On the function of the *Dictyostelium* Argonaute A protein (AgnA) in epigenetic gene regulation

Inaugural-Dissertation zur Erlangung

des akademischen Grades eines Doktors der Naturwissenschaften

(Dr. rer. nat.)

Vorgelegt im Fachbereich 18 Naturwissenschaften

der Universität Kassel

von

Xiaoxiao Zhang

Kassel, Juli 2006

Supervisors:	**Prof. Dr. Wolfgang Nellen**
	Prof. Dr. Markus Maniak
Dissertation Committee:	**Prof. Dr. Wolfgang Nellen**
	Prof. Dr. Markus Maniak
	Prof. Dr. Friedrich W. Herberg
	Prof. Dr. Mireille A. Schäfer
Dissertation date:	**21th July, 2006**

Erklaerung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstaendig und ohne unerlaubte Hilfe angefertigt und andere als die in der Dissertation angegebenen Hilfsmittel nicht benutzt habe. Alle Stellen, die woertlich oder sinngemaess aus veroeffentlichten oder unveroeffentlichten Schriften anderer Personen entnommen sind, habe ich als solche kenntlich gemacht.

Kein Teil dieser Arbeit ist in einem anderen Promotions-oder Habilitationsverfahren verwendet worden

Kassel, den

Zusammenfassung

Mit molekularbiologischen und bioinformatischen Methoden wurden die Argonauten Proteine aus *Dictyostelium discoideum* charakterisiert und die Funktion des AgnA Proteins in RNAi und DNA Methylierung untersucht, ebenso wie zelluläre Eigenschaften. Auch wurden Interaktionspartner der PAZ-Piwi Domäne von AgnA (PAZ-PiwiAgnA) entdeckt.

Das *Dictyostelium* Genom kodiert für fünf Argonauten Proteine, genannt AgnA/B/C/D/E. Die Expressionsstärke der Argonauten Proteine war AgnB/D/E > AgnA > AgnC. Alle diese Proteine enthalten die charakteristischen, konservierten PAZ und Piwi Domänen. Mittels Fluoreszenz Mikroskopie wurde gezeigt, dass eine C-terminale GFP-fusion von PAZ-PiwiAgnA (PPWa-GFP) im Cytoplasma lokalisierte.

Die Überexpression des PPWa-GFP führte zu erhöhter Effizienz der Genstilllegung durch RNAi, aber nicht durch antisense RNA. Dies deutet darauf hin, dass PAZ-PiwiAgnA in der RNAi, aber nicht in der antisense RNA vermittelten Genstilllegung involviert ist.

Eine Analyse von Protein-Protein Interaktionen durch einen Yeast-two-hybrid Screen auf eine cDNA Bibliothek von vegetativ gewachsenen *Dictyostelium* Zellen zeigte, dass mehrere Proteine, wie FF2, EF1-I, IfdA, SahA, SamS, RANBP1, UAE1, CapA, and GpdA, mit PAZ-PiwiAgnA interagieren können. Für PAZ-PiwiAgnA und HP1, HelF oder DnmA konnte eine Interaktion durch direkte Yeast-Two-Hybrid Analyse nicht gezeigt werden.

Fluoreszenzmikroskopische Aufnahmen zeigten, dass überexprimierte GFP-SahA oder -IfdA Fusionsproteine sowohl im Zytoplasma als auch in Kernen lokalisierten,

während überexprimiertes GFP-SamS im Zytoplasma gefunden wurde. Die Expression von SamS in AgnA knock down Mutanten war auf cDNA und mRNA Ebene stark reduziert, während die Expression von SahA nur gering reduziert war.

AgnA knock down Mutanten zeigten Defekte in Wachstum und Phagocytose, was darauf hindeutet, dass AgnA auch zellbiologische Eigenschaften beeinflusst. Die Inhibierung der DNA Methylierung von DIRS-1 und Skipper Retroelementen, sowie der endogenen mvpB und telA Gene im gleichen Stamm zeigte, dass AgnA in DNA Methylierung involviert ist.

Northern Blot Analysen zeigten, dass Skipper and DIRS-1 kaum in Ax2 exprimiert sind, dass aber die Expression von Skipper in AgnA knock down Mutanten hochreguliert war, während die DIRS-1 Expression nicht verändert war.

Ein Knock out des agnA Gens war nicht erfolgreich, obwohl die Integration des Konstrukts an der korrekten Position, durch homologe Rekombination, stattfand. Dies deutet auf eine Duplikation des agnA Gens im Genom hin. Das gleiche Phänomen wurde auch in ifdA knock out Experimenten beobachtet.

Stichwörter: Argonaute Protein, RNA Interferenz (RNAi), DNA Methylierung, Protein-Protein Interaktionen, *Dictyostelium discoideum*

Summary

With molecular biology methods and bioinformatics, the Argonaute proteins in *Dictyostelium discoideum* were characterized, and the function of the AgnA protein in RNAi and DNA methylation was investigated, as well as cellular features. Also interaction partners of the PAZ-Piwi domain of AgnA (PAZ-PiwiAgnA) were discovered.

The *Dictyostelium* genome encodes five Argonaute proteins, termed AgnA/B/C/D/E. The expression level of Argonaute proteins was AgnB/D/E > AgnA > AgnC. All these proteins contain the characteristic conserved of PAZ and Piwi domains. Fluorescence microscopy revealed that the overexpressed C-terminal GFP-fusion of PAZ-PiwiAgnA (PPWa-GFP) localized to the cytoplasm.

Overexpression of PPWa-GFP leaded to an increased gene silencing efficiency mediated by RNAi but not by antisense RNA. This indicated that PAZ-PiwiAgnA is involved in the RNAi pathway, but not in the antisense pathway.

An analysis of protein-protein interactions by a yeast-two-hybrid screen on a cDNA library from vegetatively grown *Dictyostelium* revealed that several proteins, such as EF2, EF1-I, IfdA, SahA, SamS, RANBP1, UAE1, CapA, and GpdA could interact with PAZ-PiwiAgnA. There was no interaction between PAZ-PiwiAgnA and HP1, HelF and DnmA detected by direct yeast-two-hybrid analysis.

The fluorescence microscopy images showed that the overexpressed GFP-SahA or –IfdA fusion proteins localized to both cytoplasm and nuclei, while the overexpressed GFP-SamS localized to the cytoplasm. The expression of SamS in AgnA knock down mutants was strongly down regulated on cDNA and mRNA level in, while the expression of SahA was only slightly down regulated.

AgnA knock down mutants displayed defects in growth and phagocytosis, which suggested that AgnA affects also cell biological features. The inhibition of DNA methylation on DIRS-1 and Skipper retroelements, as well as the endogenous mvpB and telA gene, observed for the same strains, revealed that AgnA is involved in the DNA methylation pathway.

Northern blot analysis showed that Skipper and DIRS-1 were rarely expressed in Ax2, but the expression of Skipper was upregulated in AgnA knock down mutants, while the expression of DIRS-1 was not changed.

A knock out of the agnA gene failed even though the homologous recombination of the disruption construct occurred at the correct site, which indicated that there was a duplication of the agnA gene in the genome. The same phenomenon was also observed in ifdA knock out experiments.

Key words: Argonaute protein, RNA interference (RNAi), DNA methylation, protein-protein interaction, *Dictyostelium discoideum*

Table of Contents

Abbreviations

aa	amino acid
Ab	antibody
AgnAas	Antisense construct of AgnA
Amp	ampicillin
Aps	ammonium persulphate
A260 (280)	absorbance at 260 (280) nm
bp	base pair
BCIP	5-bromo-4-chloro-3-indolyphosphate
BSA	bovin serum albumin
Bs^r	blasticidin resistance cassett
cDNA	complementary DNA
DEPC	1,4-diazabicyclo 2.2.2. octane
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ddNTP	dideoxyribonucleotide: ddATP/CTP/GTP/TTP
dNTP	deoxyribonucleotide: dATP/CTP/GTP/TTP
DTT	1,4-dithiotreitol
EDTA	ethyendiamintetraacetic acid
g	gram, gravitation constant
GFP	green fluorescent protein
G418	geneticin
GFP-ifdA	GFP N-terminally fused ifdA
GFP-SahA	GFP N-terminally fused SahA
GFP-SamS	GFP N-terminally fused SamS
GTC	guanidine thiocyanate
hr	hour
HEPES	N-2-hydroxyethylpiperazine-N’-2-ethasulfonic acid

IPTG	isopropyl-ß-D-t
KA	Klebsiella aerogenes
kb	kilo base pair
kDa	kilo Dalton
K.O.	knock out
mRNA	messenger RNA
min	minute
NP40	ethylenphenylpolyethylenglycol
OD	optical density
OLB	oligo labeling
ORF	open reading frame
PAGE	Polyacrylmide gel electrophoresis
PCR	Polymerase chain reaction
PPWa	PAZ piwi domain of AgnA
PPWa-GFP	GFP C-terminally fused PPWa protein
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	Reverse transcription PCR
SAP	Shrimp alkaline phosphatase
TEMED	N', N', N', N'-tetramethyl-ethylendiamine
Tris	Tris-(hydroxymethyl-aminomethane)
Triton X 100	Octylphenylpoly-(ethylenglycolether)
trx	thioredoxin
Tween 20	Polyxyethylen-sorbitan-monolaurate
vol	volume
x-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

1. Introduction

1.1 The introduction of *Dictyostelium discoideum*

Dictyostelium discoideum is a simple eukaryotic microorganism located at the base of the phylogenetic tree. It grows as free- living amoebae in the litter stratum lacking of flagellated stage and aggregates to form fruiting body. *Dictyostelium discoideum* naturally habitats in deciduous forest soil and decaying leaves, where the amoebae feed on bacteria by phagocytosis and grow as separate, independent, single cells, but develop as multicellular organisms when food supply is exhausted.

In the complex developmental cycle, about 10.000-50.000 individual amoebae aggregate to form a multicellular fruiting body (**Fig.1-1**). The aggregation of individual *Dictyostelium* amoebae occurs by chemotaxis to periodic cAMP signals. Under laboratory conditions, the developmental cycle from starving amoebae to the formation of mature fruiting bodies is completed within 24hours. Alternatively, under suitable environmental conditions and in the presence of proper mating-type cells, the amoebae may enter a sexual life cycle (Urushihara 1996).

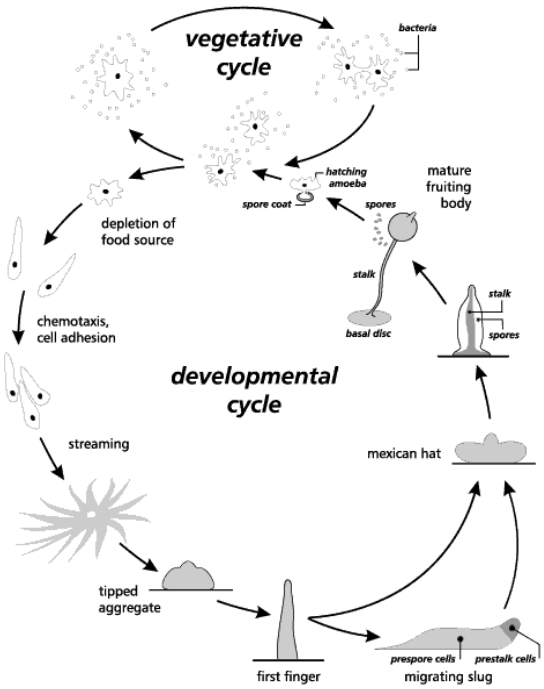


Fig.1-1 Life and development cycle of *Dictyostelium discoideum*.
(Internet: http//www.uni-kassel.de/fb19/genetics)

Dictyostelium cells can be grown axenically in liquid culture (AX) or on solid substratum in the presence of bacteria called *Klebsiella aerogenes* (KA). The generation time is approximately 8 hours in liquid culture and around 4 hours on bacterial lawns.

The organism provides unique advantages for studying fundamental cellular processes with powerful molecular genetic, biochemical, and cell biological tools. These processes include cell motility, chemotaxis, cytokinesis, phagocytosis, endocytosis, signal transduction, and aspects of development such as cell sorting, pattern formation, and cell-type differentiation (Parent and Devreotes 1999; Thomason et al. 1999; Gerisch and Weber 2000). Recently, *Dictyostelium* was also described as a suitable host for pathogenic bacteria to study the process of infection conveniently (Skriwan et al. 2002). In addition, *Dictyostelium* has many of the experimental conveniences of *S. cerevisiae* and is probably the best experimentally manipulatable protozoan. It has a short life cycle, is easy to cultivate, and can be grown in large quantities, which facilitates biochemical analysis.

The main advantages of the organism are:
(i) The amoebae are easy to lyse. The cells can be harvested from growth or any of the developmental stages. The high levels of overexpressed protein obtained in transformed cells makes this system suitable for protein purification.

(ii) The genome is relatively small with rare introns and short promoters. There are six chromosomes with sizes ranging from 4 to 7 Mb, which result in a total of about 34 Mb of DNA, a multicopy 90 kb extrachromosomal element that harbors the rRNA genes, and the 55 kb mitochondrial genome. The estimated number of genes in the genome is 12000 and many of the known genes show a high degree of sequence similarity to genes in vertebrate species.

(iii) A series of cell biological assays and molecular genetic tools can been used to

study gene function (Eichinger et al. 1999). Basically, these techniques are devised to: (1) disrupt or silence a known gene, (2) overexpress and/or tag a known gene, or (3) recover a mutated unknown gene. Most of these techniques were made possible after the establishment of efficient transformation systems in the 1980s (Nellen et al. 1984; Howard et al. 1988). The fact that *D. discoideum* is haploid constitutes a huge advantage and enormously facilitates the isolation and characterization of mutants.

1.2 RNA interference

Double-stranded RNA-induced posttranscriptional gene silencing (PTGS) is known as RNA interference (RNAi) in animals, as quelling in the filamentous fungus Neurospora crassa (Fire et al. 1998; Hutvagner and Zamore 2002; Tijsterman et al. 2002), and was first described in plants termed co-suppression (Napoli et al. 1990). The RNAi silencing mechanism plays role in cellular based innate immunity to protect against transposable elements or retrotransposons and RNA viruses, the regulation of cell identity during development, the epigenetic control of chromatin structure (Hannon 2002; Tijsterman et al. 2002; Martinez and Tuschl 2004; Mello and Conte 2004; Soifer et al. 2005). In most instances, RNAi mediates gene silencing at the posttranscriptional level, but in some cases it acts at the DNA and chromatin level. For example, it mediates DNA methylation and histone H3K9 methylation in plants (Zilberman et al. 2003)

Based on genetic and biochemical studies, the RNAi pathway has been divided into two stages: initiation and effector (Hammond et al. 2001). The initiation stage involves the generation of siRNAs (small interfering RNA) (Hamilton et al. 2002) and miRNAs (microRNAs) (Park et al. 2002; Reinhart and Weinstein 2002) from long dsRNA or hairpin RNA precursors respectively. The effector stage requires the transfer of the si/miRNAs into ribonucleoprotein complexes known as RISCs (RNA-induced silencing complexes)/ miRNP (microRNA containing ribonucleoprotein) (Hammond et al. 2000). Functional ribonucleoprotein complexes contain only single-stranded siRNAs or miRNAs (Martinez et al. 2002), and silence genes via homology-dependent mRNA degradation (Tuschl et al. 1999; Zamore et al. 2000), translational repression (Grishok et al. 2001) or transcriptional gene silencing (Hall et al. 2002; Pal-Bhadra et al. 2002; Volpe et al. 2002).

1.2.1 siRNA-induced PTGS

siRNA is generated by the RNase III endonuclease termed Dicer by a multistep

process. Following the action of Dicer, the ~22 bp siRNAs are incorporated into the RISC (Hammond et al. 2000), which identifies and silences mRNA. The silencing is performed by cleaving the mRNAs complementary to the siRNA through interactions with Argonaute 2 (Liu et al. 2004), which specifically localizes to P-bodies in the cytoplasm (Liu et al. 2005). RISC initially recognizes a double-stranded short interfering RNA (siRNA), but only one strand is finally retained in the functional ribonucleoprotein complex (Leuschner et al. 2006). The non-incorporated strand, or 'passenger' strand, is removed during the assembly process and most probably degraded thereafter. Leuschner et al. (Leuschner et al. 2006) showed that the passenger strand is cleaved during the course of RISC assembly following the same rules established for the siRNA-guided cleavage of a target RNA.

1.2.2 miRNA-induced PTGS

microRNAs (miRNAs), first discovered in *Caenorhabditis elegans*, are a large family of ~22-nucleotide (nt)-long endogenously encoded non-coding RNAs widely expressed in metazoan eukaryotes (Lee et al. 1993, Lee et al. 2004). It is generally believed that miRNAs regulate gene expression at the post-transcriptional level by inhibiting the expression of mRNAs bearing fully or partly homologous target sequences (Carrington and Ambros 2003). In addition, many miRNAs are conserved across species, strongly suggesting that they have an evolutionarily conserved role in gene regulation (Carrington and Ambros 2003).

miRNAs are initially expressed as part of one arm of an imperfect ~80-nt RNA hairpin that forms part of a longer transcript termed a primary miRNA (pri-miRNA) (Lee et al. 2003). In miRNA biogenesis, the upper part of this RNA hairpin is excised by the nuclear RNase III enzyme Drosha to produce an ~65-nt intermediate with a 3′ 2 nt overhang, termed a pre-miRNA (Lee et al. 2002; Zeng and Cullen 2003a). The pre-miRNA is then exported to the cytoplasm by the nuclear export

factor Exportin 5 and the Ran-GTP cofactor (Yi et al. 2003; Lund et al. 2004). As occurred in siRNA pathway, Dicer in the cytoplasm removes the terminal loop of the pre-miRNA to generate an ~20-bp imperfect RNA duplex with 2-nt 3′ overhangs at both ends (Grishok et al. 2001; Lee et al. 2003). The mature miRNA, which forms one strand of this duplex, is then incorporated into miRNP complex, which is guided to complementary mRNA targets and silence the targets (Hammond et al. 2000; Martinez et al. 2002). In most cases, the mature single-stranded miRNA incorporate into an miRNP (microRNA containing ribonucleoprotein) complex and guide the complex to the 3'UTR of the partially complementary mRNAs to repress translation (Mallory and Vaucheret 2004).

1.2.3 Similarities and differences between miRNA and siRNA pathway

In animals, primary miRNA derived from intergenic regions are processed into precursor RNAs in the nucleus by the RNaseIII Drosha. These precursor RNAs are transported out of the nucleus and processed by Dicer. Mature single-stranded miRNAs incorporate into an miRNP and guide the complex to the 3′ UTR of partially complementary mRNAs to repress translation (**Fig. 1-2**). In the siRNA pathway, exogenously provided double-stranded RNAs are cleaved by Dicer to generate double-stranded siRNAs. Single-stranded siRNAs incorporate into RISCs and guide these complexes to perfectly complementary mRNA, where they mediate cleavage (**Fig. 1-2**). These two processes conclude that miRNAs silence genes generally by inhibiting the translation of mRNAs bearing partially complementary target sequences; in contrast, siRNAs by degrading mRNAs bearing fully complementary sequences.

miRNA can also lead to a cleavage of perfectly complementary mRNA, and siRNA can also repress the translation of mRNA if that has short segments complementary to the siRNA in its 3′ UTR (**Fig. 1-2**). Zeng (Zeng et al. 2003b) found that an

endogenously encoded human miRNA could cleave an mRNA bearing fully complementary target sites, whereas an exogenously supplied siRNA could inhibit the expression of an mRNA bearing partially complementary sequences without inducing detectable RNA cleavage. The data suggest that miRNAs and siRNAs can repress mRNA expression by similar mechanisms, and which mechanism to follow may be largely or entirely determined by the degree of complementary of the RNA target (Zeng et al. 2003b).

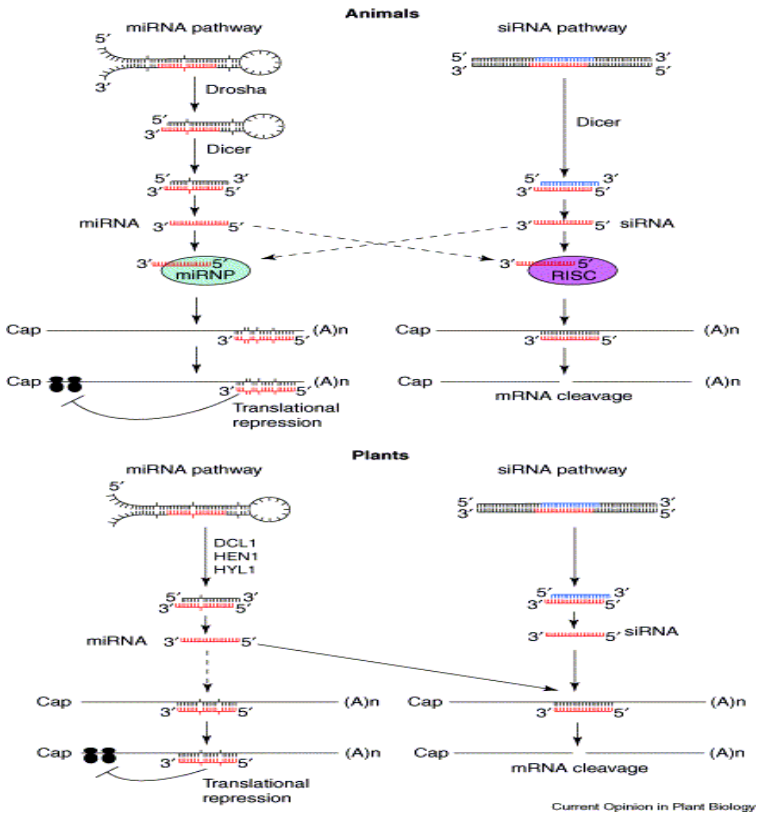


Fig.1-2: Similarities and differences between miRNA and siRNA pathways in animals and plants. This figure was taken from (Mallory and Vaucheret 2004).

In plants, the accumulation of miRNA depends on the activity of DCL1, HEN1, and HYL1, but it is unclear how dsRNA is cleaved into siRNA (Mallory and Vaucheret 2004). microRNAs and siRNAs are thought to associate with a protein complex (miRNP/RISC) to mediate mRNA cleavage, and AGO1 is suggested to form part of the plant RISC and to be a target of miRNA regulation (Vaucheret et al., 2004). Translational repression has been observed in plants, suggesting that plant miRNAs

can also repress translation by binding to a single site within the mRNA coding sequence (**Fig. 1-2**).

