated knockdown of SRP9/14

2.2.1 Design of siRNAs for SRP9/14 knockdown

A second strategy to assess whether SRP14 is a potential candidate for inhibition of miRNA maturation was to knockdown SRP9 and SRP14 in HeLa using RNA interference. For this, siRNAs targeting the genes under investigation were designed using two different strategies: first, it should be checked whether knockdown of SRP14 alone was sufficient to regain processing activity. Second, a double knockdown of SRP9 and SRP14 was performed to assure that both components of the heterodimer were removed. For SRP9/14 knockdown, two self-designed siRNAs against SRP14 and one commercial

siRNA SMARTpool against each SRP9 and SRP14 were ordered at Dharmacon. Sequences for self-designed siRNAs were obtained from a program assessing mRNA target site accessibility called RNAxs (http://rna.tbi.univie.ac.at/cgi-bin/RNAxs)[67] and chosen according to the optimal accessibility data. Table 1shows the sequences of siRNAs used including a control siRNA against GFP (Proligo).

siRNA name	Target gene	Sequence
siSRP9	SRP9	SMARTpool
siSRP14	SRP14	SMARTpool
siSRP14-1	SRP14	GCUGAAGAAGAGAGACAAAUU
siSRP14-2	SRP14	UCUUUCCCAACCCAUUUUUU
siGFP	-	ACUUCAGGGUCAGCUUGCCU

Table 1: siRNA sequences used in the experiments. Dh1, Dh2: Dharmacon smartpools 1, 2. siGFP served as a control, targeting no gene in HeLa cells.

2.2.2 SRP9/14 Knockdown in HeLa cells

To reduce the expression of SRP9 and SRP14 in HeLa cells, 4 different siRNAs were transfected into HeLa cells for 3 days, or 7 days in case a re-transfection was performed. Knockdown efficacy of the siRNAs was tested using Western Blot analysis. For this, 20 µg protein extracts of HeLa cells transfected with different siRNAs were loaded on a polyacrylamide gel. After blotting, the membrane was probed with antisera against SRP14. Figure 15 shows a robust reduction in SRP14 protein levels upon transfection of siSRP9, siSRP14, both, and siSRP14-1. Transfection of siSRP-14-Dh2 yielded a less strong reduction of SRP14. Interestingly, knockdown of SRP9 resulted in a decrease of SRP14 protein levels, possibly because SRP14 exists in complex with SRP9 and is degraded faster as a monomer. It has been observed that SRP14 was rather sensitive to proteolytic degradation (Bovia 1995). A siRNA against GFP not targeting any gene in HeLa cells was used as a control. Higher knockdown efficacy was achieved in cell extracts harvested three days after transfection than in those harvested after 7 days and a second transfection. This could have resulted from a higher replication rate of untransfected, unstressed cells, which might have overgrown SRP9/14 depleted cells. The experiments indicate that siSRP9, siSRP14, both, and siSRP14-1 cause knockdown of endogenously expressed SRP14 protein levels in transfected HeLa cells.

2.2.3 Analysis of processing activity in depleted HeLa cells

To examine whether SRP9/14-depleted HeLa cell extracts were able to recover Dicer activity and therefore produce the mature miR-138, *in vitro* processing assays using recombinant Dicer enzyme (rDcr) and radiolabeled pre-miR-138 were performed. If SRP14 were the candidate, one would expect to detect cleavage by Dicer in the SRP14-depleted cell extracts or at least stronger bands than in control cells. The result given in Figure 15 indicates that knockdown of SRP9, SRP14 or both did not influence the inhibition of processing in HeLa cell extracts as the resulting band pattern revealed no difference between SRP14-depleted and control cells (siGFP, siSRP14-Dh2). As a positive control for Dicer activity, labeled substrate was incubated with rDcr and RNAi buffer. Based on this and previous results of the recombinant protein, SRP14 was excluded from the list of putative Dicer inhibitors.

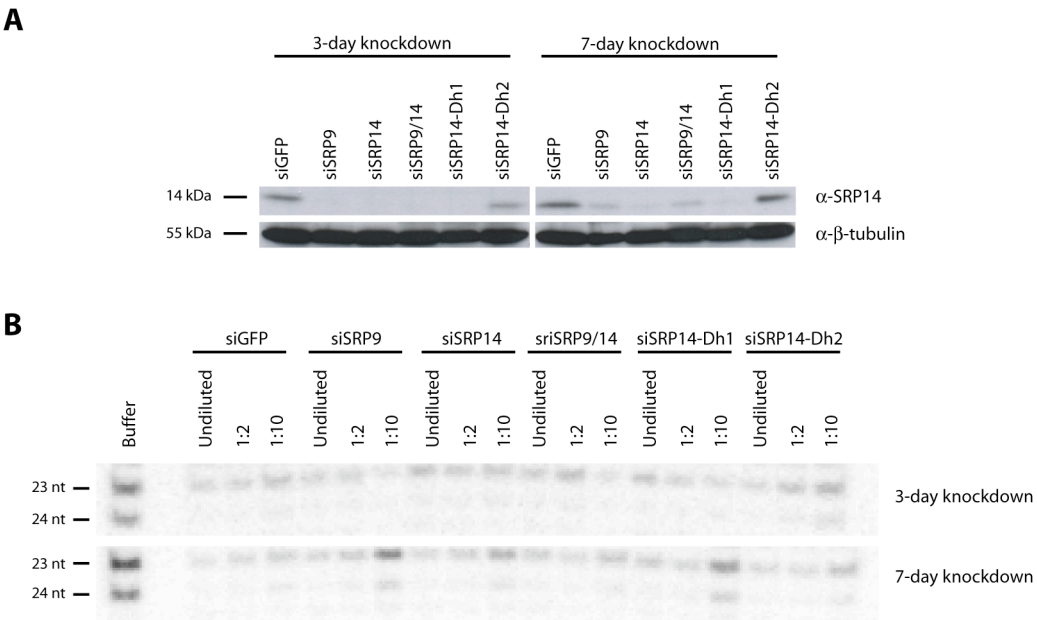


Figure 15: (A) Evaluation of siRNAs targeting SRP9 and SRP14. 20 pmol of different siRNAs including control siGFP were transfected into 70% confluent HeLa cells. Cells were harvested 3 or 7 days after transfection. SRP14 expression levels were monitored by Western Blot using commercial antisera against SRP14 (1:1000). For loading control, the blot was probed with antisera against β-tubulin. (B) Radiolabeled pre-miR-138 was incubated with rDcr and depleted HeLa extracts as indicated on top. Undiluted extracts or 1:2 and 1:10 dilutions were used. Buffer alone served as positive control (first lane). No difference of processing activity between control extracts (siGFP, siSRP14-Dh2) and SRP14-depleted extracts were observed.

3. Discussion

miR-138 is a brain-specific miRNA. In kidney, liver and lung tissues as well as HeLa cells the precursor of miR-138 accumulates and is never processed to the mature and functional miRNA[60]. This maturation step fails to take place due to the intervention of a proteinaceous inhibitory factor[58]. The aim of this thesis was to test one protein candidate for its putative role in inhibition of miR-138 maturation in HeLa cells. A search for candidate proteins by comparing mass-spectrometry results from two different purification strategies yielded a single promising candidate: signal recognition particle 14 (SRP14). SRP14 forms a complex with SRP9 and exerts its only known function as part of the multi-domain protein complex SRP, co-translationally sorting proteins to the endoplasmic reticulum. Interestingly, in primate cells, SRP9/14 exists also in a free cytoplasmic form, bound to *Alu*-like RNAs. While murine SRP9/14 does not exist in excess over SRP, primate SRP9/14 was found to be in more than 20-fold excess in HeLa cells and three other primate cell lines. Furthermore, human SRP14 contains a C-terminal repeat domain not present in murine SRP14. Thus, an additional function for this heterodimer has been assumed (Bovia 1995). The fact that murine and primate SRP14 differ in several aspects challenged the hypothesis that SRP9/14 was involved in the miRNA pathway. Obernosterer *et al.* detected accumulation of precursor and lack of mature product both in mouse tissues and HeLa cell extracts[60]. Nevertheless, the congruence in mass-spectrometry data and the RNA-binding ability of the candidate protein prompted us to examine the role of SRP14 in inhibition of pre-miR-138 processing by addition of the recombinant SRP14 to the Dicer *in vitro* processing assay, and by RNAi-mediated knockdown and subsequently testing cellular extracts for inhibitory activity.

In the last years, much work has been done to elaborate the exact binding conditions and three-dimensional structure of SRP domains[68, 69]. One very promising hint that SRP14 might be the inhibitor lies in the structure of the RNA binding protein and its known interactor 7 SL RNA in SRP.

Both SRP9 and SRP14 bind to the 5' end 3' ends of 7SL RNA in mammalian SRP, forming the *Alu* domain (Figure 16). The SRP9/14 heterodimer clamps together the open ends of the RNA by folding into a six-stranded β-sheet with a positively charged concave surface, which binds the negatively charged *Alu* domain RNA. SRP9/14 also interacts

with rRNA of the ribosome[68, 69]. The secondary structure of the *Alu* RNA contains a three-way junction of stems known as τ-junction in three-dimensional arrangement. A central U-turn in this substructure is specifically recognized by SRP9/14 and characterized by the conserved sequence UG**U**URR, where the second U forms the U-turn and R stands for a conserved purine[69]. Examination of the sequence of pre-miR-138 revealed that the consensus sequence required for binding of SRP9/14 is nearly completely present on the 5' arm of pre-miR-138 (Figure 6): UGUUG*U*.

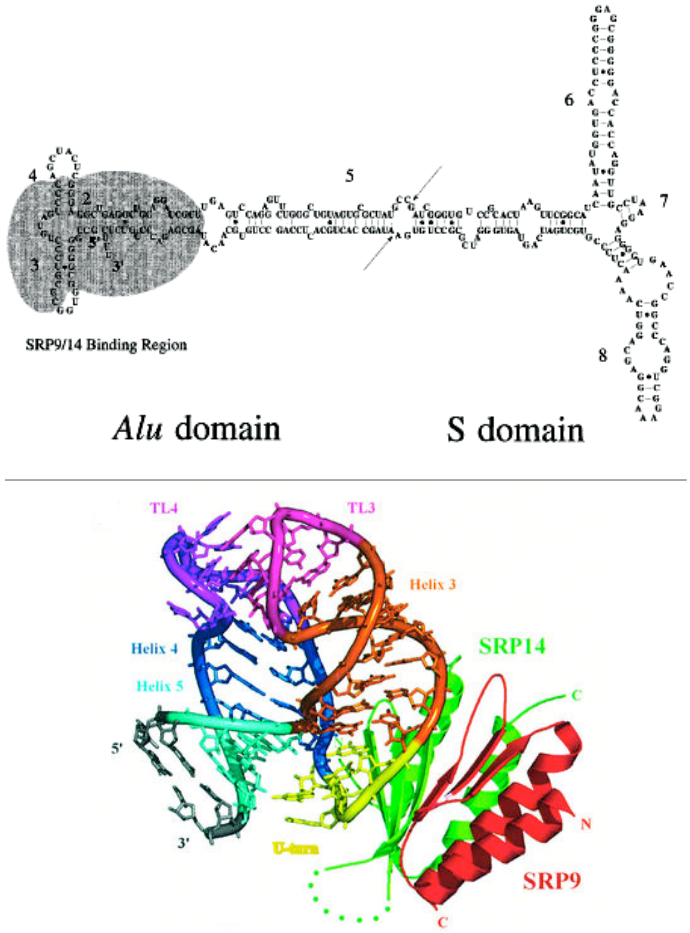


Figure 16: (Top) Secondary structure of human SRP RNA with the heterodimer SRP9/14 bound. Adapted from Birse 1997. (Bottom) Three-dimensional representation of *Alu* domain crystal structure containing SRP9 (red) and SRP14 (green) bound to RNA. Adapted from Nagai 2003.

