Artificial micro RNA system in *Dictyostelium discoideum*

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Abbreviations

Amp	ampicillin
ARE	AU-rich element
BS	blasticidin
cDNA	complementary DNA
CDS	coding sequence
ChIP	chromatin immunoprecipiation
cpm	counts per minute
dsRNA	double-stranded RNA
G418	geniticin
GFP	green fluorescent protein
h	hour
kb	kilobase
KD	knock-down
KO	knock-out
miRNA	microRNA
miRNP	micro-ribonucleoprotein
mRNA	messenger RNA
natsiRNA	natural-antisense transcript-derived siRNA
NP40	Nonident P40
nt	nucleotide
P-body	processing body
piRNA	Piwi-associated RNA
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA transcript
PTGS	post-transcriptional gene silencing
rasiRNA	repeat-associated siRNA
rNTP	ribonucleotide
rpm	rounds per minute
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference

RPA	RNase protection assay
semiq-PCR	semiquantitative PCR
RT	room temperature
RT-PCR	reverse transcriptase PCR
shRNA	short haipin RNA
siRNA	short interfering RNA
snoRNA	small nucleolar RNA
ta-siRNA	trans-acting siRNA
TE	Tris-EDTA
tRNA	transfer RNA
TSS	transcription start site
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
V	volt
W	watt

1. ZUSAMMENFASSUNG

Bei der *Dictyostelium discoideum* handelt es sich um eine soziale Amöbe, die als Modellsystem für die Beeinflussung von RNS so wie die zusammenhängenden Mechanismen dient. Ihre Position zwischen Pflanzen und Tieren erlaubt einen Einblick in die Mechanismen und die Proteinmaschinerie, die mit den bearbeiteten Themen verbunden sind. Mikro RNS sind kleine, regulierende RNS, die durch die Evolution erhalten wurden und in Tieren, Pflanzen, Viren so wie manchen Prokaryoten vorhanden sind. Sie spielen eine Rolle in der Entwicklung, Apoptosis, Zellwachstum und ihre Fehlregulation ist mit vielen Krankheiten wie Krebs, Diabetes oder neurodegenerativen Funktionsstörungen verbunden. Vor kurzem wurden durch die Sequenzierung von DNS-Bibliotheken miRNS in *D. discoideum* entdeckt.

In dieser Arbeit konnte es gezeigt werden, dass heterologue miRNS *let-7* in *D. discoideum* verarbeitet werden kann. Die expression von *let-7* miRNS in *D. discoideum* führt nach einem starken Phenotyp, zeigt während Entwicklung. Dieser Umstand kann einen Überschuss des Verarbeitungs-/Silencingsystems und/oder wegen endogenen Zielen present sein. Die verschiedenen Effekte auf dem *prel-7* Strain wurden beobachtet und charakterisiert und dienen als Hintergrund für die mögliche Rollen der miRNS.

Ein 'artificial' (künstliches) miRNS-System wurde erstellt und in *D. Discoideum* eingeführt, wodurch bewiesen wurde, dass die miRNS in der Amöbe Genexpression durch der mRNS-Stabilität und auf dem posttranskriptionalen Level vermitteln können. Weiterhin wurde zum ersten mal in diesem Organismus nachgewiesen, dass Genexpression durch die Translationkontrolle reguliert ist. Dadurch wurden neue Strukturen entdeckt, die Kolokalitäten der miRNS und Ziel (target)-RNS repräsentieren.

Zusammengefasst zeigt diese Arbeit das funktionelle künstliche miRNS-System und postuliert die Rollen von endogenen, kleinen RNS in sozialer Amöbe.

CONTENT

1. SUMMARY

Dictyostelium discoideum is a social amoeba that serves as a model system for RNA interference and related mechanisms. Its position between plants and animals enables evolutionary snapshot of mechanisms and protein machinery involved in investigated subjects. MiRNAs are small regulatory RNAs that are evolutionary conserved and present in animals, plants, viruses and some prokaryotes. They have roles in development, cell growth and differentiation, apoptosis and their miss-regulation is associated with many diseases such as cancer, neurodegenerative disorders and diabetes. Recently, through sequencing of DNA libraries miRNAs have been discovered in *D. discoideum.*

In this work, it has been shown that heterologues miRNA *let-7* can be expressed and processed in *D. discoideum*. Expression of *let-7* miRNA in social amoeba resulted in a strong developmental phenotype suggesting an overload of the processing/silencing system or/and endogenous targets. The various effects on *prel-7* strain have been observed and characterized, serving as a background for postulation of miRNA roles.

An artificial miRNA system has been established and imposed to *D. discoideum*, showing that miRNAs in *Dictyostelium* could mediate gene expression on the level of mRNA stability and on the posttranscriptional level. Furthermore, presence of translational inhibition as a type of gene control was shown for the first time in this organism. Due to it new structures representing co-localities of miRNA and target mRNA have been detected.

Taken together, this work shows functional artificial miRNA system and postulates roles of endogenous small RNA in social amoeba.

2. INTRODUCTION

2.1 Small RNAs

Non-coding RNAs play important roles in cells. Our understanding and idea of their roles started to emerge with discovery and investigation of small regulatory RNAs (21- to 30- not in length) during the past two decades. The starting point was discovery of posttranscriptional gene silencing (PTGS) in plants, where expression of transgene led to silencing of homologues sequences (Napoli et al. 1990). The next milestone was discovery of RNA interference (RNAi), showing double stranded RNA (dsRNA) as a powerful inducer of gene silencing in *C. elegans* (Fire et al. 1998). This put regulatory RNAs into a spot light and showed that dsRNA was processed into short interfering RNAs (siRNA, 21- to 25- nts in length) that guide cleavage of their cognate target RNAs (Hammond et al. 2000). Discovery of siRNA was tip of an iceberg and led to identifying many other small regulatory RNAs. Among them are micro RNAs (miRNAs) involved in control of *C. elegans* development (Lee et al. 1993; Lagos-Quintana et al. 2001; Lee and Ambros, 2001). Still, it was years after their original discovery and after the discovery of RNAi that miRNA came to a spotlight of research interest.

MiRNAs are evolutionary conserved and endogenously encoded small RNAs (21- to 25- nts in length) regulating translation of their target mRNAs (Lee et al. 1993; Lagos-Quintana et al. 2001; Lee and Ambros, 2001). They are presently confirmed in all eukaryotes (*D. melanogaster*, humans, *N. crassa*, plants etc.), some prokaryotes such as green alga *Chlamidomonas* (Zhao et al. 2008; Molnar et al. 2009) and animal viruses, such as herpes viruses, adenoviruses and retroviruses (Pfeffer et al. 2004).

The number of genes coding for miRNAs is an open question. In humans are presently registered 721 miRNA (www.mirbase.org, January 2010), but estimated number is more then a thousand. In *Dictyostelium discoideum* are reported two putative miRNAs (Hinas et al. 2007), but the estimated number is much higher.

Beside siRNA and miRNA there are as well endogenously encoded siRNA, whose main function is repression of retrotransposons and other repetitive sequences. In plants and fission yeast endogenous siRNAs can direct transcriptional silencing and chromatin condensation at the homologues sites of the genome (Mette et al. 2002).

In plants endogenous siRNAs differ based on their biogenesis and function into: trans-acting siRNAs (ta-siRNAs), natural antisense transcript derived siRNAs (nat-siRNAs) and repeat associated siRNAs (ra-siRNAs) (Vazguez, 2006). In *Dictyostelium* are present two such classes deriving from DIRS and Skipper retrotransposons (Kuhlmann et al. 2005; Hinas et al. 2007).

Other recently discovered class of small regulatory RNAs are Piwi-associated RNAs (piRNAs), whose biogenesis differs from siRNA and miRNA. They are a bit longer (24- to 30- nts in length) and produced from single-stranded precursors (Brennecke et al. 2007). piRNAs are expressed in germ cells and seem to be associated with Piwi-proteins, Argonaute related effectors proteins. They are suggested to be necessary for germ line development and needed for retrotransposone silencing (Aravin et al. 2004).

2.2 Biogenesis of miRNA and siRNA

MiRNAs are RNAII-polymerase transcripts of variable size that are 5'-end capped and polyadenilated (Cai et al. 2004). Majority of them derives from introns of protein coding genes (Kim and Kim, 2007).

The production of miRNAs is a two step process: the first step takes place in the nucleus and the second step in the cytoplasm. The primary miRNAs (pri-miRNA) transcripts are several kb long and contain hairpin structures. They are cleaved into precursor miRNA (pre-miRNAs) by RNase III protein family member Drosha (Lee et al. 2003; Filippov et al. 2000; Fortin et al. 2002; Wu et al. 2000). Drosha is ~ 160 KD large enzyme that is conserved in animals. It contains two tandem RNaseIII domains (RIIIDs) and double stranded RNA-binding domain (dsRBD) crucial for catalyses, together with the central region of protein (Han et al. 2004). Drosha is a part of a complex named Microprocessor that is ~500 KDa large in *Drosophila melanogaster* and ~650 KDa in humans. In Microprocessor, Drosha interacts with cofactor called Pasha in *D. melanogaster* and *C. elegans* or DGCR8 (DiGeorge Syndrome Critical region gene 8) in humans. DGCR8/Pasha is a ~120 KDa protein with two dsRBD and is postulated to assist Drosha in substrate recognition (Denli et al. 2004; Gregory et al. 2004; Landthaler et al. 2004). Tertiary structure of miRNA seems to be crucial for Microprocesor recognition (Lee et al. 2004; Zeng et al. 2004; Kim et al. 2004) and Drosha complex is able to measure the length of stem loops

(Kim et al. 2004), but the mechanism of this needs to be further investigated. Some miRNA derive via splicing (independently of Drosha and DGCR8) are named mirtrons (Berezikov et al. 2007).

Upon nuclear processing step by Drosha, pre-miRNAs are exported in the cytoplasm, where the second step takes place resulting in a mature ~22- nt long miRNA. This is due to Dicer activity, as well a member of RNaseIII family (Hammond et al. 2000; Lee et al. 2004; Bernstein et al. 2001; Grishok et al. 2001; Ketting et al. 2001; Knight et al. 2001). Division of maturation process is enabled by nuclear export of pre-miRNA into cytoplasm. This occurs through nuclear pore complex, large channels embedded in nuclear membrane (Lund et al. 2004) that simultaneously bind cargo and GTP-bound form of Ran-cofactor. Export of pre-miRNAs is mediated by Exportin-5 (Zeng et al. 2005; Kim et al. 2005; Nakielny et al. 1999; Yi et al. 2003; Bohnsack et al. 2002; Bohnsack et al. 2004). It is shown that upon depletion of Exportin-5 levels of pre-miRNAs and miRNAs in cytoplasm reduce, but the pre-miRNAs do not accumulate in the nucleus. This suggests that precursors are unstable and require interaction with Exportin-5 (Lund et al. 2004; Yi et al. 2003). Originally, Exportin-5 has been implicated in tRNA transport, as a substitute pathway when primary t-Exportin was depleted or overloaded (Bohnsack et al. 2002). This receptor can as well transport adenoviral RNA VA1 (a 160 nts ncRNA) and recognizes a specific structural motif known as 'minihelix' motif. In pre-miRNA this motif consists of a stem ~22 nts, a terminal loop and a 3'overhang of ~2 nts (Lee et al. 2003; Lund et al. 2004; Basyuk et al. 2003; Zeng et al. 2004).

In the cytoplasm, precursor miRNAs are processed into ~22 nt miRNA duplexes by Dicer, that was first found as a part of RNAi pathway and was later proven to be responsible for generating miRNAs (Ketting et al. 2001; Knight et al. 2001). Dicer is highly conserved protein, found in almost all eukaryotic organisms, such as *Schizosaccharomyces pombe*, plants and animals. In some organisms, there are multiple isoforms of dicer protein with distinct functions. For example, in *Drosophila melanogaster*: Dicer-1 is required for miRNA cleavage, while Dicer-2 is part of RNAi pathway (Lee et al. 2004), meaning that organism has genetically separated RNAi and miRNA pathway. Dicer homologues are multidomain proteins of ~200 KDa size that consist of: two RIIIDs and a dsRBD; have a long N-terminal segment that contains DEADBOX HELICASE DOMAIN, DUF283 domain and PAZ domain. Other associated proteins of Dicer are: RDE-4 in *C. elegans* (Tabara

et al. 2002), R2D2 (Liu et al. 2003) and FMR1 (Liu et al. 2003; Ischizuka et al. 2002; Caudy et al. 2002; Jin et al. 2004) in *D. melanogaster* and Argonaute family proteins in various organisms (Hammond et al. 2001; Carmell et al. 2002). These Dicer-interacting proteins are postulated to have role in miRNA stability and effectors complex formation; but do not seem to be required for the cleavage reaction itself (Liu et al. 2003; Zhang et al 2002; Zhang et al. 2004).

Upon genesis, miRNAs get incorporated into effectors complex, known as 'miRNP' ('miRNA-containing ribonucleoprotein complex'), 'mirgonaute' or 'miRISC' ('miRNA containing RNA induced silencing complex'). The ortholog in RNAi pathway is known as 'RISC', 'sirgonaute' or 'siRISC'.

Duplexes of ~22 nts RNAs is referred as small RNA duplex, unwound and only one strand is incorporated into protein complex. Which strand shall that be depends on thermo-dynamical stability of two ends of duplex (Schwarz et al. 2003; Khvorova et al. 2003), so that strand with relatively unstable base pairs at 5' end gets loaded. The same rule is applicable to both siRNA and miRNA duplexes.

In *D. melanogaster*, incorporation of one strand is assisted by R2D2 protein that contains two dsRBDs and forms heterodimer with Dicer, therefore orienting the complex on the RNA duplex (Tomari et al. 2004; Liu et al. 2003).

Taken together, multiple steps in miRNA biogenesis are coordinated in the following manner: in the nucleus, stem-loop precursors are specifically cropped by Drosha in order to generate a short ~2' nucleotide 3' overhangs (Lee et al. 2003; Basyuk et al. 2003). The structure of short stem-loop 3' overhang seems to be a signature motif for all dsRNAs deriving from this pathway and is recognized by Exportin-5 (Lund et al. 2004; Yi et al. 2003). Upon transport to cytoplasm, Dicer binds to pre-miRNA with preference to 3' overhangs of dsRNAs (Zhang et al. 2004). Therefore, generated 3' protruding ends by Drosha are recognized efficiently by other downstream biogenesis factor. The other end is created by Dicer that measures ~22 nts from the pre-existing terminus and cleaves duplex acting as a ruler (Lee et al. 2003; Lund et al. 2004; Zhang et al. 2004). It is possible that other additional miRNA biogenesis pathways are present in the cell as well.

2.3 The effector phase of si/miRNA pathway

Dicer together with its interacting partner TRBP (TAR RNA binding protein) recruits one of the Argonaute proteins (AGO1 to AGO4 in mammals) to form a trimeric complex. This initiates the formation of the RISC/miRNP (Gregory et al. 2005). Only one strand of the small RNA duplex, named the guide strand, is loaded on to the RISC/miRNP and into the RNA binding pocket of the Argonaute protein. The other strand, the passenger strand, is degraded. The guide strand is selected based on the stability of the base-pairing at the 5' end of the RNA duplex so that the strand with lower stability is loaded on to the RISC/miRNP (Schwarz et al. 2003). Argonautes are considered to be the effector proteins of the RISC/miRNP. This is reflected in their ability to repress protein synthesis, when they are artificially tethered to the 3' untranslated region (3' UTR) of a reporter mRNA, independently of miRNAs presence (Pillai et al. 2004). Once bound to Argonaute protein of RISC/miRNP si/miRNA finds its target due to the base pairing between mi/siRNA and target mRNA. If the base pairing is perfect, a characteristic of siRNA, endonucleolytic cleavage of the target mRNA between positions 10 and 11 of the siRNA happens. This cleavage, referred to as slicing, is catalyzed by the Rnase H fold in the Piwi-domain of the protein (Meister et al. 2004) and can be mediated only by one Argonaut protein, AGO2 in humans. Animal miRNA usually bind to their target mRNA with partial complementarities and induce repression of protein synthesis. The mechanism of repression of protein synthesis is an open question and evidence for several mechanisms has been reported. Repression by miRNA was suggested to take place after the initiation of translation (Seggerson et al. 2002). This was based on findings that cognate mRNA of *lin-4* miRNA in *C. elegans* were associated with polysomes. Degradation of nascent polypeptide was suggested as following mechanism. Later studies confirmed this, but excluded peptide degradation as possible mechanism (Maroney et al. 2006). Other interpretation was that miRNAs cause ribosome to drop off and terminate the translation of the repressed mRNA. The gap between inhibition during initiation or/and elongation phase of ribosome assembly was overcome by the suggestion that method of repression depends on origin of small RNAs involved and that both mechanism are probably active.

2.4 Regulation of miRNA expression

Most miRNAs are under the control of development or/and tissue specific signalling pathways (Miska et al. 2004; Sun et al. 2004). They might be regulated at any step of biogenesis pathway, but the transcriptional control is probably the dominant mechanism. For example, transcriptional control of *let-7* RNA in *C. elegans* depends on a transcriptional enhancer element (temporal regulating element, TRE), situates ~1200 bps upstream form *let-7* miRNA. TRE contains ~22 nts inverted repeat that might serve as a binding site for an unidentified transcriptional factor (Johnson et al. 2003). Other miRNA are controlled at posttranscriptional level, such as miR-38 in *C. elegans* (Ambros et al. 2003). The biogenesis of miRNAs can be regulated both at the level of Drosha cleavage as well as at the level of Dicer cleavage (Davis et al. 2009). The common feature of all siRNAs and miRNAs is that they require Dicer for their maturation.

There are several key differences between miRNAs and siRNAs. The first main difference is their origin. MiRNA are encoded in the genome, while siRNA represent cells response against invading nucleic acids, such as retroelements, transposons and/or viruses (Hammond et al. 2000). Although the protein machinery and processing steps of both pathways are very similar and sometimes overlapping, the second major difference is the effect of small RNA on target mRNA. In the miRNA response, the mRNA is cleaved, partially cleaved or in many cases unaffected, which means untranslated and docked in the specialized cell compartments named Processing bodies (P bodies) or GW bodies. In the siRNA response, mRNA is cleaved and in the RdRP organisms (organisms that have genes coding for RNA-directed RNA Polymerases) secondary and tertiary siRNAs are produced. This can be interpreted as an immune response of the cell towards the alien nucleic acids. Other difference is the degree of complementarily of small RNAs and mRNA target. While miRNA usually have mismatches with target sequence in the core part, siRNA align perfectly to their targets. Different interaction between miRNA and mRNA affects stability of mRNA and inhibits translation. Setting a border between miRNA and siRNA in animal system is easier then applying it on plants, which are considered to be much more complicated. In plants, miRNA lead to cleavage of the target sequences and there is no evidence of

existence of processing bodies. The pathways of small RNA biogenesis have some specificity as well.

2.5 Processing bodies

P-bodies (processing bodies) are cytoplasmic foci visible by light microscopy in somatic cells of vertebrates and invertebrates as well as in yeast, plants and trypanosomes (Kulkarni et al. 2009). At the molecular level, P-bodies represent dynamic aggregates of specific mRNAs and proteins that serve a dual function. They harbour mRNAs that are translationally silenced, and such mRNA can exit again from P-bodies to re-engage in translation. Secondly, P-bodies recruit mRNAs that are targeted for deadenylation and degradation by the decapping/Xrn1 pathway. Proteins called GW182 proteins (GW182A to GW182C in mammals, GW182 in *D. melanogaster*) and AIN-1, their homolog in *C. elegans* are essential for miRNA-mediated repression (Eulalio et al. 2008). GW182 and the Argonaute proteins interact directly in order to mediate miRNA-induced repression. Repressed mRNAs, miRNAs, as well as many components of the RNA silencing pathway, including Argonautes and GW182 proteins, accumulate in discrete cytoplasmic named GW-bodies or processing bodies (Liu et al. 2005; Pillai et al. 2005). Since the Argonaute proteins can be found distributed throughout the cytoplasm, in addition to their P-body localization, it is likely that they initiate the repression of the target mRNA in the cytoplasm outside of P-bodies, which is then later followed by accumulation into the P-bodies. The exact order of these events is unknown. Nevertheless, intact miRNA biogenesis and RNA silencing machinery are required for formation of P-bodies, supporting the idea that P-body accumulation of RISC/miRNP is a secondary effect of RNA silencing (Eulalio et al. 2007). The P-bodies were originally identified as conserved sites of mRNA storage and degradation that contain proteins required for different aspects of mRNA turnover such as decapping, deadenylation and exonucleolytic activity (Parker and Sheth, 2007).

In general, miRNAs and siRNAs are inducing repression and/or degradation of their target mRNAs, serving as a negative regulators of target mRNA. Some reports suggest that also the RNA activation is taking place under specific conditions e.g. miR-369-3p can activate translation of TNFα (Tumor necrosis factor-α) mRNA through binding to an AU-rich element (ARE) in its 3' UTR in cell cycle arrested of human cells (Vasudevan et

al. 2007). This complicates the regulatory networks of small RNAs and we just start to understand the mechanism of their work.

2.6 Biogenesis of miRNAs in plants

The miRNA biogenesis in plants differs from animals in several following features: homologues of Drosha and DGCR8/Pasha have not been found. Precursor miRNAs are quite diverse in structure and contain longer stem-loops than animal analogues. In *Arabidopsis thaliana* only one of four dicer-like proteins, DCL-1, is responsible for generating miRNAs and detection of pre-miRNAs is difficult due to their efficient processing or/and rapid turnover (Park et al. 2002; Reinhart et al. 2002; Papp et al. 2003; Kurihara et al. 2004; Xie et al. 2004). The homologue of Exportin-5 is HASTY protein (Park et al. 2005) and two additional proteins are involved in miRNA biogenesis: HYL-1, nuclear protein with two dsRBD (Han et al. 2004) and HEN-1, protein containing dsRBD and methyltransferase domain that is responsible for methylating miRNA duplex at the 2' hydroxyl groups of 3'-end nucleotides (Park et al. 2002; Yu et al. 2005).
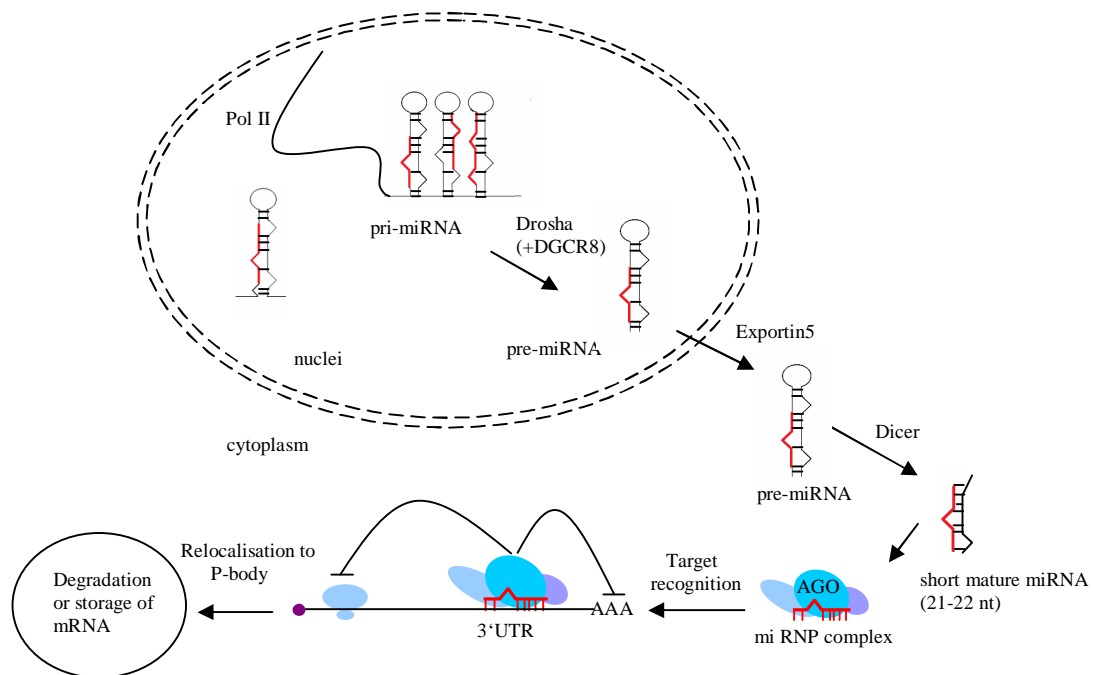
Figure 1. MiRNA processing pathway. Primary miRNA (pri-miRNA) are transcribed by RNA Pol II in the nucleus where they are processed by Drosha and its partner DGCR8 (Pasha) into precursors miRNA (pre-miRNA). Precursor is exported to the cytoplasm by Exportin5, where it is further processed by Dicer into a mature miRNA duplex. The strand with lower stability at its 5' end (in red) is selected to be loaded on to the miRNP complex containing the Argonaute protein. Target mRNAs become translationally repressed and destabilized, event accompanied by mRNA relocalization to a P-body.