1.2.4 RNA interference in *Dictyostelium discoideum*

In *Dictyostelium*, a gene construct expressing a dsRNA can induce RNA interference. The nuclease termed Dicer digests dsRNA into ~21mers sequence specific siRNAs (**Fig. 1-3**). Similar to *Neurospora, C. elegans* and others, an RNA dependent RNA polymerase (RdRP) is essential for efficient RNAi in *Dictyostelium* (Martens et al. 2002b)**.** Martens proposed that amplification process by RrpA (RNA dependent RNA polymerase A) is required for efficient gene silencing. Small amounts of ~23mers produced by the dsRNase may serve as "primers" for RrpA, which synthesizes the antisense strand using the mRNA as a template (**Fig. 1-3**). The resulting dsRNA could then again be degraded by the dsRNase into ~23mers. These could reinitiate the amplification cycle or mediate mRNA degradation by a putative RISC homolog (Martens et al. 2002b) (**Fig. 1-3**). There is no experiment to confirm that if there is a RISC in *Dictyostelium* or not.

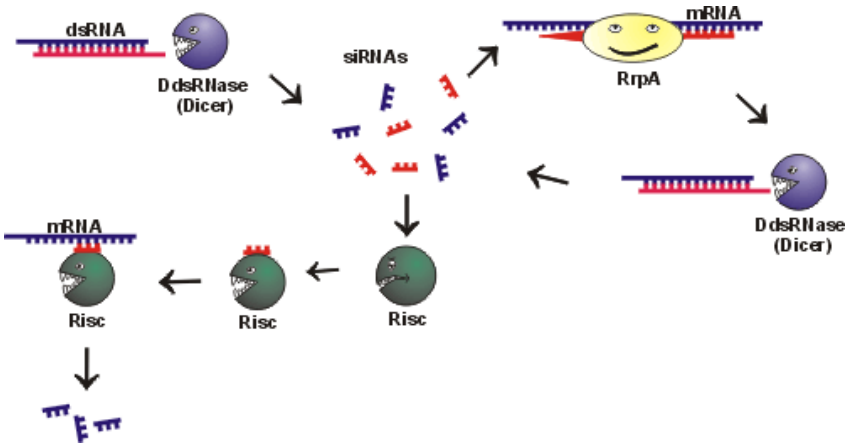


Fig. 1-3 The RNAi pathway in *Dictyostelium* cells. The picture is taken from (Martens and Nellen 2002a)

1.3 Epigenetics

Epigenetics is the study of meiotically and mitotically heritable changes in gene expression that do not involve changes in DNA sequence (Eggar et al. 2004)

Epigenetics impact many areas of biomedicine like development biology, somatic gene therapy, cloning and genomic imprinting (Wolffe and Matzke 1999). Epigenetic changes can be influenced by the environment (Bjornsson et al. 2004) and these changes can be inherited by the daughter cells during cell division and can also be inherited through the germline (Holliday 1987).

There are three distinct mechanisms related and implicated in initiating and sustaining epigenetic modifications: RNA-associated transcriptional gene silencing, DNA methylation and histone modifications (Eggar et al. 2004), that are known to interact with each other (**Fig.1-4**) (Peedicayil 2006).

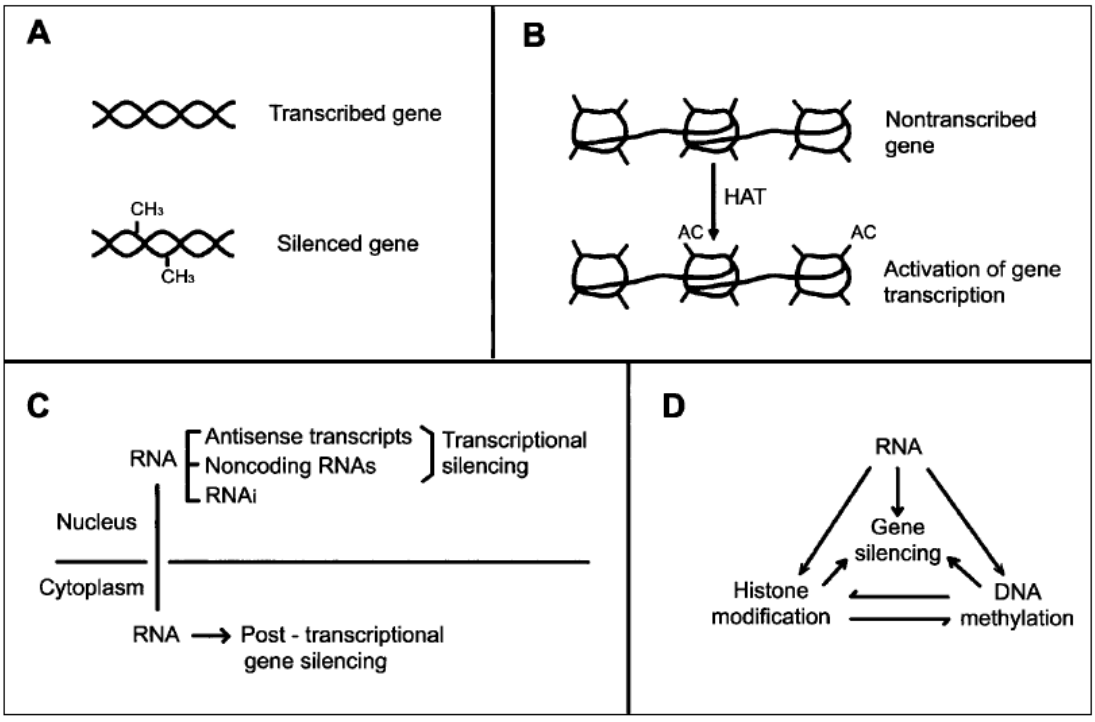


Fig.1-4 schematic representation of the various epigenetic mechanisms. A Methylation of DNA leading to silencing of a gene. B. Acetylation of histone in chromatin leading to activation of gene transcription. C. RNA-mediated transcriptional and post-transcriptional gene silencing. D. Interrelationships of the 3 epigenetic mechanisms. HAT, histone acetyltransferase. RNAi, RNA interference. The picture is taken from Peedicayil (2006).

1.3.1 RNA- associated transcriptional gene silencing

It is becoming clear that RNA plays a role in regulating DNA and the expression

profile of the genome. RNA in various forms such as antisense transcripts, noncoding RNAs, or RNA interference (RNAi) can also induce transcriptional silencing (TGS) of genes by histone modification and DNA methylation (Eggar et al. 2004).

Transcriptional gene silencing was first observed in doubly transformed tobacco when plants exhibited a suppressed phenotype of a transgene (Matzke et al. 1989), and was further substantiated in viriod-infected plant due to RNA-dependent DNA methylation (RdDM) (Wassenegger et al. 1994). The RdDM requires a dsRNA to target DNA and subsequently yields short RNAs (Wassenegger et al. 1994; Mette et al. 2000). These short dsRNA happened to include identical sequences to genomic promoter regions and proved to induce methylation of the homologous promoter and subsequent transcriptional gene silencing (Morris et al. 2004).

RNAi-mediated TGS in *Schizosaccharomyces Pombe* has been implicated in regulating heterochromatic silencing through histone 3 lysine 9 methylation (H3K9) (Volpe et al. 2002). dsRNAs are generated from bi-directional transcription of the centromeric DNA repeats, which are then processed by Dicer to siRNAs and loaded into the RITS (RNA-induced initiation of transcriptional gene silencing) complex. The RITS complex then recruits the methyltransferase Clr4 to methylate H3K9 and cause the silencing of the *cen* DNA repeats and/or swi-6-dependent heterochromatin formation (**Fig.1-4)** (Motamedi et al. 2004). The reduced H3K9 methylation in centromeric repeat in *S. pombe* dcr1 (Dicer homolog) and ago1 (Argonaute homolog) mutants denote a link between siRNA-specific targeting of histone modifications to specific genomic sequences which subsequently recruit or interact with Swi6, resulting in regulation of the heterochromatic state (Volpe et al. 2002). The association of RDRC (RNA-dependent RNA polymerase complex) with the RITS complex is Dcr1 and Clr4 dependent and correlates with the presence of siRNAs in RITS, suggesting that both siRNA-based target recognition and chromatin association are involved in mediating this interaction (Motamedi et al. 2004).

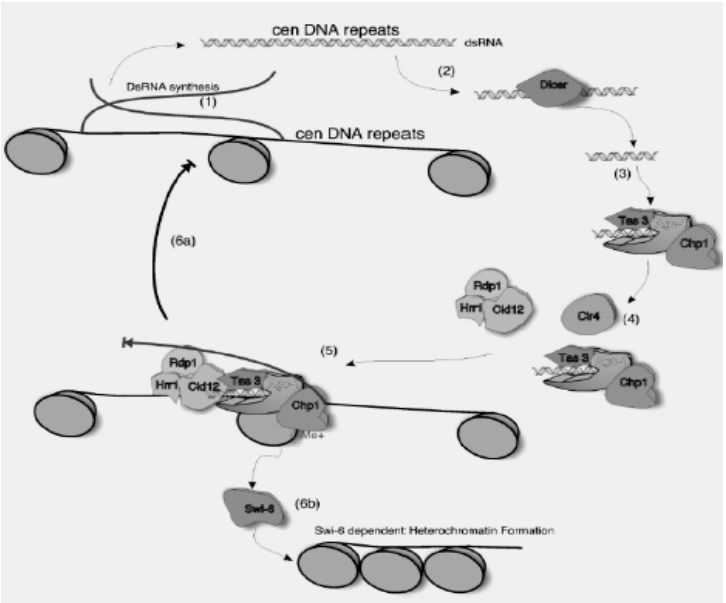


Fig. 1-4. **dsRNA-mediated TGS in *S. pombe*.** (1) *cen* DNA repeats generate dsRNAs, which are then processed by (2) Dicer to (3) siRNAs and loaded into the (4) RITS complex. The RITS complex then interacts with Clr4 and RDRC, leading to (5) H3K9 methylation and (6a) silencing of the *cen* DNA repeats and / or (6b) swi-6-dependent heterochromatin formation. This figure was taken from (Motamedi et al. 2004).

While dsRNAs can induce sequence-specific methylation of DNA in plants and histone methylation in yeast, it is unknown until recently how applicable this phenomenon was in mammalian cells. Some reports have shown that siRNAs targeted to three different genes EF1A, RASSF1, and E-cadherin, specifically the promoter regions, can induce transcriptional silencing via DNA methylation in human cells (Morris et al. 2004; Castanotto et al. 2005). The role that DNA methylation plays in the observed silencing is questionable, as siRNA-mediated TGS has been shown to occur in the absence of increased DNA methylation at the targeted promoter (Castanotto et al. 2005; Ting et al. 2005), while others find that siRNA mediated TGS correlates with increased histone methylation (Hutvagner and Zamore 2002; Buhler et al. 2005). Recent reports showed that siRNA treatment increases both H3K9 and H3K27 methylation of the targeted EF1A promoter and that this increase is dependent on nuclear specific delivery of the siRNA, meanwhile TGS can be directed by the antisense strand alone (Weinberg et al. 2006).

1.3.2 DNA methylation

DNA methylation is found in the genomes of diverse organisms including both prokaryotes and eukaryotes. In prokaryotes, DNA methylation occurs on both cytosine and adenine bases and encompasses part of the host restriction system (Wilson and Murray 1991). In multicellular eukaryotes, methylation seems to be confined to cytosine bases and is mostly associated with a repressed chromatin state and inhibition of gene expression (Bird and Wolffe 1999).

There are two general mechanisms by which DNA methylation inhibits gene expression: first, modification of cytosine bases inhibits the association of some DNA-binding factors with their cognate DNA recognition sequences (Watt et al. 1988); and second, Methyl-CpG-binding proteins repress transcription directly (Boyes and Bird 1991).

DNA methyltransferase

The DNA methyltansferases play a role in addition of a methyl group to the 5-carbon of a cytosine located proximal to a guanine (5-CpG-3). The DNA methyltransferase family (DNMT1, 3a and 3b) function to establish and maintain genomic methylation patterns which are established during embryogenesis. A fourth DNA methyltransferase, DNMT2, shows weak DNA methyltransferase activity in vitro (Hermann et al. 2003). In *Dictyostelium*, the DNA methyltransferase of the Dnmt2 family denominated DnmA was found to function on DNA methylation (Kuhlmann et al. 2005). There is ～0.2% of the cytosine residues are methylated in the *Dictyostelium* genome, which indicates that the methylation is restricted to a limited set of genomic loci. Bisulfite sequencing of specific sites revealed that DnmA is responsible for methylation of mostly asymmetric C-residues in the retrotransposons DIRS-1 and Skipper (Kuhlmann et al 2005).

1.4 Argonaute protein (PPD protein)

One of the core components in RISC complex is the Argonaute protein (Hammond et al. 2001). The Argonaute protein was first defined by the *Arabidopsis* Argonaute gene, later homologues were found in diverse organisms from erchea to humans (Bohmert et al. 1998). The Argonaute proteins are also known as PPD proteins because they are characterized by the presence of PAZ and Piwi domains.

The PAZ domain locates centrally within Argonaute proteins, binds the 2nt 3'-overhang of the siRNA duplex, and facilitates transfer of this guide siRNA or miRNA into the RISC/ miRNP complex (Zammore 2002; Ma et al. 2004) in the initiation step of RNAi. The Piwi domain has been shown to mediate the interaction of PPD domains with Dicer (Tahbaz et al. 2004) and has similar structure as RNaseH (Song et al. 2004). The shared characteristics of RNaseH and Argonaute protein, such as Mg^{+2} dependence and production of 3'-OH and 5' phosphate products indicated that this protein is directly involved in cleavage of targeted mRNAs as slicer. (Song et al 2004; Meister et al 2004, Liu et al., 2004) The mechanism by which PPD proteins mediate translational suppression and chromatin silencing are not known at this time (Jaronczyk et al. 2005).

Argonaute proteins are key components in RISC complex and thus function at the initiation and effector steps in RNAi (Hammond et al., 2001), they also play roles in the control of stem cell differentiation (Caplen et al. 2002), tissue development (Carmell et al. 2002), and chromatin modification (Verdel et al. 2004).

1.4.1 Function of Argonaute Proteins as Slicer

Slicer is the term given to the RISC-associated component that catalyses the cleavage of siRNA-targeted mRNAs. The Slicer entity was predicted to be an endoribonuclease that cleaves dsRNA molecules. As mentioned before, the PAZ domain adopts an OB (oligosaccharide/ oligonucleotide-binding)-like fold that binds

to the 3′ overhangs of siRNAs (Ma et al. 2004), the Piwi domain structure is similar to endonucleases such as RNase H and endonuclease V (Song et al. 2003; Rand et al. 2004). The magnesium-dependent activities of RNase H and endonuclease V are in agreement with the reported characteristics of RISC (Martinez and Tuschl 2004; Schwarz et al. 2004). Moreover, two aspartate residues that are required for mRNA target cleavage are spatially conserved between the archaeal Ago protein catalytic centre and the active sites of endonucleases (Song et al. 2003; Liu et al. 2004). By analogy to RNase H enzymes that cleave single-stranded RNA guided by the DNA strand in an RNA/DNA hybrid, the Piwi domain can be inferred to cleave single-stranded RNA, for example mRNA, guided by double stranded siRNA.

Each member of Argonaute protein family seems functionally distinct. In humans, the Ago2 (hAgo2) is associated with both siRNA and miRNA, and mediate RNA cleavage targeted by small RNA as slicer. However, other Argonaute subfamily members such as hAgo1, hAgo3, and hAgo4, do not mediate such RNA cleavage, although they show high similarity to hAgo2 at peptide sequence level (Liu et al. 2004). In *Drosophila*, both AGO2 and AGO1 have the capacity to show slicer activity when associated with small guide RNA, and AGO2 is directly involved in RISC formation as “slicer” of the passenger strand of the siRNA duplex (Miyoshi et al. 2006).

1.4.2 Function of Argonaute protein in chromatin modification

Indeed, some of the other proteins in the AGO family are associated with specific functions or processes. AGO4 is linked to transposon siRNAs as well as DNA and histone methylation in *Arabidopsis* (Zilberman et al. 2003).

In unicellular organisms, it seems that Argonaute proteins are multifunctional. The *Schizosaccharomyces pombe* Ago1 is required for silencing of pericentric chromatin, and for accurate chromosome segregation during mitosis and meiosis (Hall et al.

2003; Volpe et al. 2003; Ekwall 2004). Ago1 is also reported to function in concert with short heterochromatic RNAs, which are derived from repetitive sequences, to guide the chromatin-silencing machinery to sites of heterochromatin formation (Reinhart and Bartel 2002a). It was later demonstrated that this protein also functions in the classic RNAi pathway that culminates in siRNA-directed cleavage of mRNAs (Sigova et al. 2004).

1.4.3 Function of Argonaute protein in siRNA and miRNA pathways

In plants, AGO1 which controls development in *Arabidopsis*, was shown to be specifically required for siRNA accumulation and DNA methylation triggered by sense transgenes (S-PTGS) but not inverted-repeat transgenes (IR-PTGS; (Beclin et al. 2002; Boutet et al. 2003). These results indicated that AGO1 is not part of RISC but rather is acting upstream of the mRNA degradation step in the S-PTGS pathway, and that S-PTGS and IR-PTGS are two branches of the PTGS pathway that converge toward a common RISC that contains other AGO proteins (Beclin et al. 2002). Indeed, some of the other proteins in the AGO family are associated with specific functions or processes.

In *D. melanogaster*, the slicer-Argonaute2 (*d*Ago2) is required for the incorporation of siRNAs into RISC (Okamura et al. 2004). In contrast, *d*Ago1 is required for miRNA biogenesis (Okamura et al., 2004), but not siRNA-mediated RISC activities.

In humans, four Argonaute proteins (Argonautes1–4) were shown to bind miRNAs, but only hAgo2 is associated with the catalytic activity required for mRNA cleavage (Meister et al. 2004).

The *Caenorhabditis elegans* genome encodes 24 Argonaute proteins, which perform highly specialized functions. For example, RDE-1 and PPW-1 are required for efficient siRNA-mediated mRNA cleavage (Tabara et al. 1999; Fagard et al. 2000;

Tijsterman et al. 2002), whereas ALG-1 and ALG-2 are not required for this process but function in maturation and translational inhibition activities of miRNAs that regulate developmental timing pathways (Grishok et al. 2001).

These examples reinforce that in metazoans, Argonaute proteins exhibit isoform-specific functions in gene-silencing pathways. Since sequence conservation among family members is greatest in the C-termini, it is likely that their N-terminal domains determine the isoform-specific roles of PPD proteins.

1.5 Retrotransposons

Transposons are mobile genetic elements that can jump around in the genome. Since Barbara McClintock discovered “jumping genes” in the maize genome, transposable elements have been identified in many organisms. Transposons and retrotransposons are frequent targets for epigenetic chromatin silencing, which leads to naturally occurring siRNAs (Lippman et al. 2003; Vastenhouw et al. 2003). Transposons are reactivated (Lippman et al. 2003) and may be mobilized when components of the RNAi pathway are disrupted (Vastenhouw et al. 2003; Kuhlmann et al. 2005). The transposon silencing and RNAi pathways not only share components with each other, but they also share key components with the pathway controlling transgene-induced cosuppression (Ketting and Plasterk 2000; Tabara et al. 1999; Dernburg et al. 2000).

1.5.1 Transposons in *Dictyostelium discoideum*

DIRS-1 and Skipper are two transposon families in *Dictyostelium discoideum*. DIRS-1, also called Tdd-1 (Cappello et al. 1985), occurs in 40 complete and ～200 incomplete copies in the genome. It consists of inverted and nonidentical long terminal repeats (LTRs) and three overlapping open reading frames encoding protein1, reverse transcriptase and a recombinase.

Skipper is represented in approximately 15-20 copies and is abundantly transcribed

(Leng et al. 1998). It contains three open reading frames (ORFs) with an unusual sequence organization. ORFs 1 and 3 correspond to gag and pol genes; ORFs 2, pro, corresponding to protease

1.6 Introduction of the Yeast-two-hybrid system

Protein-protein interactions are essential to cellular mechanisms at all levels in biologically responsive systems. The yeast two-hybrid method, introduced by Fields and Song (Fields and Song 1989) is a powerful technique to analyze these protein-protein interactions quickly and versatilely *in vivo*. This method is carried out in yeast cell. The proteins of interest are fused with GAL4 DNA binding domain and Activation domain respectively, once these two fusion proteins are co-expressed in yeast cells, and if they can interact with each other, the expression of reporter gene is driven by this functional pair, thus a change in yeast cell phenotype linked to protein-protein interaction(s) is observed. (Young 1998).

1.6.1 Mechanism of the Yeast-two-hybrid

The "Matchmaker" Two-Hybrid system by Clontech is based on the transcription factor GAL4 of *Saccharomyces cerevisiae*. The GAL4 protein consists of two protein domains that have specific functions: the DNA binding domain (DNA-BD), which binds to DNA by interacting with a specific upstream activator domain (UAS) of the DNA, and the activator domain (AD) that activates transcription of DNA.