SRP9/14 represented an attractive candidate for the inhibitor based on the arguments mentioned above. As SRP plays a crucial role in the maintenance of cellular function, all SRP subunits including SRP9/14 are expressed in every cell type comprising neural cells. Therefore, an explanation for the lack of inhibitory function of SRP9/14 in neural tissues is indispensable.

One possibility why the ubiquitously expressed protein SRP9/14 does not act as an inhibitor in neural cells could be the existence of an RNA transcript in brain, which serves as an energetically more favorable RNA binding substrate for SRP9/14 than pre-miR-138. This RNA transcript could titrate out the inhibitor and release a free pre-miR-138, enabling precursor maturation. A second explanation could be that SRP9/14 is expressed in 20-fold excess only in tissues others than brain and that the expression level in neural cells is not sufficient for heterodimer formation independent of SRP.

To test the candidate protein, recombinant SRP9/14 was generated and added to the Dicer processing assay. Processing of pre-miR-138 was indeed inhibited upon addition of recombinant SRP9/14 fusion protein. However, processing of control precursors was inhibited as well, indicating that unspecific binding of SRP9/14 to RNA sequences blocked the access of Dicer to all tested precursors similarly (Figure 13). Inhibition was only achieved with high concentrations of recombinant protein. Addition of BSA to the assay was performed to test for concentration-dependent unspecific RNA binding. No difference in processing activity was observed with increasing BSA concentrations in both control precursor and pre-miR-138 (Figure 14).

Expressing functional recombinant proteins is often prone to failure. In principle, expression can be performed in prokaryotic or eukaryotic systems. Prokaryotic systems like the *E. coli* system used in this study provide several advantages: ease of culture, fast growth, easy induction (e.g. IPTG), simple and numerous purification options. On the other hand, recombinant expression of proteins particularly from eukaryotic genes may result in partial or complete loss of enzymatic activity due to the lack of post-translational modifications or misfolding of the protein as the bacterial cellular interior does not provide the folding environment possibly required. Eukaryotic systems (yeast, mammalian cells, insect cells) are advantageous in many ways but more difficult to handle. In this study, SRP9, SRP14 and the fusion protein were expressed in bacterial systems. As the proteins do not possess an enzymatic function and proved to be soluble, it was decided to stay with the bacterial system.

A weak point of the experimental setup is the fact that the SRP RNA binding characteristics of the recombinant proteins were never checked after purification from the lysate as has been done in previous studies[66]. Consequently, due to the denaturing conditions of the purification and elution with high concentrations of imidazole, it is possible that proper refolding of the fusion protein was not accomplished, resulting in impaired SRP9/14 function.

Furthermore, an antibody against SRP14 was available at time of the experiments, but successful enrichment of SRP14 or the fusion protein was concluded only from the detection of a strong band of expected size emerging after IPTG induction (Figure 9, Figure 12). To confirm the expression of SRP14 or the fusion protein, a Western Blot probing for SRP14 should have been performed.

BSA is used in numerous biochemical applications to reduce unspecific binding of proteins to surfaces (Western blot, immunostainings) or to stabilize enzymes during restriction digests, but is less well known for RNA binding activity. To examine whether a candidate protein has a specific function on miRNA processing, other groups have used hnRNP A1, YBX-2, Msi-2 or hnRNPL as control RNA binding proteins[54]. Nevertheless, in this study BSA added to the *in vitro* Dicer processing assay to block unspecific protein binding sites on control precursors.

The results of the assay involving recombinant candidate protein were not clear enough to allow exclusion of SRP9/14 from the list of putative inhibitors. Therefore, a second approach was pursued to check for inhibitory activity of SRP9/14.

For this, RNAi-mediated knockdown of SRP9/14 was performed. Depleted HeLa cell extracts were then added to the Dicer *in vitro* processing assay. However, removal of SRP14 from cellular extracts did not yield mature miR-138, implying that SRP14 is not involved in inhibition of Dicer processing and that the sought-after inhibitor was still present in the SRP14-depleted extracts.

3.1 Perspectives

Once the inhibitor of miRNA maturation has been identified, several experiments could be devised for validation as well as further investigation of the regulatory mechanism. Validation experiments include *in vitro* studies with recombinant protein and inhibitor-depleted HeLa cell extracts as described in this thesis. Furthermore, an immunoprecipitate of the inhibitor from HeLa cells could be added to the *in vitro* processing assay and checked for its effect on miRNA maturation. Inhibitor-depleted HeLa cell extract could be complemented with recombinant inhibitor and should regain the inhibitory effect.

The role of the inhibitor on processing should be investigated not only *in vitro* but also *in vivo*. One possibility is to ectopically express the protein factor and pre-miR-138 in cell lines normally processing pre-miR-138 and checking for impaired maturation by Northern Blotting. Ectopical expression of only the protein factor followed by measurement of endogenous miRNA levels by quantitative PCR would provide further evidence for *in vivo* relevance of the protein factor.

To confirm a direct interaction of the inhibitor with pre-miR-138, one could perform a co-sedimentation assay or an electromobility shift assay (EMSA) using radiolabelled pre-miR-138. However, to gain structural insights into the regulatory mechanism, fragments of pre-miR-138 would have to be generated and tested for inhibitor binding sites by EMSA. In addition, sequence analysis by single-nucleotide mutations could reveal conserved nucleotides crucial for inhibitor binding. The role of loop 1 in pre-miR-138 maturation has to be investigated further.

Next, to assess the influence of inhibitory activity on miRNA target regulation, one could construct a Luciferase-based reporter assay where target sites for miR-138 are cloned downstream of the luciferase gene into a vector. Overexpression of miR-138 in HeLa cells transfected with this vector should lead to a decrease of luciferase activity as miR-138 binds to its target sites on the vector and down-regulates luciferase gene expression. In contrast, RNAi-mediated knockdown of the inhibitor in HeLa cells would result in enhancement of luciferase gene expression as the inhibitor blocks generation of a mature and functional miR-138.

Finally, domain analysis of the inhibitor could provide information on RNA-binding or catalytic domains. Single amino acid substitutions in the protein factor could reveal amino acid residues required for proper function of the inhibitor.

However, it is not known whether the inhibitor of pre-miR-138 is a factor solely destined for miR-138 regulation or plays a role in the biogenesis of dozens of miRNAs. Investigation of the specificity of the inhibitor poses another interesting research topic.

4. Materials

All standard chemicals for preparing buffers and solutions were purchased from Fluka, Sigma Aldrich, or AppliChem. Specific chemicals, kits and consumables were purchased as listed below. Solutions and buffers were prepared as described below.

4.1 Kits and Consumables

Item	Supplier	Order No.
Amersham Hybond™ - N+	GE Healthcare	RPN303B
Amersham Hyperfilm™ ECL 18x24 cm	GE Healthcare	28906937
AmpliScribe™ T7-Flash™ Transcription Kit	Biozym Scientific	150.407
BioRad Protein Assay	BioRad Laboratories GmbH	500-0006
ECL™ Western Blot Detection Kit	GE Healthcare	RPN2106V1/V2
Kodak Biomax XAR film	Sigma Aldrich	F5513-50EA
Lipofectamine® 2000 Reagent	Invitrogen	11668-019
MF™ Membrane Filtern 0.025 µm	Millipore	VSWP02500
PhastGel Blue R	Pharmacia Biotech	17-0518-01
Plasmid Maxi Kit	Qiagen	12163
Plasmid Mini Kit	Qiagen	27106
QiaEx II Agarose Gel Extraction Kit	Qiagen	28704

4.2 Chemicals

4.2.1 Protein applications

Reagent	Supplier	Order No.
1,4-Dithiothreitol (DTT)	Roche Diagnostics	10708984001
Ammonium persulfate (APS)	National Diagnostics	EC-504
complete, EDTA-free	Roche Diagnostics	11873580001
Ni-NTA Agarose	Qiagen	1018244
Precision Plus Protein™ Dual Color Standards	BioRad Laboratories	161-0374
ProtoGel	National Diagnostics	EC-890
Purified BSA 10 mg/ml 100x	New England Biolabs	B9001S
N, N, N', N'-tetramethylethylenediamine (TEMED(	National Diagnostics	EC-503
Tween® 20	Fluka	93779 250 g

4.2.2 Nucleic acid applications

Reagent	Supplier	Order No.
Ammonium persulfate (APS)	National Diagnostics	EC-504
Bromophenol blue	Merck	1.08122.0005
Chloroform-Isoamyl alcohol	AppliChem	A1935,0250
Ethidium Bromide	Fluka	46065
Gener Ruler 1 kb	Fermentas	SM0313
GeneRuler 100 bp	Fermentas	SM0243
N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)	AppliChem	A3268,0250
Phenol-Chloroform-Isoamyl alcohol pH 4	AppliChem	A2279,0250
Phenol-Chloroform-Isoamyl alcohol pH 8	AppliChem	A0889,0500
SeaKem® LE Agarose	Lonza	50004L
Sequagel Buffer	National Diagnostics	EC-835
Sequagel concentrate	National Diagnostics	EC-830
Sequagel Diluent	National Diagnostics	EC-840
SIGMACOTE® (silane)	Sigma-Aldrich	SL2-100ml
N, N, N', N'-tetramethylethylenediamine (TEMED)	National Diagnostics	EC-503

4.3 Antibodies

Reagent	Host	Supplier	Order No.
α-β-tubulin	Mouse	Sigma-Aldrich	T4026
Goat anti-mouse IgG (H+L) - HRP	Goat	Invitrogen	62-6520
Goat anti-rabbit IgG (H+L) - HRP	Goat	Invitrogen	62-6120
α-SRP14	Rabbit	Proteintech Group	11528-1-AP

4.4 Enzymes

Reagent	Concentration	Supplier
Alkaline Phosphatase	20 U/µl	Roche Diagnostics
BamHI	20,000 U/µl	New England Biolabs
Gateway® BP Clonase® II enzyme mix	-	Invitrogen
Gateway® LR Clonase® II enzyme mix	-	Invitrogen
EcoRI	20,000 U/µl	New England Biolabs
GIBCO™ 0.5% Trypsin-EDTA 10x	-	Invitrogen
HpaI	5,000 U/µl	New England Biolabs
Phusion™ Polymerase	2 U/µl	New England Biolabs
Proteinase K	-	Sigma-Aldrich
Recombinant Dicer	1 U/µl	Ambion

T4 DNA Ligase	400,000 U/µl	New England Biolabs
T4 Polynucleotide Kinase	10,000 U/µl	New England Biolabs
Taq Polymerase	5U/µl	Eppendorf
Taq Polymerase	5U/µl	5 PRIME

4.5 Plasmids

Plasmid	**Supplier**	**Order No.**
pDONR201	Invitrogen	11798014
Gateway pDEST 17 Vector	Invitrogen	11803012

4.6 Oligonucleotides and radionucleotides

4.6.1 General Oligonucleotides

attB	AAAAAGCAGGCTNN
attB2	AGAAAGCTGGGTN
SRP914-LNK-AS	GATCGCCCCGGCTGTCGTCCTCCTCGCTGATCAGCTTCTGCT CGCCGCCC
SRP914-LNK-SE	AATTGGGCGGCGAGCAGAAGCTGATCAGCGAGGAGGACGAC AGCCGGGGC
T7 promoter	TTAATACGACTCACTATAGG

4.6.2 Cloning Oligonucleotides (Primers)

attB1-fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCT
attB2-rev	GGGGACCACTTTGTACAAGAAAGCTGGGT
SRP9-fwd	AAAAAGCAGGCTCAATGCCTCAGTTCCAGACCTGGG
SRP9-rev	AGAAAGCTGGGTCTCATTCTGTTTCCATAGTGACATTGCGG
SRP9-rev-EcoRI	CCGGAATTCTGTTTCCATAGTGACATTGCGG
SRP14-fwd	AAAAAGCAGGCTCAATGGTGTTGCTCGAGAGCGAGC
SRP14-fwd-BamHI	CGCGGATCCCATGGTGTTGCTCGAGAGCGAGC
SRP14-rev	AGAAAGCTGGGTCTCACTGTGCTGGTTTGCTCTTCTTAC