2.7 Interaction between miRNA and target mRNA

MiRNAs recognize their target mRNAs by complementary base pairing (Brennecke et al. 2005). The binding sites for miRNAs are usually located in the 3' UTRs of the target mRNAs and are called miRNA response elements (MRE). Insertion of a binding site to the 5' UTR or/and the coding sequence (CDS) also induces silencing. In the longer 3' UTRs (> 1300- nts) the MRE seem to localize to the 5' and 3' ends of the 3' UTR rather than the center, but the binding site are further than 15 nts from a stop codon. Number of MREs attributes to the extent of silencing and their close proximity in the 3' UTR enhances the silencing. This is the case for MRE of the same miRNA and for MREs of two different miRNAs. SiRNAs bind their targets with perfect complementarity, while miRNAs show imperfect base pairing. The 5' end of the miRNA is most important for the miRNA::mRNA interaction, especially positions 2-8 of miRNA are critical for efficient target repression. This region is termed as the seed region of the miRNA. Nevertheless, the opposite case is possible, where imperfect base-pairing or weaker G-U base-pairing in the seed region can stimulate effective silencing. This is due to an increased base-pairing in the 3' half of the miRNA (at the positions 13 to 16).

Defining rules for miRNA::mRNA interaction has been crucial for generating different tools for miRNA targets prediction. Currently, most prediction programs rely on the presence and conservation of an intact complement for the seed sequence in the target mRNA. In general, the seed sequence is the most critical determinant of miRNA target recognition, but it seems that in many special cases the seed sequence does not play a crucial role (Selbach et al. 2008).

Many of the mature miRNAs are conserved across animal species, particularly in their seed regions. MiRNA have their homologs in other species, but can also have multiple paralogs expressed from within the same genome. These paralogs can derive from the

same primary transcript or from separate transcripts and have probably been generated through gene duplications during the evolution.

MiRNA families are formed by miRNA with similar sequence at their seed region and/or beyond it. Members of miRNA families are often functionally redundant, meaning that members of the same family can regulate the same target mRNAs. Removal of a single member of a family is often insufficient to cause major regulatory defects, showing additive type of regulation by miRNAs in *C. elegans* and mouse (Ventura et al. 2009). On the other hand, the redundancy between miRNAs allows multicellular organisms an additional level of regulation by altering the number of miRNA family members expressed in a given tissue. These ways of control can give a wide and precise spectrum of gene expression, but lead to the complicated networks in the miRNA-mediated regulation.

2.8 What is actually miRNA and how to find a new one?

By present convention, miRNA is defined as a single-stranded 22- nts long RNA that is generated by RNaseIII family protein member Dicer from endogenously transcribed hairpin structures. This definition represents a wide spectrum of criteria that have to be fulfilled in order to 'assign' a new miRNA to the genome.

There are a few rules, such as: confirmed expression of miRNA by molecular biology techniques, origin of miRNA from hairpin precursor structure (miRNA sequence must be present in one of precursor arms without any large loops or bulges), sequence of miRNA should be philogenetically conserved (this is taken as a factor for hairpin sequence as well, but to a lesser content) and a precursor miRNA should accumulate in a case of Dicer reduced function.

It is regarded as adequate that only some of these rules are fulfilled if miRNA has been found via cDNA cloning technique e.g. expression of miRNA together with specific hairpin structure or expression with conserved miRNA sequence. If the expression level of miRNA is too low to be detected, but miRNA is conserved in genomes, then it is annotated as miRNA. In the case that methods other then cDNA library led to discovery of miRNA, expression and precursor rule, together with conservation rule must bee confirmed (Ambros et al. 2003).

2.9 Where are genes for miRNAs situated in the genome?

Genes for miRNAs can be situated in different genomic sequences: in intergenic regions (usually >1 kb away from annotated/predicted genes), in intronic regions in both sense and antisense orientation (Lau et al. 2001; Lagos-Quintana et al. 2001; Mourelatos et al. 2002) or as clusters of miRNA (Lee et al. 2004; Cai et al. 2004). MiRNA clusters are transcribed from their own promoters and generate polycistronic transcripts, so called primary miRNA (pri-miRNAs). These clusters have been discover via observation that 50% of known miRNAs in human genome are situated close to each other leading to assumption that they might be transcribed as a single transcription unit as well. miRNA generated from intergenic or intragenic regions are transcribed as autonomous transcription units. For miRNA transcription is responsible RNA Pol II in most cases, but there are some miRNAs that are transcribed by Pol III. miRNA transcripts of Pol II have the following characteristics: a cap m_7pppG structure and poli(A) tails (Lee et al. 2004; Cai et al. 2004), transcription activity is sensitive to L-amanitin (in contrast to Pol I and Pol III activity) and Pol II physically interacts with promoters of some miRNAs such as miR-23a-27a-2, shown by chromatin immunoprecipitation analyses. When miRNA genes are transcribed by Pol II, that is as well responsible for transcription of protein coding genes, then these two processes can be better coordinated (especially if they reside from single transcripts). Due to the Pol II associated regulatory factors, miRNA expression can be more precisely controlled in order to express specific set of miRNAs during development or in certain tissues.

Based on their genomic location miRNA genes can be grouped into following categories: exonic miRNAs in non-coding transcription units; intronic miRNAs in non-coding transcription units and intronic miRNAs in protein-coding transcription units. In mammals (Rodriguez et al. 2004) miRNA can be located in introns of proteins as well as in introns of non-protein genes, in both, sense and antisense orientations. miRNAs can overlap with exons and introns in which case are called 'mixed' variants. This way of gene organisation leads to many questions how are the steps of splicing process regulated and coordinated. The present opinion is that splicing precedes miRNA processing and released intron lariats get processed to release precursor miRNAs. This process of miRNA maturation would be analogues to genesis of small nucleolar RNAs (snoRNA). If the same transcript encodes

protein and miRNA, both are being expressed, but it is not familiar if both protein and miRNA originate from the same transcript or each primary transcript chooses between two synthetic pathways.

2.10 Proteins involved in RNAi pathway in *Dictyostelium discoideum*

Chronology of non-coding RNAs in *D. discoideum* starts with antisense mediated gene silencing (Nellen et al. 1992) and one of few endogenous antisense RNAs discovered in eukaryotes (Hildebrandt and Nellen 1992). It was the psvA (prespore-vesicle-A or EB4) gene that was developmentally regulated by expression of a *cis*-encoded antisense RNA. Afterwards, RNAi mediated gene silencing was characterized for transgenes and endogenous genes that were successfully silenced by *in vivo* expression of a hairpin RNA and the silencing events were accompanied by sequence-specific siRNA production (Martens et al. 2002). Homologues of many components of the RNAi mechanism were identified and functionally characterized in *D. disocideum*. There are two Dicer-like proteins, Dicer A (DrnA) and Dicer B (DrnB), five Argonaute proteins (Agn A-E), three RdRP (RNA-dependant RNA polymerase) homologues – RrpA, RrpB and RrpC and Helicase F (HelF), a negative regulator of RNAi. Only one of RdRPs (RrpA) is strictly required for RNAi (Martens et al. 2002), while all three are needed for antisense-mediated gene silencing (Martens and Nellen, unpublished). RdRPs are required for RNA interference in most of the model organisms. *C. elegans* (Sijen et al. 2001, Smardon et al. 2000), *N. crassa* (Cogoni and Macino, 1999), *S. pombe* (Hall et al. 2002), *A. thaliana* (Dalmay et al. 2000; Mourrain et al. 2000) and *D. discoideum* (Martens et al. 2002) are group of organisms encoding RdRPs that are required for RNAi. In *C. elegans*, *Arabidopsis*, and fungi, silencing requires an RNA-dependent RNA polymerase (RdRP), raising the possibility, that RdRP activity generates dsRNA from single-stranded RNA transcripts. Synthesis of dsRNA using the target mRNA itself as a template (using siRNA as a primer, or via de novo synthesis) could explain the phenomenon of 'spreading' of RNAi, the production of siRNAs encoded by the target gene but not by the trigger RNA. Spreading is associated with 'systemic silencing' seen in *C. elegans* and plants, in which silencing is inherited or is spread to distant parts of the organism. RdRP enzymes have not been found in *Drosophila* and mammals, and the spreading and systemic silencing phenomena are not seen in these organisms.

RdRPs share a common sequence motif, that is distantly related to the catalytic domain of DNA-dependant RNA polymerases. RdRPs can mediate primer-dependant and primer-independent RNA silencing by synthesis of complementary RNA, process in which the dsRNA is an intermediate more than the trigger. In *Dictyostelium* the RdRP recognizes transgene transcripts or antisense transcripts as 'aberrant' and converts it to dsRNA and this RdRP-derived dsRNAs is then cleaved by Dicer (Kuhlmann et al. 2005, Martens et al. 2002).

Dicer-like proteins in *D. discoideum* differ from the typical domain structure of other Dicer homologues, because they lack the N-terminal helicase domain, the PAZ and DUF domains. Helicase domain of Dicers is encoded in the N-terminal part of the RdRPs. dsRBD of Dicers is at the very beginning of the proteins (N-terminally) and both Dicers contain nuclear localization signals (NLS), suggesting their roles in processing pri-miRNAs. This is further supported by findings that DicerB is required for generation of mature miRNA *ddi-mir-1177* (Hinas et al. 2005).

The original assumption was that all these proteins might reside in one complex, contributing with different domains and activities for executing the silencing mechanism (Kuhlmann et al. 2005). Taking into consideration Argonaute homologues whose involvement in RNAi and miRNA pathways is currently investigated; this topic is an open question. It is to be investigated whether functional microprocessor in *Dictyostelium* exists and what are the components. In the investigation of RNAi mechanism no effect of small RNA spreading has been detected in transgenes nor endogenes (Nellen et al. unpublished) which differs from observations in other 'RdRP-organisms' e.g. *C. elegans*.

2.11 Biological roles of miRNAs

MiRNAs have been associated with different processes in cell, starting from developmental regulation, cell growth, genome integrity maintenance, apoptosis etc. Therefore, their up-regulation or down-regulation has been implicated with many diseases, such as cancer (Sassen et al. 2008), neurodegeneration (Hebert et al. 2007), immune defence and metabolism (Esau et al. 2006). miRNAs appear to be fine-tuners for the genome and support the transition from one transcriptional program to another e.g. during development. Here are some examples of miRNA roles studied in different organisms.

miRNAs are known to be necessary for proliferation and proper cell cycle control in many species (Grishok and Sharp 2005) and loss of miRNAs seem to cause decreased proliferation in many different cell types, starting from *Drosophila* cells to mouse ESC (Martinez et al. 2010). Therefore, it is speculated that one of many possible miRNA roles is inhibition of some conserved pathways responsible for stalling the cell cycle progression.

One of the first discovered miRNAs shown to have that function was *bantam* miRNA of *D. melanogaster*, where *bantam* null mutants were lethal and *bantam* miRNA was necessary for growth of imaginal discs through regulation of cell proliferation (Brennecke et al. 2003). Cells overexpressing *bantam* show a strong increase in growth rate and *bantam* has also some anti-apoptotic activity.

Two miRNAs with the same seed sequence, miR-221 and miR-222, are able to induce proliferation of human cancer (Galardi et al. 2007) and point mutations in their binding sites are sufficient to reduce the growth rate of the cancer cells.

Missregulation of miRNA activity has been associated with different forms of cancer and based on their activity they are grouped as 'oncomires' or 'supremeres'. Many miRNAs can increase cell proliferation and act as oncogenes ('oncomires') and the outcome of total loss of miRNAs appears to slower growth rate. There are also some miRNAs that do the opposite i.e. inhibit cell cycle progression and in this way function as tumor suppressors ('supremeres'). miR-17-92 is overexpressed in many rapidly dividing cancers and named as oncomir-1 (Hayashita et al. 2005). Expression of miR-17-92 is regulated by c-Myc, a transcription factor equally up-regulated in many human cancers (O'Donnell et al. 2005). Recently, targets for these miRNAs have been identified and also the mechanisms that allow them to accelerate the cell cycle. For instance, miR-17 and miR-20a can silence mRNAs encoding transcription factors E2F1, E2F2 and E2F3 (O'Donnell et al. 2005), but all of these transcription factors were found to regulate the expression of miR-17-92 itself, creating a self-regulatory loop. These miRNAs regulate the decision between cell cycling and cell cycle exit (Litovchick et al. 2007).

On the other side are miRNAs that are acting as tumor suppressors and one of the first miRNAs to be identified as a potential growth repressor was: let-7 and miR-84, a member of let-7 miRNA family. They regulate protein levels of RAS, a kinase signalling protein and a known oncogene, both in *C. elegans* and in humans (Johnson et al. 2005). RAS and let-7 showed inverse expression patterns in lung cancer cells, and consistently, increased expression of let-7 was sufficient to decrease proliferation of these cells. Further follow-up of the original discovery of RAS regulation in lung cancer showed that also

proliferation of human liver cancer cells could be reduced by let-7 expression and that any of the let-7 family members could trigger this reduction (Johnson et al. 2007). The growth defect was suggested to be mediated by delaying G1- to S-phase transition. The number of cell cycle regulators were found to be inhibited by let-7 in microarray studies e.g. cyclin-dependent kinase 6 and cyclin D. It was found that miRNAs sharing similar seed sequences were causing similar transcription changes (Linsley et al. 2007).

In some cases miRNAs have been described as an important part of signalling cascades. TP53 (Tumor protein p53) is a DNA-binding transcription factor that responds to various cellular stress conditions such as DNA damage by activation of numerous target genes that can, for example, induce apoptosis and stall cell cycle progression. Members of miR-34 family are targets of TP53 (Bommer et al. 2007). TP53 was shown to bind to conserved binding sites in the gene promoters of these miRNA family and upregulate their transcription. Then, increased expression of miRNAs was leading to altered expression of various genes functionally related to TP53 target genes (cell cycle, apoptosis, DNA repair etc.). Other examples for miRNA controlled proliferation come from study of cancer cells, since in cancer the miRNAs are often misregulated, therefore pointing to their roles in cell cycle. Actually, miRNA expression analysis has become an useful diagnostic tool for classification of tumours (Rosenfeld et al. 2008).

The misexpression of miRNAs is often a major contribute to the abnormal behaviour of a cancerous cell: miRNA genes are repeatedly located at fragile genomic sites that undergo amplifications or deletions in different cancers (Calin et al. 2004). For example, miR-21 and miR-17-92 cluster are amplified in neuroblastoma and follicular lymphoma, respectively, while many let-7 family members, miR-34a and miR15a/miR-16 cluster have been deleted in diverse cancers. The significance of miRNA-mediated regulation for cancer highlights the importance of miRNAs in control of endogenous processes, coordinating the balance between proliferation and differentiation, and allowing normal development of an organism. miRNAs are also needed to adjust gene expression and to support the transcriptional regulation during development in all studied animal species. The development is a sequence of coordinated cell divisions from one totipotent cell to a multicellular organism followed by differentiation from one cell type to another. At molecular level this is determined by the transcriptome and the proteome expressed by the cells and any sort of miss-regulation challenges the normal development. The significance of miRNA for development comes from animal experiments lacking the protein components involved in miRNA biogenesis e.g. *dcr-1* or the *alg-1* and *alg-2* gene deletions in *C. elegans* lead to several defects in larval development (Grishok et al. 2001;

Ketting et al. 2001; Knight and Bass 2001). Double mutations of *ago1* and *ago2* or of *ago1* and *dcr-1* lead to segmentation defects in the embryo of *D. melanogaster* (Meyer et al. 2006). In zebrafish the loss of Dicer is leading to a growth arrest one week after fertilization and death in two weeks (Wienholds et al. 2003). *Dicer* knock-out mice show morphological abnormalities by embryonic day 7.5, die before embryonic day 8.5 and the embryos do not have stem cells (Bernstein et al. 2003).

In *Dictyostelium* the multicellular organism is produced by cell aggregation and not by growth and division of a single cell (Loomis et al. 1975) and KOs of different Argonaute proteins show developmental phenotype (Nellen et al, unpublished data).

MiRNAs are also important for proper germ cell development and meiosis. *Dcr-1* null mutants of *C. elegans* are sterile, and their oocytes are abnormal, but the fertility of these worms can be restored by expression of transgenic *dcr-1* (Ketting et al. 2001). Loquacious, a dsRNA-binding partner of Dicer in pre-miRNA processing, is necessary for oogenesis and fertility in *D. melanogaster* (Forstemann et al. 2005). MiRNAs are involved in division and self-renewal in *D.melanogaster* (Yang et al. 2007).

In mammals exploring the haematopoiesis is of great interest, since the largest group of cancer diseases come from misregulation of haematopoiesis. From one haematopoietic stem cell rises a variety of progenitor cells that further differentiate to mature blood cells. Haematopoiesis also serves as a model system for studying miRNAs in cell differentiation. Haematopoietic cells express more than one hundred different miRNAs of which five are fairly specific, miR-142, -144, -150, -155 and -223 (Landgraf et al. 2007).

Another developmental process is lung development where the miR-17-92 cluster is fundamental and the mice lacking this cluster die immediately after birth, due to underdeveloped lungs (Ventura et al. 2008). miRNAs have also a specific role in limb development (Hornstein et al. 2005). Expression of the signalling gene *Shh* (*Sonic hedgehog*) is an important determinant of anterior-posterior polarity of fore- and hind limbs in mice. The forelimb-specific induction of *Shh* is mediated by Hox protein HOXB8 (Homeobox B8). miR-196 expression inhibits *Shh* in hind limbs, which in turn can regulate HOXB8 levels by mediating cleavage of its mRNA (Hornstein et al. 2005). miRNAs are now known to be important for other developmental processes such as skin morphogenesis, hair follicle formation and development of heart and muscle in mice (Yi et al. 2008). miRNAs seem to contribute to development by regulating the balance between proliferation and differentiation. They suppress cell death by serving as switches for lineage selection. On the other side they are needed for maintaining the potential of cells to differentiate into a variety of other cell types. Actually, the key questions for

understanding developmental processes are to determine roles of miRNA in this process and regulatory networks and to understand their roles and involvement in diseases such as cancer and neurodegeneration.

2.12 Evolution and miRNAs

Gene duplication, exon shuffling and alternative splicing are events that allow organisms to acquire new functions and have provided powerful means to increase coding complexity and drive genetic evolution in eukaryotes. However, these mechanisms are not sufficient to justify the huge evolutionary gap between the different eukaryotic classes (invertebrates, fishes, amphibians, reptiles, birds and mammals). The diversity among eukaryotes cannot be explained neither by the sheer number of genes (e.g. the number of *D. melanogaster* genes is only half of the human ones), nor by the number of transcripts (the alternative splicing rate is quantitatively similar for all considered eukaryotes). Furthermore, a fraction of human alternative splicing transcripts does not lead to functional products. Therefore, it has been suggested that evolutionary jumps might be due to the combinatorial ability to use the same basic elements to produce new functional entities and to tune gene expression in a fine manner in response to increasing varieties of stimuli. miRNAs are good candidates to be placed in this network as possible players in the 'evolutionary override' towards more complex functions. miRNA are suggested to be the prime players in the combinatorial view of evolution (Cacchiarelli et al. 2008).

2.13 *Let-7* miRNA

Let-7 miRNA was first discovered in *C. elegans,* as the second miRNA after *lin-4* (Reinhardt et al. 2000). It is functionally conserved and present throughout genomes from warms to humans (Pasquinelly et al. 2000). *Let-7* family in humans has 13 members on 9 different chromosomes and has been associated with many forms of cancer. *Let-7* is not encoded in te genome of *Dictyosteium. Let-7* family members are believed to have redundant roles during the development. *Let-7* is considered to be a tumor suppressor miRNA, since it is down regulated in cancer cells (O'Hara et al. 2009). The name of this miRNA is a shorter version for *lethal-7*, since *let-7* mutants of *C. elegans* can not proceed from the last larval stage to adults and

eventually dye (Reinhardt et al. 2000). In *C. elegans* the maturation of *let-7* miRNA includes a trans-splicing step, in which the sequence upstream of let-7 stem loop is replaced with spliced leader (SL1) sequence, therefore alternating secondary structure around the stem-loop. This enables trans-spliced transcripts to be more efficiently processed.

Recent studies in *Drosophila* have shown that *let-7* also functions as a heterochronic gene in this species as well (Sokol et al. 2008). A single *let-7* gene (Lagos-Quintana et al. 2001) becomes expressed at the end of the third larval instar stage and peaks in pupae during metamorphogenesis (Pasquinelli et al. 2000). *Let-7* mutants display a temporal delay in the terminal cell cycle exit in the wing and also have defects in maturation of neuromuscular junctions at adult abdominal muscles (Sokol et al. 2008).

2.14 miRNA-mediated mechanism of repression

At the moment are in focus three explanations of miRNA-mediated mechanism of repression based on experimental findings. The first explanation is that miRNAs inhibit the initiation phase of protein translation, the second is that miRNA inhibit polysome aggregation (so called ribosome drop-off effect) and the third is that miRNA mediate deanylation and decapping of mRNA (via interaction with CAF1/CCR4 deanylase complex and Dcp1/2 decapping complex). The model of initiation inhibition serves as a background for explaining the formation of structures known as pseudo-polysomes (Hentze et al. 2007). The initiation phase of translation starts upon eIF4E binding to m_7G cap of mRNA, after which, eIF4G connects eIF4E to PABP situated at poliA sequence of mRNA, forming a structure known as closed loop. This is a necessary step before 43S ribosomal unit lands and translation actively begins. In the presence of miRNA, the miRISC complex contains Argonaute proteins that push out eIF4E and eIF4G and compete for binding m_7G cap of mRNA. When this event occurs, the process of translation is inhibited and ribonucleoprotein complexes are formed. These specific complexes are named *pseudo-polysomes*, since on sucrose gradients aggregate in the same cellular fraction with polysomes. Pseudo-polysomes are postulated to be biochemical homologues of P bodies.

2.15 Small RNAs in *D. discoideum*

There are four groups of small RNAs in *Dictyostelium discoideum*, divided upon their source of origin (Hinas et al. 2007):

1. small RNAs ~21 nts in length derived from DIRS retrotransposone
2. Skipper derived small RNAs
3. small RNAs derived from antisense mRNA transcripts
4. two putative micro RNA, named *ddi-mir-1176* and *ddi-mir-1177*.

All four species of small RNAs are developmentally up-regulated, but as well expressed in vegetative cells. Small RNAs originated from DIRS retrotransposone are mostly ~21nts long, constitute around 3% of sequences and are not affected by downregulation of any known proteins of RNAi pathway. These small RNA have 5' monophosphate ends implicating Dicer as a main processing enzyme. Since DIRS constitutes centromeres of chromosome, they are suggested to have roles in centromere constitution (like small RNA in *S. pombe* and *A. thaliana*) and in prevention of transposone mobilisation.

Skipper derived small RNAs are found to be only eight in number and postulated to target sequences outside of retrotransposone, acting as miRNAs.

Small RNAs derived from antisense mRNA transcripts originated from hatA, rpmF and DDB0230011 gene and are suggested to post-transcriptionally control their mRNA levels.

All three species of small RNA represent the endogenous small interference (endogenous si) RNA since they are not encoded in the genome, but their biogenesis is a response to transcription of retrotransposons and antisense mRNA.

Two putative micro RNAs (*ddi-mir-1176* and *ddi-mir-1177*) are developmentally up-regulated and maturation of *ddi-mir-1177* is dependant of DrnB presence, suggesting that DrnB in *Dictyostelium* has a role of Drosha protein. This implicates that small RNAs biogenesis pathways in *Dictyostelium* might be genetically separated.