DNA-BD and AD are separated genetically and expressed on two different plasmids without transcriptional activity. The GAL4 transcription factor is activated to start the transcription activity when the two domains are fused to proteins that can interact. In the two-hybrid assay, a "X" gene is cloned in frame with the DNA binding domain in the *bait* vector, which also codes for a selection marker in yeast. In the second, a cDNA library or a cDNA fragment in frame (protein "Y") is cloned into the *prey* vector fused with the activator domain. This vector codes for a different selection marker. In this research, the cDNA library represents all

transcribed mRNAs from the vegetative stage of *Dictyostelium discoideum* and thus offers to investigate all protein-protein interaction that protein "X" is engaged in. If the protein X interacts with protein Y, the binding of these two will form an intact and functional transcriptional activator (TA) (Fields and Song 1989). The GAL4 BD in this newly formed TA interacts with the promoter, the AD interacts with RNA polymerase, thus the TA transcribes a reporter gene (**Fig 1-5**). If proteins X and Y do not interact, the system remains silent (**Fig.1-5**)

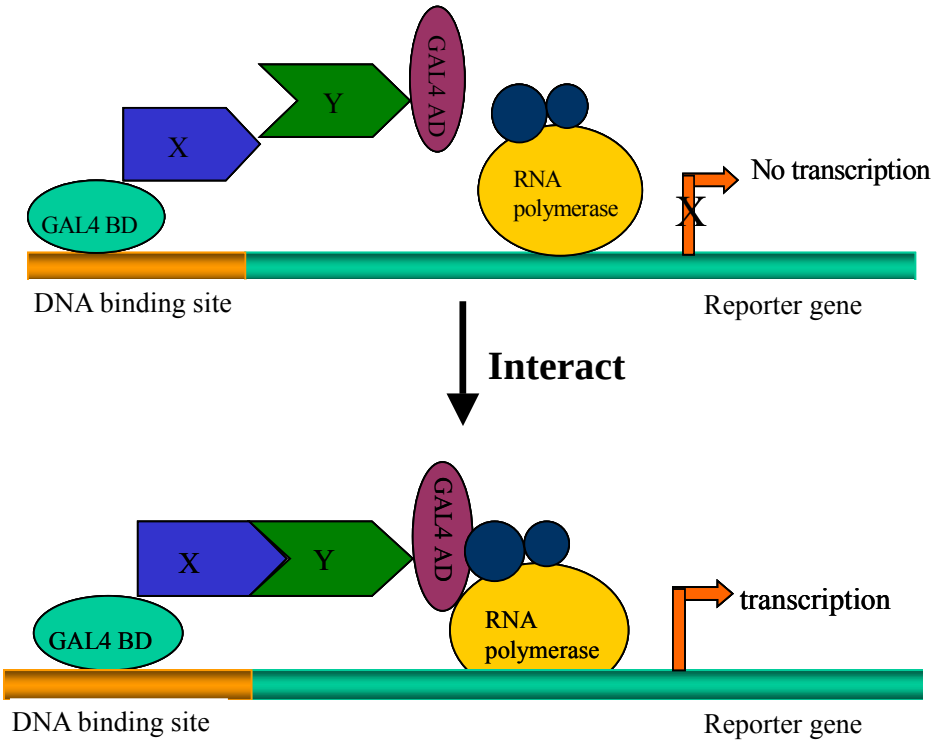


Fig.1-5 The principle of yeast-two-hybrid system. The proteins X and Y of interest are fused to GAL4 BD (binding domain) and AD (activation domain) respectively. Once these two fusion proteins are co-expressed in yeast, if they can interact with each other, the reporter gene is transcribed; otherwise the reporter gene is silent.

1.6.2 Reporter system

The interaction of the “X” protein and the translation products of the cDNA library can be detected by the lacZ reporter system. The lacZ gene is under the control of the GAL1 promoter which itself is stimulated through GAL4. If a protein from the cDNA library can interact with bait protein, the GAL4 function is restored and the transcription of the reporter gene is stimulated so that the positive interaction is

observed by the activity of β-galactosidase as blue coloured yeast colonies.

1.7 Aims

Since 1990, when horticultural researchers tried to create more purple petunias, but achieved an unexpected opposite result, first indications of the phenomenon, later called RNA interference (RNAi) were obtained (Napoli et al. 1990). Now RNAi is known to operate in humans, mice and other mammals, as well as in fungi, flies, and plants (Fire et al. 1998; Hutvagner and Zamore 2002; Tijsterman et al. 2002). This mechanism is widely used as a powerful tool to study gene function by generating gene "knockdowns" in various cell types. There are hopes to be able to treat animal and also human diseases, such as cancer or HIV by RNAi.

RNAi can effectively and specifically suppress gene expression, but many results showed that suppression is transient or unstable or the target gene is just partially silenced. In order to resolve these problems, it is necessary to study the mechanism of RNAi in details as it is done in different model systems, such as mice, flies, fungi, plants and humans. *Dictyostelium discoideum* is a powerful system for basic biomedical research in cell and developmental biology, and it is a new model organism for studying epigenetic gene silencing. The organism has unique advantages for studying fundamental cellular processes.

Known from many studies, Argonaute proteins are key components in RNAi pathway and function directly in cleavage of the mRNA as slicer, or play a role in chromatin modification, in control of stem cell differentiation, and in tissue development in different organisms, such as human, mice, flies, worms, plants (Liu et al. 2004; Caplen et al. 2002; Carmell et al. 2002). The functions of Argonaute proteins in *Dictyostelium* cells have not been investigated yet, and the RNAi mechanism in *Dictyostelium* is not fully understood.

The aim of this study was to investigate the function of *Dictyostelium* AgnA and to compare it to known Argonaute proteins from other organisms, particularly with respect to RNAi in *Dictyostelium*. To understand the cellular function of the protein, also other aspects, such as protein interaction partners, growth, development, phagocytosis, and the influence on other *Dictyostelium* Argonaute proteins were of interest.

2. Results

Members of Argonaute protein family have been found to be genetically required for RNA silencing in all organisms where their function has been studied, but the exact role of this family has generally not been determined (Buchon and Vaury 2006). The function of the Argonaute family in *Dictyostelium* has not been reported yet. To investigate the function of Argonaute proteins in *Dictyostelium discoideum,* the Argonaute A is characterized and functionally studied in this thesis.

2.1 Characterization of Argonaute proteins in *Dictyostelium discoideum*

2.1.1 The *D. discoideum* genome encodes five Argonaute genes

In *Dictyostelium* genome there are five argonaute proteins identified by the amino acid sequence of PAZ and Piwi domains in blast searches, named as AgnA, AgnB, AgnC, AgnD, and AgnE. Additionally, a small open reading frame named as AgnF that encodes a PAZ domain only was also identified. As all Argonaute proteins in other organisms, the PAZ domain of the *Dictyostelium* localizes centrally, and the Piwi localizes to the C-terminal (**Fig. 2-1**).

Fig.2-1 Schematic representation of functional domains of Argonaute proteins in *Dictyostelium discoideum* Ax2 strain

Tab. 2-1 characterization of Argonaute proteins in *Dictyostelium* cells

Gene name	Gene ID	Chromosome location	Length (aa)		
			Full length	PAZ	Piwi
AgnA	DDB0220136	2	979	84	296
AgnB	DDB0220437	5	900	123	296
AgnC	DDB0220438	2	1208	83	298
AgnD	DDB0233052	3	1295	85	219
AgnE	DDB0220439	5	1200	167	299

http://www.genedb.org/genedb/

2.1.2 The expression of Argonaute proteins in *Dictyostelium*

To investigate the expression of these five Argonaute genes, specific primers on oligo-dT primed cDNA were used for RT-PCR. All five genes are expressed, but the expression level of AgnC is lower than for the other genes and cannot always be detected. AgnA expression levels were also lower than those of AgnB, AgnD and AgnE as determined by RT-PCR (**Fig 2-2**), and the expression on RNA level is not detectable by Northern blot (data not shown). The expression of the thioredoxin gene family is shown for comparison (**Fig 2-2**).

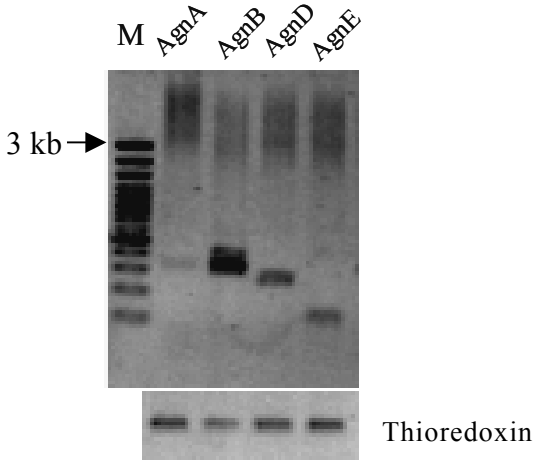


Fig.2-2 The expression of Argonaute genes in *Dictyostelium discoideum* by semi-quantitave RT-PCR The total RNA was prepared from the wild type Ax2 cells at the density of $1x10^6$ cell/ml, the 26nt oligo-dT primed cDNA was used for RT-PCR. The primers used to amplify AgnA,B,D,E are AgnAi sense 5', PPW3'; #790 agnb2, #791 agnb3; #798 agnd2, #799 agnd3; #802 agne2, #803 agne3 respectively. The thioredoxin gene is amplified with #688 Trx-1F and #689 Trx-1R primers as comparison for cDNA quantity and genomic DNA contamination.

2.1.3 The subcellular localization of PAZ-PiwiAgnA (PPWa) in *Dictyostelium* cells

The PAZ-Piwi domain of AgnA is the first Argonaute like homologue from *Dictyostelium* to be cloned. To determine the subcellular localization of the PAZ-Piwi domain in *Dictyostelium discoideum* cells, a GFP fused protein was constructed and overexpressed in *Dictyostelium* vegetative cells (**Fig.2-3A**). The pDd-GFP expression vector encoding green fluorescence protein (GFP) was used to

construct the PAZ-PiwiAgnA fused with GFP C-terminally (**Fig.2-3A**). The pictures (**Fig.2-3 B, C**) show that the PAZ-Piwi domain localizes to mainly distinct spots in the cytoplasm of the cells in the vegetative stage.

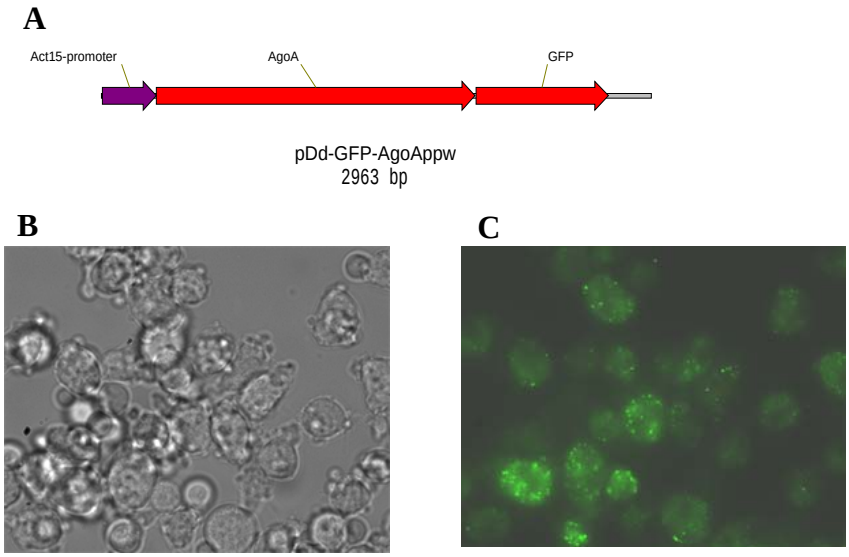
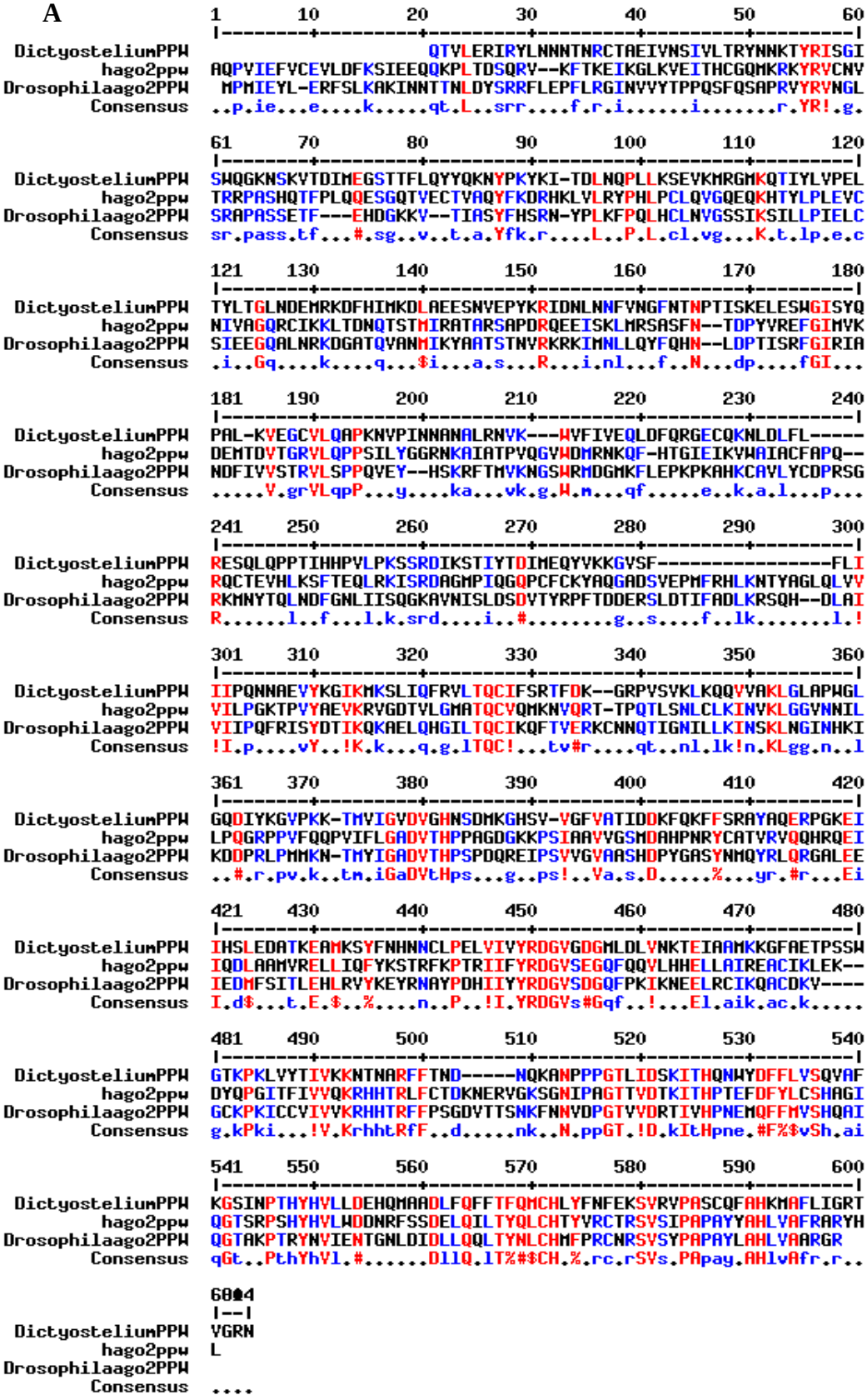


Fig.2-3 Over-expression of PAZ-PiwiAgnA fused with GFP in *Dictyostelium* vegetative cell. (A) The construct of the PPWa-GFP. The PPWa cDNA fragment was amplified with PPW-GFP 5' and PPW-GFP 3' primers, cloned into pGem T-easy vector, subsequently cloned into pDd-GFP vector with digestion of BamHI/ EcoRI.
(B) Contrast of PPWa-GFP expressed cells. **(C)** Image of PPWa-GFP fluorescent cells.

2.1.4 The similarity of PAZ-PiwiAgnA to PAZ Piwi domains in other organisms

The *Dictyostelium discoideum* agnA gene defines a putative 3.1kb open reading frame encoding a 979 amino acid protein with a predicted molecular weight of 111.5kDa. The PAZ-Piwi domain defines as a 527 amino acid open reading frame. As mentioned before, human AGO2 (hago2) and *Drosophila* AGO2 (dago2) function as slicer in RNAi pathway, especially the PAZ and Piwi are the main functional domains in RNAi mechanism. An alignment is done to compare the identity of *Dictyostelium* PPWa to human and *Drosophila* AGO2 PAZ Piwi domain. The *Dictyostelium* PAZ domain shows 17% identity to human orthologous, and 18% to *Drosophila* orthologous (**Fig.2-4A, B**). The *Dictyostelium* Piwi domain shows higher identity (36%) to human Piwi domain. (**Fig.2-4C**).


A
DictyosteliumPPW
hago2ppw
Drosophilaago2PPW
Consensus

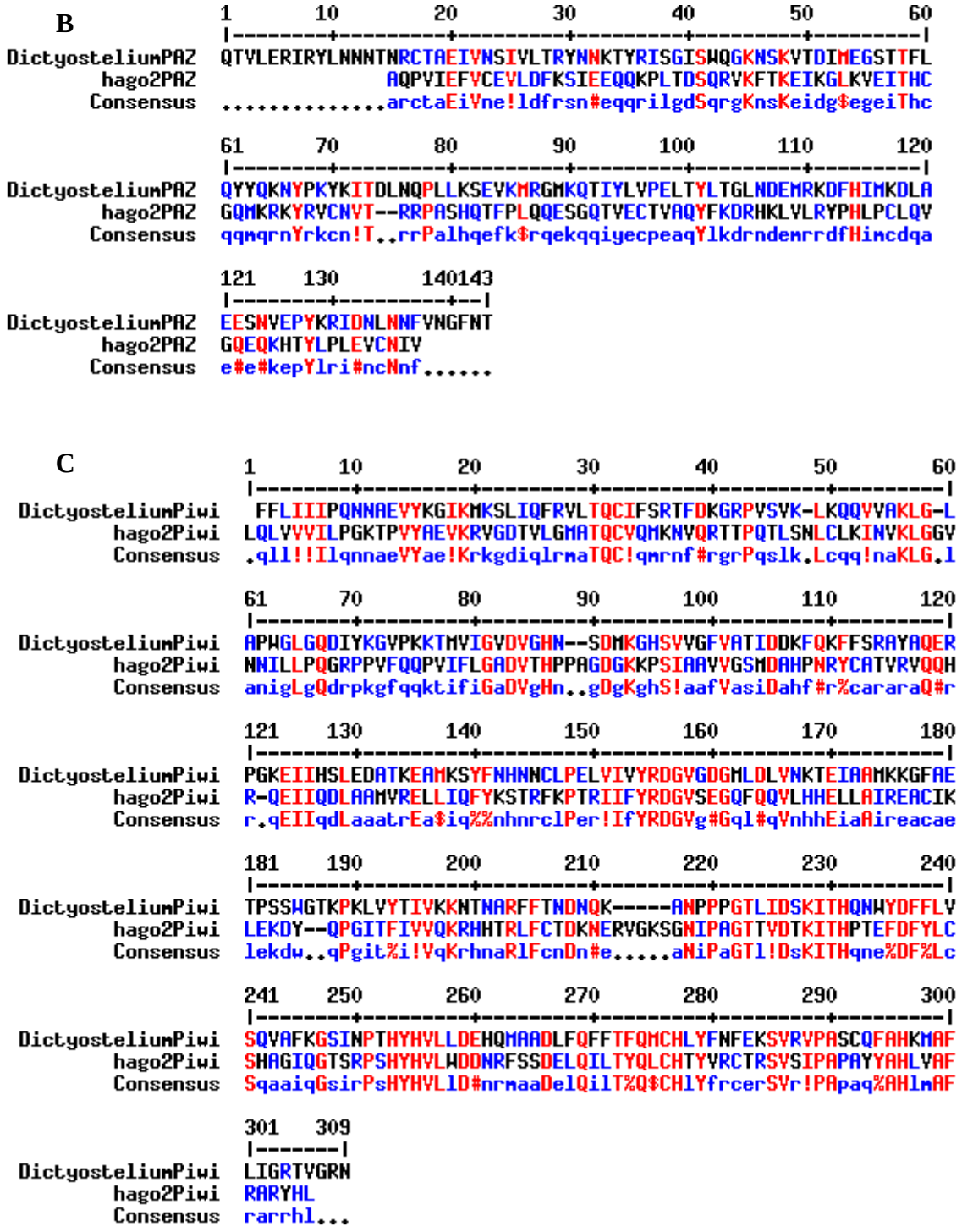


Fig.2-4 Multiple alignment of PAZ Piwi from different organisms.

A. Comparison of PAZ Piwi from *Dictyostelium discoideum* (DDB0220136), *Drosophila melanogaster* (DQ228772), and *Homo sapiens* (gi|38372888).

B. Alignment of PAZ domain from *Dictyostelium discoideum* (DDB0220136) and *Homo sapiens* (gi|38372888). **C**. Alignment of Piwi from *Dictyostelium Discoideum* (DDB0220136) and *Homo sapiens* (gi|38372888).

Red: identity. Blue: similarity. MultiAlign tool is used to perform the alignment. (http://prodes.toulouse.inra.fr/multalin/multalin.html)

2.1.5 The effect of PAZ-Piwi-AgnA on development

The strain expressing GFP fused PAZ-PiwiAgnA is used in this study to investigate whether the ectopic overexpression gives rise to any putative phenotypic abnormalities. There is no obvious difference between Ax2 and overexpression strain in the vegetative cells, as demonstrated by identical growth velocity and cell morphology. In contrast to this, development seems delayed in the overexpression strain compared to wild type (**Fig. 2-5**). Both cell lines are allowed to develop for the same time period. When Ax2 formed already fruiting bodies, most cells in the overexpression line still stayed in the first finger stage (**Fig. 2-5A, B, C, D**). Comparing with the wild type, the overexpression strains show smaller and thinner stalks and fruiting bodies **(Fig. 2-5B**, **E**), and the cell aggregates look less transparent **(Fig. 2-5B**, **D**).

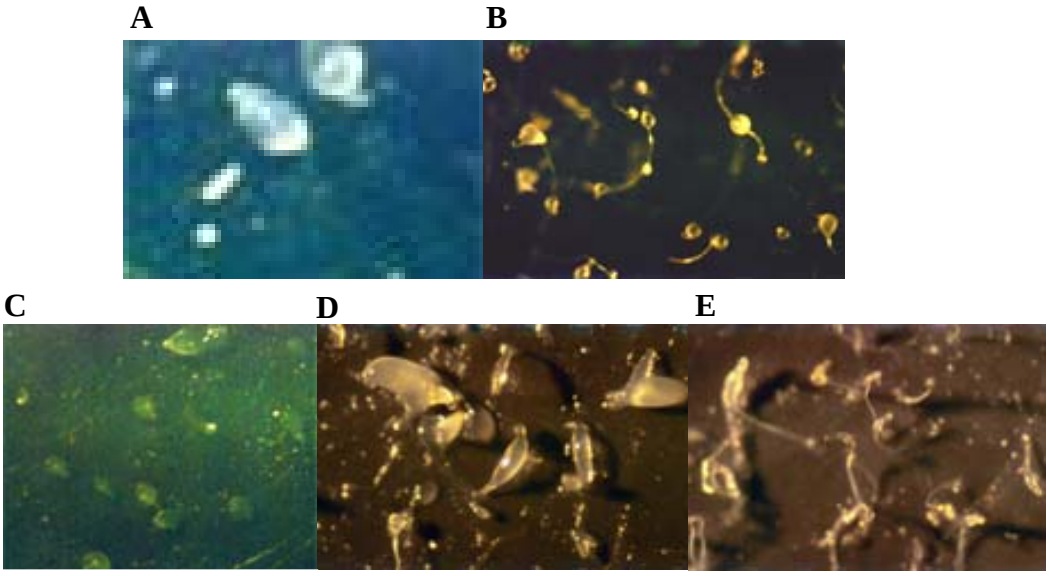


Fig. 2-5 The developmental course of Ax2 and PAZ-PiwiAgnA overexpressor. Development of Ax2 strain after 16 hrs **(A)** and 18 hrs. **(B).** Development of PPWa-GFP overexpressor after 16 hrs **(C)** and 18 hrs (**D, E).** The pictures of **(A)** and **(C)** were taken with the same camera settings, and **(B)**, **(D)**, **(E)** are with the same settings.