4.6.3 Sequencing Oligonucleotides

SRP9-fwd	AAAAAGCAGGCTCAATGCCTCAGTTCCAGACCTGGG
SRP9-rev	AGAAAGCTGGGTCTCATTCTGTTTCCATAGTGACATTGCGG
SRP9-rev-EcoRI	CCGGAATTCTGTTTCCATAGTGACATTGCGG
SRP14-fwd	AAAAAGCAGGCTCAATGGTGTTGCTCGAGAGCGAGC
SRP14-fwd-BamHI	CGCGGATCCCATGGTGTTGCTCGAGAGCGAGC

SRP14-rev	AGAAAGCTGGGTCTCACTGTGCTGGTTTGCTCTTCTTAC

4.6.4 miRNA precursor sequences

pre-miR-19	AGUUUGCAUAGUUGCACUACAAGAAGAAUGUAGUUGUGCAAAUCUA UGCAAAACUGA
pre-miR-138	AGCUGGUGUUGUGAAUCAGGCCGACGAGCAGCGCAUCCUCUUACCC GGCUAUUUCACGACACCAGGGUU
pre-miR-138L1	AGCUGGUGUUGUGAAUCAGGCCGACGAGCAGCGCAUCCUCUUACCC GGCUUGAUUCACGACACCAGGGUU

4.6.5 Radionucleotides

Reagent	**Concentration**	**Supplier**
[γ-^{32}P] dATP	3000 Ci/mmol, 10mCi/ml	Perkin-Elmer (Easytides)

4.7 Buffers and Solutions

4.7.1 Protein applications

1 M DTT	7.71 g in 50 ml dH_2O
1 M IPTG	2.38 g in 10 ml dH_2O
10% APS	1 g APS in 10 ml dH_2O
10x PBS	80 g NaCl, 2 g KCl, 11.6 g $Na_2HPO_4.7H_2O$, 2 g KH_2PO_4, Dissolve in 1000 ml dH_2O, pH 7.3 (HCl)
10x SDS-PAGE Running Buffer	250 mM Tris Base, 1.92 M Glycine, 1% w/v SDS
10x Semidry-Blot Buffer	57.6 g Glycin, 12 g Tris, 4 ml 20% SDS, add H_2O to 400 ml; 10x Buffer:MeOH:H_2O = 1:1:8
1x PBS-T	1 ml Tween® 20 in 1000 ml 1x PBS
2x Cracking buffer	125 mM Tris pH 6.8, 25% v/v Glycerol, 5% w/v SDS, 0.01% w/v bromophenol blue, 1:10 1 M DTT (fresh): 5 ml 1M Tris, 11.5 ml 87% Glycerol, 20 ml 10% SDS 1 tip B-blue, ad 40 ml dH_2O
5x Sample buffer	62,5 mM Tris pH 6.8, 25 mM EDTA pH 8.0, 5% w/v SDS, 5% v/v b-mercaptoethanol, 0.025% w/v bromophenol blue, 50% v/V glycerol
Blocking solution	4 g non-fat dry milk in 100 ml PBST
Coomassie staining solution	8 ml 20% Acetic Acid, 8 ml PhastGel Blue R (1 tablet in 4:6dH_2O:MeOH)
Coomassing destaining solution	60 ml dH_2O, 10 ml acetic acid, 30 ml methanol
HeLa Lysis Buffer	50 mM HEPES pH 7.4, 1% NP-40, 100 mM KCl, 5 mM $MgCl_2$, 10% Glycerol, 0.1 mM AEBSF, 0.5 mM DTT (fresh); 10 ml 1M HEPES, 2 ml
Washbuffer His-tag	100 mM NaCl, 50 mM Tris-Cl pH 8, 5 mM β-Mercaptoethanol, 0.1 mM AEBSF Protease Inhibitor, ad 1000 ml dH_2O
Washbuffer His-tag 25 mM imidazole	100 mM NaCl, 50 mM Tris-Cl pH 8, 5 mM β-Mercaptoethanol, 0.1 mM AEBSF Protease Inhibitor, 25 mM imidazole, ad 1000 ml dH_2O
Washbuffer His-tag 50 mM imidazole	100 mM NaCl, 50 mM Tris-Cl pH 8, 5 mM β-Mercaptoethanol, 0.1 mM AEBSF Protease Inhibitor, 50 mM imidazole, ad 1000 ml dH_2O
Washbuffer His-tag 100 mM imidazole	100 mM NaCl, 50 mM Tris-Cl pH 8, 5 mM β-Mercaptoethanol, 0.1 mM AEBSF Protease Inhibitor, 100 mM imidazole, ad 1000 ml dH_2O

Washbuffer His-tag 300 mM imidazole	100 mM NaCl, 50 mM Tris-Cl pH 8, 5 mM β-Mercaptoethanol, 0.1 mM AEBSF Protease Inhibitor, 300 mM imidazole, ad 1000 ml dH_2O

4.7.2 RNA and DNA applications

1 M HEPES	Dissolve 24 g in 100 ml water, adjust pH with 5 M KOH to pH 7.4
100 mM ATP	4,236 g in 14 ml dH_2O (500 mM), dilute to 100 mM
10x TBE	108 g Tris, 9.3 g EDTA, 55 g Boric acid, ad 1 L dH_2O
2x Formamide	1 ml 0,5 M EDTA, 9 ml formamide, 10 ng bromophenol blue, 10 ng xylene cyanol
2x STOP Buffer	24 g urea, 4 ml 0.5 M EDTA, 10 ng bromophenol blue, dH_2O ad 50 ml
Dicer reaction buffer	30 mM Tris, 50 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 10% glycerol
Orange G Loading Buffer	0.5% Orange G in 50% glycerol
RNAi buffer (100 mM KCl)	30 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol; 30 ml 1 M HEPES, 5 ml 1 M $MgCl_2$, 33 ml 3 M KCl, 113 ml 87% Glycerol, 819 ml dH_2O
TE Buffer	10 ml 1M Tris, 2 ml 0.5 M EDTA, ad 1 L dH_2O
TN Buffer	10 mM Tris pH 8.0, 100 mM NaCl

4.8 Cells

4.8.1 Mammalian cells

Cell line	**Supplier**	**Order No.**
HeLa	IMBA collection	-

4.8.2 Bacteria

Strain	**Supplier**	**Order No.**
E. coli DH5α	IMBA collection	-
E. coli Rosetta™	IMBA collection (Novagen)	-

4.9 Media

4.9.1 Basic cell culture medium

Reagent	**Supplier**	**Order No.**
GIBCO™ DMEM 1x	Invitrogen	10938-025 500 ml
GIBCO™ Opti-MEM I 1x	Invitrogen	31985-047 500 ml
Penicillin-Streptomycin Solution	Sigma-Aldrich	P0781

L-Glutamine 200 mM	Sigma-Aldrich	G7513

DMEM for HeLa cells	Add 50 ml FCS, 5 ml Penicillin-Streptomycin, 7.5 ml Glutamine, 10 ml 1 M HEPES pH 7.4 to 500 ml DMEM

4.9.2 Special cell culture medium

Freezing Medium	90% DMEM with 15% FCS, 10% DMSO

4.9.3 Bacterial medium

LB	10 g Bacto Tryptone, 5 g Yeast Extract, 5 g NaCl, ad 1 L dH_2O, pH 7.47 (NaOH)
2x TY-MED	16 g Tryptone, 10 g Yeast Extract, 5 g NaCol, ad 1 L dH2O, pH 7.4 (NaOH)

4.9.4 Antibiotics

Antibiotic	**Supplier**	**Order No.**
Ampicillin sodium salt	AppliChem	A0839,0010
Kanamycin sulfate	AppliChem	A1493,0025

4.10 Appliances

Appliance	**Supplier**
Adjustable nucleic acid sequencing unit	C.B.S. Scientific
AlphaImager	Biozym Scientific
Analytical Balance CP64	Sartorius
Biofuge Pico Benchtop Centrifuge	Heraeus
DNA Engine Peltier Thermal Cycler	Bio-Rad Laboratories
Dual adjustable nucleic acid sequencer	C.B.S. Scientific
Image Eraser	Amersham Biosciences
Isotemp 210	Fisher Scientific
Model 583 Gel Dryer	Bio-Rad Laboratories
Multifuge 3	Heraeus
Multitron II Incubator Shaker	Infors
NanoDrop 1000	Thermo Fisher Scientific
pH Meter Level 1	inoLab
PicoFuge Microcentrifuge	Stratagene
Power Pac™ 3000 / HV	Bio-Rad Laboratories
PTC-200 Peltier Thermal Cycler	MJ Research
RC 5C Plus Superspeed Centrifuge	Sorvall

Rocketing Platform	Biometra
Rotator SB3	Stuart®
SmartSpec Plus Spectrophotometer	Bio-Rad Laboratories
Thermomixer® Comfort	Eppendorf
Top Loading Balance CP2201	Sartorius
Trans-Blot® SD Semi-dry Transfer Cell	Bio-Rad Laboratories
Typhoon TRIO	Amersham Biosciences

4.11 Software

4Peaks	http://mekentosj.com/4peaks/
AlphaImager	Alpha Innotech
Excel	Microsoft
Illustrator 10	Adobe
Multalin	http://bioinfo.genotoul.fr/multalin/multalin.html
Photoshop 7.0	Adobe
PlasmaDNA	http://research.med.helsinki.fi/plasmadna/
Reverse Complement	http://www.bioinformatics.org/SMS/rev_comp.html
RNAxs	http://rna.tbi.univie.ac.at/cgi-bin/RNAxs
T_m Calculator	http://www.appliedbiosystems.com/support/techtools/calc/
Word	Microsoft
XPlasMap	http://www.panix.com/~iayork/XPlasMap/

5. Methods

Relevant chemicals, buffers, and solutions are listed in section 4 ("Materials").

5.1 Mammalian Cell Culture

5.1.1 Standard cell culture

Standard condition for incubation of HeLa cells was 37°C in a humidified incubator supplemented with 5% CO_2.

5.1.2 Splitting of cells

Fresh plates with warm DMEM were prepared. Old medium was removed and cells were washed once with 1x PBS. After removing PBS, an appropriate volume of 1x Trypsin-EDTA was added (see Table 2) and the plate tilted to spread trypsin over the cells. Incubation of the cells for 2-5 minutes at 37°C resulted in the loss of cell adherence to the plate and the reaction was stopped by adding warm DMEM starting from the bottom of the plate to avoid the formation of cell clumps. Rotation of the plate should be avoided due to accumulation of cells at the plate walls. Cells were incubated at 37°C over night. HeLa cells growing on a 10 cm dish were split twice a week 1:10.

	Surface area	**Plating medium**	**PBS wash**	**Trypsin**	**DMEM[1)]**	**1:10[2)]**	**1:5[3)]**
10 cm plate	60 cm^2	10 ml	10 ml	0,5 ml	5 ml	0,5 ml	1 ml
6-well plate	10 cm^2	2 ml	2 ml	0,1 ml	1 ml	0,1 ml	0,2 ml

Table 2: Overview of volumes needed for splitting cells.

[1)] Volume of medium to stop reaction

[2)] Volume of cells transferred to the new plate / well for 1:10 split

[3)] Volume of cells transferred to the new plate / well for 1:5 split

5.1.3 Freezing of cells

A confluent 10 cm dish was trypsinized and washed once with 1x PBS. The cells were resuspended in freezing medium to 2x 10^6 cells/0.5 ml medium and stored in 1 ml cryo-vials at -80°C using a freezing box filled with isopropanol. After 24 hours, the cryo-vials were placed into a liquid-nitrogen container for long-term storage.