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-     aa              c       a       a  u        u c uc
 ccca  gucguaucaggugg caauuuuu ucaaggaa gcug aucaucaag g c  c
 ||||  |||||||||||||| |||||||| |||||||| |||| ||||||||| | |  c
 gggu  cagcauaguccacc guuaaaaa aguuccuu cgac uaguaguuc c g  u
a     --              c       c       c  u        u u uc
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a    c       --ac    c   uu              a cu a            u   cc   uu   u    aa
 uca caaauaa     uaaa cag  aggguuuaaugguucu u  ca uuguuuuuuu ggau  caaa  uaauc auuuu
u
 ||| ||||||      |||| |||  |||||||||||||||| |  || |||||||||| ||||  ||||  ||||| |||||
u
 agu guuuauu     auuu guc  ucccgaguugccaaga a  gu aauaaaaagga ccua  guuu  auuag uaaaa
u
c    a        aauu    a   uu              a ag  c          -   uu   --   -    aa
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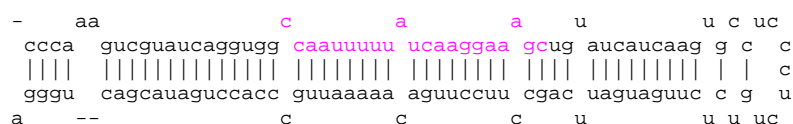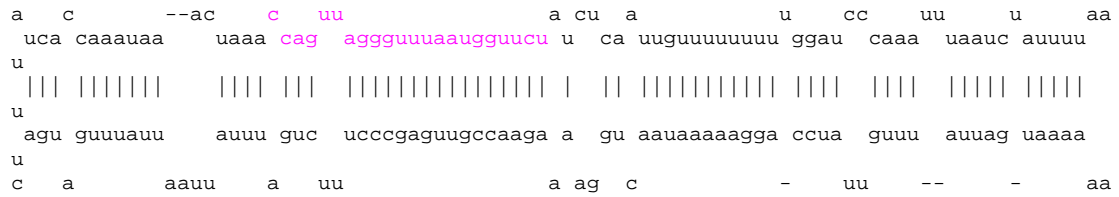
Figure 2. Sequence and hairpin structure of *ddi-mir-1176* and *ddi-mir-1177* in *Dictyostelium* (*www.mirbase.org*).

2.16 Roles of small RNA in *D. discoideum*

It is assumed that upon specific pattern of up-regulation during the development, miRNAs can be involved in controlling the development of *D. discoideum*. Since putative miRNA and other small RNAs are also present in the vegetative cells, it is assumed that they play role in this phase of life cycle, but it is unknown which (Hinas et al. 2007). I will be focussing on roles and significance of miRNA in *D. discoideum* in the results chapter of my work.

2.17 *Dictyostelium discoideum* as a model system

D. discoideum is an established model system for signal transduction, chemotaxis, cell differentiation and development and RNAi and related mechanisms.

The genome of *D. discoideum* is sequenced and has 34Mb and ~12000 genes (Eichinger et al. 2005). Its haploid genome makes it suitable system for genetic studies. The fast growth and cell doubling time in approximate eight hours enable fast selection of mutants and provide enough biological material for experiments. Since it has two life phases, vegetative and developmental, it is suitable for investigating chemotaxis, signal transduction and cell differentiation and development. On the other hand genome of the organism is highly AT rich with more then 70% of sequences coded and has many short introns.

This organism enables applying of integration vectors (Firtel et al. 1984; Nellen and Firtel 1985; Nellen et al. 1985), extrachromosomal vectors (Firtel et al. 1985), ectopic expression of genes (Witke et al. 1985), usage of marker genes such as GFP

or b.galactosidase, gene knockouts by homologues recombination (De Lozanne et al. 1987; Knecht and Loomis 1985) and by RNAi (Martens et al. 2002).

Life cycle of *D. discoideum* starts in a soil ground, where unicellular amoeba feeds on present bacteria. The availability of food source and density of present cells is constantly monitored via secretion of glycoprotein called pre-starvation factor (PSF) (Clarke et al. 1998). Once the threshold levels are exceeded cells start forming multicellular aggregates. This process of transition from unicellular to multicellular organism is initiated by starvation and starts few hours after secretion of 3'-5'cyclin adenosine monophosphate (cAMP). cAMP is secreted in pulses and diffuses to neighbour cells (Konijn et al. 1968), binds to G-coupled protein receptor and leads to cell movement up the gradient. cAMP binds to receptors and triggers cAMP phosphorilation (via activation of adenylat cyclase) and production of new cAMP. Approximately 10^5 cells form aggregates (Gerisch et al. 1975) by tight adhesion to surface. They proceed into standing slugs, fall to the surface and then migrate towards light source. On this stage 20% of cells in the anterior part of the slug give heterologues prestalk population while the rest 80% of cells form prespore population. During mid-development, *Dictyostelium discoideum* can choose between two different pathways. The finger stage can either proceed directly to culmination, or it can fall over to form a migratory, phototactic slug. This migratory stage is thought to enable *Dictyostelium* to find an appropriate site for fruiting body formation (Fey et al. 2007). Slugs form preferably in low ionic strength and in the dark, approximately 16 h after onset of starvation. Prestalk cells vacuolise, secrete cellulose wall and eventually give stalk cells that hold the sporahead. Sporeheads with formed spores originates from prespore cells that have shrunk and secreted spore coat in order to be resistant to environment conditions. The developmental cycle lasts around 24 h. Upon availability of food and moisture, spores will germinate and form unicellular amoebas (Loomis et al. 1975).
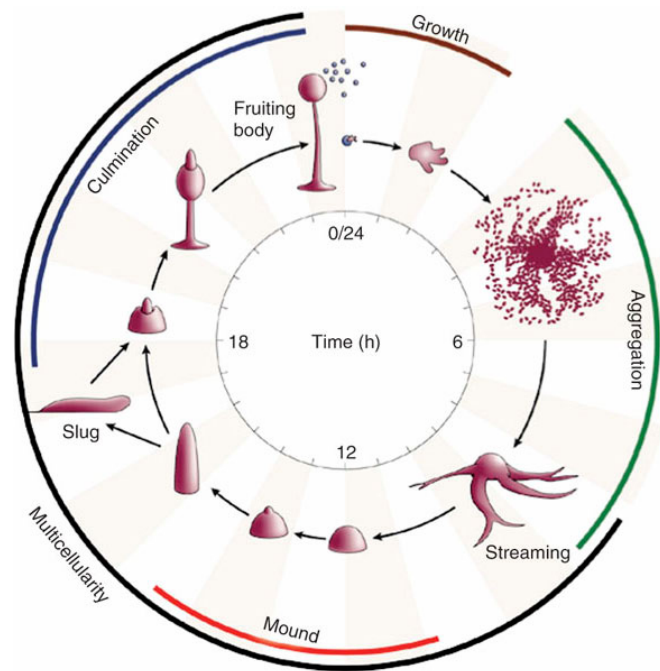
(Fey et al. 2007)

Figure 3. Developmental morphogenesis of *D. discoideum* starts from single (vegetative) amoebae that aggregate and form the mature fruiting body that consists of a stalk and a sorus of spores on top of it. Aggregation is mediated by the chemotaxis of cells toward cAMP to form a multicellular aggregate. During this process, cells can be seen streaming toward a central domain or aggregation centre. Outcome of aggregation is the formation of a multicellular organism, a mound, which then forms a tipped mound. As development proceeds, the tip extends and forms a finger. The extended finger might fall over to form a phototactic migrating slug. Eventually, the finger or slug contracts and the anterior tip begin to rise to form a fruiting body. During culmination, the cells differentiate into vacuolated stalk cells that support a spore head containing spore cells. The spores can tolerate a wider range of environmental conditions than the amoebae and germinate following dispersal therefore renewing the cycle. The entire process from starvation of vegetative cells to the formation of a mature fruiting body takes around 24 h. (Loomis et al. 1975, Fey at al. 2007).

3. MATERIALS AND METHODS

3. 1. Devices

Autoclave	Zirbus, Bad Grund
Bio Imaging Analyzer	Raytest, Straubenhardt
BAS cassette 2025	Raytest, Straubenhardt
Biological Workstation (FPLC)	Biorad, München
cell counter (Coulter Counter ZM)	Coulter Electronics, Krefeld
centrifuges:	
- Avanti™ 30	Beckmann, München
- Centrifuge 5417 C	Eppendorf, Hamburg
- Rotina 48R	Hettich, Tuttlingen
- ultracentrifuge L3-50	Beckman, München
E.A.S.Y. gel documenting system	Herolab, Wiesloch
elektroporator (Gene PulserII®)	Biorad, München
elektrophoresis chambers:	
- agarose gels mechanical workshop,	Uni Kassel
- protein gels (SE 250)	Hoefer Pharmacia, SF, USA
- PAA gels mechanical workshop,	Uni Kassel
Fluorescent microscope (Leica DM IRB)	Leica, Wetzlar
Geiger counter (Mini-Monitor)	Mini-Instruments, GB
gel casting chambers mechanical workshop,	Uni Kassel
gel dryer	Bachofer, Reutlingen
heating block electronic workshop,	Uni Kassel
hybridization oven	Bachofer, Reutlingen
imager: Fuji X Bas 1500	Raytest, Straubenhardt
laminar flow hood	Nunc, Wiesbaden
magnetic stirring plate	Bachofer, Reutlingen
microscope	Zeiss, Jena
microwave oven	Aldi, Essen
PCR-Mastercycler personal	Eppendorf, Hamburg
pH-Meter 320	Bachofer, Reutlingen

eim

pipettes (20µl, 200 µl, 1000 µl)	Gilson, Langenfeld
Photometer (Ultrospec® 2000)	Pharmacia Biotech, Freiburg
power supplies:	
-Power Pac 3000	Bio-Rad, Canada
-EPS	Pharmacia, Freiburg
rocking platfrom	Heidolf, Germany
scale	Mettler, Giessen
scale analytical (BP 210 S)	Sartorius, Göttingen
semidry blotting apparatus von Kreuz,	Reiskirchen
Speed Vac concentrator	Savant, USA
ultra-sonicator UP 200S	Dr. Hielscher GmbH, Stansdorf
UV- table	Bachofer, Reutlingen
Vortex Genie Bender	Hohbein AG, Germany

3. 2. Consumables

3MM paper	Whatman, Göttingen
Becher glasses	Schott, Mainz
Costar-plates	Schütt, Göttingen
Cryo-tubes	Nunc, Wiesbaden
disposable pipette tips	Sarstedt, Nürnbrecht
EP-cuvettes (Gene Pulser® 0,4 cm)	Biorad, München
Falcon-tubes (15 ml, 50 ml)	Sarstedt, Nürnbrecht
glass pipettes	Hirschmann, Germany
Hybond nylon membranes (NX, N+)	Amersham
HisTrap®, Ni-NTA columns	Pharmacia, Freiburg
injection needles	B.Braun, Melsungen
injection syringes	B.Braun, Melsungen
nitrocellulose membrane (porablot™ NCP)	Macherey-Nagel, Düren
parafilm	Schütt, Göttingen
petri-dishes	Sarstedt, Nümbrecht
PCR-tubes	Sarstedt, Nürnbrecht
sterile-filter (0.22 µm, 0.45 µm pores)	Millipore, Eschborn

scalpels	C.Bruno Bayha GmbH, Tuttlingen
tubes (1,5 ml; 2 ml)	Sarstedt, Nürnbrecht

3. 3. Chemicals and reagents

acetic acid 100%	Fluka, Deisenhofen
acetone	Fluka, Deisenhofen
acrylamide/bis-acrylamide (30%, 40%)	Roth, Karlsruhe
agar-agar	Euler, Frankfurt am Main
agarose	Sigma, Taufkirchen
ammonium peroxodisulfate (APS)	Merck, Darmstadt
ammonium sulphate	Roth, Karlsruhe
bacto-peptone	Difco, Augsburg
bacto-tryptone	Difco, Augsburg
biotin-11-ATP	PerkinElmer
biotinamidocaproyl hydrazide	Sigma-Aldrich
ß-mercaptoethanol	Fluka, Deisenhofen
boric acid	Roth, Karlsruhe
Bradford solution Bio-Rad	
Bromphenolblue	Fluka, Deisenhofen
BSA	Roth, Karlsruhe
calcium chloride ($CaCl_2$)	Roth, Karlsruhe
Calcofluor	Roth, Karlsruhe
CHAPS	Roth, Karlsruhe
Complete-mini (Protease Inhibitor Tablets)	Roche, Mannheim
Coomassie Brilliant Blue G-250	Serva, Heidelberg
dATP MBI	Fermentas, St. Leon-Rot
dCTP MBI	Fermentas, St. Leon-Rot
dGTP MBI	Fermentas, St. Leon-Rot
dTTP MBI	Fermentas, St. Leon-Rot
DAPI	Roth, Karlsruhe
DMSO	Sigma, Taufkirchen
DTT	Roth, Karlsruhe
EDTA	Roth, Karlsruhe

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ethanol 99.8%	Roth, Karlsruhe
ethidium bromide	Fluka, Deisenhofen
formaldehyde 37%	Riedel-de-Haen, Seelze
formamide	Roth, Karlsru
gelvatol	Fisher Scientific, Nidderau
glycerol, 86%	Roth, Karlsruhe
glycine	Roth, Karlsruhe
guanidine thiocyanate	Roth, Karlsruhe
HEPES	Roth, Karlsruhe
IPTG	Roth, Karlsruhe
Imidazol	Roth, Karlsruhe
ImmunoPure Immobilized Streptavidin	Pierce, USA
isopropanol	Roth, Karlsruhe
liquid nitrogen Messer	Griesheim, Krefeld
lithium chloride (LiCl)	Roth, Karlsruhe
magnesium chloride ($MgCl_2$)	Roth, Karlsruhe
magnesium sulphate (Mg_2SO_4)	Roth, Karlsruhe
methanol	Roth, Karlsruhe
methylene blue	Roth, Karlsruhe
milk powder	TSI, Zeven
MOPS (γ-(morpholino)-propansulfonic acid)	Roth, Karlsruhe
NBT BTS, St. Leon-Rot	
Neutral Red	Roth, Karlsruhe
N-lauroylsarcosine	Roth, Karlsruhe
Ni-Sepharose™	Amersham, Freiburg
phenol	Roth, Karlsruhe
phenol/chloroform	Roth, Karlsruhe
Protein A-Sepharose beads	Amersham
polyethylene glycol (PEG) 6000	Roth, Karlsruhe
potassium (meta)periodate (KIO_4)	Sigma-Aldrich
potassium acetate (KAc)	Riedel-de-Haen, Seelze
potassium chloride (KCl)	Roth, Karlsruhe
potassium hydrogenphosphate (KH_2PO_4)	Fluka, Deisenhofen
PMSF (phenylmethylsulfonylfluoride)	Roth, Karlsruhe

rATP MBI	Fermentas, St. Leon-Rot
rCTP MBI	Fermentas, St. Leon-Rot
rGTP MBI	Fermentas, St. Leon-Rot
rUTP MBI	Fermentas, St. Leon-Rot
sucrose	Roth, Karlsruhe
SDS -sodium dodecyl (lauryl) sulphate	Roth, Karlsruhe
sephadex (G25, G50)	Fluka, Deisenhofen
sodium borohydride (NaBH$_4$)	Sigma-Aldrich
sodium acetate (NaAc)	Fluka, Deisenhofen
sodium azide (NaN$_3$)	Merck, Darmstadt
sodium carbonate (NaCO$_3$)	Roth, Karlsruhe
sodium chloride (NaCl)	Fluke, Deisenhofen
sodium citrate	Roth, Karlsruhe
sodium dihydrogenphosphate (NaH$_2$PO$_4$)	Fluka, Deisenhofen
disodium hydrogenphosphate (Na$_2$HPO$_4$)	Fluka, Deisenhofen
sodium hydroxide (NaOH)	Fluka, Deisenhofen
TEMED	Roth, Karlsruhe
Tris	Roth, Karlsruhe
Triton-X-100	Roth, Karlsruhe
Tween 20	Roth, Karlsruhe
urea	Roth, Karlsruhe
X-gal	Fermentas, St. Leon-Rot
xylene cyanol	FF Fluka, Deisenhofen

3. 4. Radioactive materials

[α-^{32}P] dATP (110 TBq/mmol)	Hartmann Analytic, Braunschweig
[α-^{32}P] ATP (110 TBq/mmol)	Hartmann Analytic, Braunschweig
[γ-^{32}P] ATP (110 TBq/mmol)	Hartmann Analytic, Braunschweig
[α-^{32}P] UTP (110 TBq/mmol)	Hartmann Analytic, Braunschweig

3. 5. Antibiotics

ampicillin	Roth, Karlsruhe

amphotericin	PAA, Cölbe
blasticidin MP	Biomedicals, Eschwege
geneticin (G418)	PAA, Cölbe
penicillin/streptomycin	PAA, Cölbe
kanamycin	Sigma, Deisenhofen
chloramphenicol	Sigma, Deisenhofen
tetracyclin	Serva, Heidelberg

3. 6. Antibodies

coronin antibody (176-3-6)	University of Kassel
discoidinI antibody (80-52-13)	University of Kassel
GFP antibody (264-449-2)	University of Kassel
EB4 (psvA) antibody	University of Kassel
IgG, goat-anti-mouse,	
alkaline phosphatase-coupled	Dianova, Hamburg
IgG, goat-anti-rabbit	
alkaline phosphatase-coupled	Dianova, Hamburg

3. 7. Enzymes, kits and molecular weight standards

DNase I, RNase free MBI	Fermentas, St. Leon-Rot
Klenow DNA polymerase MBI	Fermentas, St. Leon-Rot
proteinase K	Boehringer Mannheim, Mannheim
restriction endonucleases	MBI Fermentas, St. Leon-Rot,
	Gibco BRL, Eggenstein
	New England Biolabs
	Boehringer Mannheim, Mannheim
reverse transcriptase (Mu-MLV)	MBI Fermentas, St. Leon-Rot
RNase A	Merck Biosciences, Bad Soden
RNase-inhibitor (RNasin)	MBI Fermentas, St. Leon-Rot
shrimp alkaline phosphatase (SAP)	USB
SP6 RNA-polymerase	MBI Fermentas, St. Leon-Rot
T4 DNA-ligase	MBI Fermentas, St. Leon-Rot

T4- polynucleotide kinase	MBI Fermentas, St. Leon-Rot
T7 RNA-polymerase	Dept. of Genetics, Uni-Kassel
MBI	Fermentas, St. Leon-Rot
Taq-DNA-polymerase	Dept. of Genetics, Uni-Kassel
MBI	Fermentas, St. Leon-Rot
NucleoSpin® ExtractII	Macherey-Nagel, Düren
NucleoBond® PC 100	Macherey-Nagel, Düren
pGEM -T-easy cloning kit	Promega, USA

Molecular weight standards

Protein marker (Roti-Mark prestained)	Roth, Karlsruhe
1 kb DNA- ladder MBI	Fermentas, St. Leon-Rot
100 bp DNA- ladder MBI	Fermentas, St. Leon-Rot
50 bp DNA-ladder MBI	Fermentas, St. Leon-Rot
Decade marker	Ambion

3. 8. Buffers and solutions

All buffers and reagents were prepared using bidistilled water or sterile Millipore water. Here are stated buffers and solutions commonly used.

Blocking solution	5 % milk powder in 1 x PBS
BCIP solution	50 mg/ml BCIP-T in DMF
Coomassie solution	10% acetic acid
	0.1% Coomassie Brilliant
	Blue (CBB) G250
Coomassie destainer	5% methanol
	7% acetic acid

Comassie solution (improved protocol)   20 ml 85 % H_3PO_4

60 g ammonium sulfate

1 g Coomassie Brillant Blue G-250

ad 1000 ml H_2O

destaining with H_2O

DAPI solution   1 mg/ml DAPI in MP-Wasser

DEPC water   0.1% diethylpyrocarbonate in H_2O

incubated overnight, autoclaved

DNA loading buffer   30% glycerol

50 mM EDTA

20 mM Tris/HCl, pH 8,0

0,1% bromphenolblue

0,1% xylene cyanol

Ethidium bromide solution   10 mg/ml

2 x Laemmli buffer   62,5 mM Tris HCl (pH 6,8)

2% glycerol

2% SDS

5% ß-mercaptoethanol

0,001% bromphenolblue

9 x Laemmli buffer   3,3 ml glycerol

1,5 ml β-mercaptoethanol

0,69 g SDS

0,228 g Tris

ad 10 ml H_2O

adjust pH 6,8

0,3 mg bromphenolblue

Native RNA-loading dye   70 % glycerol

	0,1 x TBE
	0,1 % xylen cyanol
	0,1 % bromphenolblue

10 x NCP buffer
12,1 g Tris/HCl pH 8,0
87 g NaCl
5 g Tween® 20
 ad 1000 ml H_2O

NBT solution
75 mg/ml NBT in 70% DMF

10 x PBS buffer
60,12 g NaCl
15,3 g Na_2HPO_4 (2 H_2O)
4,02 g KCl
3,82 g KH_2PO_4
 ad 1000 ml H_2O, autoclaved

Phosphate buffer, pH 6,7
56,5 ml 1 M NaH_2PO_4
43,5 ml 1 M $Na_2H PO_4$

SDS- lysis buffer
0,7% SDS
 25 mg/ml proteinase K, dissolved in TE
pH 8,0

Solution I (for plasmid preparation)
25 mM Tris-HCl, (pH 7,4)
10 mM EDTA
15% sucrose

Solution II (for plasmid preparation)
200 mM NaOH
1% SDS

Solution III (for plasmid preparation)
 3 M sodium acetate, (pH 4,7)
20 x SSC
3 M NaCl

0.3 M sodium citrate

20 x TAE buffer 800 mM Tris-acetate

40 mM EDTA

10 x TBE buffer 900 mM Tris-borate

20 mM EDTA

TE buffer (pH 7,4 or 8,0) 10 mM Tris-HCl

1 mM EDTA

3.9 *Dictyostelium* media

AX-medium (pH 6.7) 18 g glucose

14,3 g bacto-peptone

7,15 g yeast extract

0,616 g Na_2HPO_4 x 2 H_2O

0,486 g KH_2PO_4

ad 1000 ml H_2O

DD20-medium, pH 6,5 20 g protease-peptone

8 g glucose

7 g yeast extract

0,47 g Na_2HPO_4 x 2 H_2O

0,33 g NaH_2PO_4 x 7 H_2O

ad 1000 ml H_2O

MES-HL5-medium, pH 7,1 10 g Glucose

10 g Protease-Peptone

5 g Hefeextract

1,3 g MOPS

ad 1000 ml H_2O

G_0- medium as AX-medium

	+ 50 µg/ml ampicillin
	+ 10 U/ml penicillin
	+ 10 µg/ml streptomycin
	+ 0.25 µg/ml amphotericin

G10 medium

as G_0-medium
+ 10 µg/ml geneticin

B10 medium

as G_0-medium
+ 10µg/ml blasticidin

Soerensen- phosphate buffer (pH 8.7)

2 mM Na_2HPO_4
15 mM KH_2PO_4

SM agar plates

15 g bacto-agar
10 g peptone
10 g glucose
1 g yeast-extract
1 g $MgSO_4$
2,2 g KH_2PO_4
1 g K_2HPO_4
ad 1000 ml with H_2O,
autoclaved;
10 ml 20% maltose, 10 ml
$MgSO_4$
20 ml/Petridish

Bacterial media

LB medium, pH 7,0

10 g Bacto-Tryptone
5 g yeast-extract
5 g NaCl
ad 1000 ml H_2O

LB-agar   LB-medium

                                               + 13 g/l Agar-Agar

LB$_{amp}$-plates   as LB-agar,

                                               + 50 µg/ml ampicillin

3.10. Biological material

Bacterial strains

Escherichia coli - DH5α™   Invitrogen, Karlsruhe

Klebsiella aerogenes   (Williams and Newell, 1976)

Dictyostelium discoideum

Dictyostelium discoideum AX2, strain 214

3.11 Plasmid mini-preparation (alkaline lysis)

1,5 ml of a bacterial culture, grown over night at 37°C was used to prepare plasmid DNA by the alkaline lysis method (Birnboim and Doly, 1979). *E. coli* cells were collected by centrifugation at 3500 rpm for 5 min and resuspended in 100 µl solution I by strong vortexing. After adding 200 µl solution II, the cells were lysed at RT for 5 min, then neutralized with 150 ml solution III. After 10 min incubation on ice, the samples were centrifuged at 14000 rpm for 15 min and the supernatant was collected and precipitated with 0,8 ml pure ethanol. The pellet was washed with 70% ethanol, dried and dissolved in 20 µl water.