These experiments were repeated several times and the described phenotypes shown in **Fig.2-5C-E** were observed repetitive occasions, but not always. The reason for this might be different levels of ectopic expression.

2.2 The Yeast-two hybrid to screen interaction partners of PAZ-PiwiAgnA *in vivo*

Since Argonaute proteins are involved in RNAi in many organisms, is Argonaute A required for RNAi in *Dictyostelium discoideum* and how? First, my research aims at finding the proteins that can interact with Argonaute A in *Dictyostelium discoideum* at the vegetative stage. To analyse systematically protein-protein interactions, most biochemical analyses of such interactions are carried out *in vitro*, thus under non-physiological conditions and are sometimes of limited value. A more systematic approach is the yeast two-hybrid system developed by Fields and Song (1989). This genetic approach, that also allows the detection of short-lived protein-protein interaction, is based on the function of eukaryotic transcription factors and allows a systematic analysis.

2.2.1 Bait and prey protein

In this research, the PAZ-Piwi domain of Argonaute A protein from *Dictyostelium discoideum* is used as the bait protein by cloning into the bait vector so called pGBKT7.

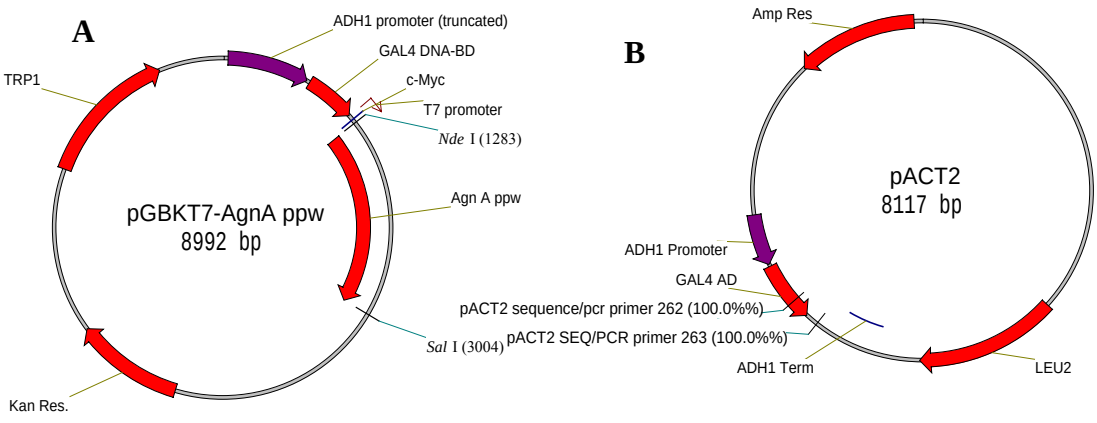


Fig 2-6 The constructs of bait vector (A) and prey vector (B). The PAZ PiwiAgnA is cloned into pGBKT7 vector by digestion of pGMT-easy-PPWa with SalI/NdeI. The PPWa cDNA fragment in pGMT-easy is amplified with PPWa5' and PPWa3' primers with the standard PCR program. The conversion of a library in λ YES-R into a pACT2 vector see methods part.

The *Dictyostelium discoideum* cDNA library from vegetative stage is used as prey protein to screen for the positive interactions by cloning into the prey vector called pACT2 vector (**Fig. 2-6**). Only cells that contain both plasmids can switch on the reporter system.

2.2.2 Verification of positive clones by different methods

The positive interactions can be identified through a β-galactosidase assay. However, also unspecific interactions might result in blue colonies. There are some tests including colony growth rate and size, more stringent growth conditions used to verify initially the specific and strong interaction between bait and prey protein. Further, some experiments, which include digestion of the prey vector from positives yeast clones, PCR on the prey vector with specific primers primed to the specific sites on pACT2, and digestion of PCR products are used to verify positive clones and eliminate duplicates.

2.2.2.1 Select co-transformants with selective medium

The SD triple dropout medium (trp-, leu-, his-) is used for the doubly transformed cells to preclude untransformed cells or those that only contain the bait vector. A further selection step can be carried out by applying 3-Amino-1,2,4-trizol (3-AT) to SD triple dropout medium. The 3-AT functions on some reporter yeast strains as competitive inhibitor of the yeast HIS3 gene product (Bartel et al. 1993). This leads to a further reduction of the already weak expression of the His3 gene via the TATA-Box (TC). By this competition, the growth of the non-interaction colonies is slowed down.

In my experiments, the Triple drop out medium, so called SD medium without Trp, Leu and His is used for verifying the initial co-transformants. The first coming out clones with big size are picked up to the stringent SD medium that contains 50mM 3-AT to eliminate weak interactions.

2.2.2.2 β-galactosidase assay by colony-lift filter

Clones grown on the stringent SD medium are picked up to a master plate. After two or three days growth, the β-galactosidase assay is performed by colony-lift to observe the activity of β-galactosidase which is stimulated by the interactions between bait and prey protein (**Fig. 2-7**).

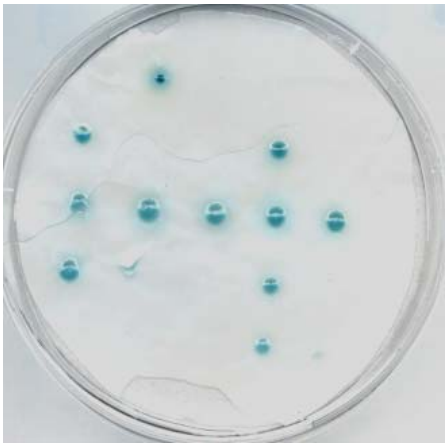


Fig.2-7 β-galactosidase assay verifies positive co-transformants which contain interacting bait and prey proteins. All clones are picked up from 50mM 3-AT SD plates, only several clones can activate reporter system.

2.2.2.3 Analysis of Yeast plasmid inserts by digesting the prey vector

In order to analyze the interaction partners of the bait protein, the first is to check the recombination of the gene from cDNA library to the prey vector. Because of the relatively large size (>6kb) and the low copy number (about 50/cell) of some yeast plasmids, the DNA yields as low. In addition, plasmid DNA from yeast is often contaminated by genomic DNA because yeast contains about 3 times as much genomic DNA as *E. coli*. It therefore is recommended to transform the yeast plasmid to *E. coli* for working. Since the bait and prey vectors have different selective markers, e.g. the bait is kanamycin resistant, and the prey is resistant to ampcillin, these two vectors can be selected by different medium after transformation into *E. cloi*. To analyze the inserts of the prey vector, the restriction digestions can be used to check whether the plasmids are truncated or not. As shown in **Fig2-8A,** the colony marked as 021, 138, 144 have truncated plasmid DNA which should give 7.7+0.758kb bands when digested by HindIII. The complete plasmid DNA shows

different digestion patters comparing with the pACT2, and different plasmid DNA shows different digestion pattern (**Fig2-8B**). The Nr.131, 171 and 211 show the same digestion pattern but different from the others. It is assumed that Nr.131, 171 and 211 might be duplicates.

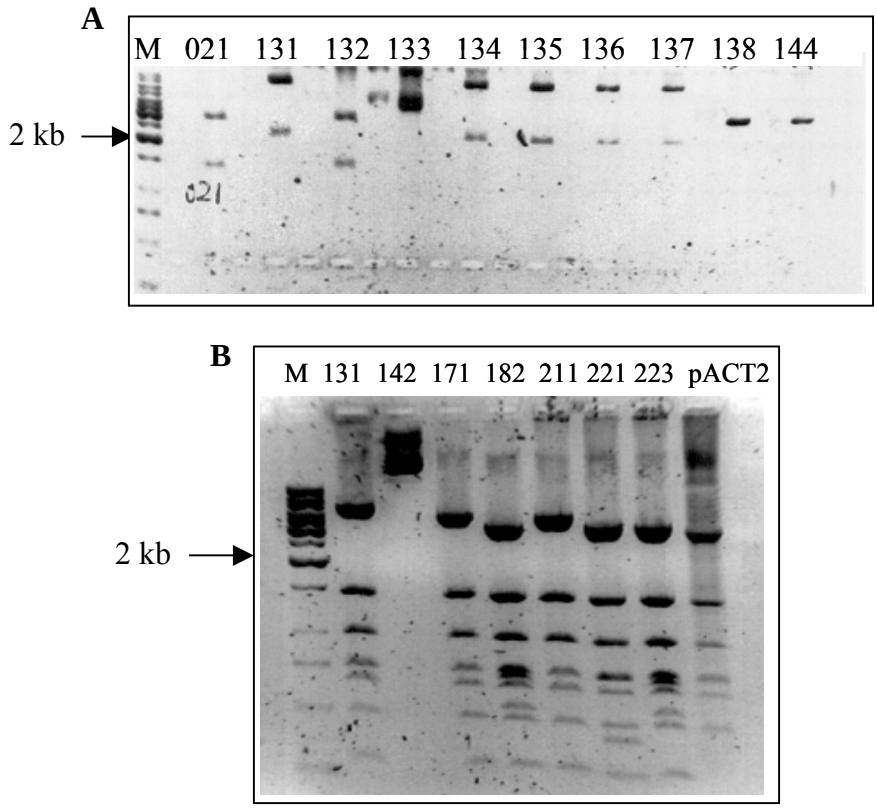


Fig. 2-8 Digestion of plasmid DNA of prey from different clones with (**A**) HindIII and (**B**) HincII. The numbers present different samples.

2.2.2.4 Analysis of Yeast plasmids insert by PCR

Sometimes a two-hybrid library screening results in many, even hundreds of positive candidate clones. However, a few abundant insert sequences may account for the majority. The cDNA inserts from all plasmids encoding candidate interacting proteins can be amplified by PCR and sorted into groups based on the restriction digestion patterns. After colonies have been sorted, a representative clone from each group can be transferred to a new master plate for further analysis. The primers flanking the multiple cloning sites in the pACT2 prey vector are used to amplify the inserts (**Fig.2-9A**). By comparing the PCR products with pACT2 control, it enables

to exclude some false positives, such as Nr. 223 which has no cDNA inserts (**Fig.2-9B**) or which has smaller inserts than pACT2 control (data not shown).

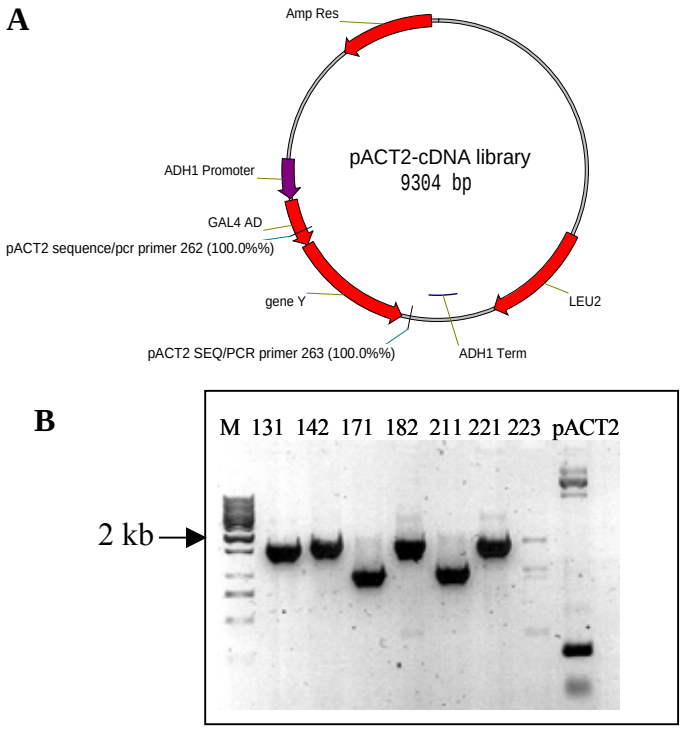


Fig.2-9 Analysis of yeast plasmid inserts with PCR from different colonies. A. Map of prey vector. **B.** PCR on prey plasmid DNA from differnt blue colonies with indicated primers on the map **(A)**.

2.2.2.5 Analysis of Yeast plasmid inserts by digesting the PCR products

By the digestion pattern of different PCR products with a frequent-cutter restriction enzymes, such as AluI or HaeIII, the inserts in the pACT2 vector can be sorted into different groups as shown in **Fig.2-10**.

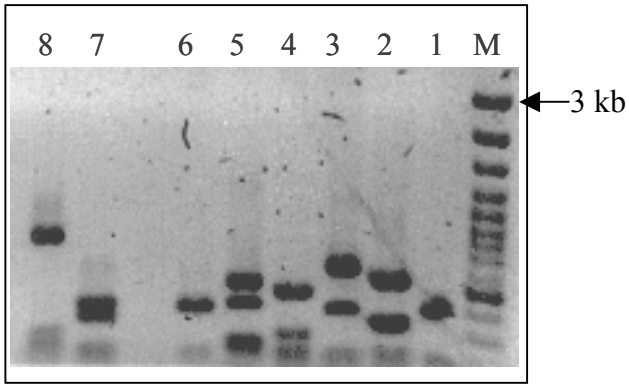


Fig.2-10. Digestion of PCR products from different clones with AluI.

8 PCR products are digested with AluI, and every one shows different digestion

pattern (**Fig. 2-10**). This result indicates that these 8 inserts are from 8 different genes.

2.2.2.6 Elimination of false positives by setting up different controls

The Yeast two-hybrid system, although it is a highly useful system to study protein and protein interaction, has many limitations. This system presents low throughput, high rates of false positives, low detection levels and poor expression stability. In order to eliminate the false positives, a series of controls were set up to exclude the false interactions which were caused from the binding domain and activation domain. In this research, the prey plasmids from putative positive yeast clones are transformed into yeast containing empty pGBKT7 vector or pGBKT7-PPWa vector individually, and then the β-galactosidase assay is re-performed.

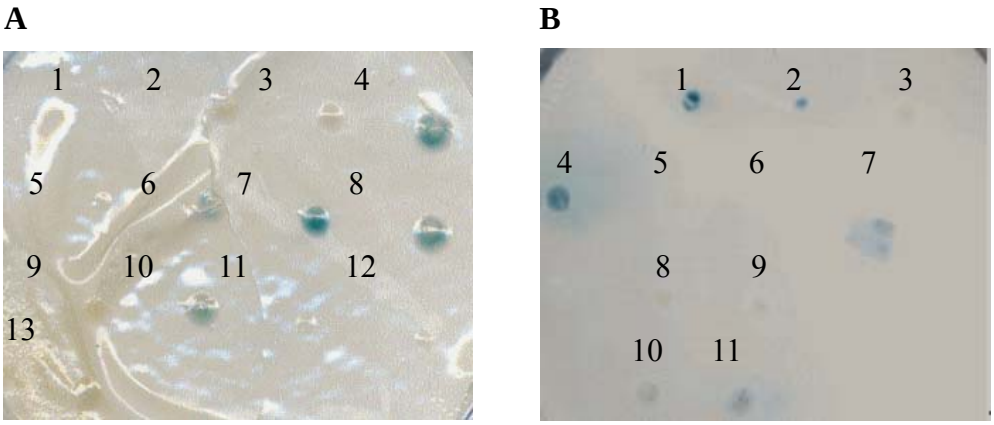


Fig.2-11 Control experiments to eliminate false positives by β-galactosidase assay. Plasmids from prey are transformed into both pGBKT7 and pGBKT7-PPWa individually, and then perform the β-galactosidase assay.
P=pGBKT7, W=pGBKT7-PPWa, the numbers and letters following P and W present different prey plasmids.
A:1=Pc17, 2=Pc17, 3=Pc6B, 4=Wc6B, 5=Pc26, 6=Wc26, 7=Wc7-1, 8=Wc7-1, 9=Pc24, 10=Wc24, 11=Wc3, 12=Wc7-2, 13=Wc29
B:1=W34, 2=W56, 3=Pc7-1, 4=Pc10, 5=Wc10, 6=Pc23, 7=Wc23, 8=Pc7-2, 9=Pc22, 10=Pc29, 11=Pc3, 12=Wc14, 13=Wc17. W34=pACT2

The pictures (**Fig.2-11**) show that the reporter gene cannot be activated when the bait cannot interact with prey (**Fig2-11A**1, 2), but it can be activated by the interaction between GAL2 DNA binding domain and activation domain in false positive clones (**Fig2-11B**4, 5). In real positive clones, the reporter gene is only

activated when the bait and prey proteins can interacts, but keeps silent in clones containing empty bait vector and prey protein (**Fig2-11A** 3,4; 5,6; 9,10). The results show that the experiments are suitable to eliminate false positives.

2.2.2.7 Sequence

The prey plasmid DNA from real positive clones is sequenced to get information about the interaction proteins. For the optimal results, it is recommended to sequence the plasmid DNA prepared from DH5α *E. coli* cells.

2.2.3 Screening for interaction proteins of *Dictyostelium* PAZ-PiwiAgnA protein

2.2.3.1 Expression of PAZ-PiwiAgnA in yeast

In order to screen the interaction partners of *Dictyostelium* PPWa protein by Yeast-two-hybrid system, the PPWa protein must be fused into DNA binding domain of bait vector and the fusion protein must be expressed correctly in yeast.

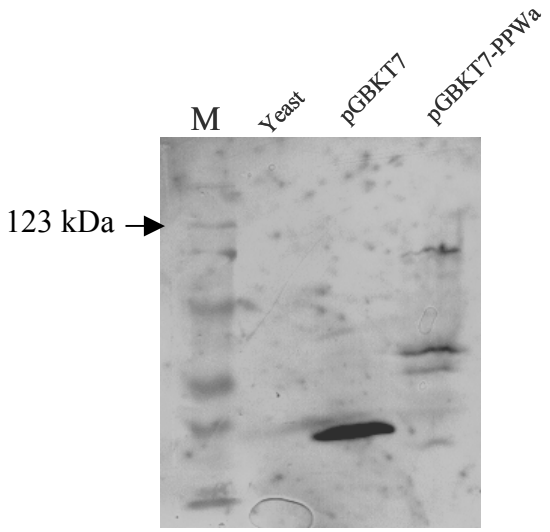


Fig. 2-12 Western blot analysis of PPWa expression in Yeast. The protein extract from wild type yeast is used as negative control, the protein from the strain containing pGBKT7 is used as comparison. The expression of proteins is detected with c-myc antibody.

To get the PPWa fusion protein, the pGBKT7-PPWa construct (**Fig.2-6A**) is transformed into yeast strain, after several days growing, the protein is extracted and

a western blot is performed to check the expression of fusion protein. The western blot result shows that PPWa fused into DNA binding domain and is expressed in the correct size in yeast strain (**Fig.2-12**).

2.2.3.2 Control experiments and screen statistics to verify the Yeast-two-hybrid with *Dictyostelium* cDNA library

The yeasts containing different bait and prey constructs are grown on different selective medium (**Table 2-2**). The results show that the SD medium is suitable for selecting the co-transformants, while the SD containing 3-AT medium is suitable for selecting the co-transformant comprising the bait protein and the prey protein. This indicates that SD containing 3-AT medium might exclude some false positives during screen. PPWa and *Dictyostelium* cDNA library are not auto-activated.

Table2-2 Control experiments to detect yeast-two-hybrid

Constructs		Selection by	
Bait	**Prey**	**SD**	**SD+50mM 3-AT**
pGBKT7	-	-	-
pGBKT7-PPWa	-	-	-
-	Library	-	-
-	PACT2	-	-
pGBKT7-PPWa	PACT2	+	-
pGBKT7-PPWa	Library	+	+

(+) growth, (-) no growth

Table2-3 Statistic Results of co-transformation of PPWa and *Dictyostelium* library

		Library	
		50 (µg)	**250 (µg)**
Colony Nr.	SD	181	700
	SD+30mM 3-AT	11	60
	X-gal assay	9	42
	PCR	6	31
Colony %	3-AT% of all	6.1	8.57
	Blue% of all	5	6
	Blue% of 3-AT	81.8	70
	Positive% of blue	66.7	71

Different amounts of cDNA library are transformed into the yeast containing pGBKT7-PPWa. The co-transformants are grown on SD medium, the resulting clones are selected by SD containing 3-AT medium again, and the successful clones are tested by X-gal assay and PCR analysis. The statistic results (**Table2-3**) prove that selection by SD containing 3-AT medium, X-gal assay and analysis of prey plasmids with PCR stepwise are feasible methods to verify positive clones of the first step.

2.2.3.3 Sequencing results from Yeast-two-hybrid

After having transformed 63.9 x 10^8 cells and screened 900 clones, the DNA inserts from putative positive clones are sequenced. From all the selected clones of the yeast-two-hybrid, 27 sequence data are grouped (**Table 2-4**) according to the nucleotide bases.

Table 2-4 Sequence results from yeast-two-hybrid

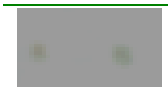
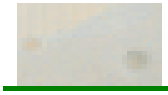
Sample	Prod. ID	Descriptions	Hits	E
241	Gi167815	Ribosomal L3	6	0.0
031	gi2251091 (DDB0055953)	COX3 cytochrome c oxidase subunit III	4	0.0
111	Gi1262182	pACT2	3	3e-90
401	gi7200	Act8	3	0.0
56	DDB0189856	SahA (s-adenosyl-L-homocystein hydrolase)	2	0.0
081	DDB0206002	Esterase_Lipase (Acetylcholinesterase)	2	0.0
211	gi2251091	NADH dehydrogenase	2	7e-86
142	DDB0185998	ERG4_ERG24 (Lamin B receptor)	2	0.0
271	DDB0189379	JC1V2_0_00171	2	0.0
201	DDB0218659	BC4V2_0_01043	2	0.0
331	DDB0216319	mitochondrial large subunit rRNA	3	0.0
A8	DDB0187464	RANBP1	2	e-159
071	gi4958873 (DDB0075694)	COX1 cytochrome c and Quinal oxidase polypeptide I	1	e-159 (0.0)
121	gi41059705	abpE	1	e-128
131	DDB0186654	Nop	1	0.0

261	Gi167787	Elongation factor II	1	0.0
411	gi1688325	IfdA(RNA helicase)	1	e-123
421	gi7274	EF1-I gene for elongation factor 1 alpha	1	0.0
A1	DDB0186729	Ribosomal L18a	1	0.0
A40	Gi167579	Dicty Act15	1	e-111
161	Gi1788975	E.coli K12 MG1655	1	e-158
C17	DDB0189295	BC5V2_0_01967	1	0.0
C24	DDB0230070	S-adenosylmethionine synthetase	1	0.0
C6B	DDB0167376	gpdA	1	0.0
C23	DDB0190927	UAE1	1	0.0
31	DDB0185022	capA	1	0.0

2.2.3.4 Candidate genes from Yeast-two-hybrid

To eliminate false positives, a series of control experiments are carried out to screen the real interacting proteins. The prey vectors comprising cDNA inserts (pACT2-gene) are transformed into the yeast strains containing bait (pGBKT7-PPWa) and empty bait (pGBKT7) vectors individually, and the co-transformants are analyzed by the X-gal assay again. Only the colonies turning blue which are co-transformants of pGKT7-PPWa and pACT2-gene are regarded as positive interactions. The results (**Table 2-5**) show that 11 proteins from *Dictyostelium* vegetative cells can interact with PAZ-PiwiAgnA. Excluded are UAE1, capA, gpdA, COX3, BC5V2_0_01967, three candidate genes ifdA, sahA, samS are interesting for research. Being the translation initiation factor 4A, IfdA is considered taking part in protein synthesis, and the similarity to RNA helicase is supposed to unwind RNA in RNAi pathway. SahA and SamS are two enzymes involved in SAM metabolism, which indicate that AgnA may influence DNA methylation through the interactions with these two enzymes.