5.1.4 Thawing of cells

Aliquots containing HeLa cells were stored at -186°C in liquid nitrogen. The tube was thawed in a water bath heated to 37°C until only a little block of ice was left and then immediately put on ice. 10 ml warm DMEM were added dropwise. The cells were spinned down (2', 200 x g, RT). The supernatant was removed and after resuspending the cell pellet in 10 ml warm DMEM the cells were seeded on a 10 cm dish.

5.1.5 Transfection of adherent cells with siRNAs

Cells were seeded on a 6-well plate at a density of ~ 2x 10^5 cells per well in 2 ml DMEM and incubated until the cells were 70-80% confluent (18-24 hours). For the transfection, two solutions per siRNA were separately prepared. In solution 1, 250 µl Opti-MEM were mixed with 100 pmol siRNA and the tube vortexed. In solution 2, 250 µl Opti-MEM were mixed with 5 µl Lipofectamine™ 2000, the tube was vortexed and incubated for 5 minutes (RT). Preparation of a master mix (x times 250 µl Opti-MEM + x times 5 µl Lipofectamine™ 2000) is recommended. Both solutions were vortexed and incubated for 5 minutes at room temperature. Then the two solutions were combined, mixed carefully and incubated for 20 minutes at room temperature. In the meantime, cells were washed once with 1x PBS and incubated in 2 ml Opti-MEM per well until transfection time. For the transfection, the siRNA-containing transfection reagent was added to the wells dropwise and the cells were incubated for 6 hours. Then Opti-MEM was removed and 2 ml DMEM were added per well. After three days 75% of the cells per well were harvested for generating whole cell extracts, while the remaining 25% of the cells were seeded on a new 6 well-plate and transfected a second time in the same way like before. Three days later the cells were harvested for whole cell extracts.

To knockdown SRP9/14 in HeLa cells, the following volumes were used for transfection:

Solution 1: 6 x 250 µl Opti-MEM + 6 x 5 µl LipofectamineTM

Solution 2: 6 different solutions according to Table 3.

Opti-MEM	siRNA	Stock	Final Amount RNA	Volume pipetted
250 µl	siGFP	10 mM	100 pmol	10 µl
250 µl	siSRP9	20 mM	100 pmol	5 µl
250 µl	siSRP14	20 mM	100 pmol	5 µl
250 µl	siSRP9/14	20 mM/20 mM	100 pmol	2.5µl/2.5µl
250 µl	siSRP14-1	10 mM	100 pmol	10 µl
250 µl	siSRP14-Dh2	10 mM	100 pmol	10 µl

Table 3: Transfection scheme and reagent concentration for SRP9/14 knockdown.

The volume of reagents needed depends on the surface area of the cell culture plate. An overview of the used volumes based on the manufacturer's instructions and experience with transfection in the laboratory is given in Table 4.

Culture vessel	Surf. area per well	Shared reagents		DNA transfection		RNA transfection	
		Vol. of plating medium	Vol. of dilution medium	DNA	Lipofect-amine™ 2000	RNA	Lipofect-amine™ 2000
96-well	0.3 cm^2	100 µl	2 x 25 µl	0.2 µg	0.5 µl	5 pmol	0.25 µl
24-well	2 cm^2	500 µl	2 x 50 µl	0.8 µg	2 µl	20 pmol	1 µl
12-well	4 cm^2	1 µl	2 x 100 µl	1.6 µg	4 µl	40 pmol	2 µl
6-well	10 cm^2	2 µl	2 x 250 µl	4 µg	10 µl	100 pmol	5 µl
60-mm	20 cm^2	5 µl	2 x 500 µl	8 µg	20 µl	200 pmol	10 µl
10-cm	60 cm^2	10 µl	2 x 1.5 ml	24 µg	60 µl	600 pmol	30 µl

Table 4: Transfection scheme and reagent concentration for different plates.

5.1.6 Lysis of adherent cells

Freshly harvested cells or frozen pellets were resuspended in an appropriate volume of HeLa Lysis Buffer (e.g. 6 x 50 µl for 6-well plate). The cell suspension was pipetted up and down a few times to induce rupture of the cell membranes, followed by centrifugation of the lysate (15', 16 000 x g, RT). The supernatant was used immediately or snap-frozen and stored at -20°C as whole cell extract.

5.2 Bacterial culture techniques

5.2.1 Standard cell culture

For all experiments, *Escherichia coli* bacteria from the strain DH5α or BL21 (Rosetta™, Novagen) were used. Bacteria were generally cultured 12-16 hours either as suspension in LB medium using a shaking incubator (220 rpm, 37°C) or on agar-containing LB plates (37°C incubator). For fast growing needs, bacteria were incubated in 2x TY-MED for 8-10 hours. When required, antibiotics were added to the media in the following concentrations:

Antibiotic	Concentration Stock	Dilution	Final Concentration
Ampicillin	100 mg/ml	1:1000	100 µg/ml
Kanamycin	30 mg/ml	1:1000	30 µg/ml

5.2.2 Generation of chemically competent *E. coli*

Chemically competent *E. coli* were prepared and provided by Ivan Botto from the IMBA service department.

5.2.3 Transformation of plasmids into *E. coli*

For the standard transformation protocol, 100 µl of competent *E. coli* stored at -80°C were thawed on ice and mixed with varying volumes of the ligation mix or 50 ng of plasmid DNA (standard amount). After incubation on ice for 30 minutes, the cells were subjected to a heat shock at 42°C for two minutes and cooled down on ice for 3 minutes. For regeneration, they were then incubated in 1 ml pre-warmed LB medium without resistances for at least 60 minutes in a shaker (750 rpm, 37°C). After centrifugation (2', 3400 x g, RT) using a tabletop centrifuge 900 µl supernatant were removed, the pellet was resuspended in the remaining medium and plated out on a LB plate with the corresponding resistance (LB_{Amp} or LB_{Kan}). The plate was incubated over night at 37°C.

5.3 DNA techniques

5.3.1 General DNA protocols

Standard DNA methods (restriction digestion, cloning methods) were performed according to the manufacturer's instructions. Modifications are indicated where necessary. Plasmid preparation was performed using the QIAprep Spin Miniprep Kit (Qiagen). For standard plasmid isolation (miniprep), 5 ml LB or 2x TY-MED with the appropriate selective antibiotics were inoculated with a single colony from a LB plate. The purification protocol was performed according to the instructions given in the manual. To elute the plasmid DNA, 30 µl elution buffer or dH_2O were added to the center of the column and DNA concentration was determined by photometrical measurement at 260/280 nm (1:50 dilution in dH_2O).

5.3.2 Agarose gel electrophoresis

To separate DNA on agarose gels, agarose was mixed with 0.5x TBE to the desired concentration (e.g. 1.5 g agarose in 100 ml TBE for a 1.5% gel) and heated in a microwave until the powder was completely dissolved. After cooling down of the solution using running tap water, ethidium bromide was added in a 1:10 000 dilution. The gel was poured into a casting tray for polymerization at RT. DNA samples were pipetted into sample wells after mixing with 5x Orange G loading buffer. An appropriate DNA marker was loaded beside the samples (2-5 µl). A typical 100 ml gel was run in 0.5x TBE at 100 V for one hour. DNA fragments were visualized by exposing the gel to UV-light.

5.3.3 Conventional Polymerase Chain Reaction

PCRs were usually run with three different concentrations of template DNA: undiluted sample, 1:10 and 1:100. The concentration of the primer stock solution was always 10 µM. Dilutions of template or primer were made with dH_2O. The PCR reaction was carried out in a volume of 50 µl using a thermal cycler. Two different protocols described in the following were performed.

5.3.3.1 PCR using *Taq* DNA Polymerase

The *Taq* DNA Polymerase from *Thermus aquaticus* possesses a 5′–3′ DNA-dependent DNA polymerase activity and a 5′–3' exonuclease activity. The extension rate of *Taq* is 1 kb per

minute. Depending on the length of the product, the extension time of the PCR cycle needed to be adjusted (e.g. 2 minutes for 2 kb). The annealing time was either calculated on the basis of the melting temperature (T_m) of the primers used ($T_a = T_m - 5°C$) or several annealing temperatures were tried out in parallel.

Reaction mix

Volume	Stock	Final conc.
1 µl	Template (undil., 1:10, 1:100)	-
5 µl	10x buffer	1x
1 µl	dNTP mix (10 mM)	0.2 mM
1 µl	Forward primer (10 µM)	0.2 µM
1 µl	Reverse primer (10 µM)	0.2 µM
40.5 µl	dH_2O	-
0.5 µl	*Taq* DNA Polymerase (5 U/µl)	2.5 U
50 µl	Total volume	

PCR Program

	Time	Temp.
	5'	95°C
29 cycles	20"	95°C
	45"	x °C
	x "	72°C
	6'	72°C
	∞	4°C

5.3.3.2 PCR using Phusion™ DNA Polymerase

The Phusion™ DNA Polymerase from *Pyrococcus* is a proofreading polymerase with 3'-5' exonuclease activity (error rate: 4.4 x 10^{-7}). The extension time is 15 seconds per kb. Depending on the length of the product, the extension time in the PCR cycle needed to be adjusted (e.g. 30" for 2 kb). The annealing time was either calculated on the basis of the melting temperature of the primers used ($T_a = T_m - 5°C$) or several annealing temperatures were tried out in parallel.

Reaction mix

Volume	Stock	Final conc.
1 µl	Template (undil., 1:10, 1:100)	-
10 µl	5x buffer	1x
1 µl	dNTP mix (10 mM)	0.2 mM
0.5 µl	$MgCl_2$	
1 µl	Forward primer (10 µM)	0.2 µM

PCR Program

	Time	Temp.
	2'	98°C
29 cycles	30"	98°C
	45"	x °C
	x "	72°C
	10'	72°C

1 µl	Reverse primer (10 µM)	0.2 µM		∞	4°C
35 µl	dH_2O	-			
0.5 µl	Phusion™ DNA Polymerase (2 U/µl)	1 U			
50 µl	Total volume				

5.3.4 Sequencing analysis

To check cloned fragments or vectors 500 ng of DNA together with corresponding sequencing primers (see section 4.6.3) was sent to the in-house sequencing department. Sequence files were obtained online in ABI-format and analyzed using "4Peaks".

5.4 RNA techniques

5.4.1 RNA polyacrylamide gel electrophoresis

For RNA gels, special glass plates, combs and spacers were cleaned with detergent and 70% ethanol. The plates were dried and siliconized using Sigmacote® to facilitate removal of the gel from the glass plates after running. For processing assays, 0.4 mm spacers were used, for labeling and purification gels 1 mm spacers. Prior to loading the gel, sample wells were rinsed using a syringe filled with 0.5x TBE buffer to remove traces of urea. Volumes and reagents for casting gels are given in Table 5. RNA samples to be analyzed were mixed with 2x STOP buffer, denaturated (95°C, 1') and loaded into the sample wells. The gel was run in a vertical gel electrophoresis chamber in 0.5x TBE. For 0.4 mm gels, pre-run was at 30 W for 60 minutes, 1 mm gels were pre-run at 10 W for 10 minutes. Electrophoresis was stopped when the bromophenol blue marker reached the end of the gel.