3.12 Plasmid maxi-preparation

For the preparation of bigger amounts of plasmid DNA 50 - 100 ml *E. coli* cultures were used. The mini-preparation method was up-scaled respectively. All plasmids,

used for transformation in *Dictyostelium* or for *in vitro* transcription, were prepared with the Nucleobond midi-columns Macherey&Nagel kit, used according to the manual of the manufacturer.

3.13 Genomic DNA preparation from *Dictyostelium discoideum* - fast mini preparation (Barth *et al.*, 1998)

Dictyostelium cells, grown on Costar 24-well plates (5 x 106 cells), were collected by centrifugation at 4000 rpm for 5 min The cells were resuspended in 300 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, 0,7% SDS) and 30 µg Proteinase K (in TE buffer) was added, followed by incubation at 45°C for one hour. The genomic DNA was extracted with phenol/chloroform and precipitated with ethanol. The genomic DNA, prepared by this method, was used for PCR experiments

3.14 Genomic DNA preparation from *Dictyostelium discoideum* - maxi preparation

$1-2 \times 10^{8}$ cells were collected from axenic culture with high cell density (approximately $4-6 \times 10^{6}$ cells/ml) by centrifugation at 1800 rpm for 10 min at 4°C, washed once with ice-cold phosphate buffer and resuspended in 50 ml nuclear lysis buffer. The cells were lysed by addition of NP40 to a final concentration of 1%. The nuclear fraction was obtained by centrifugation at 4000 rpm for 15 min. The nuclear pellet was resuspended in 5 ml SDS-lysis buffer and incubated with 100 µl Proteinase K solution (25 mg/ml in H_2O) at 50°C for 3 hours. The genomic DNA was extracted twice with phenol/chloroform (1:1 vol/vol) and precipitated by adding 1/10 vol. 3M sodium acetate and 2 vol. pure ethanol. The DNA precipitate was washed with 70% ethanol, dried and carefully resuspended in 200-300 µl water.

Nuclear lysis buffer SDS-lysis buffer
50 mM HEPES, pH 7,5 0,7% SDS in TE-buffer
40 mM MgCl2
20 mM KCl
5% Sucrose
1% NP 40

3. 15 Isolation of total RNA from *Dictyostelium discoideum* (Maniak *et al*., 1989)

$1–3 \times 10^7$ cells were pelleted by 1700 rpm for 10 min at 4°C and lysed in 500 µl solution D. After adding 50 µl of 3 M sodium acetate (pH 4,7) and 500 µl phenol/chloroform, the sample was vortexed and centrifuged 15 min at 14000 rpm. The upper phase was collected and precipitated by adding 1 vol. isopropanol. The RNA was then pelleted, washed with 70% ethanol, dried in the speed-vac and dissolved in 100 µl DEPC water or formamide.

Solution D (incomplete)
4 mM GTC
25 mM sodium citrate
0,5% sarcosyl
To prepare a complete solution D, before use 360 µl β-mercaptoethanol were
added to 50 ml solution (0,1 M).

3. 16 Enrichment of small RNAs from total cellular RNA

To a total RNA preparation, dissolved in 5 ml DEPC water, PEG (MW=6000) was added to a final concentration of 5% and NaCl to a final concentration of 0,5 M. The sample was mixed, incubated on ice for 30 min and then high molecular weight nucleic acids were pelleted at 10000 x g for 15 min. The resulting supernatant, containing tRNA, small rRNAs and siRNAs, was precipitated with three volumes of ethanol after incubation at –20°C over night. The RNA was washed with 75% ethanol, dried and dissolved in 200 µl formamide.

3. 17 Polymerase chain reaction (PCR)

The polymerase chain reaction (Mullis *et al*., 1986) was used to amplify DNA *in vitro*. The following general protocol was used for amplification on plasmid and genomic DNA templates:

Reaction mixture

100 ng template DNA

10 pmol each primer

5 µl dNTP mix (2 mM each)

5 µl 10xPCR buffer (100 mM Tris/HCl pH 8; 0, 1% Triton X-100;

50 mM KCl, 10-25 mM MgCl$_2$)

2 µl Taq polymerase

ad 50 µl H$_2$O

The following typical cycling protocol was used, however modified with respect to the annealing temperature, the elongation time and/or the elongation temperature depending on the primer pair used and on the length of the PCR fragment.

1. step 3-5 min 95°C

2. step (30-40 cycles) 30 s 95°C denaturation

30 s 50°C annealing of primers

30 s 72°C polymerization (elongation)

3. step 15 min 72°C

3.18 RT-PCR (Reverse transcription – PCR)

The RT-PCR method is used to produce a cDNA using RNA as a template. The reverse transcriptase, an RNA-dependant DNA polymerase, was used. As a primer for the first strand synthesis, an Oligo(dT) primer or a sequence specific primer was used. In order to prevent DNA contaminations, the RNA, diluted in DEPC water, was precipitated with 1 vol 8 M LiCl and 2,5 vol ethanol for 30 min at –20°C and then centrifuged at 14000 rpm for 30 min, washed in 70% ethanol, dried and dissolved in DEPC water. Alternatively, the RNA sample was treated with Dnase I (RNase-free) for 30 min at 37°C.

Reaction mixture for DNase treatment

10 µl RNA in DEPC water (~ 5 µg)

2 µl 10xDNase I buffer

2 µl DNase I (RNase free) (4 U)

1 µl RNasin (10 U)

ad 20 µl H$_2$O

Reaction mixture for cDNA synthesis

3 µl total RNA (~1-2 µg)

2 µl Oligo(dT) primer or sequence specific primer (5 pmol/µl)

ad 10 µl DEPC H$_2$O

For denaturing of the RNA, the reaction mixture was incubated for 5 min at 65°C and then immediately placed on ice.

Then 4 µl 5xRT buffer (MBI Fermentas) were added,

   5 µl dNTPs (each 2 mM) and

   1 µl RNasin (10 U).

The reaction was incubated for 5 min at 42°C, allowing for the annealing of the primer, and after the addition of 1 µl (10-20 U) M-MuLV reverse transcriptase further incubated for 60 min at 42°C for the first strand synthesis. The reaction was stopped by heat inactivation of the enzyme at 70°C.

3 µl were used for a standard PCR, using sequence specific primers.

3.19 Gelelectrophoresis of nucleic acid samples

Generally, DNA fragments were separated on 0,8 – 1,8 % agarose gels. The corresponding amount of agarose was dissolved in 1xTBE buffer, melted in a microwave, then ethidium bromide was added to a final concentration of 0,5 µg/ml and the gel was poured into a horizontal gel-forming chamber. The gels were run using 1xTBE buffer and were documented using UV light (256 nm) and the E.A.S.Y. system. Total RNA was separated on 1,8 – 2 % denaturing GTC (20 mM) agarose gel. The agarose was melted in 100 ml 1 x TBE buffer using a microwave and after cooling to 60°C 500 µl of 1M guanidium thiocyanate was added (Goda and Minton, 1995). The amount of RNA to be run on the gel was mixed with 1,5 – 2 volumes of denaturing RNA loading buffer (95% Formamide, 2 mM EDTA), heated for 5 min at 85$_o$C and put immediately on ice. The gels were run in the cold room at 90-100 V.

For separation of *in vitro* transcripts and $_{32}$P-labelled or non-labelled nucleic acids (RNA and DNA), the samples were run on 8 to 15% denaturing polyacrylamide/urea gels, depending on the length of the nucleic acid fragments. The gels were run in 0,5 x TBE buffer by 500 V for 1-3 hours.

Polyacrylamide / urea gel:

Urea 14,4 g (8 M)

10xTBE 1,5 ml

40% Polyacrylamide x ml

ad 30 ml H_2O

The polymerization of the gel was achieved by the addition of 80 µl TEMED and 80 µl 20% APS.

3.20 Gel elution of DNA fragments

For elution of DNA fragment from the agarose gels, the desired band was cut under UV light (366 nm). The DNA was purified using Nucleospin™ (Macherey & Nagel) purification kit according to the manual of the manufacturer. Alternatively, the "home" method was used. A small piece of Whatman paper was cut and was pressed against the walls of a 1.5 ml Eppendorf tube with the upper side of an yellow tip.

The tip was cut shorter, a hole was made at the bottom of the tube and the small "column" was placed in another Eppendorf tube. The Whatman paper was wetted with 100 µl 1 x TBE buffer and centrifuged for 1 min at 14000 rpm. After discharging the flow-through, the agarose band was cut into small pieces, placed around the yellow tip and centrifuged for 1 min at 14000 rpm. The DNA containing flow-through was precipitated, washed with 70% ethanol and diluted in H_2O.

3.21 Gel elution of RNA fragments from polyacrylamide gels

The RNA fragment was subjected to PAGE and the desired band was cut under UV light (for non-radioactive RNA). When gel elution of radioactively labelled RNA was performed, the gel was exposed shortly on an imaging plate for analysis in a Fuji Phosphorimager and the position of the corresponding band was marked by overlapping the gel, packed in a plastic sheet, and the printed image. The RNA was extracted from the excised band by shacking in water overnight at room temperature. The RNA was ethanol precipitated, washed with 70% ethanol, dried and diluted in DEPC H_2O.

3.22 Cloning of DNA fragments

Cloning of DNA fragments was performed by standard molecular biology techniques. Restriction digestion was made using appropriate buffer and temperature according to the supplier (1-2 units of enzyme/ µg of plasmid DNA, 1-2 hours incubation in a total volume of 20 µl). For preparative restriction digestions the reaction was up-scaled respectively. Religation of linearized plasmid vectors was prevented by removal of the 5` terminal phosphate group with shrimp alkaline phosphatase (SAP): 1-2 units of SAP were added to the restriction digestion and the reaction was incubated at 37°C for 30 min The enzyme was inactivated for 10 min at 85°C and the dephosphorylated DNA was used further for ligation. The ligation of DNA fragments was performed in 20 µl volume overnight at 16°C using T4 Ligase (2 - 4 U). In a standard ligation reaction a molar ratio of 1:5 between linearized vector and insert was used. Blunt end ligations were carried out at room temperature for 4-6 hours. PCR fragments were cloned using T-cloning kit (MBI-Fermentas) and pGEM-T-Easy vector system.

3.23 Fill-in reaction to produce blunt ends from 5'-overhangs with Klenow-Polymerase

Sticky DNA ends with 5'-overhangs can be filled-in with complementary nucleotides with the help of Klenow-Polymerase, obtaining blunt-ends, which can be ligated in a following ligation procedure. The method can also be used to end-label DNA fragments with 5'-overhangs.

Klenow reaction
x µl DNA
2 µl 10xY-Tango buffer (MBI)
1 µl 2 mM dNTP mix
1-2 U Klenow-Polymerase
ad 20 µl H$_2$O
The reaction was incubated at 37°C for 1 h, extracted with phenol and precipitated with ethanol.

3.24 T4-DNA-Polymerase Reaction

Sticky DNA ends with either 5' or 3'-overhangs can be blunted using the 3'- 5' exonuclease activity of T4 DNA polymerase. In presence of high dNTP concentrations the T4 polymerase stops the degradation of the DNA by reaching the dsDNA region, since the polymerase and the exonuclease activities of the enzyme are in equilibrium.

T4-DNA-Polymerase reaction mixture

4 µl 5 x reaction buffer (MBI)

2 µg DNA

1 µl 2 mM dNTP mix

2 U T4 DNA Polymerase

ad 20 µl H$_2$O

The reaction was incubated for 20 min at 11°C, extracted with phenol/chloroform, the DNA was precipitated with ethanol, washed with 70% ethanol, dried and diluted in 20 µl H$_2$O.

3.25 Transformation of competent *E. coli* cells

Competent *E. coli* cells were prepared using the CaCl$_2$-method (Dagert and Ehrlich, 1979). One aliquot (200 µl) was thawed on ice and a ligation mixture (20µl) or 1 µl plasmid DNA was added by gently stirring the cells while pipetting. After 40 min incubation on ice, a heat shock was done for 90 s at 42°C and the cells were put on ice for 10 min. Then 1 ml LB medium was added and the cells were incubated for 30 min at 37°C. After 5 min centrifugation by 3500 rpm, the cell pellet was resuspended in ~200 µl LB medium, plated on LB-Amp agar plate and incubated overnight at 37°C.

3.26 Oligolabeling

The 'oligo-labeling' method was used for labelling DNA fragments. 0.1-0.3 µg purified DNA template was denatured by heating at 95°C for 5 min followed by annealing of random DNA hexamers. The Klenow fragment elongated the primers, incorporating α^{32}P-dNTPs.

Reaction mixture

10 µl OLB-mix

2 µl BSA (10 mg/ml)

5 µg template DNA

5 µl α_{32}P-dATP

2 –3 U Klenow fragment

ad 50 µl H_2O

The reaction was incubated at 37°C for 1 h and then stopped by addition of 100 µl phenol. Free nucleotides were separated by centrifugation through a Sephadex G50 spin column. The purified radioactive probe was then denatured by heating at 90°C for 5 min, cooled on ice, and used for hybridization.

OLB-Mix

200 mM Tris/HCl, pH 7.5

25 mM $MgCl_2$

10 mM ß-mercaptoethanol

1 M HEPES pH 6.6

13.5 U A_{260} oligos-hexamers (MBI)

0.25 mM dCTP, dGTP, dTTP

3.27 In vitro transcription

In vitro transcription is used to generate radio-labelled or non-labelled RNA sequences that can be used as probes for hybridization, antisense RNAs, substrates for studies of RNA-protein interactions, etc. As templates different vectors can be used (for example pGEM 3Z, pGEM T-Easy), which possess promoter sequences for T7 or SP6 Polymerases, where the sequence to be transcribed is cloned after the promoter. The plasmid DNA was cut with an appropriate restriction enzyme (run-off transcription), separated on an agarose gel and gel eluted. Alternative, PCR fragments were used as templates for *in vitro* transcription, where T7 or SP6 promoter sequence was designed in the primers. The PCR fragments were gel eluted and precipitated with ethanol.

Reaction mixture

1–5 µg template DNA

10 µl 5 x transcription buffer (MBI)

5 µl rNTP mix (5 mM ATP, CTP, GTP, 2 mM UTP)

3-5 µl α_{32}P-UTP

1 µl RNase inhibitor RNasin

1-2 µl T7 /SP6 Polymerase

ad 50 µl H_2O

For non-radioactive *in vitro* transcriptions, rNTP mixtures were used, where the concentration of all rNTPs was 5 mM. The reaction was incubated for 30 min at 37^oC and stopped by addition of an equal volume of phenol/chloroform. Non-incorporated nucleotides were removed by a gel filtration over a Sephadex G-50 column.

3.28 End labeling with T4 Polynucleotide Kinase (PNK)

T4 PNK is a polynucleotide 5'-hydroxyl kinase that catalyzes the transfer of the γ-phosphate from ATP to the 5'-OH group of single and double stranded DNA and RNAs. The reaction is reversible and in the presence of ADP, T4 PNK exhibits 5'-phosphatase activity and catalyzes the exchange of terminal 5' phosphate group (exchange reaction).

Labeling of 5'-termini of DNA/RNA by forward reaction

20 pmol 5'-termini dephosphorylated DNA/RNA

2 µl 10 x reaction buffer A (MBI)

3 – 5 µl γ $_{32}$P-ATP

10 U T4 PNK

ad 20 µl H_2O

The reaction was incubated at 37^oC for 30 min, extracted with an equal volume of phenol/chloroform, processed over a Sephadex G-50 spin column and precipitated with ethanol.

For labeling of 5'-protruding termini of DNA by the exchange reaction, the same protocol was applied. Instead of buffer A, buffer B (MBI) was used, and additionally 4 µl 24% PEG 6000 were added to the reaction mixture.

Hybridization solution

50 % formamide

50 mM sodium phosphate (pH 7,2)

5xSSC buffer

0,1% N-lauroylsarcosine

7% SDS

3.29 Northern blot analysis

Total RNA (~10 µg) was separated by gel-electrophoresis in a denaturing (GTC) agarose gels at 90 V in the cold room. The gel was photographed under UV light and the capillary transfer of RNA on a nylon membrane and the hybridization of the membrane were conducted in the same way as in the Southern blot. The hybridization was performed overnight at 40-60° C for an RNA radioactive probe and at 42-50°C for a DNA radioactive probe.

3.30 Northern blot of small RNAs

Northern blot of small RNAs was carried out as described in (Hamilton et al., 2008). The following procedure was used: 20-40 µg total RNA from *Dictyostelium* were mixed 1:1 with 2xRNA dye, denatured for 5 min at 95°C and put on ice for 5 min. The RNA was separated on a 12% polyacrylamide gel, containing 7M urea in 1xMOPS (20x20 cm glass plates) and run at 25 W for 1h. The gel was electroblotted in DEPC water at 20 V at 4°C for 15 min (BioRad TransBlot Cell) to Hybond Nx membrane (Amersham) and the RNA was chemically crosslinked for two hours at 50-60^{0}C during which time RNA becomes crosslinked to nylon. As a probe, a random primed labelled PCR derived probe was used.

Chemical-crosslinking solution
(0.16M EDC in 127mM 1-methylimidazole, pH8)

245 µl 12.5 M 1-methylimidazole stock solution

9 ml RNAse-free water

1M HCl (usually about 300 µl) to adjust the pH to 8.0

0.753 g EDC

ad 24ml working solution

Oligo labeling reaction

15 µl PCR product

5 µl γ_{32}P-ATP (100 µCi)

10 ul 10mM dCTP, dGTP, dTTP mix

5 ul 10x Klenov buffer

2 ul Klenov enzyme

ad 50µl H_2O

The reaction was incubated at 37°C for 1 h, unincorporated nucleotides were removed by sephadex column centrifugation. The membrane was prehybridized with Church buffer (20-30 ml depending on membrane size) at 42°C for 1 h. The prehybridization buffer was changed with 20-30 ml fresh prewarmed (42°C) Church buffer and the denatured labelled probe was added. The hybridization was carried out at 42°C overnight. The membrane was washed with pre-warmed (45°C) wash solutions (same volume as for hybridization):

I. Rinse with 2xSSC / 0,1% SDS

II. Wash 1 x 5 min at 42°C with 2xSSC / 0,1% SDS

III. Wash 2 x 5 min at 42°C with 0,5xSSC / 0,1% SDS

IV. Wash 2 x 5 min at 42°C with 0,2xSSC / 0,1% SDS

The washing was controlled by monitoring or exposure for a short time in the Phosphor Imager. The damp membrane was put into a plastic hybridization bag and sealed. The membrane was then exposed on an imaging plate for analysis in a Fuji X Bas 1500 Bioimaging analyzer over night. The membranes were stripped for further use by shaking in strip buffer 2-3 x 20 min at 80°C.

Church buffer

1% BSA

1 mM EDTA

0,5 M NaPO$_4$ buffer pH 7,2

7% SDS

2xRNA dye (1 ml)

916 µl formamide

34 µl 0,5 M EDTA

25 µl 1% brome phenol blue

25 µl 1% xylene cyanol

Strip buffer

0,1 x SSC

1% SDS

25 µl 1% xylene cyanol

Strip buffer

0,1 x SSC

1% SDS

Nuclear lysis buffer:

50 mM HEPES, pH 7,5

40 mM MgCl$_2$

20 mM KCl

5% Sucrose

14 mM β-mercaptoethanol

Storage buffer:

40 mM Tris pH 8

10 mM MgCl$_2$

1 mM EDTA

50% glycerol

14 mM β-mercaptoethanol

5 x reaction buffer:

200 mM Tris, pH 7,9

50 mM MgCl$_2$

250 mM KCl

0,5 mM DTT

25% glycerol

Pre-hybridization buffer EB:

0,5 M NaP04, pH 6,5

7% SDS

Hybridization buffer EB:

50% Formamide

5 x SSPE

50 mM NaP04, pH 6,5

Washing solution 1:

2xSSC

0,1% SDS

Washing solution 2:

0.2xSSC

0,1% SDS

3.31 Sucrose gradient separation

Preparation of sucrose gradients

Sucrose gradients of 10-40% were prepared in 12 ml Beckman tubes using hand made mixing apparatus (University of Kassel) or alternatively, putting decreasing sucrose layers on top of each other, then freezing and melting tubes. Sucrose was diluted in STKM buffer.

10x STKM (0.25 M sucrose, 20 mM Tris, 0.15 M KCl, 5-15 mM MgCl$_2$ buffer, 0,5% NP40, pH 7.4)

Isolation of fractions

Cells lysates of 5-15 x 10^7 cells were put on top of gradients and ran at 39 000 rcf using Ti40 Beckman Rotor at Beckman ultracentrifuge for 180 min at 4^0C. 1-1,5ml fractions were collected from top to bottom and used further for protein and RNA detection. RNA preparation was done using acidic phenol preparation protocol and absorbance for measured at 260 nm.

3.32 SDS polyacrylamide gelelectrophoresis (SDS-PAGE) of protein samples (Laemmli, 1970)

Protein samples were separated with the help of discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE). Depending on the molecular mass of the proteins, different resolving gels were used (7-12%). Protein samples were supplemented with 2 x Laemmli buffer, heat-denatured for 5 min at 95^oC and then separated on SDS-polyacrylamide gels. The gels were prepared using glass plates and gel-casting chambers. Electrophoresis was carried out in 1 x running buffer at 20 – 40 mA. The gels were electro-blotted or stained with Coomassie brilliant blue G 250.

12% resolving gel Stacking gel
Acrylamide/bis-acrylamide
30% / 0,8%
4,4 ml / 450 µl

Lower Buffer 2,64 ml 1 ml

Upper Buffer
H2O 3,4 ml 2,5 ml
250 mM EDTA 43,2 µl 16 µl
TEMED 6 µl 4 µl
20% APS 120 µl 60 µl

5x Running Buffer

1,5 M Tris pH 8,8 0,5 M Tris pH 6,8 151 g Tris

0,4% SDS 0,4% SDS 72 g Glycin

5 g SDS

ad 1000 ml with H_2O

3.33 Western blotting (electroblotting, semidry-blotting)

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes using a semidry blotting system. The transfer was performed with the 'Semi-dry' Blot apparatus at 2 mA/cm^2 membrane (max. 40 V) for 1 h in a transfer buffer.

Semi-dry Blot buffer

5,8 g Tris

2,92 g Glycin

0.38 g SDS

200 ml Methanol/Ethanol

ad 1000 ml H_2O

3.34 Immunodetection with enzyme conjugated secondary antibodies

The Western blots were immersed in blocking buffer (1xPBS or 1xNCP + 5% milk), then incubated with the primary antibody at a proper dilution. The blots were washed 3 x 5 min each with 1xPBS (or 1xNCP) and incubated with an alkaline phosphatase (AP) conjugated secondary antibody directed against the primary antibody (e.g. goat anti-mouse, goat antirabbit IgG). Antigens were identified by chromogenic visualization in BCIP substrate solution (0,2 mg/ml in 0,1 M Na_2CO_3).

3.35 GFP measurement on fluorimeter

Presence of fluorescent protein was detected using fluorimeter. The sample for GFP was exited on 490 nm for 4 s and relative absorbance was measured at 510 nm.

3.36 *Dictyostelium* axenic cell growth

Dictyostelium discoideum AX2 strain and the derived transformants were grown in AX or selection media. *Dictyostelium* cells were inoculated at 1-5 x 10^4 cells/ml and shaken at 150 rpm at 22°C, then harvested at indicated cell densities.

3.37 Cloning of *Dictyostelium* on SM plates

KA suspension was prepared by washing one KA plate with 5 ml phosphate buffer. To obtain single clones of *Dictyostelium*, around 50-200 cells were resuspended in 100 µl fresh prepared KA suspension and plated on SM plates. Plates were grown at 22°C for 3 days until colony plaques appeared on the bacterial lawn. Single clones were picked up with tooth picks and grown on 24-well Costar plates.