Table 2-5 candidate genes from yeast –two-hybrid

Colonies[A]	Gene Name		Function
	efbA1 (EF1-I)	Elongation factor1-I	Protein synthesis
	efbA (EF2)	Elongation factor 2	Protein synthesis
	ifdA	Translation initiation factor 4A (similar to ATP-dependent RNA helicase)	Protein synthesis, nucleic acid binding
	RANBP1	Ran binding protein 1	Cell signaling
	sahA	s-adenosyl-L-homocystein hydrolase	SAM metabolism
	samS	s-adenosylmethionine synthetase	SAM metabolism
	UAE1	ubiquitin activating enzyme E1	
	capA	cAMP-binding protein	
	BC5V2_0_01967		
	gpdA	glyceraldehyde-3-phosphate dehydrogenase	NAD binding
	cox3	cytochrome oxidase subunit 3	

[A]The left colonies on the pictures are the co-transformants of pGBKT7 and pACT2-gene, the right colonies on the pictures are the co-transformants of pGBKT7-PPWa and pACT2-gene. The genes in pACT2 are shown on the Gene Name column.

2.2.4 Researches on sahA, samS, and ifdA candidate genes

2.2.4.1 Characterization of SahA, SamS, IfdA

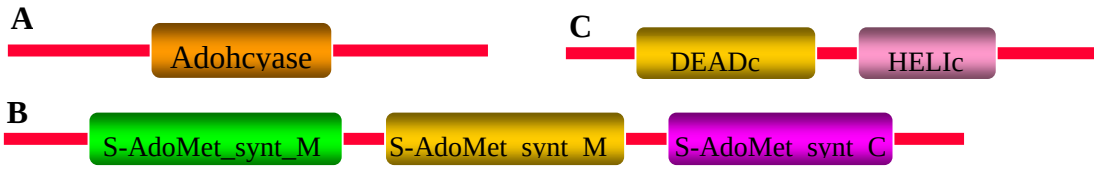


Fig.2-13 Schematic representation of SahA (A), SamS (B), IfdA (C)

The functional domains of these three genes shown in **Fig 2-13** are based on the blasting results from blast tools (http:// www.ncbi.nlm.nih.gov/blast/).

Sequences of sahA, samS, and ifdA genes were blasted by NCBI blast tools to obtain insight of gene details.

SahA:

SahA is highly similar to mammalian SAHH (AdoHyc hydrolase) (**Fig. 2-13 A**). It functions in catalyzing the hydrolysis of S-adenosyl-L-homocysteine into adenosine and homocysteine.

SamS:

SamS has the methionine adenosyltransferase activity (**Fig. 2-13 B**), which catalyzes the formation of S-adenosylmethionine (AdoMet) from methionine and ATP.

ATP + L-methionine + H2O = phosphate + pyrophosphate + S-adenosyl-L-methionine

The S-adenosyl-methionine is an important methyl donor for transmethylation and is also the propylamino donor in polyamine biosynthesis

IfdA:

IfdA is highly homologous to *Arabidopsis* translation initiation factor 4A which is the component of the translation initiation factor 4F complex. IfdA is similar to ATP-dependent RNA helicase, which comprises the DEAD-box and Helicase superfamily c-terminal domain (HELICc) (**Fig 2-13C**).

DEAD-box helicases are a diverse family of proteins involved in ATP-dependent RNA unwinding, needed in a variety of cellular processes including splicing, ribosome biogenesis and RNA degradation.

Helicase superfamily c-terminal domain is associated with DEAD-box protein, and is found in a wide variety of helicases and helicase related proteins. Some DEAD-box helicases have been shown to unwind nucleic acid duplexes utilizing the free energy from nucleoside triphosphate hydrolysis to fuel their translocation along

DNA, unwinding the duplex in the process.

2.2.4.2 Expression of SahA, SamS and IfdA in *Dictyostelium*

To analyze the expression of SahA, SamS and IfdA in *Dictyostelium*, RT-PCR reactions are performed on cDNA prepared from a vegetatively grown Ax2 strain. These three genes are amplified from one batch of cDNA shown by the thioredoxin control (**Fig.2-14**). The results from RT-PCR reactions show that SahA, SamS and IfdA are expressed in *Dictyostelium* vegetative cells, and the expression level is almost the same (**Fig.2-14**).

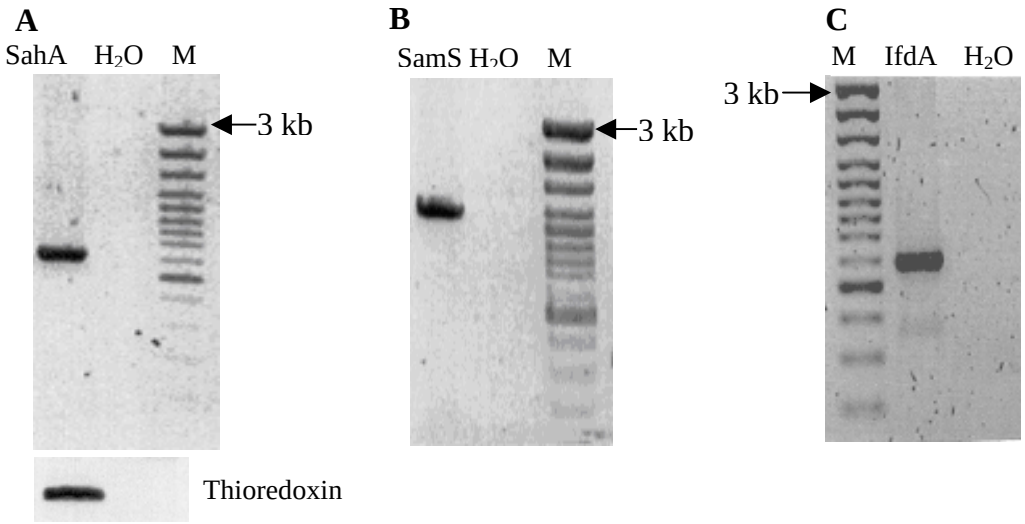


Fig. 2-14 The expression of SahA, SamS, and IfdA in *Dictyostelium* as monitored by RT-PCR. All RT-PCR reactions use the same cDNA prepared from *Dictyostelium* wild type cells, the thioredoxin gene is used as a control to evaluate the quality of the cDNA and genomic DNA contamination.(**A**): cDNA fragment of SahA is amplified with Saha RA 5' and Saha 3' BamHI primers by the standard PCR program; (**B**) cDNA fragment of SamS is amplified with SamS 5' and 3' primers. The elongation step uses 60℃ and 2 min insted of 72℃ in the standard program. (**C**) cDNA fragment of IfdA is amplified with ifdA RA5' and ifdA 3' BamHI primers by the standard PCR program.

2.2.4.3 Subcellular localization of SahA, SamS, IfdA in *Dictyostelium* cells

Since RT-PCR showed the expression of these three candidate genes in *Dictyostelium* Ax2 cells, their subcellular localization is investigated next by fluorescently labeled proteins. The pdneo-GFP vector encoding for N-terminal GFP fusion protein is used to construct the overexpression of SahA/ SamS/ IfdA in

Dictyostelium cells (**Fig. 2-15 A**). Western Blot using total protein extract of transformed *Dictyostelium* cells reveals that all three GFP-fused proteins (GFP-IfdA/ SahA/ SamS) are expressed in the expected size (**Fig. 2-15 B, C**). Thus, cells expressing the fusion proteins can be used to detect the protein localization in the cell by fluorescence microscopy.

The fluorescence microscope images (**Fig 2-16**) show that SahA and IfdA localize to the cytoplasm and nuclei, while SamS localizes to the cytoplasm only. Interestingly, some of the cells overexpressing GFP-ifdA display several nuclei as a phenotype (**Fig 2-16 B**).

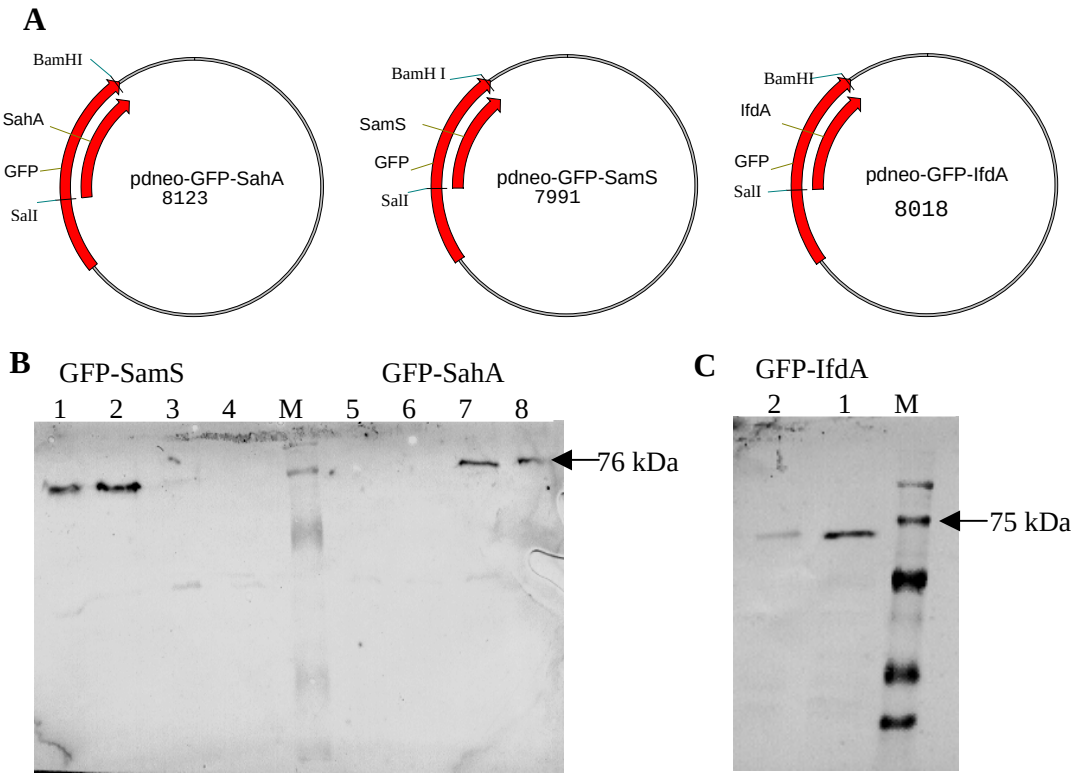


Fig. 2-1t Overexpression of SahA, SamS, and IfdA fused with GFP in *Dictyostelium* cells. (A) The constructs of SahA, SamS, and IfdA fused with GFP**.** All cDNA fragments of SahA, SamS and IfdA are digested from pet-SahA/ SamS/ IfdA plasmid DNA with BamHI/ SalI, then ligated into pdneo-GFP vector which is digested with BamHI/ SalI. **(B)** The western blot of GFP-SahA/ SamS/ (1-2, 7-8 are expression clones, 3-4, 5-6 are nonexpression clones) and (**C**) IfdA overexpressed in *Dictyostelium* cells. The GFP fused proteins are detected by GFP antibody. The expected molecular weight of GFP-SahA is 76kD, the GFP-SamS is 71kD, and the GFP-IfdA is 74kD.

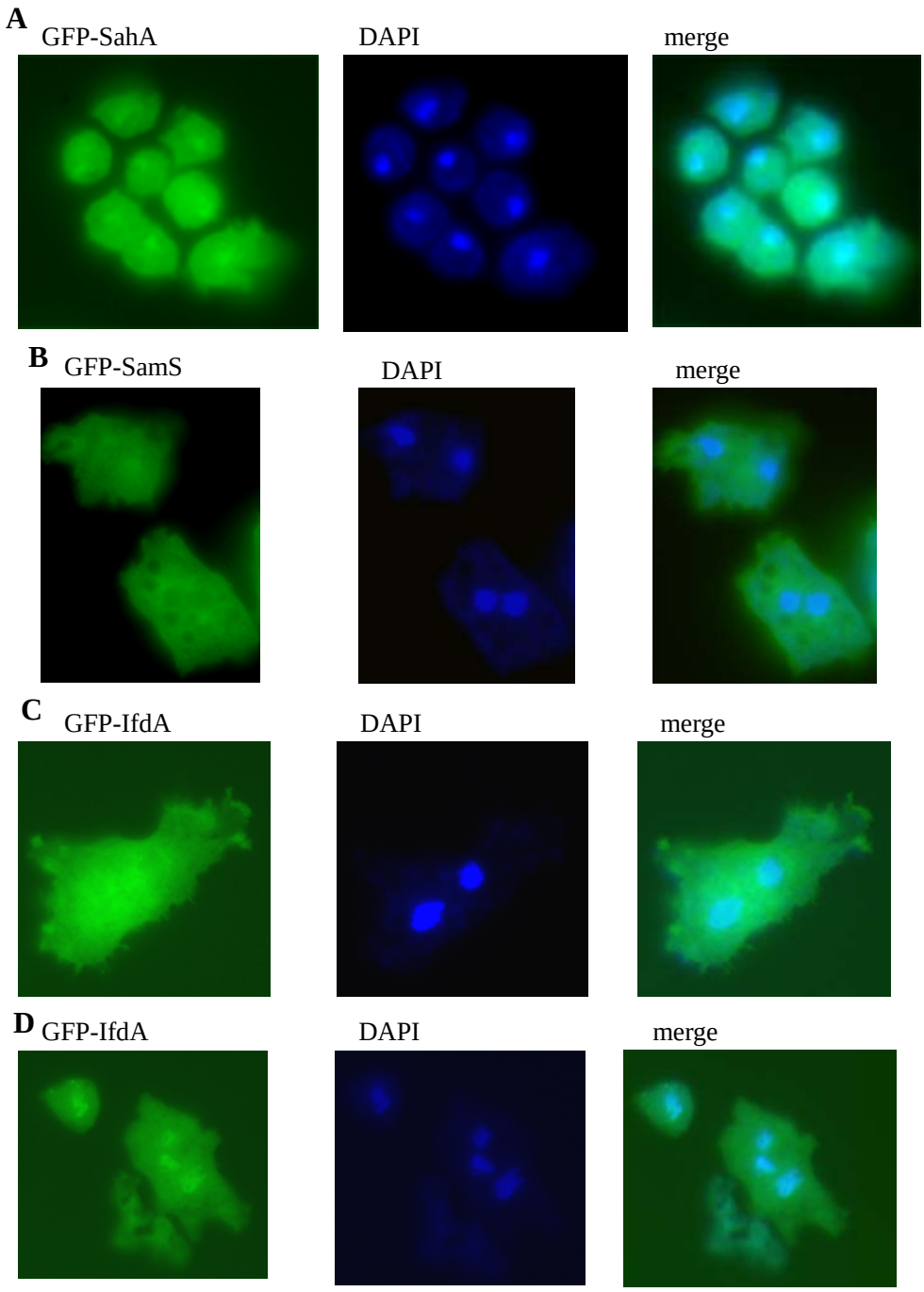


Fig.2-16 Subcellular localization of overexpressed SahA (A), SamS (B), IfdA (C, D) in *Dictyostelium* cells. SahA and IfdA localize to cytoplasm and nuclei, SamS is cytoplasmic. The GFP-IfdA (**D**) images show that there are several nuclei in IfdA overexpressed cells.

2.2.5 Attempt to knock out sahA, samS and ifdA genes using the pLPBLP cre-lox vector

To study the function of sahA, samS, and ifdA *in vivo*, knock out constructs for these three genes are created that are based on the pLPBLP cre-lox vector (**Fig. 2-17A**). The left arm (LA) of sahA is amplified with sahA LA 5' and sahA RA 3' primers, the right arm (RA) is amplified with sahA RA 5' and sahA RA 3' primers with the standard PCR program. Both fragments are cloned into pGem-Teasy vector, then the LA is digested with BamHI/ NdeI and ligated into pLPBLP cre-lox vector digested with the same enzymes; and the RA is digested with HindIII/ SalI and ligated into the formed pLPBLP cre-lox-LA vector digested with the same enzymes. The LA of samS is amplified with SamS LA 5' and SamS LA 5' primers, the RA is amplified with SamS RA 5' and SamS RA 3' primers with the standard PCR program. Both fragments are cloned into pGem-Teasy vector, and then follow the same procedure as for sahA knock out, but with BamHI/ PstI instead of BamHI/ NdeI. The LA of ifdA is amplified with IfdA LA 5' and IfdA LA 3' primers, the RA is amplified with IfdA RA 5' and IfdA RA 3' primers with the standard PCR program. The procedure for sahA knock out is used to construct the ifdA knock out but with the NcoI/ SalI restriction enzymes instead of HindIII/ SalI.

Successfully cloned vectors are transformed into the Ax2 wild type strain and transformants are selected by growth on BS10 medium. PCR reactions with specific primers on genomic DNA from the transformant are performed to check the disruption of each target gene. For this purpose, the 5' outer with Bsr 3' and 3' outer with Bsr 5' primers are used in the PCR reactions to check the homologous recombination of Bs^r cassette into the genome. There is no gene disruption observed in hundreds of the transformants of the pLPBLP SahA and pLPBLP SamS constructs (data not shown). One clone shows the homologous recombination out of hundreds of pLPBLP IfdA transformants in PCR reactions on genomic DNA (**Fig. 2-17B, C**).

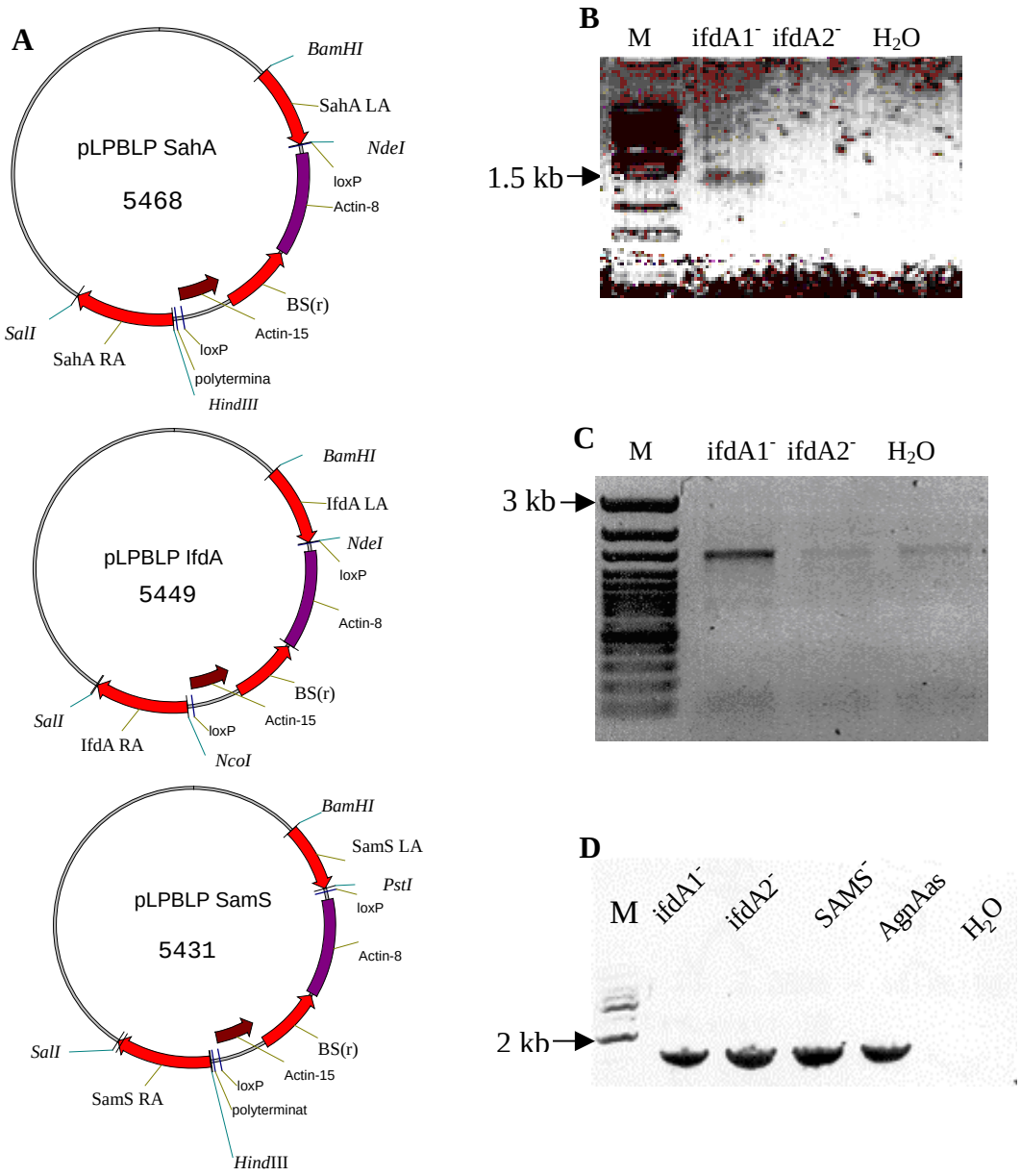


Fig. 2-17 knock out experiment on sahA, samS, and ifdA.
(A) The maps of sahA, samS, and ifdA knock out constructs.
(B) PCR on genomic DNA of pLPBLP IfdA transformants (ifdA$^-$) with ifdA 3'outer and Bsr 5' primers (1.4kb). The PCR signal of ifdA1$^-$ showed the recombination of Bs^r cassette on correct sites. **(C)** PCR on genomic DNA of pLPBLP IfdA transformants (ifdA$^-$) with ifdA 5'outer and Bsr 3' primers (2.3kb). The PCR signal of ifdA1$^-$ showed the recombination of Bs^r cassette on correct sites **(D)** PCR on genomic DNA of pLPBLP IfdA transformants (ifdA$^-$) with ifdA 5' and BamHI 3' primers (1.6kb). SAMS$^-$ and AgnAas mutants are used as controls. ifdA1$^-$ showed the PCR signal of homologous recombination in (**B**) and (**C**), ifdA2$^-$ is used as control here because of no homologous recombination observed in (**B**) and (**C**). All PCR reactions use 60˚C 2min for elongation based on the standard PCR program. The PCR signals showed the amplified ifdA gene with the same phenotype in different cell lines.