		6%	**8%**	**10%**	**12%**	**15%**	**20%**
0.4 mm gel	**Concentrate**	7.2 ml		12 ml		18 ml	24 ml
	Diluent	20 ml		15 ml		9 ml	3 ml
	Buffer	3 ml		3 ml		3 ml	3 ml
	10% APS	240 µl		240 µl		240 µl	240 µl
	TEMED	12 µl		12 µl		12 µl	12 µl
	Volume	30 ml		30 ml		30 ml	30 ml

1 mm gel	**Concentrate**	12 ml	16 ml	20 ml	24 ml	20 ml	40 ml
	Diluent	33 ml	29 ml	25 ml	21 ml	15 ml	5 ml
	Buffer	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml
	10% APS	400 µl	400 µl	400 µl	400 µl	400 µl	400 µl
	TEMED	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl
	Volume	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml
Long gels	**Concentrate**	17 ml		28 ml		42 ml	56 ml
	Diluent	46 ml		35 ml		21 ml	7 ml
	Buffer	7 ml		7 ml		7 ml	7 ml
	10% APS	560 µl	l	560 µl		560 µl	560 µl
	TEMED	28 µl		28 µl		28 µl	28 µl
	Volume	70 ml		70 ml		70 ml	70 ml

Table 5: Reagents and volumes needed for RNA polyacrylamide gels.

5.4.2 Generation of miRNA precursor probes

5.4.2.1 *in vitro* transcription by T7 RNA polymerase

Substrates for the processing assay were generated by *in vitro* transcription using the AmpliScribe™ T7-Flash™ Transcription Kit. To create the transcription template, DNA sequences of the desired miRNA precursors obtained from the miRNA registry miRBase were fused at their 5-end to the T7 minimum consensus promoter sequence (TTAATACGACTCACTATAGG). For *in vitro* transcription, the following reaction components were combined at room temperature in the order given and incubated (37°C, 90').

Volume	Reagent	Final concentration
x µl	RNase-free water	-
1 µg	Linearized template DNA	50 ng/µl
2 µl	AmpliScribe™ T7-Flash 10x Reaction Buffer	1x
1.8 µl	100 mM ATP	9 mM

1.8 µl	100 mM CTP	9 mM
1.8 µl	100 mM GTP	9 mM
1.8 µl	100 mM UTP	9 mM
2 µl	100 mM DTT	10 mM
2 µl	AmpliScribe™ T7-Flash Enzyme Solution	-
20 µl	Total volume	

5.4.2.2 Ethanol precipitation of RNA transcripts

After *in vitro* transcription and prior to the purification of the RNA transcripts on polyacrylamide gels, the miRNA precursors were precipitated from the enzyme-buffer solution using 100% ethanol to reduce salt content and by this avoid formation of a smearing salt bubble on the polyacrylamide gel. The precipitation was performed according as follows: the volume of the sample was adjusted to 300 µl with dH_2O. 1/10 volume of 3 M sodium acetate (NaOAc) and 3 volumes of -20°C cold 100% ethanol were added. RNA precipitated at -20°C for at least 60 minutes. Then, the sample was centrifuged at 4°C for 30 minutes at maximum speed (16 000 x g) and the supernatant was removed. To wash the pellet, 1 ml of 70% ethanol was added and after inverting the tube centrifugation was repeated. The supernatant was removed to let the pellet dry. 40 µl of 1x STOP buffer were added to resuspend the pellet. The mixture was denaturated (95°C, 1') and then loaded on a 1 mm 8% polyacrylamide gel.

5.4.2.3 PAGE-purification of RNA transcripts

Transcribed RNAs can be purified using gel or column purification, depending on the heterogeneity of the sample. If other transcripts than desired exist in the sample, gel purification is preferred as this allows excising only the correct band after electrophoretic separation.

The miRNA precursors were separated on an 8% denaturing polyacrylamide gel with 1 mm slots (for conditions see 5.4.1). RNA was detected by taking advantage of the UV epi-illumniation effect of RNA strands: the gel was placed between a handheld lamp with a wavelength of 254 nm and a white sheet of paper. RNA transcripts in the gel absorbed the emitted UV rays, being visualized on the sheet of paper. Transcripts of correct size were marked and excised from the gel using scalpels. The gel slices were incubated in 300 µl of 0.3 M NaCl overnight in a shaker (4°C, 1000 rpm) to elute the transcripts. The

eluate was precipitated using 3 volumes of -20°C cold 100% ethanol at -20°C for at least 60 minutes. Then, the sample was centrifuged at 4°C for 30 minutes at maximum speed (16 000 x g) and the supernatant was removed. To wash the pellet, 1 ml of 70% ethanol was added and after inverting the tube centrifugation was repeated. The pellet was air-dried and resuspended in an appropriate volume of TN buffer to a final concentration of 10 pmol/µl (10 µM). The actual concentration of the transcript was determined by photometrical measurement at 260/280 nm using SmartSpec™ Plus (Bio-Rad).

5.4.2.4 Dephosphorylation of miRNA precursors

5'-end labeling of miRNA precursors with the radioactive isotope γ-^{32}P-ATP enables detection in processing assays. For this, the RNA transcripts were first dephosphorylated at their 5'-ends with alkaline phosphatase (20 U/µl, Roche) using 1 U per pmol RNA at 50°C for 60 minutes. To remove the enzyme from the reaction afterwards, an RNA extraction was performed.

5.4.2.5 Phenol/chloroform/isoamyl alcohol extraction of RNA transcripts

The dephosphorylation reaction was adjusted to 300 µl with dH_2O and extracted once with phenol/chloroform/isoamyl alcohol (pH 4.0, P:C:I = 25:24:1) and once with chloroform/isoamyl alcohol (C:I = 24:1), followed by ethanol precipitation according to the instructions given in 5.4.2.2. The pellet was resuspended in an appropriate volume of dH_2O to a final concentration of 10 pmol/µl.

5.4.2.6 Labeling of miRNA precursors with radioactive γ-^{32}P-ATP

For the 5'-end labeling, the following reagents and amounts were combined at RT:

Volume	Reagent	Final concentration
1 µl (= 10 pmol)	miRNA precursor	1 pmol/µl
1 µl	10x T4 PNK buffer	1x
1.5 µl	^{32}P- γ -ATP	
6.4 µl	dH_2O	-
0.1 µl	T4 PNK	
10 µl	Total volume	

After incubating the reaction mix for 25 minutes at 37°C, the reaction was quenched by adding 1 µl of 100 mM cold ATP. After 5 minutes the reaction was stopped by adding 11 µl of 2x STOP-buffer. The radioactively labeled transcripts were then purified on a polyacrylamide gel according to the instructions given in section 5.4.1. The correct band was detected by autoradiography on X-ray films (Kodak) and excised using scalpels. Elution of the 5'-end labeled miRNA precursors was performed as described before (5.4.2.3). The purified transcripts were resuspended using TN buffer to a final concentration of 100 nM, denatured (95°C, 1'), reannealed (37°C, 60') and stored at -20°C.

5.5 Functional assays

5.5.1 Dicer processing assay for miRNA precursors

This assay was developed to monitor Dicer activity on specific miRNA precursors (Leuschner 2007). In general, 0.0156 µl recombinant Dicer enzyme (rDcr, 1 U/µl) was added to 2.5 µl 2x reaction buffer. The reaction mix was incubated 10 minutes at 37°C with 1 µl of a 1:500 diluted proteinase K solution (stock: 14 mg/ml) to proteolytically activate the rDcr enzyme. 2.5 µl of the enzyme solution were mixed with 0.5 µl substrate and 2 µl dH_2O, RNAi-Buffer or SRP9/14-depleted cell extracts to check for Dicer activity. The reaction was stopped after 5 minutes at 37°C by adding 5 µl 2x STOP-buffer. RNA was denatured (95°C, 1') and loaded on a 15% polyacrylamide gel (0.4 mm slots, 30 W) to separate the cleavage products. The gel was exposed over night using X-ray films and signals detected using Typhoon TRIO.

5.5.2 Processing assay for miRNA precursors using recombinant protein

To analyze the effect of SRP9, SRP14, both or SRP9-myc-14 on Dicer processing 2.5 µl pre-activated rDcr in 2x reaction buffer, 0.5 µl substrate and 2 µl of the recombinant candidate protein in varying concentrations were mixed (SRP9, SRP14: undiluted, 1:5, 1:10, 1:50, 1:100; SRP9-myc-14: undiluted, 1:3, 1:9, 1:27, 1:81, 1:243, 1:729). The incubation procedure and electrophoretic separation was performed as stated in section 5.5.1.

5.5.3 Processing assay for miRNA precursors using SRP9/14 and BSA

To eliminate putative unspecific RNA-binding effects of proteins, 0.5 µl of a 10 mg/ml bovine serum albumin solution were incubated with 2 µl SRP9/14, 0.5 µl substrate, and 3 µl 2x reaction buffer containing 0.0156 µl pre-activated rDcr for 5 minutes. The reaction was stopped by adding 6 µl 2x STOP-buffer, denatured (95°C, 1') and loaded on a 15% polyacrylamide gel (0.4 mm slots, 30 W, see section 5.4.1).

5.6 Preparative protein techniques

5.6.1 Expression and purification of recombinant protein

BL21 is the most commonly used bacterial strain for expression of recombinant proteins. In this study, Rosetta™ *E. coli* (Novagen), a strain derived from BL21, but better suited for expressing eukaryotic proteins, was used for expression of human recombinant proteins. Rosetta bacteria translate mRNA from eukaryotic genes more efficiently than BL21 as they possess additional tRNAs complementary to eukaryotic codons, which are usually rarely found in bacterial genes.

5.6.1.1 Transformation of plasmid into *E. coli*

50 ng of the expression vector pDEST17-SRP9, pDEST17-SRP14 or pDEST17-SRP9-myc-14 transformed in 10 µl chemically-competent Rosetta™ *E. coli* according to the transformation protocol in section 5.2.3.

5.6.1.2 Expression of recombinant protein

To set up a pre-culture, one Rosetta™ colony from a freshly streaked LB plate was inoculated in 20 ml LB medium plus antibiotics and grown until OD_{600} was higher than 3 (5-7 hours). The pre-culture was then diluted in 400 ml LB without resistances to an OD_{600} of 0.1. Cells were grown until OD_{600} of 0.4 was reached, then cooled down to 20°C for 30 minutes. For later examination, a 1 ml aliquot from the cell suspension was taken at this step and spinned down (2', 3400 x g, RT). The pellet was resuspended in 50 µl 1x Sample Buffer and denaturated (95°C, 5'). Induction of protein expression was performed by adding IPTG to a concentration of 0.1 mM and incubating at 20°C over night in a shaker. Again a 1 ml aliquot was taken from the induced culture and treated in the same way as before.

5.6.1.3 Hypotonic lysis

The cells were harvested using a Sorvall centrifuge (5600 x g, 20', 4°C). The pellet was resuspended in 30 ml cold 1x PBS and transferred to a 50 ml falcon tube. The cells were again harvested (15', 2000 x g, 4°C). The washed pellet was resuspended in 400 ml Washbuffer His-tag + 1/2 tab of protease inhibitor mix and transferred into 50 ml Sorvall centrifuge tubes. Lysis of the cells was supported by sonication of cell membranes performed in the coldroom (6 x 15", 30" pause in between). To remove the cell debris, sonicated cells were spinned down (30', 16.000 x g, 4°C).

5.6.1.4 Affinity chromatography

2 ml of the Ni-NTA beads were equilibrated by spinning down (3', 500 x g, 4°C) and washing twice with Washbuffer His-tag. To remove the supernatant, a special Pasteur pipette coupled to a vacuum pump was used. After the first washing step, the beads were separated into two 15 ml falcons to provide more space for the bacterial extract. After centrifugation of the sonicated cells, 30 µl of the supernatant and a bit of the pelleted cell debris were taken as an aliquot. The remaining lysate was transferred onto the two fractions of Ni-NTA beads and the tubes were mounted on a rotation wheel in the cold room for 90 minutes. Care had to be taken to avoid spuming. The beads were harvested by centrifugation (3', 500 x g, 4°C). At each of the next steps, an aliquot of 30 µl was taken. The beads were washed with 30 ml Washbuffer His-tag by vortexing and centrifuging (3', 500 x g, 4°C). For the second wash, 4 ml Washbuffer His-tag + 25 mM imidazole was added to each of the falcon tubes, vortexed, pooled and put onto a column. After settling of the slurry the valve was opened and the flow-through collected. Then the column was washed a third time with 10 ml Washbuffer His-tag + 50 mM imidazole and a fourth time with 10 ml Washbuffer His-tag + 100 mM imidazole, For the elution, 1 ml elution buffer with 300 mM imidazole was added on top of the column. The beads were vortexed and let settle, then the valve was opened and the first eluate collected. For the second elution the procedure was repeated. A small aliquot was also taken from the beads for later analysis.