3.38 Transformation of *Dictyostelium* using electroporation

2 x 10^7 cells, grown to a density of 1 x 10^6 were collected, washed once with ice-cold phosphate buffer and once with EP buffer, then resuspended in 800 µl EP buffer. 15-20 µg DNA was added and the cells were incubated on ice for 10 min. Electroporation was performed at 1 kV, 25 µF in a 0,4 cm electroporation cuvette (the time constant between 3 and 4 ms). The cells were plated on a Petri dish, mixed with 2 drops (8 µl each) 0,1 M $CaCl_2$ and 0,1 M $MgCl_2$ and incubated at RT for 15 min. Then 10 ml DD20 medium were added for overnight incubation. On the next day, the medium was replaced by the appropriate selection medium. The cells were kept under selection until transformants were obtained.

EP buffer, pH 6,1
10 mM Na_2HPO_4,
50 mM sucrose

3.39 Classical transformation of *Dictyostelium discoideum* (Nellen and Firtel, 1985; Nellen and Saur, 1988)

10 ml *Dictyostelium* cells, grown to a density of approx. 1 x 10^6 were pipetted in a petri dish. After 30 min the cells have settled down on the bottom and the medium was changed with 10 ml MES-HL5. Simultaneously, the DNA sample was prepared:

20 µg DNA were diluted in 600 µl 1xHBS buffer and 38 µl 2 M $CaCl_2$ were added drop-wise to the solution under rigorous mixing. The DNA precipitated as micro-crystals during the following 25 min incubation. The medium from the petri dish was removed and the DNA solution was distributed drop-wise over the cells. After 20 min incubation, 10 ml MES-HL5 medium were added and the transformation reaction was incubated for 3 h at 22°C. Then the medium was changed with 2 ml 18% glycerin in 1xHBS. After 5 min, the glycerine solution was removed carefully and 10 ml MES-HL5 medium were added. On the next day, the medium was changed with a selection medium, containing the appropriate antibiotic. For selection of resistant clones, the medium was changed every 2-3 days.

2 x HBS, pH 7,05 4 g NaCl
0,18 g KCl
0,05 g NaH_2PO_4
2,5 g HEPES
0,5 g Glucose
ad 250 ml H_2O

3.40 Development of *Dictyostelium* on nitrocellulose filters

Approximately 5 x 10^7 *Dictyostelium* cells were pelleted by 1700 rpm, washed once with phosphate buffer, resuspended and placed on a black nitrocellulose filter (d=5 cm), which had been boiled beforehand for 5 min in water. The nitrocellulose filter had been placed over two layers of Whatman 3MM paper, wetted with phosphate buffer. The development was done at 22°C in a closed chamber to prevent the drying of the filters. After 16 hours the development was checked optically and the aggregates were harvested by scratching the filters with a scalpel. The cells were disaggregated by vortexing in 10 ml phosphate buffer and the cells were processed further for isolation of total RNA according to the protocol.

3.41 Fluorescence Microscopy

Cell were fixed at -20°C in methanol for 20 min, washed three times with 1xPBS and stained with DAPI (1 mg/ml, diluted 1:15000 in 1xPBS). The fixed cells were

analyzed with a Leica DM IRB inverted fluorescence microscope. For image acquisition, a Leica DC 350F digital camera and IM50 software were used. Images were processed in AdobePhotoshop.

3. 42 Neutral Red Staining

Cells of $1x10^6$ density were collected and washed 2x in 20 ml phosphate buffer. 1ml of neutral red solution (5mg/ml in water or phospahate buffer) was added with gentle mixing on ice for 30sec, enabling the diffusion of vital dye into cells. Cells were again washed two times in 20 ml phosphate buffer and plated on PA plates with density of $5x10^5$ cell/cm^2. Upon development, in acidic environment vital dye turns red, labelling the tips of slugs.

3.43 psv-A (EB4) antibody staining

In appropriate developmental phase cells growing on white nitrocellulose filters were fixed with 3.7% formaldehyde in Z buffer for 20 min, permeabilized with 0.3% NP40 in Z buffer for 10 min and washed carefully 2x2 min with Z buffer. The first antibody (rabbit serum against EB4 potein dissolved in Z buffer, 10ul/ml) was incubated 2h on RT or overnight at 4^0C, washed away and the second antibody (G&R in Z buffer, 1ul/2ml) was added for 1-2 hrs. Cells were washed and soaked in BCIP/AP buffer mixure and upon the development of color the reaction was stopped with water. Cells were dried and photographed on microscope.

3.44 Calcofluor staining of spores

Spores were washed in phosphate buffer, stained with calcofluor (2ul/ml) for 10 min and observed on microscope under UV light. Calcofluor is vital dye penetrating into cellulose walls and enabling visualisation of spore morphology.

3.45 Chimeras

Chimeras were made mixing *wt* and *prelet-7* strains, of which one was carrying GFP under constitutive actin-15 promoter expression. Strains were mixed in proportion

1:1, plated on PA and nitrocellulose philtres and observed on fluorescent and light microscope.

3.46 Primers and constructs used

Sequencing primers

M13 Seq CGCCAGGGTTTTCCCAGTCACGAC
M13 rev GAGCGGATAACAATTTCACACAGG
T7 Primer TGTAATACGACTCACTATAGGG
SP6-Primer ATTTAGGTGACACTATAGAATAC

BSr primers

BSrG1 5´ CGCTACTTCTACTAATTCTAGA
BSr G1 3' TCTAGAATTAGTAGAAGTAGCG

Other primers

prelet-7 forw TGGGATGAGGTAGTAGGTTGTATAG
prelet-7 rev TAGGAAAGACAGTAGATTGTATAGTT
prelet-7HHR forw GGGAGACCTACTACCTCACTG

3'UTR lin-41 forw CACAATAGCACCTCTTTTCCT
3'UTR lin-41 rev ACACAGAAAAACTCTCAGAAAAGT

prelin-4 forw ATGCTTCCGGCCTGTTCCC
prelin-4 rev ATCTGCTCAAACCGTCCTGGTA

ddi-mir-1177 forw ATCACCAAATAAACTAAACCAGTTAG
ddi-mir-1177 rev GTCATCAAATAATTAATAAATCAGAAAG

gfp forw ATGAGTAAAGGAGAAGAACTTTTC
gfp rev CTTTGTATAGTTCATCCATGCC

actin forw GTGTAAAGCCGGTTTTGCTGG
actin rev GATTGGTACAGTGTGGGAG

Plasmids

Commercial plasmids

pGEM 3Z   Promega, Mannheim
pGEM T-easy   Promega, Mannheim
pDneo2   (Witke et al. 1987)
pDneo2a   (modification by Manu Dubin, 2006)
pdD-GFP

pTX-GFP   (Egelhoff et al. 2000)
pTX-BsR
pveII
MB38   (Blaauw et al. 2000)
MB35

3'UTR lin-41 sequence was cloned into pDneo2aGFP vector using PstI and BamHI sites or SalI and XhoI sites, respectively.

Prelet-7, *prelin-4* and *ddi-mi1177* were cloned into pveII vector using BamHI and SacI sites.

Prelet-7 was cloned into pTX-BsR vector using KpnI and BamHI sites.

Prelet-7 was cloned into MB38 vector using BglII and PaeI sites.
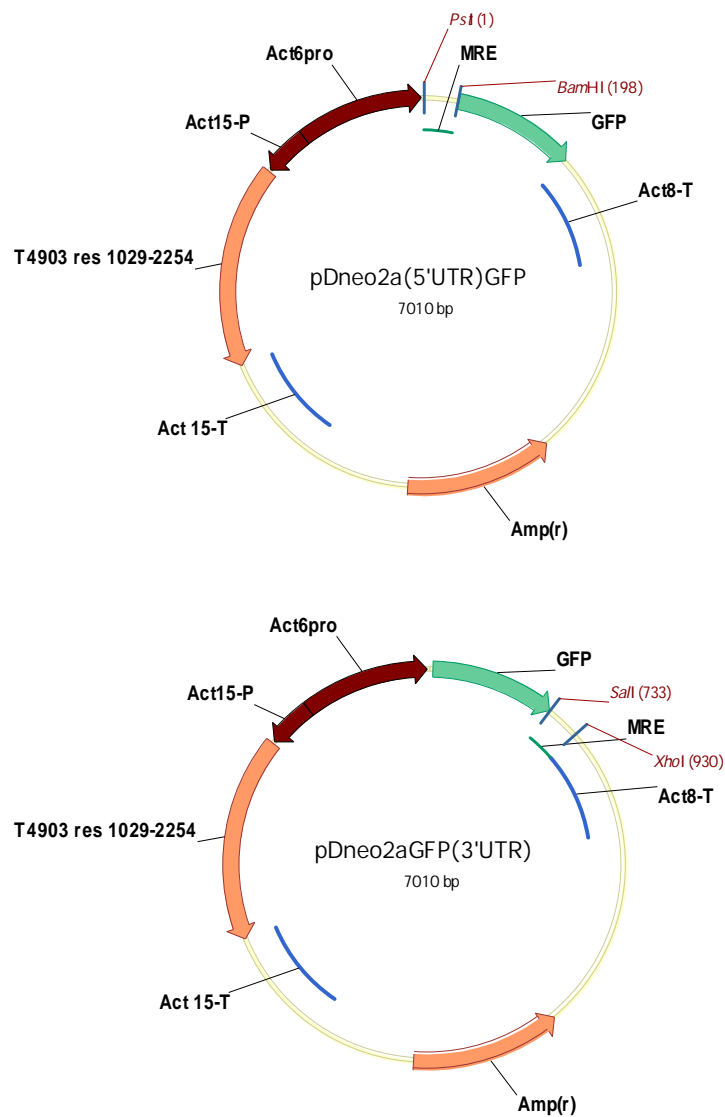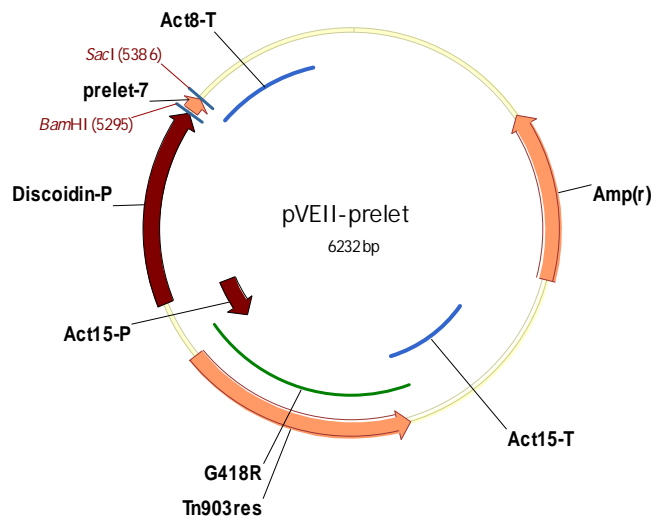
4. RESULTS

4.1 Expression of *let-7* miRNA

Dictyostelium discoideum is an established model system for RNAi and related mechanisms. It possesses a protein machinery with two Dicer-like proteins, five members of Argonaute family proteins, three RNA-directed RNA polymerases (RdRPs) (Martens et al. 2002) and an inhibitor of RNAi, HelicaseF (HelF) (Popova et al. 2006). Though originally discovered as a component of RNAi pathway, Dicers are as well responsible for miRNA production, as a part of genetically separated pathway such as in *D. melanogaster* or overlapping with RNAi pathway such as in humans (Kolb et al. 2005). Taking these facts into consideration, the task was to investigate, whether processing of precursor miRNAs and expression of mature miRNAs would be functional in *Dictyostelium*. This would demonstrate that evolutionary distant protein machinery can work on a human precursor and thus support the assumption that the pathway is highly conserved and important. In order to investigate this, precursor miRNA prelet-7 was used. In the case of pre-miRNA signature recognition by one or both Dicer proteins of the social amoeba, detection of mature miRNA *let-7* would be expected. The pre-miRNA signature refers to the hairpin structure with stem-loop and bulges, a typically conserved feature in plants and animals (MacRae et al. 2007).

Prelet-7 is 70- nts in size which is substantially shorter than the usual RNAi constructs that are employed in *Dictyostelium*. The approximate size of an active RNAi construct in social amoeba is more than 700 nts in length (Martens et al. 2002).

Prelet-7a, cloned into pTXBsR vector, was transformed into *Dictyostellium discoideum*. This strain is further referred to as *prel-7* strain. RNA was isolated and probed on Northern blots. Prelet-7 miRNA, expressed as 70 nts long precursor, was only detected as a ~21- to 22- nts sized small RNA. The precursor itself was not detected, which is similar to the situation in plants (Yang et al. 2007). As shown in figure 1.A. human prelet-7a folds into typical hairpin structure with stem loop and bulges (predicted by mfold program). Figure 1.B. shows the detection of let7-a miRNA on Northern blot. Figure 1.D. confirms prel-7 strain by PCR detection of prelet-7 on genomic DNA.
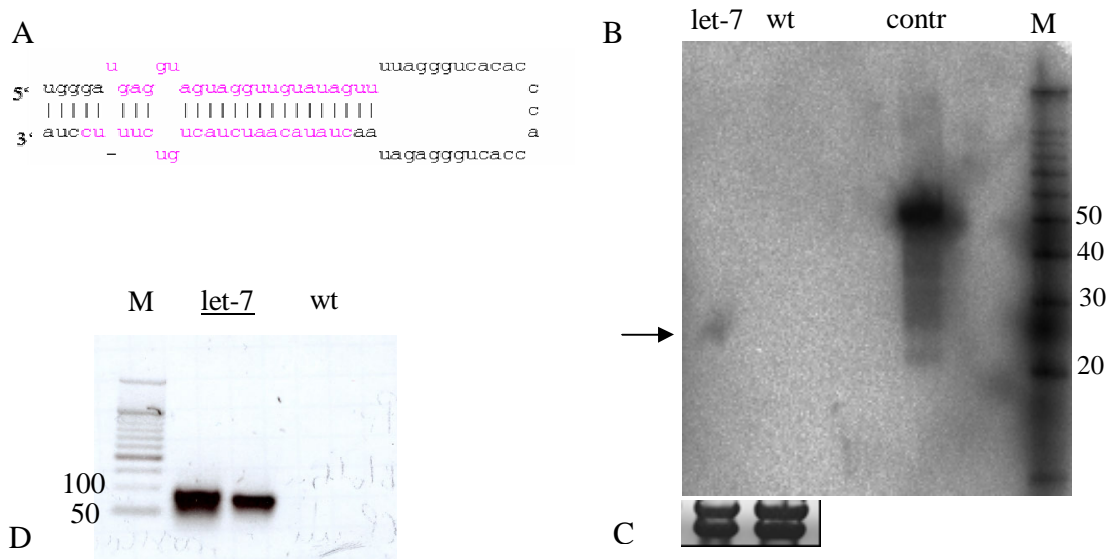
Figure 1. A. Sequence of *prelet-7a* miRNA (http://www.mirbase.org). The mature miRNA::miRNA* duplex is shown in pink color and the active miRNA strand is generated from the 5' arm of the precursor. B. Northern blot of small RNAs showing expression of *let-7* miRNA as ~ 21- to 22- nts long RNA (indicated by arrow). Wt RNA serves as negative control, M stands for Decade RNA marker (Ambion) and control (contr) is a 50 nts long DNA oligo on prelet-7 sequence. C. RNA loading control prepared from prelet-7 and wt strain. D. Confirmation of *prel-7* strain by PCR on genomic DNA.

4.2 Reporter system for miRNA characterisation

Since *Dictyostelium discoideum* was able to process the heterologues prelet-7 miRNA, further interactions of *let-7* and its natural micro RNA response elements (MRE) were investigated. For this purpose, the interaction of *let-7* with two MREs of the lin-41 UTR were used (Vella et al. 2004) (figure 3.A.). *Let-7* miRNA downregulates lin-41 (abnormal cell LINeage) gene expression during the larval development of *C. elegans* and this leads to premature death of larvas (Reihart et al. 2000).

A GFP reporter system was established, carrying two MREs of lin-41 in the 5'UTR and 3'UTR of the reporter gene, respectively (figure 3.B). The reporter system was cloned into the pDneo2a vector. The expression of constructs carrying MREs (referred to as 'bait') were checked on Western blots, showing normal expression of the GFP for both construct versions (figure 3.C.).

A


```
             GUU     A
lin-41  5'UUAUACAACC  CUAC CUCA 3'
let-7a  3'UGAUAUGUUGG  GAUG GAGU 5'
                       AU
```
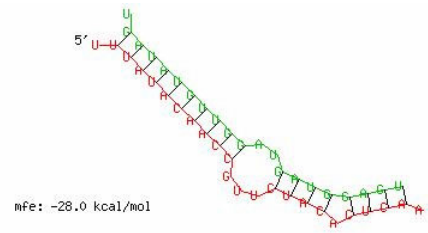
```
             AUU
lin-41  5'UUAUACAACC  CUGCCUCA 3'
let-7a  3'UGAUAUGUUGG  GAUGGAGU 5'
                       AU
```
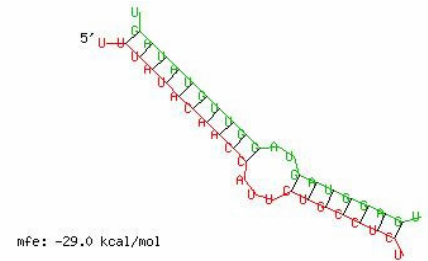
B

5'*ctgcag*gtccacaatagcacctcttttcctcaaattgcaccaactcaagtatacctt**ttatacaaccgttctacactca**ac
gcgatgtaaatatcgcaatcccttt**ttatacaaccattctgcctc**tgaaccattgaaaccttctcccgtactcccaccaataga
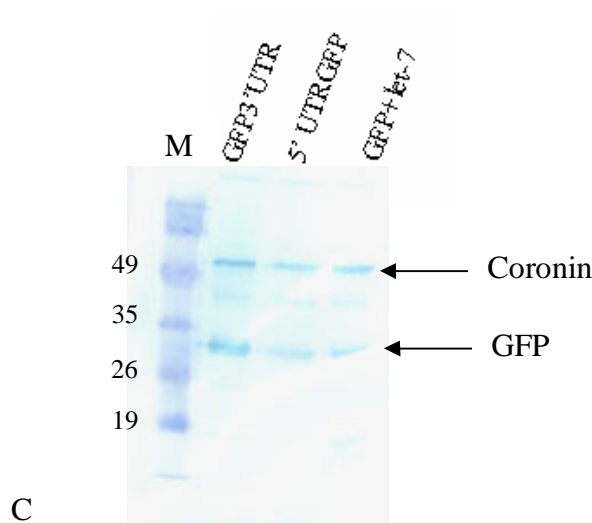ttattgcacttttctgagagtttttctgtgt*aaatg*ggatccatgagt 3'
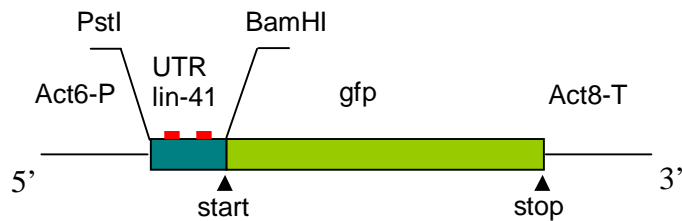
5'gtcgac*ataa*cacaatagcacctcttttcctcaaattgcaccaactcaagtatacctt**ttatacaaccgttctacactca**a
cgcgatgtaaatatcgcaatcccttt**ttatacaaccattctgcctc**tgaaccattgaaaccttctcccgtactcccaccaatag
attattgcacttttctgagagtttttctgtgt*ctcgag* 3'
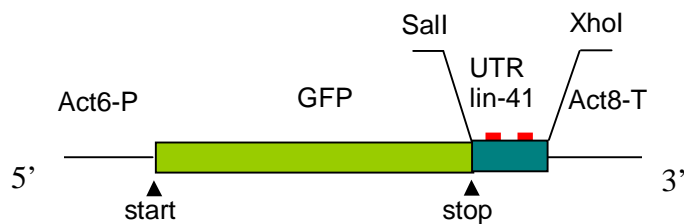
Figure 3. A. Predicted base pairing between *let-7* miRNA and 3'UTR of lin-41 RNA (miRanda software). B. Reporter constructs consisting of the gfp gene tagged with bait sequence (MRE of lin-41 gene) inserted into the 5'UTR (before the start codon) and into the 3'UTR of reporter (between stop codon and polyA sequence), respectively. Sequence of lin-41 UTR is given above and its target sites (MREs) are shown in pink colour. C. Western blot showing expression of GFP for both constructs (GFP3'UTR, 5'UTRGFP and GFP coexpressed with *prelet-7*); marker size in KD; Coronin serves as an expression controle.

4.3 Translational control of gene expression in *Dictyostelium discoideum*

After confirming the expression of the reporter system, the *prelet-7* strain was transformed with the bait constructs and expression of gfp was monitored on the protein and RNA level. As a control, the *prelet-7* strain expressing GFP without *let-7* target sites was used. Cells were first checked by microscopy (figure 4.A.), showing that co-expression of bait GFP and *prelet-7* resulted in the absence of green cells. Western blots confirmed the absence of GFP in these strains that are from now on referred to as silenced clones (figure 4.B.).

Since the RNAi mechanism in *Dictyostelium* leads to degradation of the target mRNA and production of siRNA derived from the target sequences (Popova et al. 2006), and the introduction of long hairpin constructs directed against endogenous genes or transgenes result in the absence of both target protein and mRNA (Martens et al.

2002, Popova et al 2006), further characterisation of the reporter system on RNA level followed.

In order to characterize the type of silencing caused by miRNA *let-7*, the levels of gfp mRNA were measured in silenced clones and compared to controls by Northern blots and RT-PCR. In the silenced clones, the gfp mRNA was present and not degraded.

Target mRNA was expressed on the same level in silenced clones as in controls, although the protein could not be detected (figure 5.A., figure 6).

To summarize: upon expression of *prelet-7,* the gfp mRNA levels were unaffected, whereas of the protein was absent.