However, PCR on this clone reveals also the presence of an intact copy of the ifdA gene (**Fig. 2-17D**). This is indicative of a duplication of the ifdA gene, either in this clone, or in the *Dictyostelium* genome.

2.2.6 Expression of SahA, SamS, IfdA in *E. coli*

For further studying SahA, SamS, and IfdA *in vitro*, these proteins are overexpressed as N-terminal His-tag fusions in *E. coli*. The cDNA fragments of SahA and IfdA are digested with BamHI/ NdeI and then legated into pet-15b expression vector that is digested with the same enzymes. The cDNA fragment of SamS is digested with XhoI/ BamHI and followed by the ligation into pet-15b vector that is digested with XhoI/BamHI.

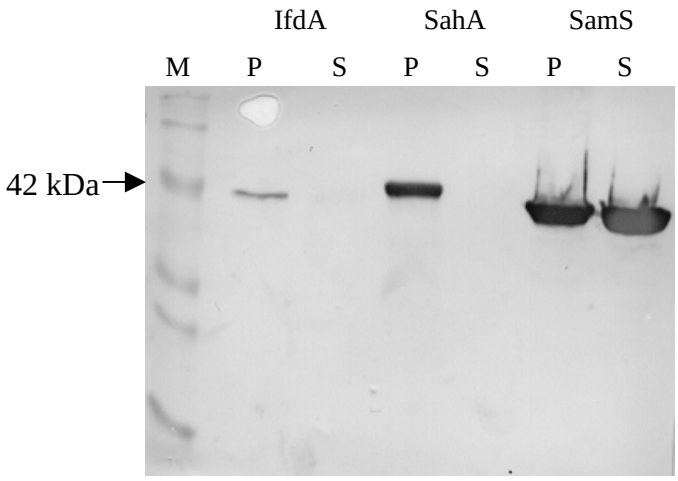


Fig. 2-18 Expression of N-terminal His tagged SahA, SamS, and IfdA in *E. coli* monitored by western blot. The BL21 *E. coli* strains expressing N-terminal His tagged SahA, SamS and IfdA were induced by 1mM IPTG for 3h, the cell lysates were separated into two parts: the supernatant (S) and pellets (P). The western blot is performed to analyse the expression of proteins with His antibody. The expected molecular weights are His-SahA 49kD, His-SamS 44kD, and His-IfdA 47kD.

These three constructs are transformed into BL21 *E. coli* strain. The His-tagged His-SahA/ SamS/ IfdA proteins are inducible by IPTG in the BL21 *E. coli* strain (**Fig. 2-18**), but only His-SamS is soluble, while the other two seem to be in inclusion bodies (**Fig. 2-18**). The induced His-SamS protein in *E. coli* will be used for pull down assay in the next chapter.

2.2.7 No interaction between PAZ-PiwiAgnA and HelF, HP1, or DnmA by direct yeast-two-hybrid experiments

It is interesting to see whether other proteins involved in gene regulatory processes that are studied in this lab can interact with the PAZ-PiwiAgnA, which would hint towards a cellular function of this protein. To study an interaction between PPWa and HP1 (Kaller M. et al. 2006), HelF (Popova B. et al. 2006) and DnmA (Kuhlmann M. et al. 2005), the cDNAs of these three proteins are cloned in the pACT2 vector based on the following strategy. The cDNA fragments of HelF and DnmA are digested from pet-15b HelF and pet-15 DnmA plasmids with BamHI/NcoI individually, then ligated into pACT2 that is cut with the same enzymes. The HP1 cDNA fragment is amplified with HP1_3'_Y2H and HP1_5'_Y2H primers, and then ligated into pACT2 with BamHI/NcoI restriction ends. The resulting vectors are used together with pGBKT7-PPWa to investigate a presumed interaction directly by the yeast-two-hybrid. The experiments of β-Gal assay cannot reveal an interaction between HelF, HP1, DnmA and PAZ-PiwiAgnA.

2.3 Study of protein-protein interaction *in vitro* by pull down assay and mass spectrometry

The yeast-two-hybrid system has been used to study protein-protein interaction *in vivo*, and then a pull down assay and mass spectrometry are used to study protein-protein interaction *in vitro* in order to confirm the results from yeast-two-hybrid screen. To perform this experiment, one protein termed bait is immobilized on a Ni-NTa column, and then a *Dictyostelium* protein extract is poured on this pre-incubated column. If the proteins from the extract interact with the bait, they can be co-eluted together from the column. The co-eluted proteins can later be analyzed by mass spectrometry (**Fig. 2-19**). For this purpose, two experiments are designed. One is to immobilize the induced His-PPWa from *E. coli* on the Ni-NTa column, on which the wild type *Dictyostelium* protein extracts are poured. The other is to immobilize the induced His-SAMS from *E. coli* on the Ni-NTa column, to which the *Dictyostelium* protein extracts of the wild type or of the PPWa-GFP overexpression strain is poured.

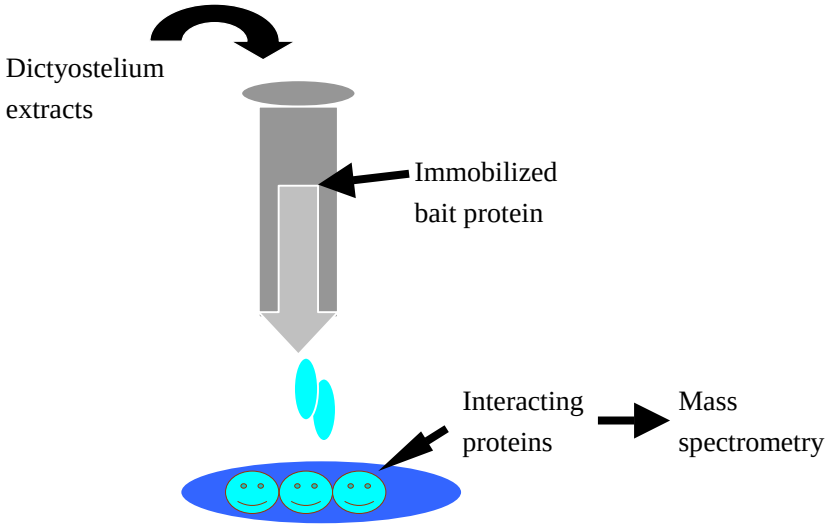


Fig. 2-19 The schematic representation of the pull down assay

2.3.1 Pull down assay of induced His-PPWa with *Dictyostelium* protein extracts

The yeast-two-hybrid experiments show that PAZ-PiwiAgnA can interact with

several *Dictyostelium* proteins *in vivo*, the pull down assay of induced His-PPWa with *Dictyostelium* extracts is used to confirm the proteins interactions with PPWa *in vitro*. Western blot analysis shows that the His-PPWa is inducible by IPTG, however the protein is found in inclusion bodies in *E. coli*, it is only soluble after treated with urea (**Fig. 2-20**). A total of 500ml culture is sonified to obtain soluble His-PPWa in the supernatant and immobilized on Ni-NTa column. The *Dictyostelium* protein extracts are poured on both pre-incubated column and empty column as a control. The same amount of eluate is loaded on the SDS-PAGE protein gel (**Fig. 2-21**), and the specific bands are cut for mass spectrometry (**Fig. 2-21**). There 18 bands are cut from both co-eluate and eluate from control respectively.

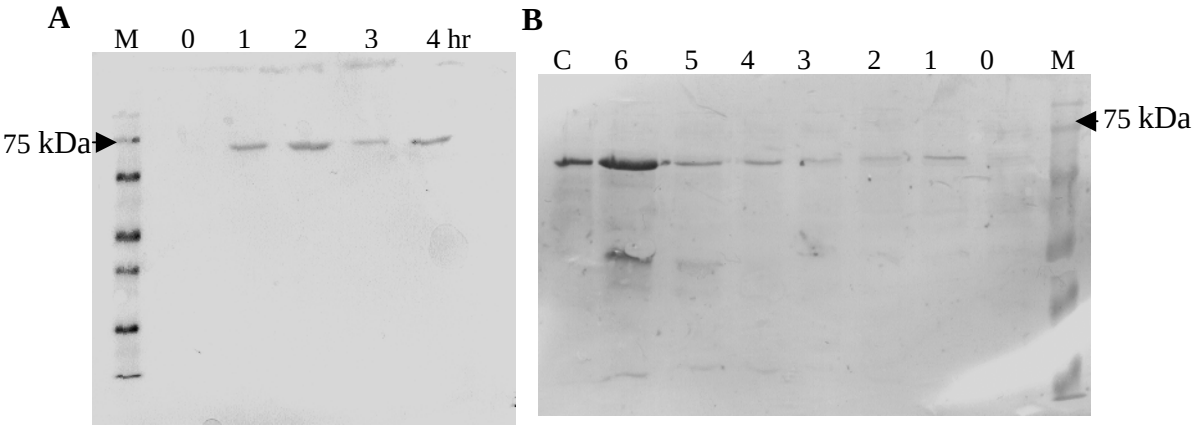


Fig. 2-20 Western blot of induced His-PPWa in *E coli.* (A) Induction of His-PPWa by IPTG at different time points. **(B)** Solubility of induced His-PPWa treated with different concentration of Urea. C: crude extract. Nr.: concentration (M) of Urea that is used to treat the supernatant of induced His-PPWa.

Data from mass spectrometry are analyzed by the Dicty Database (http://dictybase.org/). There are some specific proteins co-eluted with His-PPWa, but only sahA and elongation factor I and II are in line with the results from the yeast-two-hybrid experiments. The sahA gene is found twice in co-eluate with the total score 263 in Nr.24 band and 68 in Nr.2 band, which means it matches 9 peptides and 3 peptides respectively. But it also appears once in control with total score 130 in Nr. 23 bands, which matches 5 peptides. The elongation factors I and II are found frequently in both co-eluates and control. They appeared in co-eluate with

the highest total score of 747 in Nr. 24 band, and in control with the highest score of 704 in Nr. 23 band. It is impossible to discriminate the specific binding between SahA, elongation factors I /II and His-PPWa.

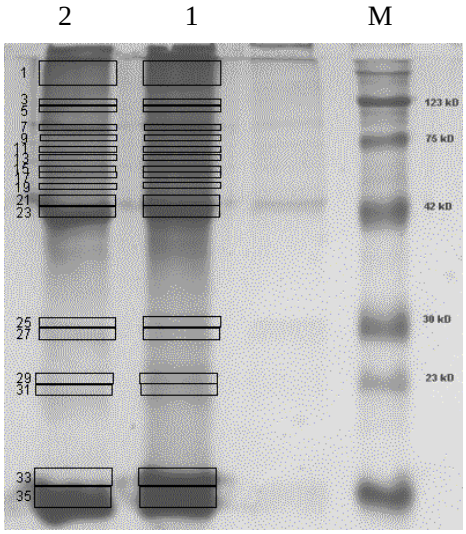


Fig. 2-21 SDS-PAGE protein gel of the induced His-PPWa with *Dictyostelium* extracts for pull down assay. 1 The co-eluate of induced His-PPWa with *Dictyostelium* extracts. 2 The eluate of induced His-PPWa from empty Ni-NTa column. The number represents the bands that are cut for Mass Spectrometry. The even number is for the co-eluate (not shown in the picture), the control is represented by the odd number.

2.3.2 Pull down assay of induced His-SamS with *Dictyostelium* protein extracts from the PPWa-GFP overexpression strain

Because of the higher solubility in *E. coli* compared to SahA and IfdA, SamS is selected for the pull down assay. First, we try to pull down the His-SamS with the specific interacting proteins from the PPWa-GFP overexpressing *Dictyostelium* extracts. The induced His-SamS is immobilized on the column, and the PPWa-GFP *Dictyostelium* extracts are poured on the pre-incubated column and an empty column as a control. The co-eluates are analyzed by western blot with His and GFP antibody. The eluates from the empty column are analyzed by western blot with the GFP antibodies. The analysis of western blot show that the SamS and PPWa can be co-eluted from the column (**Fig. 2-22 A**), but for unknown reason the GFP fused PPWa binds to the column too (**Fig. 2-22B**). It is impossible to discriminate whether

co-elution of SamS and PPWa is due to an interaction or due to the self-binding of PPWa-GFP on the column.

The binding of PPWa-GFP overexpressing protein from *Dictyostelium* strains to the Ni-NTa column is determined by elution with different concentration of imidazole. The protein start to be eluted at 100mM imidazole, but most protein is eluted at 500mM imidazole (**Fig. 2-23**).

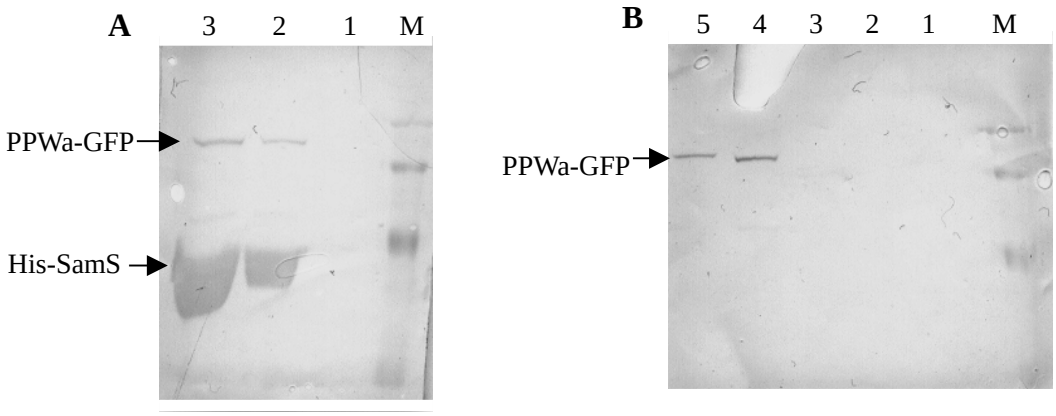


Fig. 2-22 The pull down assay of induced His-SamS with protein extracts from PPWa-GFP overexpressing *Dictyostelium* cells. (A) Western blot of co-eluates with His antibody and GFP antibody.1 –3: the first, the second and the third eluate stepwise. **(B)** The western blot of PPWa-GFP analyzed on the empty Ni-NTa column with GFP antibody. 1: flow through of PPWa-GFP, 2: the last washing fraction, 3-5: the first, the second and the third eluate.

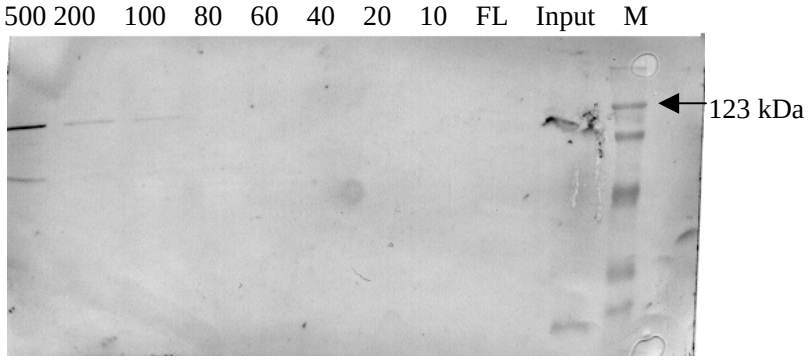


Fig. 2-23 The western blot of PPWa-GFP overexpressing *Dictyostelium* extracts eluted form Ni-NTa column with different concentration of imidazole (mM). The analysis is carried out with GFP antibody. FL flowthrough.

For this reason, we try to pour the wild type *Dictyostelium* extracts on the His-SamS immobilized column. The co-eluates are loaded on the protein gel twice with

different amount. The specific bands are cut from the SDS-PAGE protein gel, and analyzed by mass spectrometry (**Fig. 2-24**). There are some specific proteins co-eluted with His-SamS (**Table 2-6**), but the expected AgnA is not found. The very specific bands appeared (Nr.12, 13, 14, 16) in co-eluate are SamS. The experiment needs to be repeated.

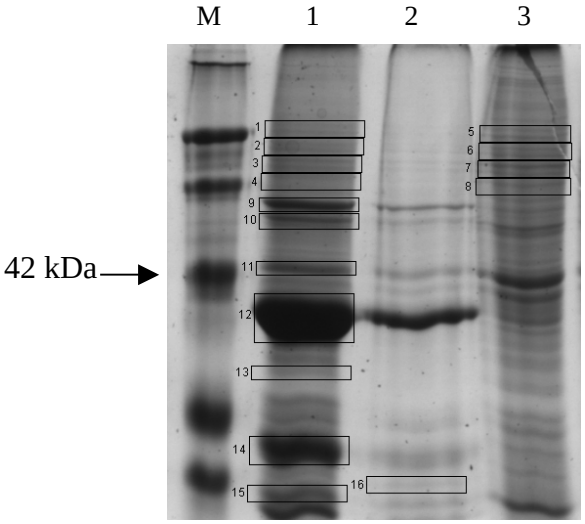


Fig. 2-24 SDS-PAGE protein gel of the induced His-SamS with *Dictyostelium* extracts for pull down assay. 1,2: 20 µl and 8µl co-eluates of induced His-SamS with *Dictyostelium* extracts. 3: The eluate of induced His-SamS from empty Ni-NTa column. The numbers represent the bands that are cut for Mass Spectrometry.

Table 2-6 Specific proteins from co-eluate of PPWa-GFP *Dictyostelium* protein extracts and His-SamS

Gene ID	Gene name	Score	Position[a]
DDB0188434	DD8-14	309	9
DDB0190540	cnrI	545	10
DDB0188461	vacAI	510	10
DDB0185833	abcF2	211	10
DDB0167044	pakB	171	10
DDB0187639	tubA	146	11
DDB0169357	thfA	270	13
DDB0167396	gpbB	127	13
DDB0218835	RPLP0	103	13
DDB0168933	rps4	369	14
DDB0167174	putative O-methyltransferase	211	14
DDB0185828	efa1B	133	14

DDB0217516	peroxiredoxin (thioredoxin peroxidase)	393	15
DDB0219325	rpl10	393	15
DDB0219325	rpl11	393	15
DDB0168933	rps 4	243	15
DDB0219467	comA	185	15
DDB0219839	rps3	151	15
DDB0191937	rasG	130	15
DDB0204610	rpl19	127	15
DDB0184102	rpl13	101	16
DDB0183803	ranA	96	16

a: the position is represented by the number appeared in **Fig.24,** which is cut for mass spectrometry.

2.4 The effect of PAZ-PiwiAgnA on RNA interference and antisense RNA mediated gene silencing in *Dictyostelium*

Argonaute proteins are the core components in the RNAi pathway in many organisms (Hammond et al. 2001). The Argonaute proteins, such as hAgo2 in human, AGO2 in *Drosophila*, influence RNAi by cleaving mRNA as slicer (Liu et al. 2004; Miyoshi et al. 2006). To study the effects of PAZ-PiwiAgnA on gene silencing in *Dictyostelium* cells, two series of experiments are designed. One studies the effect of PAZ-PiwiAgnA on RNA interference mediated gene silencing, the other studies the antisense RNA mediated gene silencing.

2.4.1 The effect of PAZ-PiwiAgnA on gene silencing mediated by RNAi

To study the function of PAZ-PiwiAgnA on RNAi mediated gene silencing, the endogenous discoidin gene family is used as a target. Post-transcriptional gene silencing can be induced by transforming *Dictyostelium* with a vector expressing a hairpin construct against discoidin. In this experiment, the GFP C-terminally fused PPWa is overexpressed in *Dictyostelium* cells to study how it influences the silencing efficiency by discoidin hairpin construct. The hairpin construct against discoidin termed Disci and the PPWa overexpression construct termed PPWa-GFP (**Fig.2-25, Fig. 2-3A**) are co-transformed into *Dictyostelium* cells by classical transformation. The wild type strain transformed with Disci construct is used as a control.

For the analysis, the wild type cells transformed with Disci are selected by Geneticin resistance in G10 selective medium directly, while the co-transformants are selected by a series of experiments because of the identical selection marker carried on both vectors. Individual co-transformants are selected by visualizing the green fluorescent cells with a fluoresce microscope (**Fig. 2-3B**) and the expression of GFP fusion protein is confirmed by western blot (**Fig. 2-26**). The PCR reaction on

genomic DNA from individual co-transformants is carried out to check the Disci integration (**Fig. 2-26**).

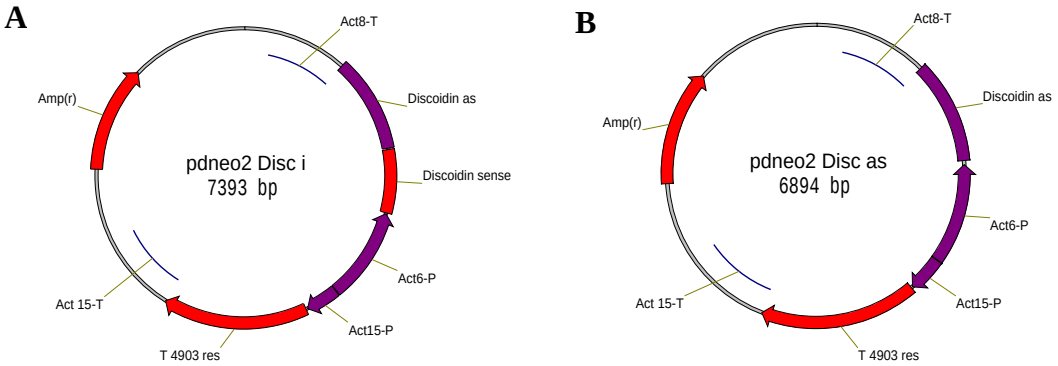


Fig. 2-25 The constructs of discoidin hairpin structure (A) and discoidin antisense RNA (B) structure (Martens et al. 2002).