5.6.1.5 Dialysis of purified proteins

Due to the high imidazole concentration of the eluates (300 mM), the purified protein samples had to be dialysed. The bottom of a Petri dish was filled with dialysis buffer

(RNAi buffer). Using forceps, Millipore membrane filter discs were floated on the surface of the buffer. Small volumes of samples (20-40 µl) were pipetted onto the membranes and dialyzed for 60 minutes. Then the samples were removed from the membrane using pipettes and used for analysis.

5.6.2 Determination of protein concentration

5.6.2.1 Bradford assay

The protein concentration of cell lysates or eluates was measured using Bradford Protein Assay according to the manufacturer's instructions. 800 µl dH_2O plus 1 µl sample were added to 200 µl Bradford solution in a plastic cuvette. For generation of the standard curve, 0, 2, 6 or 10 µl of a 1 mg/ml BSA solution were mixed with 800 µl dH_2O + 1 µl Washbuffer His-tag. The absorption of the protein-Bradford solution was measured immediately using a spectrophotometer exciting at a wavelength of 595 nm.

5.7 Analytical protein techniques

5.7.1 SDS polyacrylamide gel electrophoresis and Western blotting

5.7.1.1 Conventional one-dimensional gels

SDS-PAGE was performed on either 9 x 9 cm or 12 x 9 cm gels, comprising a 5% stacking gel (pH 6.8) and a 12% or 15% separation gel (pH 8.8). Gels were casted according to Table 6, TEMED and APS were added in the last step. After pouring the separation gel, 1 ml of butanol was added on top to avoid the formation of bubbles. Protein samples were mixed with 5x sample buffer, denatured (95°C, 5') and loaded into the wells with a protein marker.

Separation gels

15%	**For 10 ml**	**For 30 ml**	**For 50 ml**
Separation Buffer 4x	2.5 ml	7.5 ml	12.5 ml
30/0.8% acryl/bisacryl	5 ml	15 ml	25 ml
dH_2O	2.5 ml	7.5 ml	12.5 ml

10% APS	50 µl	150 µl	250 µl
TEMED	5 µl	15 µl	25 µl
12%	For 10 ml	For 30 ml	For 50 ml
Separation Buffer 4x	2.5 ml	7.5 ml	12.5 ml
30/0.8% acryl/bisacryl	4 ml	12 ml	20 ml
dH_2O	3.5 ml	10.5 ml	17.5 ml
10% APS	50 µl	150 µl	250 µl
TEMED	5 µl	15 µl	25 µl
10%	For 10 ml	For 30 ml	For 50 ml
Separation Buffer 4x	2.5 ml	7.5 ml	12.5 ml
30/0.8% acryl/bisacryl	3.33 ml	10 ml	16.6 ml
dH_2O	4 ml	12 ml	20 ml
10% APS	50 µl	150 µl	250 µl
TEMED	5 µl	15 µl	25 µl
7.5%	For 10 ml	For 30 ml	For 50 ml
Separation Buffer 4x	2.5 ml	7.5 ml	12.5 ml
30/0.8% acryl/bisacryl	2.5 ml	7.5 ml	12.5 ml
dH_2O	4.93 ml	14.8 ml	24.7 ml
10% APS	50 µl	150 µl	250 µl
TEMED	5 µl	15 µl	25 µl
Stacking gel (5%)			
	For 10 ml	For 30 ml	For 50 ml
Stacking Buffer 8x	1.25 ml	2.5 ml	5 ml
30/0.8% acryl/bisacryl	1.66 ml	3.35 ml	6.7 ml
dH_2O	7 ml	14 ml	28 ml
10% APS	50 µl	100 µl	200 µl
TEMED	10 µl	20 µl	40 µl

Table 6: Reagents and volumes needed for casting SDS-PAGE gels.

Electrophoresis was carried out at 20 mA per gel in SDS-PAGE running buffer until the bromophenol blue marker reached the stacking gel, followed by separation at 30 mA per gel for 60 minutes or until the bromophenol blue marker reached the end of the gel. The glass plates were then diassembled, the stacking gel cut off and the gel stained with Coomassie or prepared for Western Blotting.

5.7.1.2 Pre-cast one-dimensional gradient gels

When available, Novagen® 4-20% Tris-glycine gradient gels (1 mm, 15 wells) were used for separation of proteins. Electrophoresis was carried out in SDS-PAGE running buffer at 20 mA for samples in the stacking gel and 30 mA afterwards until the bromophenol blue marker reached the end of the gel.

5.7.2 Staining of SDS-PAGE gels

5.7.2.1 Conventional Coomassie staining

After disassembly of the gel, the stacking gel was removed and the separation gel immersed in Coomassie staining solution for 8 minutes. To destain, the gel was incubated in Coomassie destaining solution until single bands could be discriminated (30 minutes). The destaining solution was exchanged until the signal-background relation was good (up to one day). To wash away traces of methanol from the destaining solution, the gel was then incubated in dH_2O and dried if necessary.

5.7.2.2 Drying of stained SDS-PAGE gels

For drying of the stained gel, it was sandwiched between Whatman paper (two layers) and Saran wrap and dried for 90 minutes at 80°C using a vacuum pump-coupled gel dryer.

5.7.3 Western blot analysis

After electrophoretic separation, the gel was immersed in Semidry-Blot-Buffer. For blotting, 2x 3 Whatman papers and a PVDF membrane of gel size were prepared and moistened with Semidry-Blot-Buffer. After assembly of the blotting sandwich (3 Whatman papers + membrane + gel + 3 Whatman papers) the gel was blotted for 1.5 hours least. The power was dependent on gel size: cm^2 x 0.8 = mA. Then the membrane was blocked

one hour in 4% non-fat dry milk in PBST at RT (or at 4°C over night), followed by incubation with the first antibody in 4% milk-PBST for one hour at RT. The membrane was washed three times with milk-PBST (2x 5 minutes, 1x 10 minutes) and incubated with the corresponding HRP-coupled secondary antibody in PBST for one hour at RT. After three washing steps with PBST (2x 5 minutes, 1x 10 minutes) the membrane was developed using Enhanced Chemi-Luminescence Kit and exposed on Amersham Hyperfilm™ ECL films.

5.8 Expression cloning using Gateway® Technology

Gateway® cloning is a technology developed by Invitrogen that enables the researcher to shuttle the gene of interest into various expression and functional analysis systems. Time-consuming and/or low-efficiency steps of traditional cloning methods are avoided by using an entry clone that performs site-specific recombination with the desired destination vector (Figure 17).

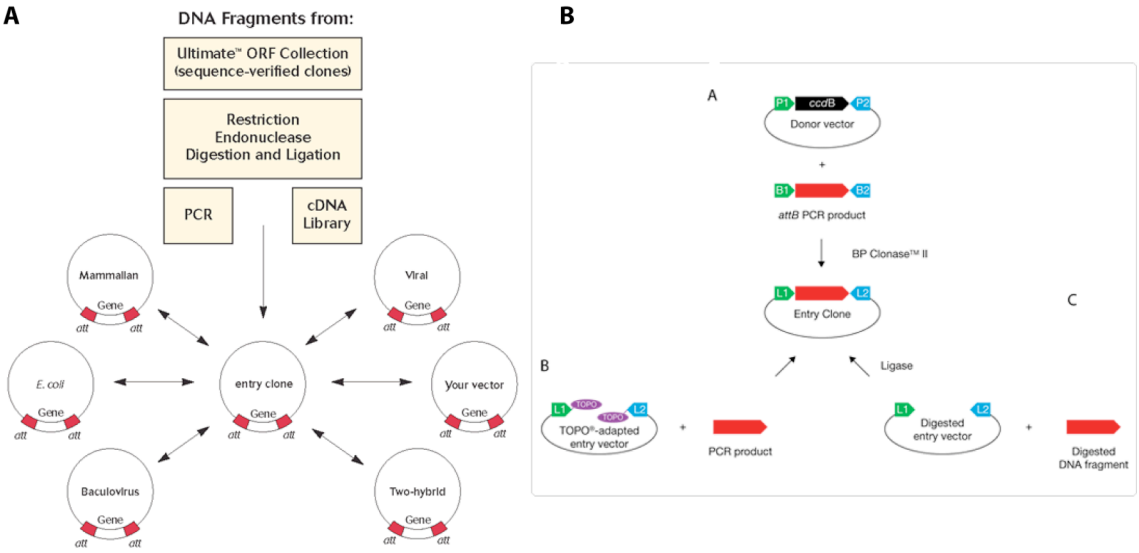


Figure 17: Gateway® cloning. (A) Overview of possible genes sources and target vectors for Gateway® cloning. After cloning the desired gene into an entry vector shuttling into various destination vectors is facilitated by site-specific recombination. (B) Top: Three options exist to generate the entry vector. In this study, the entry vector was cloned using aattB-flanked PCR product and the donor vector pDONR201. Bottom: Once the gene of interest is cloned into the entry vector, gene shuttling into different made possible by clonase enzymes. Figure modified and adapted from Invitrogen.

(http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Gateway-Cloning).

5.8.1 Cloning of SRP9 and SRP14

A 2-step PCR protocol was employed to clone SRP9 (261 bp) and SRP14 (333 bp) into the entry clone pDONR201. Both genes were amplified from RIKEN library clones 9530033C02 and F8301020O9, respectively. The following template-specific primers extended by the generic recombination sites attB1 and attB2 (12 nt) were used.

General scheme:

attB1: 5'-AA AAA GCA GGC TNN-(template-specific sequence)-3'

attB2: 5'-A GAA AGC TGG GTN-(template-specific sequence)-3'

Designed primers:

SRP9-fwd-attB1: AAAAAGCAGGCTCA**ATG**CCTCAGTTCCAGACCTGGG

SRP9-rev-attB2: AGAAAGCTGGGTC*TCA*TTCTGTTTCCATAGTGACATTGCGG

SRP14-fwd-attB1: AAAAAGCAGGCTCA**ATG**GTGTTGCTCGAGAGCGAGC

SRP14-rev-attB2: AGAAAGCTGGGTC*TCA*CTGTGCTGGTTTGCTCTTCTTAC

Legend: attB1 sites, attB2 sites, template-specific sequence, **start codon**, *stop codon*

5.8.1.1 Phusion PCR step 1

For SRP9 and SRP14, the extension time was 15", two annealing temperatures, 50°C and 56°C, were specified in parallel.

Reaction mix

Volume	Stock	Final conc.
1 µl	RIKEN template (undil., 1:10, 1:100)	-
10 µl	5x buffer	1x
1 µl	dNTP mix (10 mM)	0.2 mM
0.5 µl	$MgCl_2$	
1 µl	Forward primer (10 µM)	0.2 µM
1 µl	Reverse primer (10 µM)	0.2 µM
35 µl	dH_2O	-
0.5 µl	Phusion™ DNA Polymerase (2 U/µl)	1 U
50 µl	Total volume	

PCR Program

	Time	Temp.
	2'	98°C
29 cycles	20"	98°C
	45"	x °C
	x "	72°C
	10'	72°C
	∞	4°C

The product generated in PCR step 1 was amplified in the second 2-phased PCR using universal attB adapter primers, which align to the attB1 and attB2 site attached to the cloned genes.