In other organisms this phenomenon is referred to as translational inhibition and is associated with miRNAs. In this study it was shown for the first time that translational inhibition exists as a type of gene regulation in *Dictyostelium*.
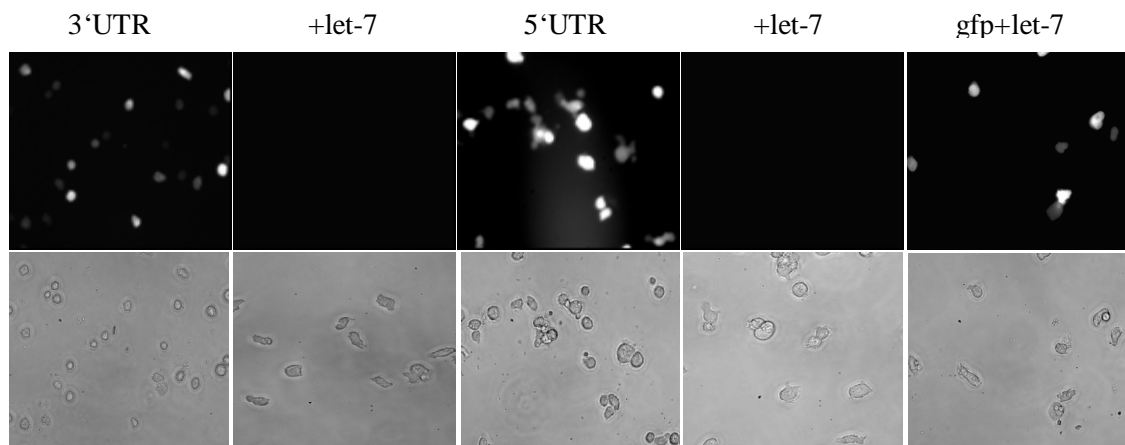
Figure 4. Cells showing silencing of GFP constructs upon *let-7* expression (silent clones).
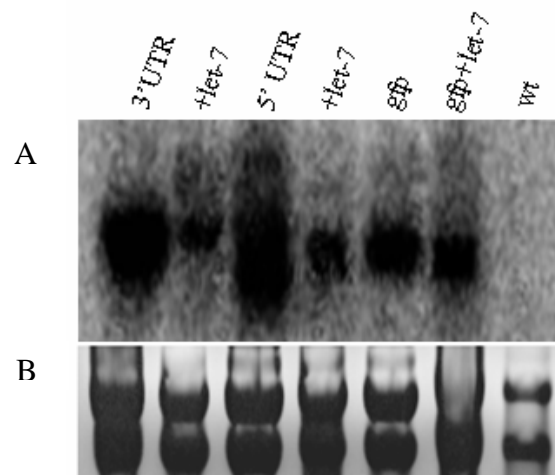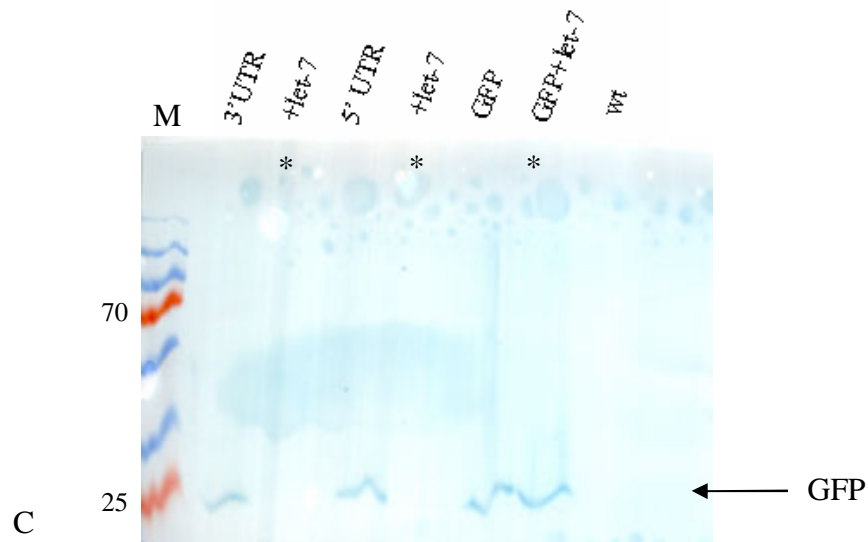
Figure 5. Detection of GFP. This panel shows presence of gfp mRNA and absence of GFP on the protein level. A. Northern blots for gfp3'UTR (3'UTR), 5'UTRgfp (5'UTR) mRNA and their silenced version (upon prelet-7 expression); controls are RNA from cell lines of gfp co-expressed with prelet-7, gfp and wt. B. RNA loading controls. C. Western blot showing no detectable protein in the silenced cells (+let-7), (* indicates the presence of prelet-7 and arrow indicates GFP).
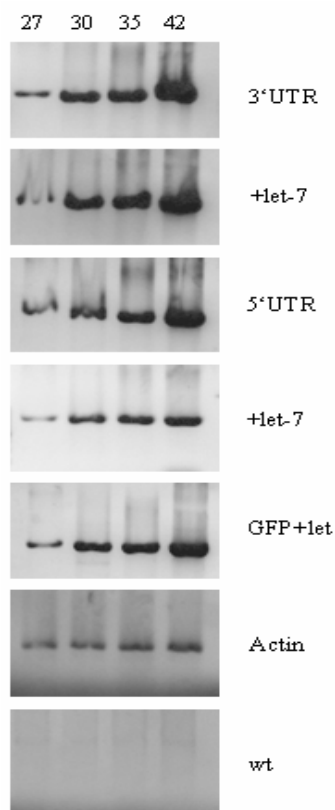
Figure 6. A. Semi-quantitative RT-PCR. Gfp mRNA levels were checked in silenced cells and their controls. The number on top indicates the number of PCR cycles. Actin8 hkg served as control for the reaction. gfp3'UTR (3'UTR), 5'UTRgfp (5'UTR) cells and their silenced version (+ *let-7*) (that show no detectable protein on Western blots), gfp co-expressed with *prelet-7* (GFP+let-7) and wt.
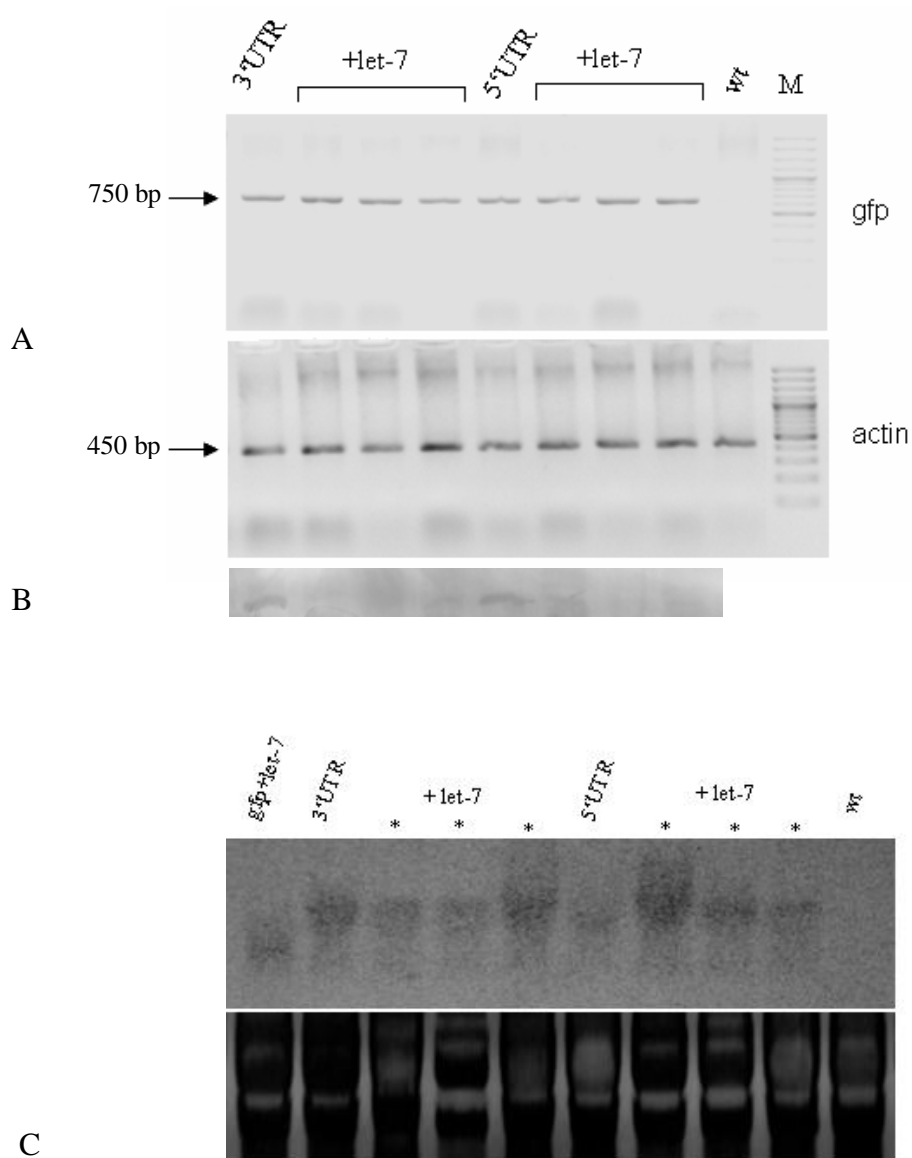

Figure 7. Three independently derived silenced cell lines checked for gfp expression on mRNA and protein level. A. RT-PCR on gfp and actin8 showed the presence of mRNA in three independently aroused silenced clones transformed with each bait construct. B. Western blot showing silencing of the GFP reporter by let-7 expression. C. Northern blot showing the presence of target mRNA in the silenced clones (indicated with *).

The mechanism of translational control for transgenes confirms that this type of regulation is present in *Dictyostelium*. A specific mechanism of action has yet not been assigned to *Dictyostelium* putative miRNAs in and it is not known wheather they are able to transitionally inhibit their targets or/and lead to mRNA degradation. From the obtained data, it is most likely that endogenous miRNA in *Dictyostelium* control the expression of target mRNA by translational arrest or it represents at least one of their mechanisms of action.

## 4.4 Co-localisation of gfp mRNA and *let-7* miRNA

The translational silencing of transgenes in the social amoeba raised the question of cellular localisation of the transcribed but untranslated mRNA. In order to investigate where gfp mRNA was located in the cells and whether it was associated with small RNA, sucrose gradients of cells lysates were performed. The RNA from each fraction was isolated and the absorbance profile was measured. Isolated RNA was used for RT-PCR and Northern blots in order to investigate the distribution of gfp mRNA and *let-7*.

The outcome was the co-localisation of gfp mRNA and *let-7* in monosome peak fraction and polysome fractions for both versions of silenced cell lines.

These 'co-localites' of gfp mRNA and *let-7*, detected to overlap in gradient fractions, suggested same cellular compartments of storage for both RNAs.

Taken together, these data showed that translational inhibition as a active mechanism of gene control had as a consequence formation of mRNA and miRNA 'co-localites' that on sucrose gradient segregated as/with polysomes.
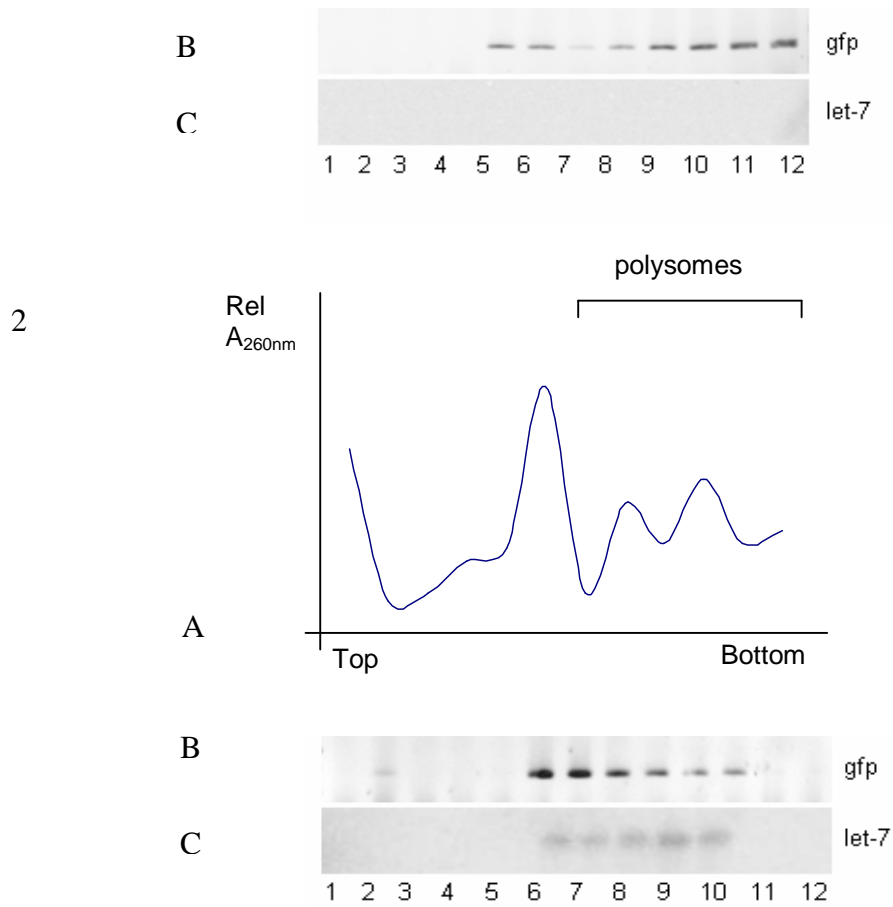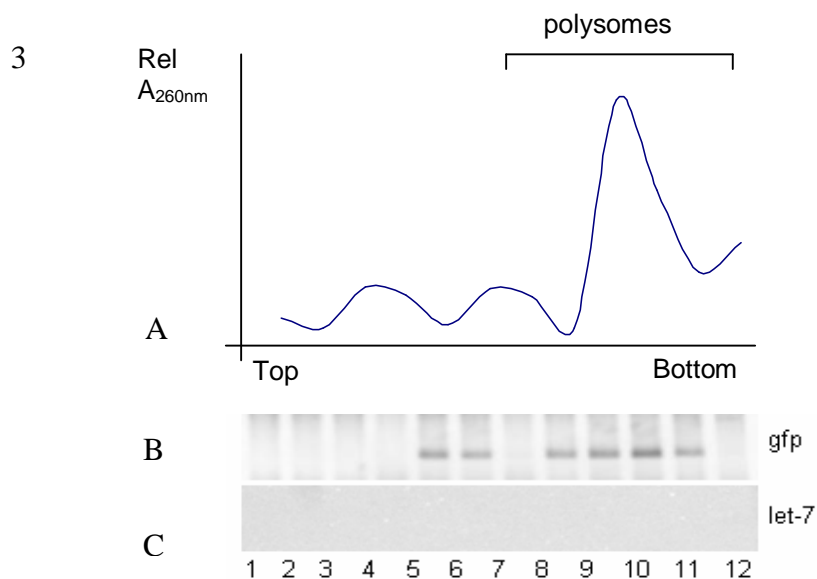
1
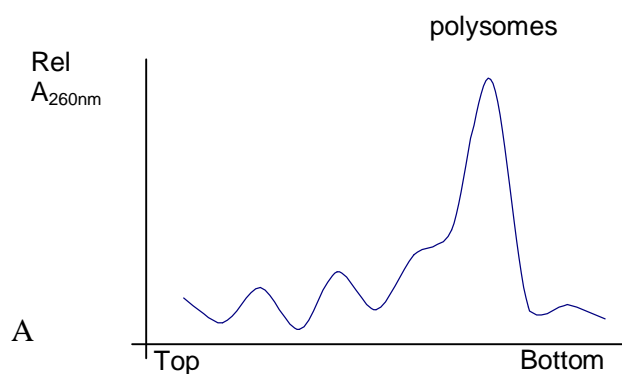
Figure 8. mRNA and miRNA colocalisation in the silenced cell line of 3'UTR strain (2) and control (1). Sucrose gradients of 10-40% were divided into fractions numbered from the top to the bottom. A. Representative profile of absorbance at 260 nm for RNA distribution. B. RT-PCR for gfp showing the distribution of mRNA in monosome peak and smaller polysomes. C. Northern blots for small RNA, showing let-7 colocalising with gfp mRNA in polysome fractions and monosome peak fraction.
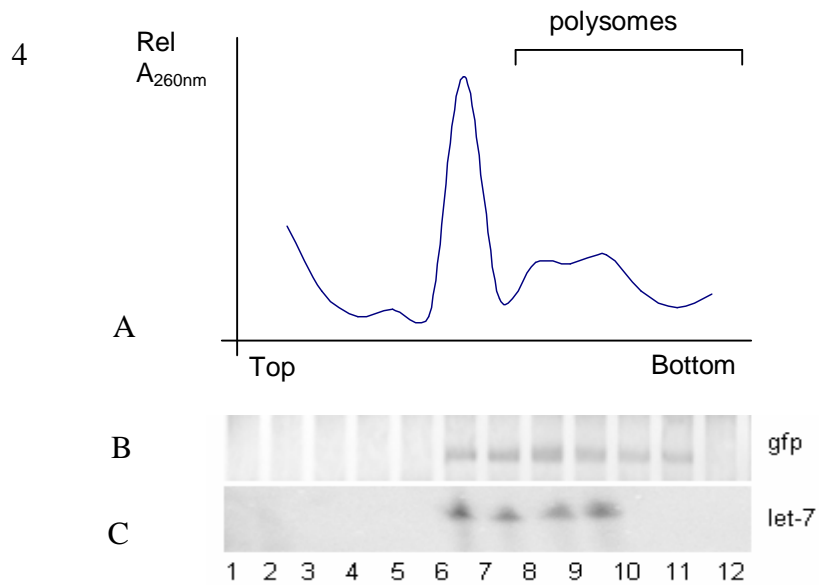
Figure 9. mRNA and miRNA colocalisation in the silenced cell line of 5'UTR strain (4) and control (3). Sucrose gradients of 10-40% were divided into fractions numbered from the top to the bottom. A. Representative profile of absorbance on 260 nm for RNA distribution. B. RT-PCR for gfp showing the distribution in the monosome peak and polysomes. C. Northern blots for small *let-7* RNA analyses, showing colocalisation with gfp mRNA in monosome and polysome fractions.

4.5 Functional role of miRNA *let-7* overexpression and processing in *Dictyostelium discoideum*

4.6 Phenotype of *prelet-7* strain

The strain transformed with *prelet-7* miRNA (*prelet-7* strain) was able to express and process *let-7* miRNA. It could efficiently silence GFP reporter constructs containing a target sequence on the protein level. In order to further characterize the *prelet-7* strain and to investigate possible effects of miRNA overexpression, the observation of strains on bacterial lawns (KA) and on phosphate agar (PA) plates was performed. Compared to wt, prel-7 showed developmental delay of several hours on PA plates. This was most severe at the phase of slug formation at ~15 hrs of development, when prel-7 cells were predominantly found at the periphery during aggregate formation. As the time progressed prel-7 continued to lag behind in development compared to wt strain, ending with the specific phenotype difference.

The *prelet-7* phenotype showed: delayed development, smaller sized spore heads, smaller fruiting bodies, reduced number of fruiting bodies and decreased sporulation efficiency (figure 10, figure 11.B.). The described phenotype in part resembles the HelF KO phenotype, a protein postulated to be involved as a negative regulator of RNAi pathway (Popova et al. 2006).

The spores production test (SPT) showed the deficiency in spore formation for prel-7 strain of around 40% compared to wt (figure 11.A.). In order to look at this more carefully, staining with an antibody against the prespore marker psvA (prespore vesicle A or EB-4 protein) was done. The psvA gene is developmentally regulated and labels the prespore population of cells within slug. Due to this, it is possible to distinguish prespore and prestalk cell population on slug stage during the development and stain spore heads in adult fruiting bodies (figure 11.B.).

Prelet-7 slugs showed reduced staining with the psvA/EB4 antibody in the prespore zone, while the staining of wt slugs gave the expected 2:8 distribution of unstained prestalk and stained prespore cells, respectively (figure 12.A.). The decreased level of staining in the prespore zone for prel-7 slugs corresponded to decreased spores production test. The SPT was calculated as an average value of starting number of developing cells and number of harvested spores from three independent measurements.
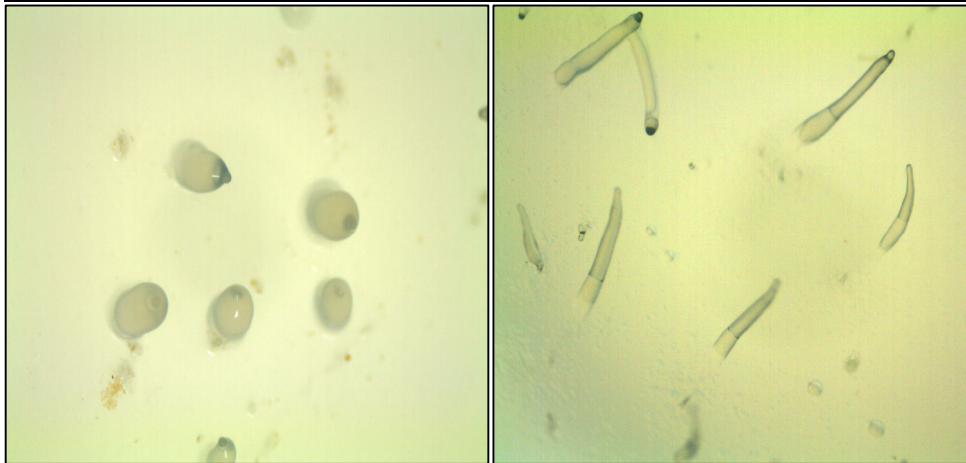
prelet-7   *wt*
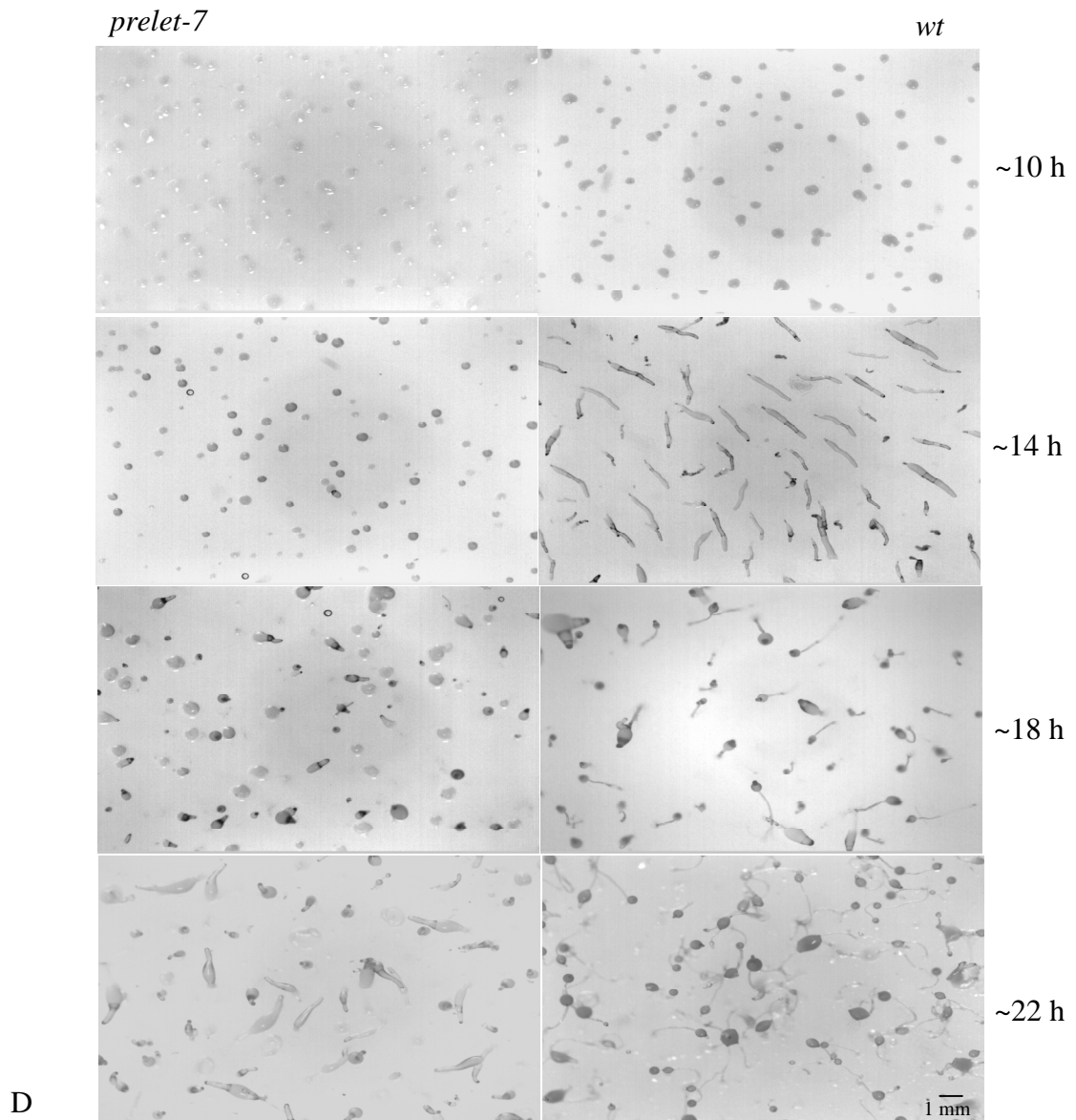
~10 h

~14 h

~18 h

~22 h

D

1 mm
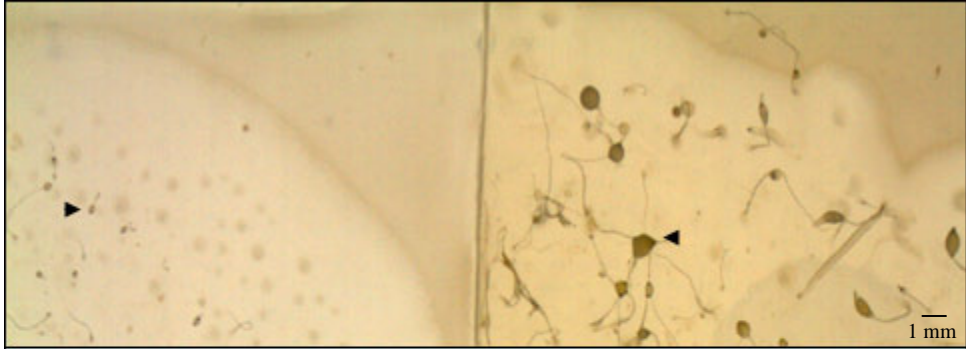
Figure 10. Phenotype difference between *prelet-7* (on left) and *wt* strain (on right). A. Plaques at 48 hrs of development on KA plates show decreased number of fruiting bodies and smaller size sporeheads (plaques were cut out, put next to each other and taken in the same frame of 4x magnification). B. Profile photo of prel-7 and wt strain showing difference in fruiting body size (plaques were cut out, placed next to each other and taken in the same frame of 4x magnification). C. Development of *Dictyostelium discoideum* at ~15 hrs showing delay in prel-7 compared to wt strain that had progressed to slugs. D. Development time course.
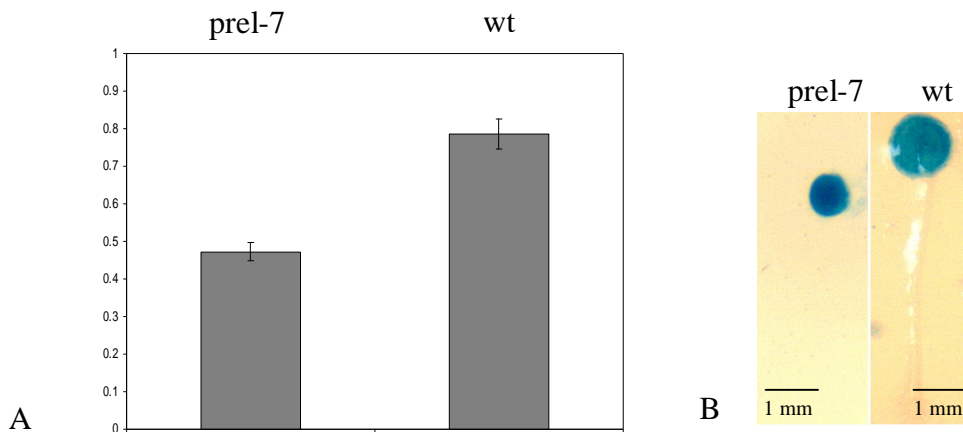
Figure 11. *Prelet-7* strain (on left) compared to *wt* (on right). A. Spores production test (SPT) shows decrease of ~40% in *prelet-7* strain compared to wt. SPT is given as relative value of plated number of cells and counted spores. B. psvA/EB4 staining of fruiting bodies with a polyclonal antibody showing difference in size of spore heads for *prelet-7* and *wt*.