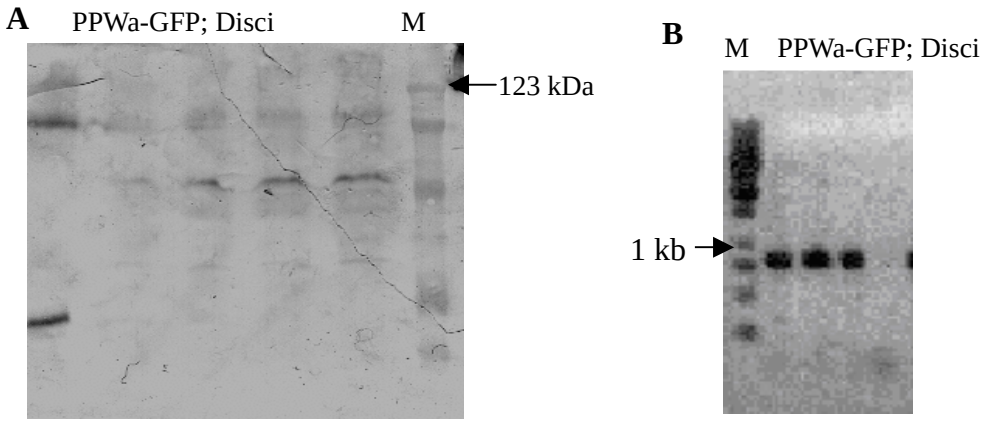


Fig. 2-26 The analysis of PPWa-GFP and Disci co-transformants (PPWa-GFP; Disci). (A) Western blot of co-transformants with GFP antibody. **(B)** PCR on genomic DNA from co-transformants with Act6-Seq-P and DISC-AS-P-ML1 primers based on the standard PCR program (802bp).

Having proven the co-transformants by these methods, individual co-transformed clones were analyzed for silencing of discoidin by western blot. The expression of the endogenous coronin gene on protein level is used to monitor equal protein quantities. Because of the cross-reaction between the antibodies against discoidin and coronin, the blotted membranes are cut into two parts, which were individually incubated with the respective antibody. Several transformations are done in parallel to get enough individual co-transformed clones, thus preventing studying duplicates. Totally 68 single co-transformants of Disci and PPWa-GFP are obtained for study.

The analysis of silencing efficiency by western blot show that the overexpressed PPWa protein in *Dictyostelium* cells increases the silencing efficiency to 100%, compared to 46% silencing, 19% partial silencing in wild type transformed with Disci (**Fig. 2-27**). This result suggests that PAZ-PiwiAgnA is involved in the RNAi mechanism in *Dictyostelium*.

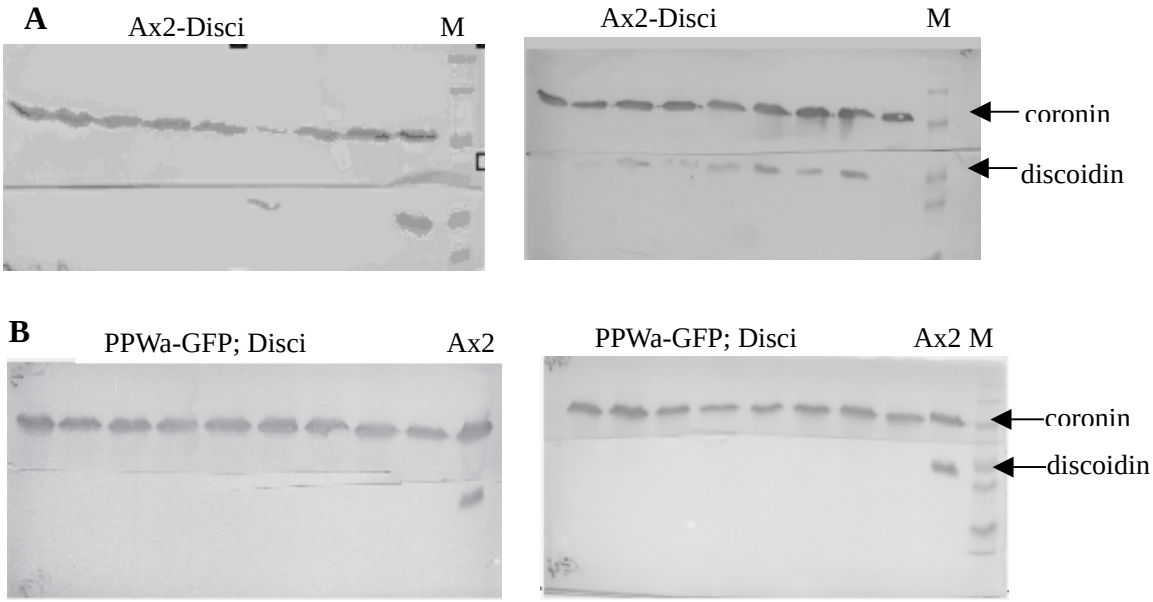


Fig. 2-27 The analysis of effects of PAZ-PiwiAgnA on RNAi mediated gene silencing in *Dictyostelium*. The expression of target gene is analyzed by western blot with discoidin antibody, and the expression of coronin detected with coronin antibody quantifies the protein amount used in the experiments.
(A) Western blot analysis of the expression of discoidin in wild type transformed with Disci construct (n=70). **(B)** Western blot analysis of the expression of discoidin in PPWa-GFP; Disci co-transformants (n=68). Ax2 wild type is used as control.

The coronin hairpin construct has been tried for the same experiment as well, but here no successful co-transformant could be obtained after several transformation experiments.

2.4.2 The effect of PAZ-PiwiAgnA on gene silencing mediated by antisense RNA

Next to RNAi, also antisense RNA can induce gene silencing as well. To study, if the overexpression of PAZ-PiwiAgnA can influence also the silencing efficiency, which

is mediated by antisense RNA pathway, the discoidin antisense (Discas) construct (**Fig.2-25 B**) is used as gene silencing trigger. The experiments are performed as above. Simply, the Discas and PPWa-GFP constructs are co-transformed into *Dictyostelium* cell by the classical method, and the wild type strain transformed with Discas construct is used as a control. The wild type cells transformed with Discas are selected by Geneticin resistance in G10 medium directly, while the co-transformants are selected by the visualization of green cells under the fluorescent microscope (**Fig. 2-3B**) and by PCR on genomic DNA (**Fig. 2-28B**).

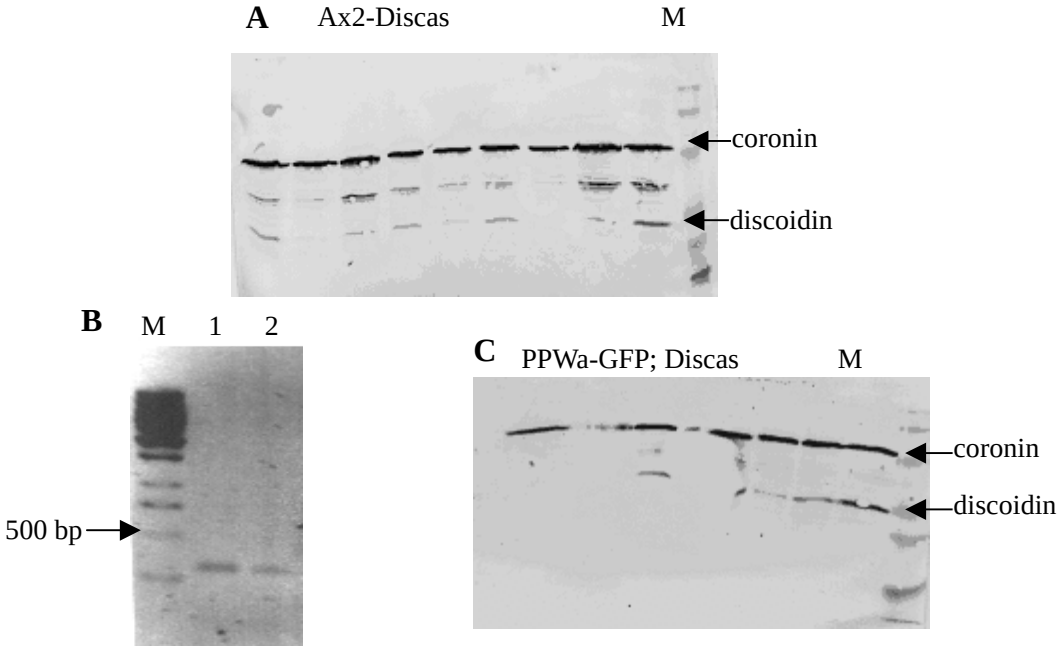


Fig. 2-28 The analysis of effects of PAZ-PiwiAgnA on antisense RNA mediated gene silencing in *Dictyostelium*. The expression of discoidin gene is analyzed by western blot with discoidin antibody. The expression of coronin detected with coronin antibody quantifies the protein used in the experiments.
(A) The expression of discoidin in the wild type Ax2 strain transformed with Discas construct by western blot (n=18). **(B)** PCR on genomic DNA from the PPWa-GFP; Discas co-transformants with Act6-Seq-P and DISC-AS-P-ML1 primers based on standard PCR program (326bp). **(C)** The expression of discoidin in PPWa-GFP; Discas co-transformants by western blot (n=13).

13 individual co-transformed clones and 18 clones from wild type transformed with Discas are obtained for study. The analysis of the silencing efficiency of Discas by western blot shows that there is no difference between the wild type and the PPWa-GFP overexpression strains (**Fig.2-28**). In both cases, silencing is observed at

varying degrees for some clones, but not in others, in line with published data (Martens et al. 2002b). This indicates that overexpression of PPWa does not influence the gene silencing mediated by antisense RNA.

2.5 Knock down of Argonaute A and its effects

2.5.1 The duplication of AgnA

To study the function of Argonaute A protein in *Dictyostelium* cells, knock out of AgnA are based on the K.O. vector (**Fig. 2-29A**) and the modified K.O. vector containing cre-lox (**Fig. 2-29B**). To construct the Dicty AgoA K.O vector (**Fig. 2-29A**), the left arm is amplified with Ago 1 5' and 3' primers, and ligated into the K.O. vector after both insert and vector are digested with SphI/ Bsp120I restriction enzymes. The right arm is amplified with Ago 1 RA 5' and 3' primers, and ligated into K.O. -AgnA LA vector following the digestion with HindIII/ BamHI on both insert and vector. The K.O. -AgnA RA vector is constructed using BspEI/ BamHI. To clone the AgoA K.O. cre-lox construct (**Fig. 2-29B**), first the cre-lox BS^r cassette is digested with HindIII/ BcuI from the pLPBLP vector, and then ligated into K.O. -AgnA LA vector that is digested with HindIII/ XbaI to replace the original BS^r cassette. Then the right arm is digested with BspEI/ HindIII from the K.O. -AgnA RA vector and ligated into the preformed vector.

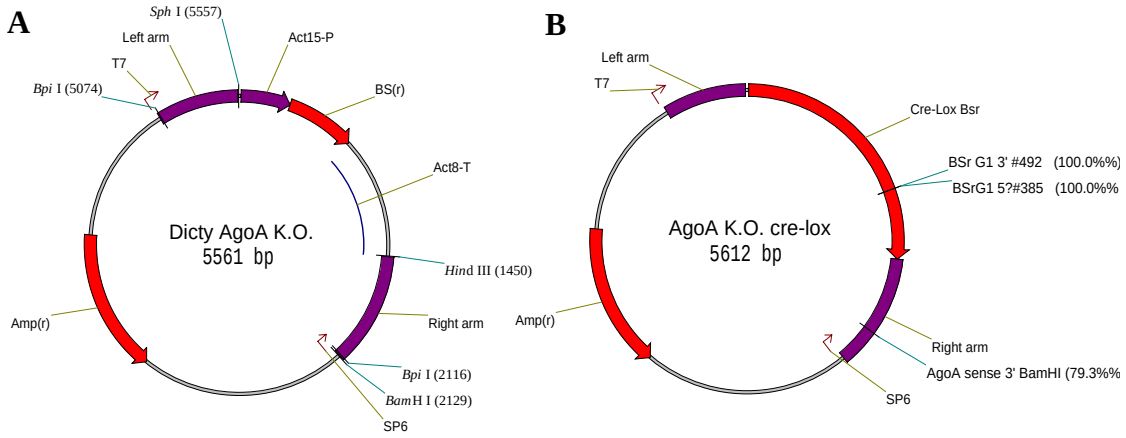


Fig. 2-29 The constructs of AgnA knock out. (A) The construct of AgnA K.O with K.O vector. **(B)** The construct of AgnA K.O. with Cre-lox cassette.

These two constructs termed Dicty AgoA K.O. and AgoA K.O. cre-lox (F**ig. 2-29**) are digested with BpiI restriction enzyme, the linear fragments containing leaf arm, BS^r cassette and right arm are transformed into *Dictyostelium* cells by electroporation. PCR reactions on genomic DNA from single transformed clones are performed with specific outer and BSr primers to detect the integration of the BS^r

cassette into the genome, and with internal primers to detect the disruption of the gene.

In the *Dictyostelium* cells transformed with Dicty K.O. AgoA vector, the AgnA 5' outer and BS^r 385 primers are used to check the integration of the BS^r cassette into the genome (**Fig 2-30A**). PCR results show that the BS^r cassette recombined at the correct sites, however, an RT-PCR on these clones reveal that the gene is still expressed in the same length as observed for the wild type (**Fig 2-30B**).

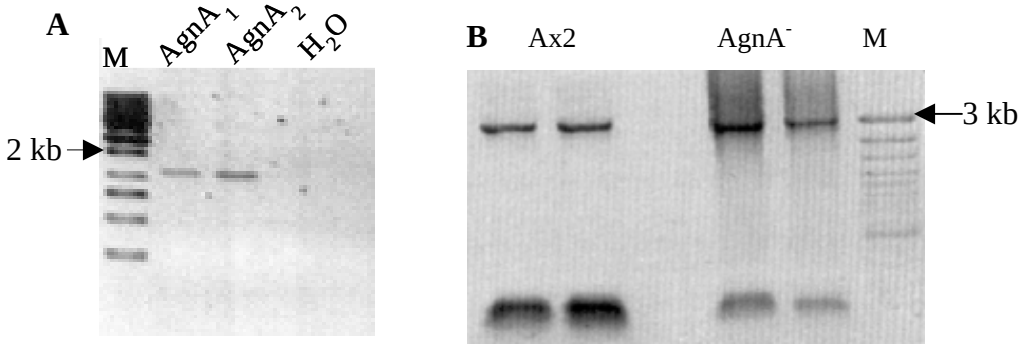


Fig. 2-30 Detection of AgnA knock out with Dicty K.O. AgoA vector.
(A) PCR on genomic DNA from AgnA knock out transformants ($AgnA^-$) with AgnA 5' outer and BS^r 385 primers, based on the standard PCR program.
(B) RT-PCR on cDNA from Ax2 wild type and AgnA knock out transformants ($AgnA^-$) with AgnA-GFP 5' and 3' primers. 60℃ and 2.5 min are used for elongation according to the standard PCR program.

In the AgoA K.O. cre-lox transformed cells, a primer termed AgnAi sense 3' primer (**Table 4-1**), which is inside the left arm, and the BS^r 385 primer are used to check if the BS^r cassette is transformed into the cells (**Fig. 2-31A**), and the PPW 3' primer is used as outer primer together with BS^r 385 primer to check the recombination of the BS^r cassette on correct sites (**Fig. 2-31B)**. Both experiments show that BS^r cassette integrates into the genome at expected sites. But the PCR results on genomic DNA from AgoA K.O. cre-lox transformed cells show that the agnA gene in transformants was not disrupted compared to wild type Ax2 (**Fig. 2-31C).** Also, RT-PCR results reveal that the gene is still expressed at a similar level as in the wild type (**Fig. 2-31D)**

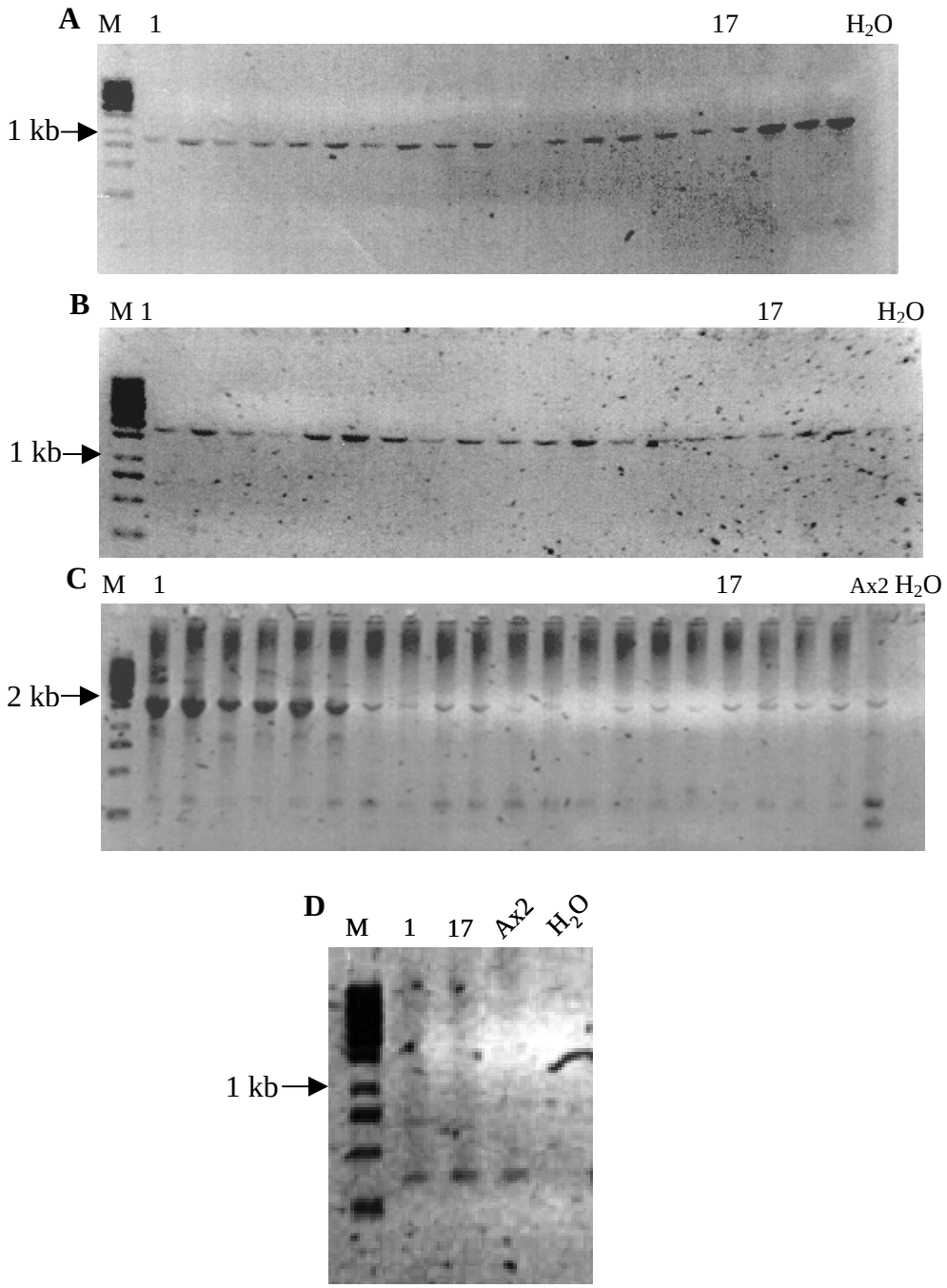


Fig. 2-31Analysis of AgnA knock out transformants with cre-lox construct in *Dictyostelium* cells. (A) PCR on genomic DNA from AgnA knock out transformants with BS^r 385 and AgnAi sense 3' primers, based on the standard PCR program (870 bp). **(B)** PCR on genomic DNA from AgnA knock out transformants with BS^r 385 and PPW 3' primers (1.7 kb). 60℃ and 2 min are used for elongation according to the standard PCR program. **(C)** PCR on genomic DNA from AgnA knock out transformants with PPW 5' and PPW 3' primers (1.7 kb). 60℃ and 2 min are used for elongation according to the standard PCR program.**(D)** RT-PCR on cDNA from AgnA knock out transformants (Nr. 1 and Nr. 17) with AgnAi sense 5' and 3' primers. Ax2 wild type is used as control.

The consistent failure of knocking out AgnA in two independent experiments suggests that the gene is duplicated in the genome of Ax2 wild type strain.

2.5.2 Knock down of AgnA by RNAi and antisense RNA constructs in *Dictyostelium* cells

Because of the duplication of the Argonaute A gene in the *Dictyostelium* genome, it was not feasible to knock out the gene. Instead, RNAi and antisense RNA constructs (**Fig. 2-32**) are created to knock down AgnA, which would allow studying its function. To obtain the AgnA hairpin construct (**Fig. 2-32 A**), the fragment of AgnAsense is amplified with AgnAi sense 5' and AgnAi sense 3' primers by the standard PCR program, then digested with PstI/ BamHI restriction enzymes and subsequently cloned into the pdneo2 vector that is digested with the same enzymes. The fragment of AgnA antisense is amplified with AgnA antis 5' and AgnA antis 3' primers by the standard PCR program, digested with BamHI/ EcoRI restriction enzymes and cloned into pdneo2–AgnAsense vector that is digested with the same enzymes. The AgnA antisense construct (**Fig. 2-32 B**) is cloned by the same method as antisense fragments did.

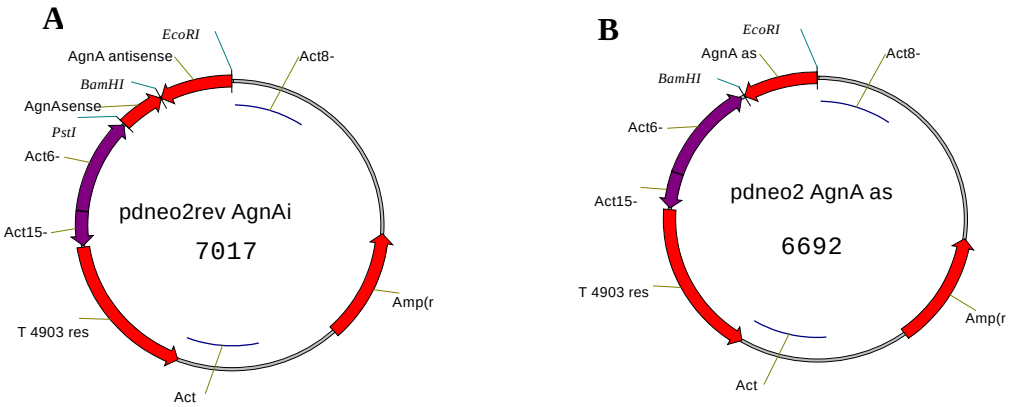


Fig. 2-32 The constructs of AgnA knock down by RNAi and antisense RNA.
(A) The construct of AgnA knock down by RNAi (AgnAi). **(B)** The construct of AgnA knock down by antisense RNA (AgnAas).