5.8.1.2 Phusion PCR step 2

Reaction mix

Volume	Stock	Final conc.
1 µl	Step 1 product (undil., 1:10, 1:100)	-
10 µl	5x buffer	1x
1 µl	dNTP mix (10 mM)	0.2 mM
0.5 µl	$MgCl_2$	
1 µl	Universal attB1 adapter primer (10 µM)	0.2 µM
1 µl	Universal attB2 adapter primer (10 µM)	0.2 µM
35 µl	dH_2O	
0.5 µl	Phusion™ DNA Polymerase (2 U/µl)	
50 µl	Total volume	

PCR Program

	Time	Temp.
	2'	98°C
1st phase:	20"	98°C
	30"	45°C
	x "	72°C
2nd phase:	20"	98°C
	30"	55°C
	x "	72°C
	10'	72°C
	∞	4°C

The amplified PCR products from step 2 contain attB-sites capable of recombination with attP-sites. In the BP reaction, Clonase catalyses the recombination of attB-flanked PCR products with the donor vector pDONR201.

5.8.1.3 BP reaction

Volume	Reagent	Final conc.
1 µl	attB-PCR product (40-100 fmol)	-
1 µl	pDONR201 vector (150 ng/µl), Kan^R	15 ng
2 µl	5x BP Clonase Reaction Buffer	1x
2 µl	Clonase	-
4 µl	TE Buffer	-
10 µl	Total volume	

The reaction mix was incubated for 60 minutes at 25°C. Then, the total reaction volume was transformed into competent *E. coli* and plated out on LB_{Kan} plates (pDONR201

carries a kanamycin resistance). A positive clone was selected for the second recombination step, the LR reaction, which allows shuttling of SRP9 and SRP14 from the pDONR clone into the chosen destination vector pDEST17.

5.8.1.4 LR reaction and transformation

Volume	Reagent	Final amount
-	pDONR201-SRP9/14	25-75 ng
0.5 µl	pDEST17 (150 ng/µl), Amp^R	15 ng
ad 4 µl	TE buffer	-
1 µl	Clonase II LR Enzyme Mix	-
5 µl	Total volume	

60 ng of pDONR vectors were used for the LR reaction. After incubation (25°C, 60') 0.5 µl Proteinase K was added for 10 minutes at 37°C to stop Clonase II activity. The reaction mix was then transformed in *E. coli* and plated out on LB_{Amp}. Positive clones were confirmed by sequencing and named pDEST17-SRP9 and pDEST17-SRP14. Expression of recombinant proteins is described in section 5.6.1.

5.8.2 Cloning of SRP9-myc-14

Cloning of the gene expressing the fusion protein SRP9-myc-14 involved two main parts:

1) Ligation of the genes for SRP9, SRP14, and the myc-tag, followed by amplification of the ligation product, and a
2) 2-step PCR protocol based on Gateway® cloning as described in section 5.8.1.

5.8.2.1 Primer design and cloning the genes

SRP9 and SRP14 were first amplified from RIKEN clones 9530033C02 and F8301020O9, respectively, using the following primers:

SRP9-fwd-attB1: AAAAAGCAGGCTCA**ATG**CCTCAGTTCCAGACCTGGG

SRP9-rev-EcoRI: CCG***G'AATTC***TGTTTCCATAGTGACATTGCGG

SRP14-fwd-BamHI: CGC***G'GATCC***C**ATG**GTGTTGCTCGAGAGCGAGC

SRP14-rev-attB2: AGAAAGCTGGGTCTCACTGTGCTGGTTTGCTCTTCTTA

Legend: attB1 site, attB2 site, template-specific primer, **start codon,** *restriction site*, stop codon

Annealing temperature was 56°C, extension time 15".

Reaction mix

Volume	Stock	Final conc.
1 µl	RIKEN clone (undil., 1:10, 1:100)	-
10 µl	5x buffer	1x
1 µl	dNTP mix (10 mM)	0.2 mM
0.5 µl	$MgCl_2$	
1 µl	Forward primer (10 µM)	0.2 µM
1 µl	Reverse primer (10 µM)	0.2 µM
35 µl	dH_2O	-
0.5 µl	Phusion™ DNA Polymerase (2 U/µl)	1 U
50 µl	Total volume	

PCR Program

	Time	Temp.
	2'	98°C
29 cycles	20"	98°C
	45"	56°C
	15"	72°C
	10'	72°C
	∞	4°C

The PCR product were then digested using *EcoRI* for SRP9 and *BamHI* for SRP14 90 minutes at 37°C.

Volume	Reagent	Final conc.
10 µl	SRP9 PCR product	-
2 µl	10x *EcoRI*-buffer	1x
0.4 µl	*EcoRI*	-
7.6 µl	dH_2O	
20 µl	Total volume	

Volume	Reagent	Final conc.
10 µl	SRP14 PCR product	-
2 µl	10x Buffer 3	1x
0.4 µl	*BamHI*	-
2 µl	10x BSA	1x
5.6 µl	dH_2O	
20 µl	Total volume	

The reaction mix was loaded on a 2% agarose gel and eluted according to the manufacturer's instructions in 30 µl elution buffer using QIAquick Gel Extraction Kit.

DNA concentrations: SRP9 = 3.7 ng/µl, SRP14 = 5.4 ng/µl.

5.8.2.2 Preparation of the linker

The linker (myc-tag) was generated from two oligo sequences:

Sense (LNK-SE):

5'-AATTGGGCGGCGAGCAGAAGCTGATCAGCGAGGAGGACGACAGCCGGGGC-3'

Antisense (LNK-AS):

5'-GATCGCCCCGGCTGTCGTCCTCCTCGCTGATCAGCTTCTGCTCGCCGCCC-3'

Legend: *BamHI*, *EcoRI*, myc-tag sequence

First step was to phosphorylate both linker strands separately at the 5'-end.

Volume		**Final concentration**
1 µl	Oligo (100 mM)	100 pmol
1 µl	ATP (100 mM)	10 mM
1 µl	10x PNK buffer	1x
0.5 µl	Polynucleotide Kinase	-
6.5 µl	dH_2O	-
10 µl	Total volume	

The reaction mix was incubated at 37°C for 60 minutes. Then both samples LNK-SE and LNK-AS were mixed an annealed one minute at 95°C. 100 fmol of double-stranded linker was ligated with total amount of eluted SRP9 and SRP14 at 16°C over night.

5.8.2.3 Ligation of linker with SRP9/14

Volume		**Final concentration**
25 µl	SRP9 (*EcoRI*)	--
25 µl	SRP14 (*BamHI*)	-
1 µl	ds LNK	100 fmol-
6 µl	10x T4 ligase buffer	1x
2 µl	T4 ligase	-

1 µl	dH_2O	
60 µl	Total volume	

5.8.2.4 Phusion PCR step 1

The ligation mix was amplified using Phusion™ PCR to increase the amount of the fusion construct.

Reaction mix

Volume	Stock	Final conc.
1 /5 / 10 µl	Ligation mix	-
10 µl	5x buffer	1x
1 µl	dNTP mix (10 mM)	0.2 mM
0.5 µl	$MgCl_2$	
1 µl	SRP9-fwd-attB1	0.2 µM
1 µl	SRP14-rev-*BamHI*	0.2 µM
x µl	dH_2O	-
0.5 µl	Phusion DNA Polymerase (2 U/µl)	-
50 µl	Total volume	

PCR Program

	Time	Temp.
	2’	98°C
29 cycles	20”	98°C
	45”	56°C
	15”	72°C
	10’	72°C
	∞	4°C

The band of expected size (approximately 650 bp) was eluted from the gel (c = 30 ng/µl) and used as a template for the second step of the Gateway® cloning protocol.

5.8.2.5 Phusion PCR step 2

Reaction mix

Volume	Stock	Final conc.
1 µl	SRP9-myc-14 (undil., 1:10, 1:100)	-
10 µl	5x buffer	1x
1 µl	dNTP mix (10 mM)	0.2 mM
0.5 µl	$MgCl_2$	
1 µl	Universal attB1 adapter primer (10 µM)	0.2 µM

PCR Program

	Time	Temp.
	2’	98°C
1st phase:	20”	98°C
	30”	45°C
	30 ”	72°C
2nd	20”	98°C

1 µl	Universal attB2 adapter primer (10 µM)	0.2 µM		30”	55°C
35 µl	dH_2O			30 ”	72°C
0.5 µl	Phusion™ DNA Polymerase (2 U/µl)			10’	72°C
50 µl	Total volume			∞	4°C

The amplified PCR products were recombinated into pDONR201 as before (BP reaction).

5.8.2.6 BP reaction

Volume		**Final concentration**
1 µl	attB-PCR product (40-100 fmol)	-
1 µl	pDONR vector (150 ng/µl)	15 ng/µl
2 µl	5x BP Clonase Reaction Buffer	1x
2 µl	Gateway® BP Clonase® II enzyme mix	
4 µl	TE Buffer	
10 µl	Total volume	

The reaction mix was incubated for 60 minutes at 25°C. Then, the total reaction volume was transformed into competent *E. coli* and plated out on LB_{Kan} plates. Sequencing analysis of these clones showed that none of them included the linker. Therefore, the cloning strategy was modified.

5.8.3 New cloning strategy for SRP9-myc-14

Amplified SRP9 and SRP14 were again digested with *EcoRI* and *BamHI*, respectively. Fragments were purified from agarose gels (30 µl) and yielded 7 ng/µl (SRP9) and 16 ng/µl (SRP14).

The linker strands were separately phosphorylated as before (5.8.2.2). Then the sense and antisense strand were subjected to phenol-chloroform-isoamylalcohol extraction using non-acid phenol to remove the polynucleotide kinase from the solution.

5.8.3.1 Phenol-chloroform-isoamyl alcohol extraction of DNA fragments

290 µl were added to the reaction to yield 300 µl solution in total. 1 volume of non-acid PCR was added and the tube shaken vigorously. After 5 minutes centrifugation at 16 000 x g the upper aqueous phase was pipetted into a new tube. 1 volume chloroform-isoamyl alcohol was added and the tube shaken vigorously. After 5 minutes centrifugation at 16 000 x g the upper phase was again pipetted into a new tube (~290 µl). 1/10 volume of 3 M NaOAc (29 µl) was added and the tube was inverted several times. 3 volumes of -20°C EtOH abs. was mixed with the solution and incubated 60 minutes least at -20°C. Then the sample was centrifuged 30 minutes (4°C, 16 000 x g). The supernatant was removed and 1 ml 70% EtOH added. The tube was inverted twice and again centrifuged for 30 minutes (4°C, 16 000 x g). After removal of the supernatant the pellet was air-dried and then resuspended in TE buffer (80 µl for ~ 80 pmol; 20% loss of DNA amount).

5.8.3.2 Preparation of the linker

Next, two different linkers were generated, only one strand of the duplex being phosphorylated as follows:

1) Phosphorylated LNK-AS + LNK-SE

2) Phosphorylated LNK-SE + LNK-AS

The singles-stranded DNA fragments were denatured (95°C, 1') and hybridized (37°C, 20'). Then, ligation was performed with SRP9 and SRP14 according to the following scheme:

1 pmol SRP9 + 3 pmol LNK1

1 pmol SRP14 + 3 pmol LNK2

Volume		**Final conc.**
22 µl	SRP9 (*EcoRI*)	1 pmol
3 µl	LNK1	3 pmol
3 µl	10x T4 ligase buffer	1x
1 µl	dH_2O	
30 µl	Total volume	

Volume		**Final conc.**
12.5 µl	SRP14 (*BamHI*)	1 pmol
3 µl	LNK2	3 pmol
0.4 µl	10x T4 ligase buffer	1x
10.5 µl	dH_2O	1x
30 µl	Total volume	

The reaction was incubated for 120 minutes at 37°C, then denatured (95°C, 1') and annealed (37°C, 20').

For the second ligation, 4µl 10x T4 ligation buffer, 2 µl T4 ligase and 34 µl dH_2O were mixed with the SRP9- and SRP14-solutions yielding 100 µl and incubated over night at 16°C.