4.7 Characterization of *prelet-7* strain

The delay in development and decrease in sporulation efficiency led further to investigation of prel-7 strain and questions of cells behaviour and destiny during development. In order to look at this more closely, neutral red staining of prel-7 and wt slugs was performed (figure 12.B.). Neutral red is vital dye that in acidic environment turns into red colour e.g. the anterior part of the slug.

Prel-7 slugs showed different staining pattern and more extensively stained zone at the anterior part compared to wt slugs. This observation corresponded to the staining pattern of slugs with psvA/EB-4 antibody (figure 17.A.) (as a mirror affect) and to STE values.

Since the increased neural red staining in the anterior part of prel-7 slugs suggested increased vacuole activity and lisosome digestion of cells, the possible effect on the foot morphology formation was examined. The foot morphology of prel-7 fruiting body showed no difference compared to wt (figure 13.A.).

Taken together the explanation for these data could be that in the slug phase and during its presorting and migration significant part of cell population was subjected to increased loss by vacuolated, being left behind or not being able to segregate.

Different patterns of staining for neutral red and psvA/EB4 antibody, decreased sporulation and smaller fruiting bodies could be the repercussion of extensive cellular vacuolisation during the development and/or different slug pattern.
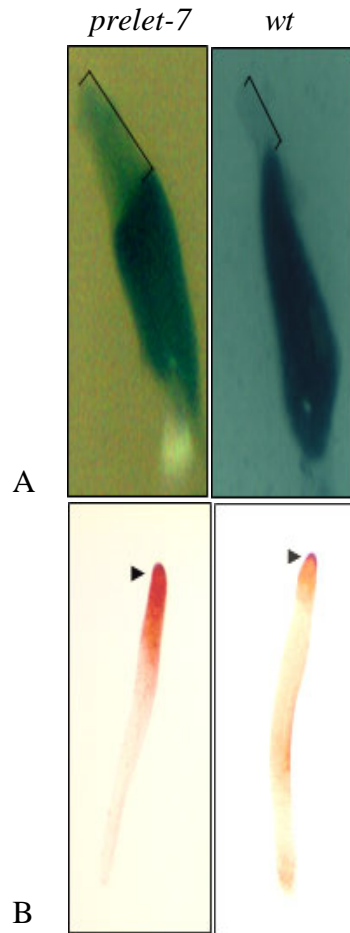
Figure 12. Analyses of *prelet-7* strain (on left) compared to *wt* (on right). A. Staining of slugs with an antibody against the prespore marker psvA/EB4 showing disproportion and decreased staining pattern in prel-7 slug compared to wt slug. B. Neutral red staining showing an extension of the anterior part of *prelet-7*slug strain compared to *wt*.

The different pattern of psvA(EB4) staining led to further investigate spore morphology, since this protein is included in its coat formation. However, *prelet-7* spores show no difference in size or morphology compared to *wt*, leading to conclusion that spore morphology was unaffected.

Figure 13. *Prelet-7* strain (on left) compared to *wt* (on right). A. Basal disc of foot showing no detectable difference between *prelet-7* and *wt*. B. Calcofluor staining of spores showing no detectable difference between *prelet-7* and *wt*.
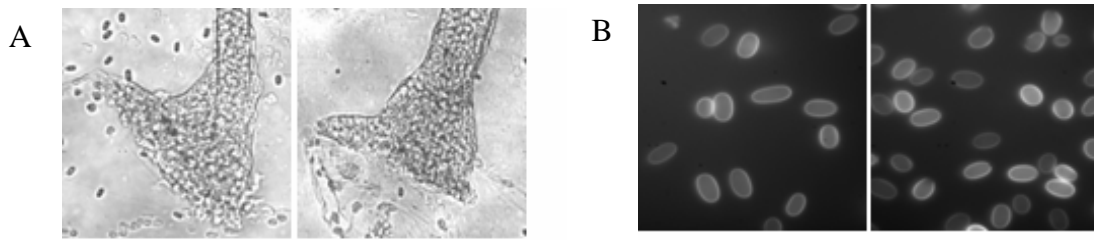
In *prelet-7* slugs the distribution of prespore and prestalk cells does not correspond to the usual 8:2 distribution. Therefore it was interesting to investigate in more detail the behaviour of prel-7 cells during development. chimeras were made from 50% *prelet-7* cells mixed with 50% GFP labelled wild type cells. As a control GFP labelled and unlabelled wild type cells were mixed.

As can be seen in figure 14 an equal distribution of green cells was observed in the control chimeras, suggesting a random distribution of labelled cells throughout the slug. In *prelet-7* chimeras, the green wild type cells tended to group at the posterior part of the slug, while *prelet-7* cells accumulated in the anterior part.

The gradual lagging of *prelet-7* cell within the chimeric slugs can be observed in figure 15. At the mound stage both populations are equally mixed, but with the progression of development, *prelet-7* cells tent to localise to the anterior part, while green wild type cells localised at the posterior part of the slug.

prelet-7 chymeras   control chymeras
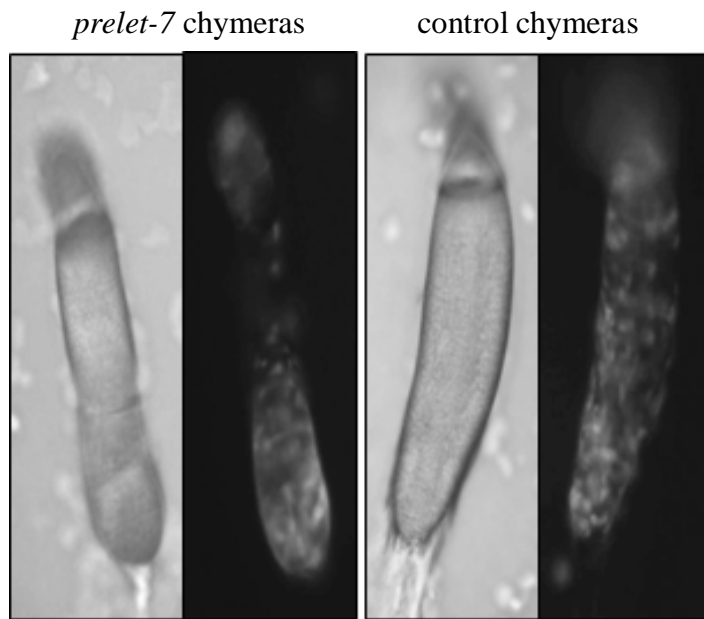
Figure 14. Analysing labelled slug chimeras. Photos were taken on light and fluorescent microscope, respectively. *Prelet-7* chimeras were made by mixing 50% gfp labelled wild type cells with 50% and unlabelled *prelet-7* cells. Green cells accumulated in the posterior part. Control chimeras were made of 50% gfp and wt cells each and showed equal distribution of green cells.
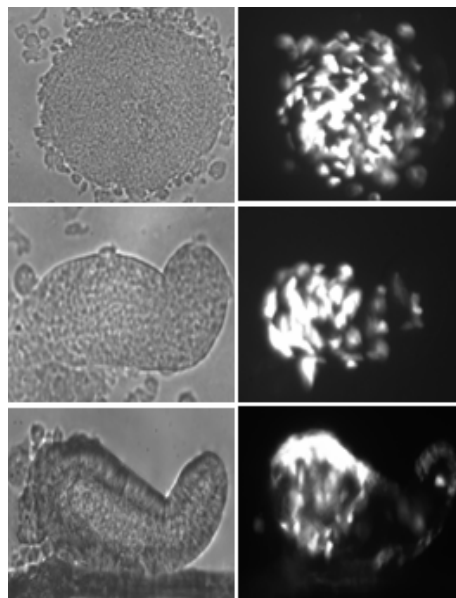
Figure 15. Distribution and migration of *prelet-7* cell within the labelled chimeric slugs shows pattern of prel-7 cells sorting to the posterior part.

The tendency of *prelet-7* cells to accumulate in the anterior part of the chimera supported the observation of *prelet-7* slugs stained with neutral red or the psvA antibody. In both cases the *prelet-7* cells displayed a preference to become stalk cells.

4.8 Why do *prelet-7* cells display a phenotype?

The observation that the mere introduction of a heterologous miRNA precursor resulted in a developmental phenotype can be explained by few hypothesis.
It is possible that the let-7 miRNA upon processing finds fortuitous complementary sequences in endogenously expressed RNAs and down-regulated them. This would be a typical off-target effect observed in *Dictyostelim* siRNA response (Popova et al. 2006). Since *Dictyostelium* can process pre-miRNA other possibility is the overload of the endogenous machinery by high expression of prelet-7.
Off-target effects could be reduced or titrated out by the introduction of its target mRNA to which it would bind. Therefore, the developmental phenotype of strains coexpressing prelet-7 strains with 'bait' mRNA was investigated.
The developmental time course of all strains: prel-7, wt, and both versions of silenced strains was observed.
As seen in figure 16, the developmental phenotype was maintained in the silenced strains, but these strains showed more asynchrone development. Comparing the morphology of plaques of four lines silenced on KA plates, similar although not identical phenotype was observed (figure 16.A.). Plaques of silenced strains showed decreased and smaller sozed sporeheads as seen in prel-7 strains, bur showed as well prolonged slug presense found even in 48 hrs old plaques (figure 16.B.).
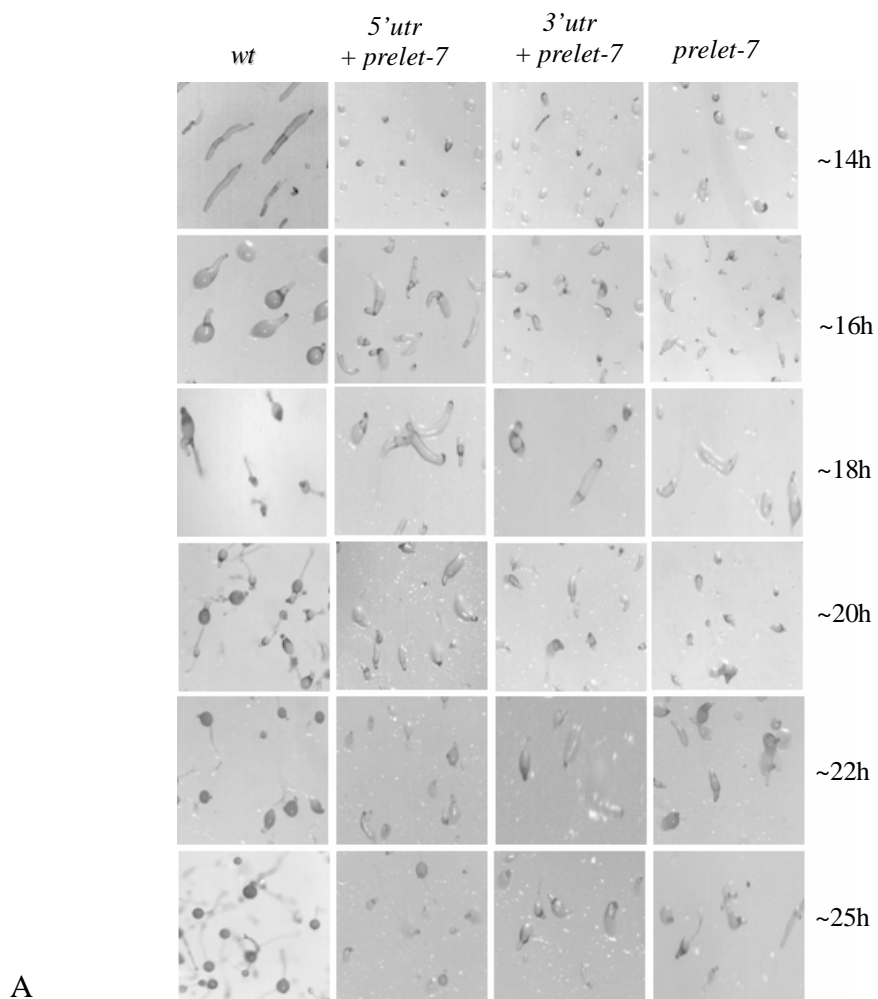
A
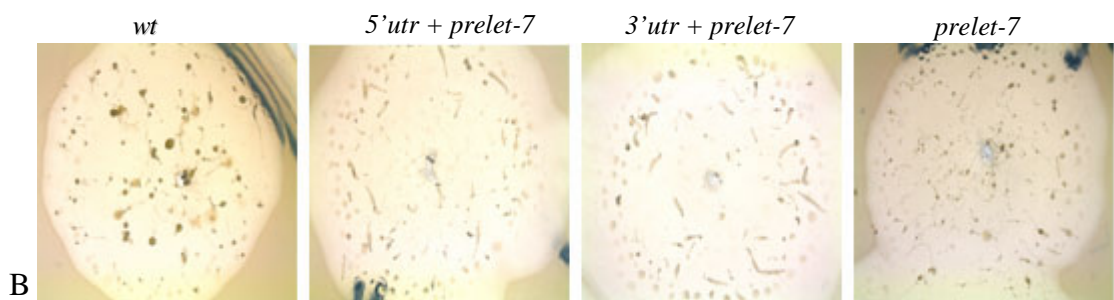
B

Figure 17. A. Developmental time course on PA plates of wt, both versions of silenced strains and *prelet-7*. B. 48 hrs old plaques on KA plates.

4.9 Inducible tet-off system for *prelet-7* expression

To more directly investigate the phenotypic effects of *prelet-7*, expression of the miRNA was controlled by the externally regulated tet-off system (Bujard et al. 1994; Blaauw et al. 2000). Cells were grown for 48 hrs in a shaking culture in the absence or presence of the tetracycline analog doxicycline and then plated for development on soaked PA plates. Comparison of treated and untreated line with the *prel-7* and wild type strain respectively showed that the developmental phenotype was almost completely reversed to wild type upon turning off the *prelet-7* expression. To assure that the expression of *prelet-7* stays turned off during the development PA plated were soaked with doxycycline.

From the obtained results (figure 18), it is suggested that there were no consequential changes present on the genome level in *prel-7* strain during the miRNA expression on the vegetative cell stage. Upon inhibition of miRNA expression the wt phenotype in development was recovered. This implies that the inducible expression of miRNA is responsible for presence of phenotype by interfering with the gene expression on the posttranscriptional level.
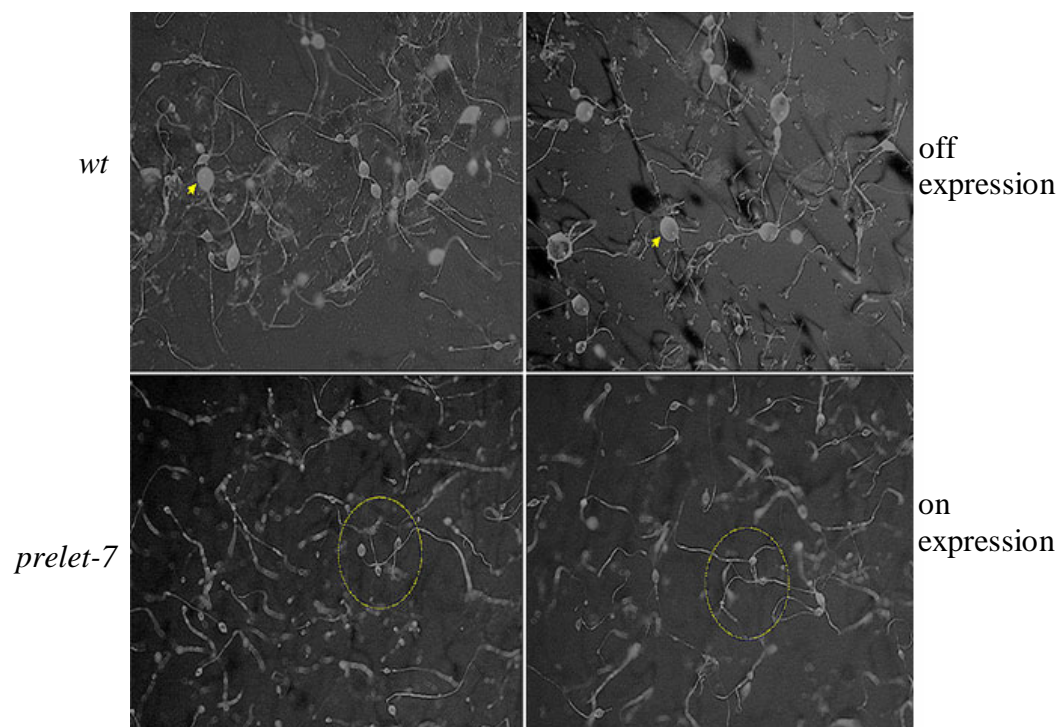

Figure 18. Inducible tet-off system controls the expression of prelet-7. Upon treatment with doxycycline (off expression) the inducible strain reverses back to wt phenotype.

4.10 Search for possible *let-7* targets in *Dictyostelium*

Transfected siRNA and miRNA regulate numerous transcirpts that have only limited complementarity to the active strand of the RNA duplex. This process reflects natural target regulation by miRNAs, but is an unintended consequence ('off-target') of siRNA-mediated silencing (Linsley et al. 2006). A sequence complementarity to the seed region of the si/miRNA is a primary determinant of regulation of unintended transcripts and their corresponding proteins by all experimentally examined si/miRNAs, as well as shRNAs (Linsley et al. 2006). Short stretches of sequence complementarity to the si/miRNA or shRNA seed sequence is the key to the silencing of unintended transcripts.

The seed sequence of *let-7* miRNA complementarily binds ATACCTC and/or CTACCTC sequences in the 3'UTR of target genes (Brennecke et al. 2005).

Applying the blast search for these sequences in *Dictyostelium* genome gave only one possible target gene DDB_G0286091, where the target sequence is coded in the ORF. There is no assigned fuction to this gene. One well-known target of *let-7* is RAS oncogene, which has a homologue in *Dictyostelium*. Bioinformatical approach, nevertheless, predicted no interaction between *let-7* and ddi-ras.

The possible target sequences for miRNA *ddi-mir-1176*: AAAATTGG, *ddi-mir-1177*: CTAACTG and heterologuos *lin-4*: CTCATTT gave no results.

Since the bioinformatical approach can give false positive, all the possible targets are to be biologically confirmed. On the other hand, it is possible that some target genes can be found only with the biological approach (i.e microarrays), because the applied rules for their search are incomplete.

4.11 Search for *Dictyostelium* protein analogs of P bodies

Until now there is not direct evidence of existence of P bodies in *Dictyostelium*. In order to address this question in more detail, here is the summerized search for analogues of P body components in *Dictyostelium*.

Table 1. shows proteins found in P bodies in various organisms (Eulalio et al. 2007) and their homologues in *Dictyostelium*. Most of proteins are present in *Dictyostelium*, including Argonaute proteins, but there is no homologue of GW182 of *D. melanogaster* (AIN-1 in *C. elegans*).

Name	Function	Organisms	Effects of depletion or overexpression on P-body integrity	Dictyostelium Gene homologe DDB0	% of identity
XRN1, Sc Kem1	5'→3' exonuclease	Human, mouse, Sc	Depletion: increase in P-body size and number (Sc)	237529	46
GW182, Ce AIN-1	In the miRNA pathway	Human, Dm, Ce	Depletion: P-body loss (human)	x	
DCP2, Ce DCAP-2	Decapping enzyme	Human, Dm, Ce, Sc	Depletion: increase in P-body size and number (human); overexpression: P-body loss (human)	251811	36
DCP1, Ce DCAP-1	Decapping-enzyme subunit	Human, Dm, Ce, Sc	Depletion: increase in P-body size and number (Sc)	187387	25
Hedls, Ge-1	Decapping co-activator	Human, Dm	Depletion: P-body loss (human); overexpression: increase in P-body size and number (human)	219320	24
Dm CG5208, Pat1	Decapping co-activator	Dm, Sc	Depletion: slight reduction in P-body size (Sc); overexpression: increase in P-body size and number (Sc)	292714	22
EDC3 (LSm16)	Decapping co-activator	Human, Dm, Sc	Overexpression: P-body loss (human)	x	
LSm1–7	Decapping co-activator complex	Human, Sc	Depletion: P-body loss (human), but increase in P-body number	269922	32

			(Sc)		
RAP55 (LSm14)	Predicted decapping co-activator	Human	Depletion: P-body loss	281279	47
RCK/p54, Dm Me31B, Ce CGH-1, Sc Dhh1	Decapping co-activator, translation regulator	Human, Dm, Ce,	ScDepletion: P-body loss (human and Sc); overexpression: P-body loss, but increase in P-body size and number (human), P-body loss (Sc)	234196 (ddx6)	69
eIF4E	Translation-initiation factor	Human, rat	Not determined	191262	66
eIF4E-T	Translational repression	Human	Depletion: P-body loss	x	
SMG7	NMD	Human	Overexpression: increase in P-body size	x	
SMG5	NMD	Human (when co-expressed with SMG7)	Not determined	x	
UPF1 Sc Nam7	NMD	Human (when co-expressed with SMG7), Sc (on depletion of DCP2, DCP1, XRN1, UPF2 or UPF3)	Depletion: no effect (Sc)	288923	51
UPF2	NMD	Sc (on depletion of DCP2, DCP1 or XRN1)	Depletion: increase in P-body size	281623	24
UPF3	NMD	Sc (on depletion of DCP2 DCP1 or XRN1)	Depletion: increase in P-body size	292240	41
Argonaute proteins	In the siRNA and miRNA	Human, Dm, Ce	Not determined	AgnA-E	

	pathways Ago2		miRISC	220136 (AgoA)	24
CCR4–CAF1–NOT complex	Deadenylation	Human, Sc	Depletion: loss of P-bodies (human and Sc)	284461 x x	31
CPEB	Translation regulator	Human	Not determined	270634	24
FAST	Fas-activated serine/threonine phosphoprotein	Human	Not determined	x	
TTP	ARE-mediated mRNA decay	Human	Not determined		
Staufen	Double-stranded RNA-binding protein, mRNA localization	Dm	Not determined	x	
Rbp1	RNA-binding protein, mediates decay of mitochondrial porin mRNA	Sc (under stress conditions)	Not determined	291966	39
Rpb4	Subunit of RNA polymerase II	Sc	Depletion: increase in P-body size and number	282739	43
Sbp1	Suppressor of decapping defects	Sc	Depletion: reduction in P-body formation under stress conditions; overexpression: increase in P-body size and number	x	
Gemin5	Component of the SMN protein complex involved in assembly of U snRNPs	Human	Not determined	288425	27
Dcs2	Stress-induced regulatory subunit of the scavenger decapping enzyme Dcs1	Sc	Not determined	x	
APOBEC3G,	Deoxycytidine	Human	Not determined	x	

APOBEC3F	deaminase with antiviral activity				

Table 1. Proteins found in P bodies and their holologues in *Dictyostelium*. Ce, *Caenorhabditis elegans*; CPEB, cytoplasmic polyadenylation element-binding protein; Dm, *Drosophila melanogaster*; EDC3, enhancer of decapping-3; eIF4E, eukaryotic translation-initiation factor-4E; eIF4E-T, eIF4E-transporter; miRNA, microRNA; NMD, nonsense-mediated mRNA decay; Sc, *Saccharomyces cerevisiae*; siRNA, small interfering RNA; SMN, survival of motor neurons; snRNP, small nuclear ribonucleoprotein; TTP, tristetraprolin.