5.8.3.3 Phusion PCR step 1

Phusion™ PCR was performed on the ligation mix using the primers for step 1 of Gateway® cloning.

Reaction mix

Volume	Stock	Final conc.
1 /5 /10 µl	Ligation mix	-
10 µl	5x buffer	1x
1 µl	dNTP mix (10 mM)	0.2 mM
0.5 µl	$MgCl_2$	
1 µl	SRP9-fwd (10 µM)	0.2 µM
1 µl	SRp14-rev (10 µM)	0.2 µM
35 µl	dH_2O	-
0.5 µl	Phusion™ DNA Polymerase (2 U/µl)	1 U
50 µl	Total volume	

PCR Program

	Time	Temp.
	2'	98°C
29 cycles	30"	98°C
	45"	56°C
	15"	72°C
	10'	72°C
	∞	4°C

The amplified product was checked on a 2% agarose gel and used for the second step.

5.8.3.4 Phusion PCR step 2

Reaction mix

Volume	Stock	Final conc.
1 µl	PCR product step 1 (undil, 1:10, 1:100)	-
10 µl	5x buffer	1x
1 µl	dNTP mix (10 mM)	0.2 mM
0.5 µl	$MgCl_2$	

PCR Program

	Time	Temp.
	2'	98°C
1st phase:	20"	98°C
	30"	45°C
	30 "	72°C

1 µl	Universal attB1 adapter primer (10 µM)	0.2 µM	2nd phase:	20”	98°C
1 µl	Universal attB2 adapter primer (10 µM)	0.2 µM		30”	55°C
35 µl	dH_2O			30 ”	72°C
0.5 µl	Phusion™ DNA Polymerase (2 U/µl)			10’	72°C
50 µl	Total volume			∞	4°C

BP reaction with the product from step 2 was performed as before (5.8.1.3) and after sequencing analysis the positive clone was subjected to LR reaction. pDEST17-SRP9-myc-14 plasmids were transformed into Rosetta *E. coli* and used for expression of the recombinant protein.

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7. Appendix

7.1 Sequences

SRP9 (RIKEN 9530033C02): 261 bp

>ri|9530033C02|PX00112O12|1319 contigs=3 ver=1 seqid=23886

GGTCTTTGCGCCGCCGGAGTGCAGCGCTGTGGGCGGTTGCGGGAGAGCGGCGGGCCGCCGGGGCGACAGCGC
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CTATGGAAACAGAATGAATGGTTTGACATGAAGACGACTGTTCCGTTATTGGGAAGTAATCAGCTTTTGAAA
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GTGCACCGGCAGATACTGACACTGATGGCTGACAGTGTGTGCGTCCTTCACGCCGGTGTGCAACGTACGCAG
TGTTTACTCAGTGTGGCTCCCGCCTCAGACTGAATTAAAGTATTACTATAGCATGCCTCTCAGAACTGCTAG
CTTCCTAATAAACCACAAATCCACCGTTTCTATGTAGCTTGTATTTGTAAAGTCTGAGTTTACCGTCTGAAT
GTAAAGTTCCGTGTTGTCCGCTCACGACGGAAGTGAAGAAGATACTGCATTCAAGTGTTTTTTGTAAAAGTT
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ATACAAGCATCCTGTTTTGTTG

Amino acid sequence:

MPQFQTWEEFSRAAEKLYLADPMKVRVVLKYRHVDGNLCIKVTDDLVCLVY
RTDQAQDVKKIEKFHSQLMRLMVAKESRNVTMETE

NCBI accession number: NM_003133

SRP14 (RIKEN F830102009): 333 bp

>ri|F830102O09|PL00017I22|2820 contigs=104 ver=1 seqid=106810

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GCCTGTTCATCAGCATCCCACTAGGAAAGATGAGACCAGATGTGACAGAGCAGAGCAGCAGGGTTACTGCCT
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Amino acid sequence:

MVLLESEQFLTELTRLFQKCRSSGSVFITLKKYDGRTKPIPRKSSVEGLEPAEN
KCLLRATDGKRKISTVVSSKEVNKFQMAYSNLLRANMDGLKKRDKKNKSKK
SKPAQ

NCBI accession number: NM_003134

7.2 Abbreviations

α	antisera
A	adenosine
Ago	Argonaute
Amp	ampicillin
APS	ammonium persulfate
AS	antisense
ATP	adenosine triphosphate
attB	bacterial attachment site
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
c	concentration
dATP	deoxyadenosine triphosphate
DGCR8	DiGeorge syndrome critical region 8
dH_2O	distilled H_2O
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dNTP	deoxynucleoside triphosphate
ds	double-stranded
DTT	1,4-dithiothreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylene-diaminetetraacetic acid
EtOH	ethanol
FCS	fetal calf serum
fwd	forward
g	gravity
G	guanine
GFP	green fluorescent protein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hnRNP	heterogeneous nuclear ribonucleoproteins
HRP	horseradish peroxidase
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan	kanamycin
kb	kilobase pair(s)
kDa	kilodalton
L1	loop 1
LB	lysogeny broth
LNK	linker
mA	milliampere
miRNA	miRNA
miRNP	miRNA-ribonucleoprotein
mRNA	messenger RNA
NaOAc	sodium acetate
Ni	Nickel
Ni-NTA	Ni^{2+}-nitrilotriacetic acid

nm	nanometer
No.	number
nt	nucleotide(s)
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBST	PBS containing 0.1% Tween® 20
PCI	phenol-chloroform-isoamyl alcohol
PCR	polymerase chain reaction
pH	power of hydrogen
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTGS	post-transcriptional gene silencing
rDcr	recombinant Dicer
rev	reverse
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RT	room temperature
SDS	sodium dodecyl sulfate
SE	sense
siRNA	small interfering RNA
SRP	signal recognition particle
stRNA	small temporal RNA
T	thymine
T_a	annealing temperature
TBE	tris-borate electrophoresis buffer
TE	tris-EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
T_m	melting temperature
TN	tris-sodium chloride
TRBP	trans-activation-responsive RNA-binding protein
Tris	tris-(hydroxymethyl)aminomethane
tRNA	transfer RNA
TY-MED	tryptone-yeast extract medium
U	unit (enzymatic activity)
U	uracil
UTR	untranslated region
UV	ultra-violet
V	Volt
W	Watt

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- My parents for constant support of my studies and my efforts in becoming part of the scientific community.

7.4 Abstract

RNA silencing is a recently discovered naturally occurring mechanism to regulate gene expression in a wide range of organisms. Key players of RNA silencing are small RNA molecules termed microRNAs (miRNAs), which act as post-transcriptional regulators on mRNAs to induce inhibition of translation or degradation. The process of how miRNAs are generated has been investigated intensely in the last years and many protein factors involved have been identified. However, regulation of miRNA biogenesis is still a field of research with many open questions. Recent studies have indicated that miR-138 displays differential expression of its precursor form in mouse tissues, while the mature miRNA is expressed solely in neural tissues. This phenomenon was shown to be due to an unknown protein factor inhibiting processing of the precursor to the mature miRNA. Several candidate proteins have been identified by chromatography methods of which the heterodimer SRP9/14 was the most promising. In this thesis, evaluation of SRP9/14 as a putative inhibitor of miRNA biogenesis was performed using two approaches: first, the heterodimer was expressed as recombinant protein and tested for its ability to inhibit processing of pre-miR-138 in *in vitro* assays. Second, SRP9/14 was knocked-down in HeLa cells using siRNAs followed by testing depleted cell extracts inhibitory activity using *in vitro* processing assays. Both approaches demonstrated that SRP9/14 does not inhibit pre-miR-138 maturation and has to be excluded from the list of putative inhibitors.

7.5 Zusammenfassung

RNA Silencing ist ein kürzlich entdeckter, natürlich vorkommender Mechanismus zur Regulation von Genexpression in zahlreichen Organismen. Die Schlüsselfunktion des RNA Silencing wird von kleinen RNA-Molekülen namens microRNAs (miRNAs) getragen, die als post-transkriptionelle Regulatoren auf mRNAs einwirken, um Translationsinhibition oder Degradierung zu induzieren. Die Entstehung von miRNAs wurde in der Vergangenheit intensiv erforscht, weshalb zahlreiche beteiligte Proteinfaktoren bereits identifiziert werden konnten. Die Regulation der miRNA-Biogenese ist allerdings noch ein Forschungsfeld mit vielen offenen Fragen. Jüngste Untersuchungen zeigen, dass die Vorstufe von miR-138 in Mausgeweben differentiell exprimiert wird, während die reife miRNA lediglich in Nervengewebe exprimiert wird. Es konnte auch gezeigt werden, dass diese Beobachtung auf einen unbekannten Proteinfaktor zurückzuführen ist, der die Prozessierung von der Vorstufe zur reifen Form blockiert. Einige Kandidaten konnten durch chromatographische Methoden identifiziert werden, wobei das Heterodimer SRP9/14 am vielversprechendsten war. In der vorliegenden Studie wurde SRP9/14 als potentieller Inhibitor für die miRNA-Biogenese mittels zweier Methoden evaluiert: einerseits wurde das Heterodimer als rekombinantes Protein exprimiert und auf seine Funktionalität als Inhibitor von miR-138 in *in vitro* Assays getestet. Andererseits wurde die Expression von SRP9/14 in HeLa-Zellen durch siRNAs abgeschaltet und die Zellextrakte funktionell auf Vorhandensein des Inhibitors getestet. Beide Methoden zeigten, dass es sich bei SRP9/14 nicht um den gesuchten Inhibitor handelt.

Curriculum Vitae

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1994 – 2002	Secondary Grammar School || Piaristengymnasium BG8, Vienna; graduated with distinction

HIGHER EDUCATION

10/2002 – present	Molecular Biology || Institute of Life Sciences, University of Vienna, Austria *1st part completed in 2005* *2nd part focussing on biochemistry, immunology, cell biology*
10/2003 – present	Sinology || Institute of East Asian Studies, University of Vienna, Austria
09/2007 – present	Diploma thesis "title" || Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Group Javier Martinez

INTERNSHIPS RELATED TO STUDY

08/2004	Internship in the research group of Rudolf Valenta || Institute of immunopathology ,Vienna General Hospital Research Centre *Topic: Cloning of birch pollen mosaic antigens*
07/2006	Internship in the research group of Andreas Hartig || Institute of Biochemistry, Max F. Perutz Laboratories, Vienna *Topic: Proteins in peroxisomal membrane of S. cerevisiae*
10/2006	Internship in the research group of Christoph Schüller || Institute of Biochemistry, Max F. Perutz Laboratories, Vienna, Austria *Topic: Msn2 and Mn5 stress signaling in S. cerevisiae*
11/2006 – 12/2006	Internship in the research group of Tamás Schweighoffer || Novartis Institute of Biomedical Research, Vienna *Topic: Molecular determinants of human mast cell degranulation*
01/2007 – 02/2007	Internship in the research group of Javier Martinez || IMBA - Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria *Topic: Analysis of miR-138 target interaction in mammalian cells and mouse brain cryo-sections*
08/2008	Internship in the research group of Andreas Hartig || Institute of Biochemistry, Max F. Perutz Laboratories, Vienna *Topic: SUC2-Tagging of peroxisomal membrane protein Pex3*

OTHER PRACTICALS & WORK EXPERIENCE

2001 – 2003	4-week internships at Tscheinig & Partner Workflow Software Consulting GmbH, Vienna
2003 – 2005	Part-time secretary at Tscheinig & Partner Workflow Software Consulting GmbH, Vienna
09/2004 – 02/2005	Restaurant IKEA Wien Nord GmbH, Vienna
2005 – present	Participation in several public events („Lange Nacht der Museen“, Christmas events) and promotions
2006 – 09/2007	Latin tutor at mobile nachhilfe and Lernquadrat, Vienna