5. DISCUSSION

Dictyostelium discoideum is a proven model system for RNAi and related mechanisms (Martens et al. 2002, Popova et al. 2006) that possesses the required protein machinery consisting of: five Argonaute proteins, two Dicer-like proteins, Helicase F (a negative regulator of RNAi), three RdRPs (RNA-dependant RNA Polymerase). The proteins discovered so far are most likely only part of the machinery involved in the si/miRNA pathways. Their roles and interacting partners as well as *Dictyostelium* homologues of newly discovered proteins from si/miRNA pathway in other organisms are under investigation. Two nucleases, an RNase III enzyme Dicer and RNase H enzyme Argonaute are essential for initiation and effector phases of RNAi in all investigated organisms (Bernstein et al. 2001, Hammond et al 2001) as well as in miRNA pathway and these proteins have their homologues in *Dictyostelium*.

There are a few antisense RNAs (dutA and psvA) in *Dictyostelium* whose expression is regulated during the development on the post-transcriptional level (Kurosava et al 2007, Nellen et al. 1992).

The organism is a host for two abundant retrotransposons, DIRS and Skipper, whose expression is controlled by different components of the RNAi machinery (Hinas et al. 2007; Kuhlmann et al 2005, Dubin et al. PhD thesis in preparation). DIRS and Skipper are source of small noncoding RNAs (endogenous siRNA) that are ~21 nt in size and developmentally upregulated (Hinas et al. 2007).

MiRNAs have been discovered in almost all organisms: starting from animal viruses (Pfeffer et al. 2004), unicellular green alga *Chlamydomonas* (Zhao et al. 2008; Molnar et al. 2009) to very well established system in plants, *D. melanogaster*, *C.elegans* and humans. Althought there are differences between species, there is an amazing conservation of miRNAs and the proteins responsible for their biogenesis. The shown presence of miRNA in *Chlamydomonas* revealed that the target mRNA is cleaved, which reflected the evolutionary relatednes to plants. Evolutionary, *D. discoideum* is positioned between plants and animals; more precisely it diverged after division of yeast but before separation of fungi (Eichinger et al. 2005). The other intriguing feature of the organism is its position between unicellular and multicellular

organisation. Even though a few putative miRNAs had been found by a deep sequencing approach in *Dictyostelium* (Hinas et al. 2007), targets could not yet be defined and the ultimate prove that they acted as miRNAs has not been established. In order to get insight into this question, an artificial miRNA system was created and characterized.

First, the processing of the hairpin precursor miRNA *prelet-7* into ~22 nt long RNA supports the capability of the organism to recognise and process pre-miRNAs. It shows the ability of the protein machinery to accept the heterologous pre-miRNA *prelet-7a* and processes it, suggesting that the miRNA structure is of primary importance for recognition and processing of precursors, rather than the sequence. It also confirms high flexibility of protein machinery of *Dictyostelium*. In other organisms Dicer cleaves pre-miRNA by recognizing the base of the pre-miRNA hairpin and measuring the distance of ~20 bp from the hairpin end. Dicer cuts of si/miRNA from the end of dsRNA by a similar distance-meassuring mechanism (Zhang et al. 2004). The Dicer-like proteins in *Dictyostelium* most probably act upon the same mechanism. It stays unknown which isoform of Dicer is responsible for miRNA maturation and if the siRNA and miRNA pathways are genetically separated in *Dictyostelium*.

Two putative miRNA, *ddi-mir-1176* and *ddi-mir-1177*, whose precursors were predicted by bioinformatical approach, were shown to be processed into small RNAs (Hinas et al. 2007). Maturation of *ddi-mir-1177* was dependant on DicerB protein suggesting that DicerB in *Dictyostelium* acts as a RNse III enzyme Drosha in humans. The same study showed that all discovered small RNA species were developmentally up-regulated and their biogenesis pathways differed.

Maturation of miRNA is a two step process of which the first one takes place in the nucleus and the second one in the cytoplasm and the exact roles of Dicer proteins in *Dictyostelium* are under investigation (Balachandar et al. PhD thesis in experimental phase). It is assumed that beside the characterized miRNAs in *Dictyostelium* there are probably many more (Söderbom et al. personal communication).

The *let-7* overexpression led to a phenotype described as the *prel-7* strain, where development and spore generation were severely affected. The overexpression of several different RNAi constructs gave no specific phenotype, although these strains showed RNAi response and production of specific siRNA (Popova et al. unpublished, Popova et al. 2006). The possibility that the phenotype is a consequence of an

oversaturation of mi and/or siRNA protein machinery upon overload with precursor expression would implicate overlapping of miRNA and siRNA pathways. Nevertheless, it is not the case, since strains of *Dictyostelium* carrying siRNA constructs against various genes, such as discoidin, GFP, coroninn, Sp96 and others (Popova et al. 2006) show no phenotype nor common phenotype of dsRNA. It was as well shown that in *Dictyostelium* exist several different pathways of small RNA production (Hinas et al. 2007), separating small RNAs based on their origin. Not only that pathways of endogenous siRNA, nat-siRNA and miRNA differed, but maturation of small RNAs of same origin differed as well e.g. the maturation of one miRNA was DicerB dependant and maturation of the othe miRNA was DicerB independent. Small RNAs deriving from DIRS retrotransposone were not affected by downregulation of any known protein in RNAi pathway, while small RNAs deriving from Skipper retrotransposone were upregulated in DcrA KO and RrpC KO (Hinas et al. 2007). Therefore it is possible that besides having several distinct pathways of small RNA biogenesis the organism could possess as well distinct mechanisms of post-transcriptional gene control by the small RNAs. The other conclusion is that miRNA system of *D. discoideum* has a highly conserved function since it can accept and process the heterologuos miRNA *let-7*. Evolutionary interpretation of this would support the theory that miRNAs evolved early in unicellular systems and during the transition to multicellular systems may have taken over new roles, such as temporal and spatial control of genes involved in development. In the case of evolution of *distyostelida* to which *Dictyostelium* belongs to is proposed that miRNAs themselves were responsible for divergent evolution of the species (Ceccerelli et al. 2007).

MiRNA were originally discovered as non-coding RNA involved in control of developmental timing of *C. elegans* (Lee et al. 1993; Ambros et al. 2000). *Let-7* overexpression might have had as a consequence a loss of endogenous miRNA roles during the development. In this case, overload of the machinery with *prelet-7* prevents and/or decreases processing of endogenous pre-miRNAs that may be involved in controle of development. Therefore, competing out the endogenous miRNAs would indirectly show their decreased and/or disabled effects.

The phenotype could as well be a consequence of a target-off effect, where a mature *let-7* could negatively down-reguated other mRNA. This is called an unintended consequence effect and is typical feature of si/miRNA-mediated silencing (Linsley et al. 2006). A sequence complementarity to the seed region of the si/miRNA is a

primary determinant when searching possible targets. In the case of *Dictyostelium* the possible target is DDB_G0286091, gene of unknown function. Nevertheless, applying the same criteria to endogenous miRNAs gave no possible targets. Therefore, it stays to be confirmed by biological approach (microarray or deep sequencing) which genes are missregulated in the *prel-7* phenotype. *Let-7* is evolutionary conserved but *per se* does not exist in *D. discoideum*. Nevertheless, it might interact directly or indirectly with its protein targets or their homologues, for example ras let-60/RAS family in humans (Johnson et al. 2005) or with RPS-14 protein of ribosomal subunit such in the case of *C. elegans* (Chan et al. 2009). Searching for target sites in their *Dictyostelium* homologuos gave no output and this could not be experimentally evaluated since the antibodies for these proteins were not available for *Dictyostelium*.

The *prel-7* strain beside delay in development and decreased sporulation efficiency showed smaller fruiting bodies and in part resembled the HelF KO strain (Popova et al. 2006). HelF is a negative regulator of RNAi and the only protein of RNAi machinery whose downregulation showed detectable phenotype effects. It could be that the knock-out of the HelF gene makes cells more sensitive to sub-threshold levels of primary siRNAs, which may disturb the balance of small regulatory RNAs in the cell and result in a phenotype. Since the overexpression of HelF could not morphologically rescue the KO phenotype, this stays in domain of speculation. Assuming that HelF also has an influence on miRNAs biogenesis may explain similarities between the HelF knock-out strain and *prelet-7* overexpression: in both cases the subtle control of small regulatory RNAs may be out of balance. The key similarity is delay in development, but other experiments e.g. labelled chymeric slugs (Mog et al. PhD experiements) show their different behaviour.

Multicellularity in Dictyostelium arises from cell aggregation and not from growth and division of a single cell. Genetically diverse amoebae may enter an aggregate and, if one lineage has the capacity to avoid the stalk cell fate, it may have a selective advantage. Such cheater mutants have been found among wild isolates and created in laboratory strains (Shaulsky and Kessin, 2007). From the experimental results with chimeric slugs, where the prel-7 strain accumulate in the anterior part of the chymeric slug that gives stalk cells, it is tempting to speculate that endogenous miRNAs that are upregulated in development (Hinas et al. 2007) might have roles in fine tuning of the genome. In case that *prelet-7* overexpression disturbs endogenous miRNAs, these cells would have a missing component in regulatory networks and would loose the

evolutionary battle with the strain that is able to adapt to multicellular way of life faster. It is as well possible that they are less productive in spore formation which gives them a disadvantage in proliferation and this is why they accumulate in anterior part of the slug. Since, multicellularity requires the 'loosers' to make stalk and only when they become too altruistic they will be lost, it would be interesting to see a pattern of distribution after a few generations and investigate if the 'loosers' get lost or become more stable in number. If this is the case they would become the 'useful idiots' that enhance the chances of the wt cells to progress to the next generation. The 'survival of the fittest' would not eliminate them since their presence means the advantage to wt cells to carry more cells to the next generation and increases fitness. The most likely conclusion is that the *prelet-7* phenotype arises as a consequence of interference with endogenous miRNA. These endogenous miRNAs might controle different genes, acting in promoting and/or suppressing target gene activity in development. Such control could include: control of developmental timing, communication among cells and directing and/or redirecting cell faith within the slug. The possibility that miRNAs could somehow be included in communication between cells after the aggregate has been formed and be transported between them (like in the case of miRNA transport throught the phloem of the plants) is not to be excluded. During the development of *Dictyostellium* different types of promoters are expressed spatially and temporally in order to achieve the specific pattern of development and cell sorting. In such way, control and expression of different sets of genes enables the formation of a multicellular organism. The pattern of cell sorting and cell fate desision is an early and precise event in development that results in several subpopulations of prestalk and prespore cells. During culmination, cells at the anterior make a stalk tube, enter it and vacuolize to form a stalk on which the prespore cells climb to get off the substratum before they encapsulate. Prestalk cells are found localized to the anterior 20% of slugs over a wide range of sizes and are not homogeneous (Maeda et al. 2003). Therefore, 20% of the cells in slug (prestalk cells) die producing a stalk, whereas the remaining 80% of the slug (prespore cells) mature into spores, progressing to the next generation.

In the case of *prelet-7* overexpression, this specific pattern was disrupted, as seen with psvA/EB4 antibody staining, a marker for the prespore zone. It showed a reduced prespore zone in the *prel-7* strain. The *prel-7* phenotype upon development showed smaller sporeheads and decreased number of spores compared to wt. Autophagic

vacuoles of prestalk cells are strongly stained because of their acidity with neutral-red (Yamamoto and Takeuchi, 2006). When the prestalk zone was investigated with neutral red staining, the stained zone of *prel-7* in the anterior part was larger compared to wt. This suggested increased activity of autophagic vacuoles in the *prel-7* strain. Since the anterior part of the slug is where formation of the basal disc begins, the morphology of basal disc was investigated, but there was no difference between *prel-7* and wt strain. It stays to be investigated in detail, what follows the pattern of increased vacuolar activity in the anterior part of the slug in *prelet-7* strain. The *prelet-7* slugs progress during development, forming multicellular structures, but show severe problems with the developmental timing and with the spore/stalk ratio.

The sporulation test efficiency showed a decrease of 40% for the prel-7 strain, which corresponds to the phenotype observed for this strain. The delay in the development starts at very early stages with the window of 4-5 hours and this time difference stayed present throughout development.

The transition from unicellular to multicellular organisation means switching off one set of genes and switching on another set. The putative miRNAs were shown to be upregulated during the development, but they are as well expressed in the vegetative life phase. MiRNA are known as fine tuners of the genomes and part of different networks that are yet to be characterized in detail. *Prelet-7* strain was transformed with GFP constructs carrying two MREs and phenotype observed. Upon processing, *let-7* binds to the MRE resulting in a loss of protein expression. In the silenced strains the *prelet-7* phenotype was still present. The silenced lines were delayed in development as *prelet-7* strain on PA, but showed difference as 48 hrs plaques in which presence of slugs was noted.

The inducible expression of miRNA showed reversible controle of phenotype suggesting that genes responsible for the phenotype presence are controlled on the posttranscriptional level.

From the obtained results it is implied that miRNAs is *Dictyostelium* appear to be required for normal cell-type proportioning and cell sorting during multicellular development. In addition, the normal prestalk/prespore ratio in the organisation of the slug is absent; sporulation efficiency is reduced in *prelet-7* line and these slugs progress through development slowlier, possibly because pattern of gene expression is affected.

The artificial miRNA system in *Dictyostelium* was characterized. First, the reporter system had been created, GFP flanked with specific 3'UTR sequence of the lin-41 gene in the actin6-5'-UTR and the actin8-3'-UTR in separate constructs. This enabled labelling of GFP with two MRE at positions before the start codon and between the stop codon and the polyA signal sequence, respectively. Both transformed strains were checked by microscopy and western blots for protein expression and then coexpressed with *prelet-7*. As expected, no GFP protein was detected in the cells, but mRNA was still present in the silenced clones. This observation that mRNA was present whereas no protein was detected is the feature of the miRNA system where miRNAs inhibit translation. In contrast, in plants miRNA targeted mRNA are mostly cleaved. This outcome differed from the siRNA response in *D. discoideum*, where the target mRNA got degraded and siRNAs derived from it detected (Martens et al. 2002, Popova et al. 2006). Gfp mRNA in silenced clones was detected with northern blots and semi-quatitative RT-PCR showing that in the absence of translated protein, levels of mRNA stayed unaffected. No siRNAs derived from gfp mRNA were detected, but it stays to be therally quantified in which degree are the target mRNAs unaffected. For this the new reporter system based on the same principle but with b.galactose as reperter gene is being established.

These experiments provide the first evidence for translational control by miRNAs in *Dictyostelium* and the established artificial miRNA system implies the existence of similar endogenous mechanims of translational control.

In addition to several different pathways for small RNA processing, there is substantial evidence to sugest that at least two different ways of posttranscriptional gene controle exist in *Dictyostelium* as well, target decay and translational inhibition.
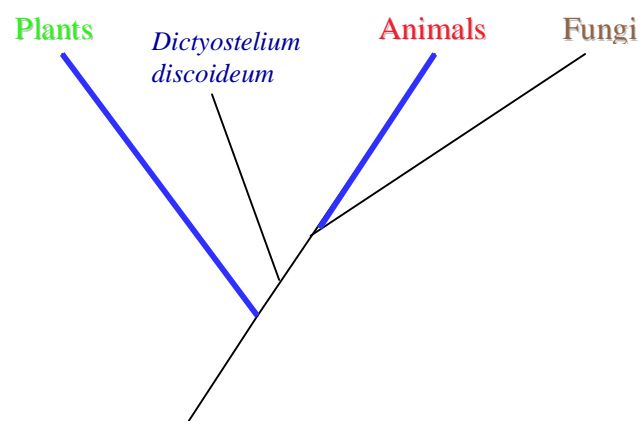
Evolutionary position of *Dictyostelium* (Eichinger et al. 2005).

The fact that the target mRNA in the cells stayed uneffected, but was not translated, opened the question where this RNA was located. In order to answer this, sucrose gradient centrifugations were performed on cell lysates and fractions were probed for gfp mRNA and *let-7* RNA. Gfp mRNA was detected in several fractions that colocalised with small RNA in the silenced lines. Additionally, in the silenced clones small RNA *let-7* was detected in several fractions that segregated with/as polysomes. This pattern was observed for both silenced version confirming that *let-7* miRNA and gfp mRNA were colocalised. Upon introducing of *prelet-7* miRNA, the pre-miRNA was processed into mature *let-7*, probably bound to target MREs on the reporter mRNA and led to inhibition of mRNA translation. The process of translation was disabled. These experiments give an insight into mechanism of transaltional inhibition. Since the antibodies for Argounate and Dicer proteins in *Dictyostelium* were not available, presence of these proteins and their possible colocalisation with endogenous and/or heterologous miRNA is under investigation with their labelled fusion forms.

The results, do not allow for an unambiguous determination at which stage translation is inhibited. Two models have been described in the literature (references to Sharp papers and Filipowicz papers): interference of the miRNA complex with translation initiation or with elongation. As has been shown by Hentze and coworkers (Hentze et al. 2007), sedimentation in polysome fractions does not necessarily mean that the mRNA is in true polysomes. It is possible that gfp mRNA and let-7 interact in a way that initiation phase of translation is disturbed, therefore causing a 'trapped' ribonucleoprotein complex in the cell. These complexes go to P-bodies (reference) in animal cells but the existence of P-bodies has not been shown yet in Dictyostelium.

Although *Dictyostelium* shares many features with plant system, such as presence of cellulose wall, the protein machinery involved in small RNA processing such as Argonaute proteins (paper name) has more similarity to animals. Most probably, several mechanisms of RNA mediated gene control exist in cells and function in separate or partially separate pathways and there is high probability that these pathways involve the same protein machinery.

One approach to further investigate this issue would be the introduction of siRNA constructs against GFP into already 'silenced' cells, to analyse reporter mRNA

stability and small RNAs distribution. Another approach would be to change the miRNA target site to obtain the complete complementarity between miRNA::mRNA bindings and observe effects on the target. This would as well give an insight about the the crucial features for recruiting protein machinery and its redundancy.

The co-localisation of gfp mRNA and *let-7* miRNA in sucrose gradients opened interesting questions, such as the mechanism of translational inhibition and the presence of cellular compartments that would sequester such complexes. In animal organism with translational control system, the specialized cellular departments named P-bodies or GW-bodies had been microscopically detected together with other involved proteins.

The previous work on Argonaute proteins in *Dictyostelium* showed that AgoA-GFP fusion constructs localised as cytoplasmatic granules (XiaoXiao, PhD thesis 2006). The interacting partners of Argonaute proteins are still unknown and it is not clear if microprocessor complex exists in *Dictyostelium*. Nevertheless, crucial proteins involved in RISC complex formation have their analoguos in this model organism.

The experiments on translational inhibition and P body formation in human cells led to the model of translational inhibition in the initiation phase. The disturbance of eIF4E and eIF4G binding in the presence of Ago proteins causes formation of "pseudopolysomes". Pseudopolysomes were suggested to be the same structures as P-bodies and on glycerol and sucrose gradients colocalise together with poyisomes themselves (Hentze et al. 2007). The same scenario is possible in *D. discoideum*, since the organism possesses five Argonaute proteins and homologues of eIF4E/G. It remains to be answered which roles do Ago proteins have in Dictyostelium. It is tempting to speculate that some of five Argonaute proteins could interacti with eIF4E and/or eIF4G homologue causing the formation of pseudopolysomes in *Dictyostelium*, as in case of *Drosophila* and humans. The setback is the open question if a functional RISC complex exists in *Dictyostelium*.

In *D. discoideum* exist several classes of small RNA. They can generally be classified as: 21 nt RNAs deriving from DIRS retrotransposone; small RNA deriving from Skipper retrotransposone; small RNA deriving from developmentaly regulated antisense mRNAs (that are named nat-siRNA in plants) and two putative miRNA of unknown functions.

Again, with evolutionary position of social amoeba, it was not possible to predict if the type of miRNA derived gene silencing would be regulated in a plant-like manner

or in animal-like manner. Approach taken in this study enabled answering the issue wheather target mRNA would be degradated or translationally repressed. It has been shown that miRNA regulating gene expression can function by translational inhibition of target mRNA. One interpretation of this would be that it represents a snap shot of evolutionary change, where a plant-like system of gene control and silencing via miRNA is being replaced with animal-model of gene control via arosal of translational repression mechanism. On the other side translational inhibition and RNA degradation by miRNAs exist side by side and are both functional in animals. There are at least two mechanisms how miRNA regulate the expression of target mRNA: by repressing translation and/or by promoting mRNA degradation. In some model organism e.g. *D. melanogaster*, both mechanism are active and require the same proteine machinery of Argonaute1 and the P-body component GW182 (Eulalio et al. 2007). *Dictyostelium* has 5 Argonaute homologues, but no GW182 homologue. It has several distinct pathways for small RNA biogenesis and as shown in this study, possibility to control target mRNA on the level of translational inhibition. It stays unclear by which mechanism endogenous miRNA effect target mRNA and which protein machinery in evolved. Nevertheless, biochemical colocalisation of miRNA and target mRNA implicates existence of cytoplasmatic ribonucleocomplexes that need to be further investigated.

Multicellularity is achieved through the coordinated action of cellular processes such as cell growth and death, cell-cell signaling, cell movements, and cell adhesion. All these events lead to differentiation of cell types and morphogenesis of a multicellular structure. The transitions from unicellular eukaryotes to multicellular ones seem to have occurred independently many times during the course of evolution (Bonner JT, 1998; Gerhart et al. 1997). What is known about the requirement for such transitions is very limited (Buss LW, 1987; Kirk DL, 2005) and many interesting questions that are imposed: how many genes are necessary and what form of networks of genes and proteins are required, are still puzzlig. *Dictyostelium* presents very usefull model to investigate this and with the emerging evidence of its miRNA system there is an additional component involved in the regulatory networks and gene controle highlighting these questions.

The fact that the organism represents a brigde between unicellular and multicellular organisation opens a wide space for speculating on miRNA roles in evolution and possible involvement in development of multicellular organism.

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Online links
DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez
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Dictyostelium discoideum information:
http://www.dictybase.org

Swiss-Prot: http://us.expasy.org/sprot

The miRNA Registry:
http://www.sanger.ac.uk/Software/Rfam/mirna
http://www.mirbase.org
http://miracle.igib.res.in/miracle

RNA & DNA Folding Applications:
http://www.bioinfo.rpi.edu/applications/mfold

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EDUCATION

1996-2000 High School Diploma. V Belgrade High School, Belgrade, Serbia

2000-2005 **Diploma** in Molecular Biology and Physiology (Biomedicine Alumni)
Faculty of Biology, University of Belgrade, Serbia
'Characterization of Homer1-a protein in the brain of transgenic mice'

2006-2010 **PhD student**. Genetics Department, Institute of Biology, University of
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HOBBIES

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