2.5.2.1. Knock down of AgnA by RNAi construct

Because Argoanute A is homologous to Argonaute B, one fragment of about 300bp

corresponding to a unique sequence of Argonaute A is used for the AgnAi construct as sense part, and another fragment with about 200bp more on the 5'end of the sense part is used as antisense part to form the hairpin structure (**Fig. 2-33A**).

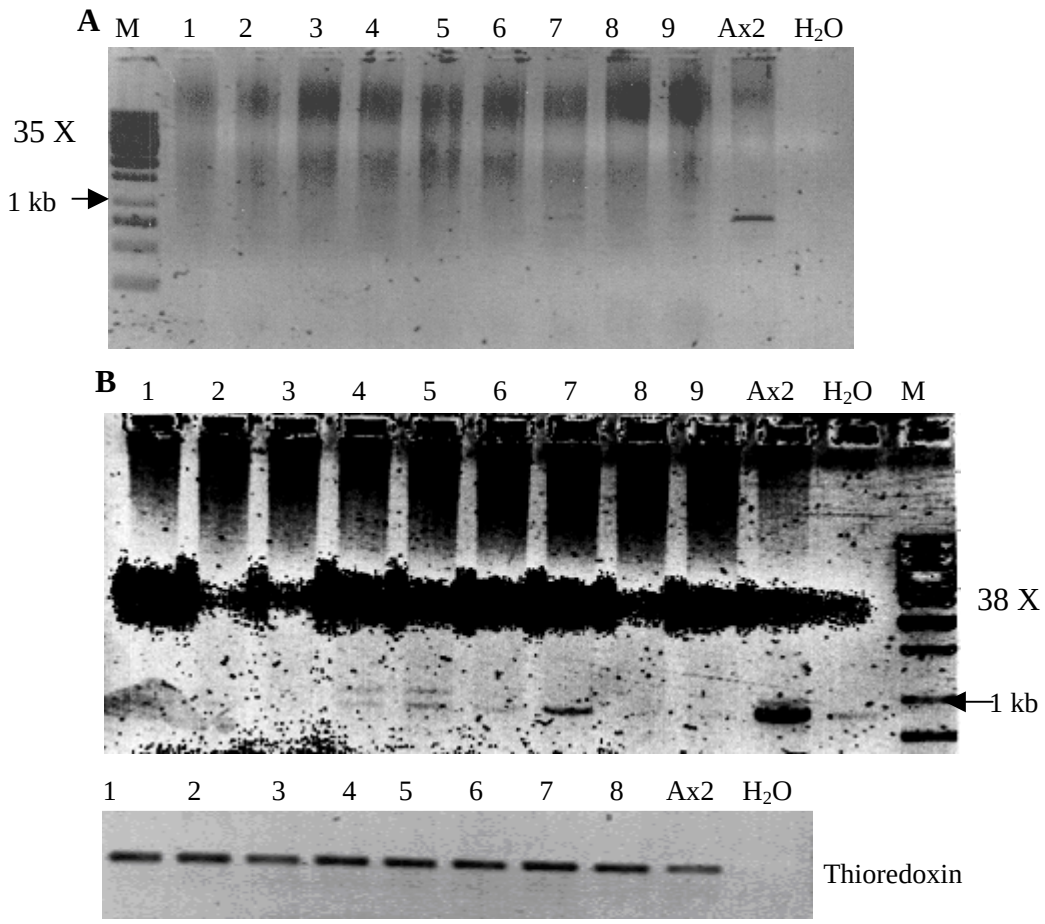


Fig. 2-33 RT-PCR on cDNA from AgnAi *Dictyostelium* cell lines. PCR is performed with PPW 5' and AgnAsense 3' primers based on standard PCR program for 35 cycles (**A**) and 38 cycles (**B**). Ax2 wild type is used as control. The 871 bp fragment is the product from cDNA, the 996 bp fragment is the product from genomic DNA. Thioredoxin is used to quantify the cDNA.

The construct termed pdneo2 rev AgnAi is transformed into *Dictyostelium* cells by the classical method. The RT-PCR reaction is carried out on cDNA from individual clones to check the silencing of Argonaute A gene. RNA from 9 single clones are extracted for RT-PCR, the PCR reaction is run for 35 and 38 cycles to detect the silencing degree (**Fig. 2-33A,B**). The RT-PCR results show that AgnA can be silenced by the hairpin construct. 5 out of 9 clones are completely silenced, 4 are not silenced (**Fig. 2-33B**).

2.5.2.2. Knock down of AgnA by an antisense RNA construct

The AgnA antisense construct termed pdneo2 AgnAas (**Fig. 2-33B**) is transformed into *Dictyostelium* cells by classical transformation. Several individual clones are selected randomly for further studies. RT-PCR is performed for 40 and 47 cycles to check the silencing of the agnA gene. The results show that 3 out of 10 clones are completely silenced by antisense RNA construct (**Fig. 2-34A, B**). The two silenced clones, termed AgnAas15 and AgnAas 16, are used for further studies in this chapter.

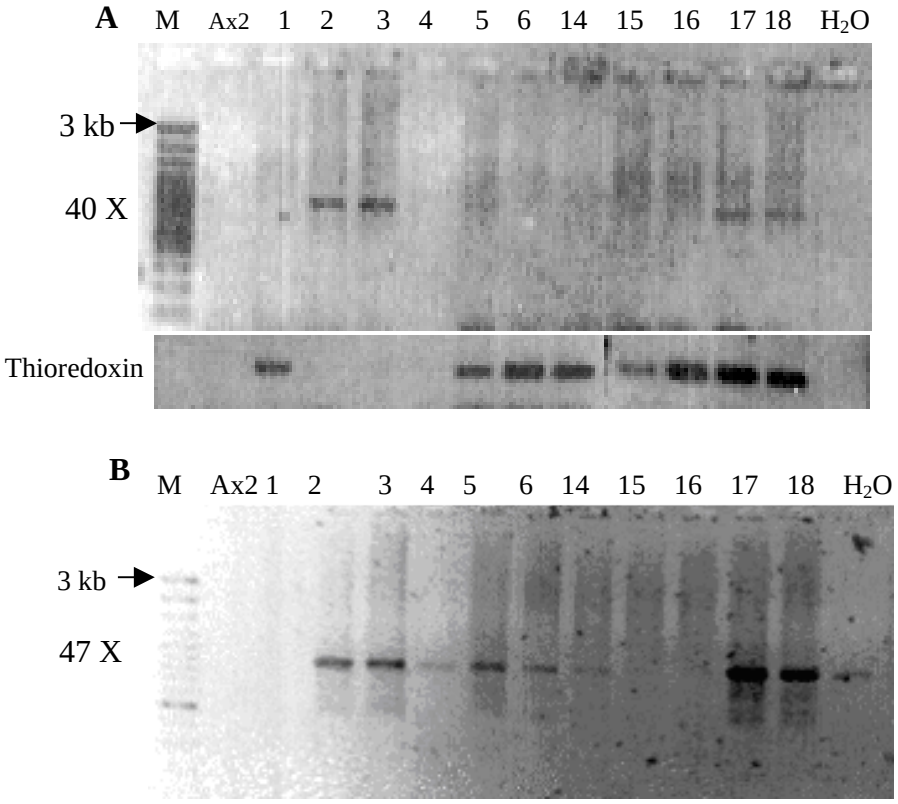


Fig. 2-34 RT-PCR on cDNA from AgnAas *Dictyostelium* cell lines. The PCR is performed with PPW 5' and AgnAsense 3' primers based on the standard PCR program for 40 cycles (**A**) and 47 cycles (**B**). Ax2 wild type is used as control. Thioredoxin is run for 30 cycles to quantify the cDNA. The Nr. 4 clone is excluded.

2.5.3 Defects on growth and phagocytosis in the AgnA knock down mutants

In order to investigate the phenotype of AgnA knock down mutants, a developmental time course is carried out and the growth process is investigated. There is no

phenotypic difference between mutants and wild type in development.

In growth process experiments, we set up two groups of experiments for analysis. One group including wild type and two AgnA knock down cell lines is shaken continuously for 90 hours, and cell density is checked at different time points. The other group is shaken under the same condition as the first group but with a dilution of cell cultures, to prevent stationary phase. For every cell line, two shaking cultures are set up in parallel, and every sample is measured twice at every time points.

The results from this analysis indicate that AgnA knock down cell lines show a decreased growth rate (**Fig. 2-35**). AgnA knock down mutants grow slower than the wild type in shaking culture (**Fig.2-35A**), and the cell number of the mutants increases 70-100 times after 90 hours which is only 33% of that observed for the wild type (**Fig.2-35B**). The doubling time of the knock down cell lines is 14.4 hours for AgnAas15 and 13 hours for AgnAas16, which prolong 2.4 and 3.8 hours respectively, compared to the 10.6 hours of the wild type. The growth curve from undiluted cultures shows that all cell lines stop growth almost at the same cell density (**Fig. 2-35C**), there is no big difference between wild type and mutants.

In an effort to understand the reduced growth rate of mutant cells, the phagocytosis process is analyzed. The fluorescently labeled yeast cells are incubated in AgnAas knock down cell suspensions and Ax2 wild type suspensions, and the relative fluorescence against time after the subtraction of fluorescence is measured at different time points by a fluorimeter. The intracellular fluorescence represents the protein content taken by *Dictyostelium* cells through phagocytosis. Meanwhile, the same amount of AgnA knock down cells and Ax2 wild type cells are grown on KA plates under the same condition, and the size of plaques are measured at different time points.

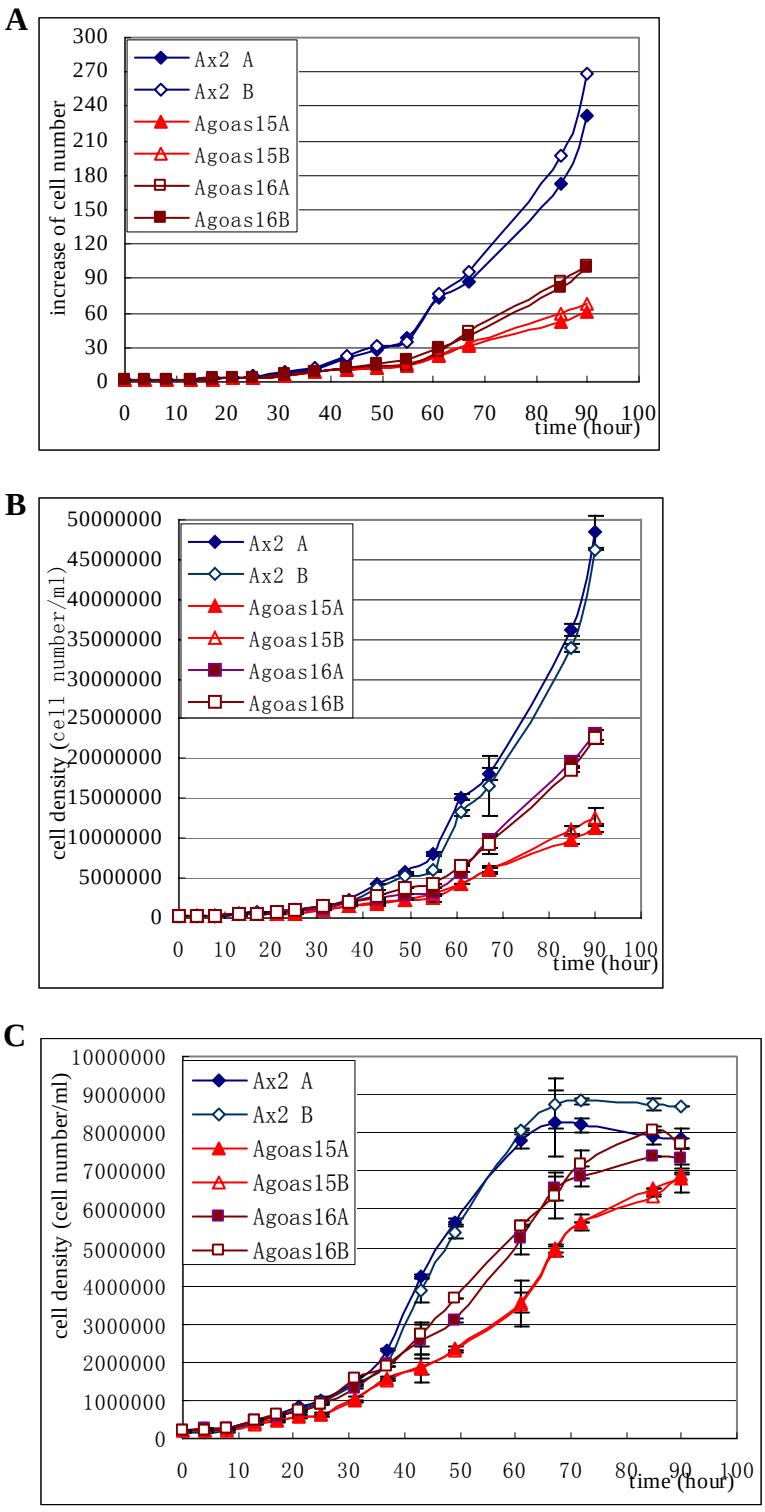


Fig. 2-35 Growth defect in AgnA know down mutants. For every cell line, two shaking cultures are incubated for experiments, and every culture measure is measured twice. **(A)** The increase of cell number of wild type and AgnA knock down cell lines in diluted culture. **(B)** The growth curve of wild type and AgnA knock down cell lines in diluted culture. **(C)** The growth curve of wild type and AgnA knock down cell lines in undiluted cell culture.

The result of intracellular fluorescence show that two AgnA knock down strains have defects on the phagocytosis process, the uptake of fluorescently labeled yeast by mutants is decreased by 40% compared to wild type Ax2 (**Fig. 2-36A**). The plaque assay shows that the AgnA knock down mutants are much smaller than the wild type (**Fig. 2-36B**). In 6 days, the diameter of the mutants is only about 40-50% of wild type Ax2 (**Fig. 2-36B**).

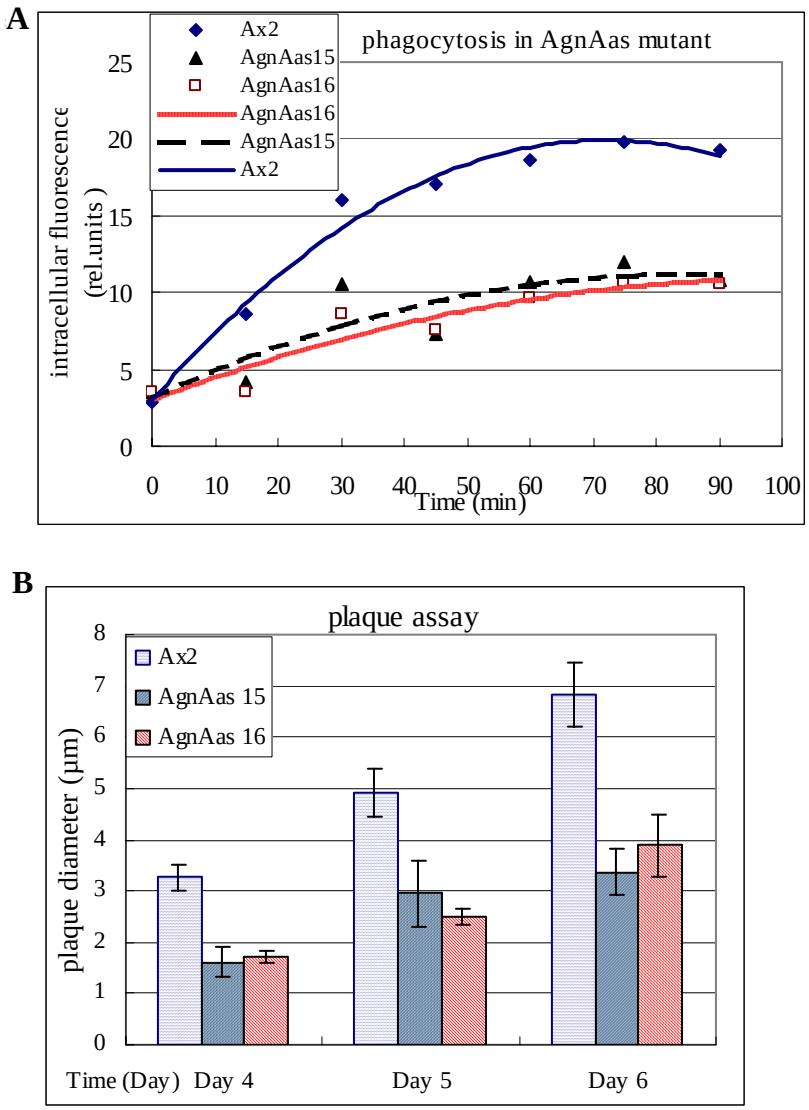


Fig. 2-36 The defect on phagocytosis process in AgnA knock down mutants and wild type. (A) The detection of phagocytosis process of AgnA knock down mutants. **(B)** The plaque assay of AgnA knock down mutants. These two experiments were done in the Department of Cell Biology.

2.5.4 The influence of AgnA on different Argonaute proteins in *Dictyostelium* cells

There are five Argonaute proteins expressed in *Dictyostelium* cells. Does AgnA influence the expression of the other Argonaute proteins? In order to answer this question, the expression of AgnB/C/D/E is investigated by semi-quantitave RT-PCR in an AgnA knock down mutant mediated by the antisense RNA construct. The cDNA from wild type Ax2 and AgnAas mutant are used as templates respectively, and AgnB/C/D/E are amplified with the same primers as used in the Result 2.1 part. The expression of thioredoxin in Ax2 and AgnAas is used to quantify the cDNA used in the experiments. The results from the RT-PCR show that there is no difference in expression level of AgnB/D/E between wild type and mutant cell lines (**Fig. 2-37**). The expression of AgnC is not obtained here after the PCR reaction was run for 30 cycles. The results demonstrate that the AgnA does not influence significantly the expression of AgnB/C/D/E in *Dictyostelium* cells.

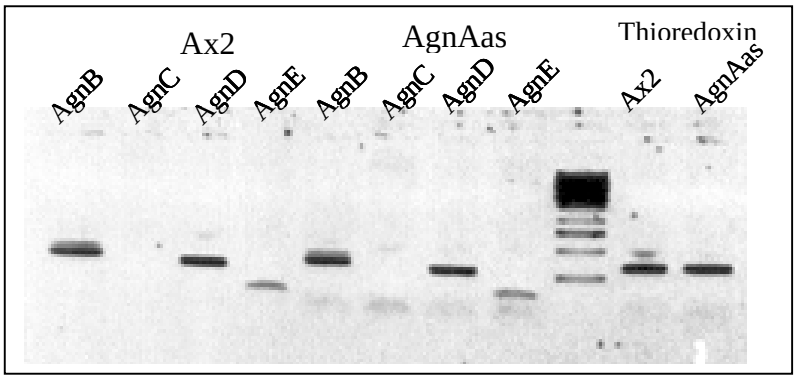


Fig. 2-37 The expression of different Argonaute proteins in AgnA knock down and wild type cell lines by semi-quantitave RT-PCR. The cDNA from Ax2 and AgnAas are used as templates, the PCR reaction of thioredoxin gene is used to quantify the cDNA used in the experiments.

2.5.5 The influence of AgnA on SamS and SahA in *Dictyostelium* cells

It is known from the yeast-two-hybrid experiments that SahA and SamS can interact with PAZ-PiwiAgnA *in vivo*. This implies that the Argonaute A protein may have effects on these two proteins. The influence on expression level of these two genes

by AgnAas mutants is studied with semi-quantitave RT-PCR and Northern blot, the wild type is used as a control.

Semi-quantitave RT-PCR is carried out on AgnAas15 and AgnAas16 knock down mutants and wild type cell lines to detect the expression of SahA and SamS on cDNA level, further Northern blot is performed to confirm the difference of the expression pattern on mRNA level.

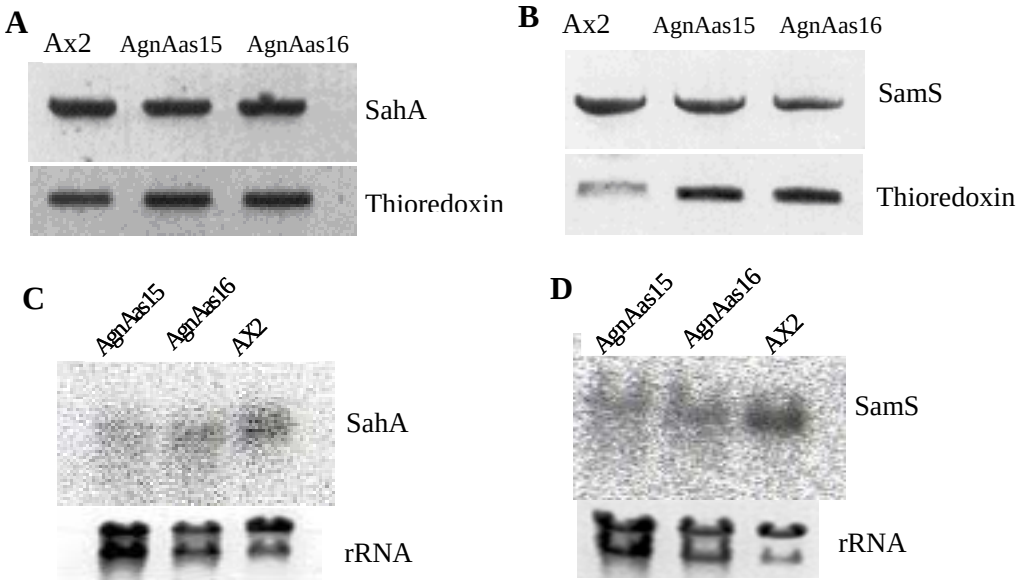


Fig. 2-38 Expression of SahA and SamS in AgnA knock down cell lines
(A) Expression of SahA in wild type and AgnA knock down mutants by semi-quantitave RT-PCR. **(B)** Expression of SamS in wild type and AgnA knock down mutants by semi-quantitave RT-PCR. **(C)** Expression of SahA in wild type and AgnA knock down mutants by Northern blot. **(D)** Expression of SamS in wild type and AgnA knock down mutants by Northern blot.

RT-PCR and Northern blot results show that the expression of SamS is strongly down regulated in AgnA knock down cell lines both on cDNA and RNA level (**Fig. 2-38B,D**). The results from RT-PCR and Northern blot show that the expression of SahA is slightly down regulated in AgnA knock down cell lines compared to the wild type Ax2 (**Fig. 2-38 A, C**). The small difference of the expression of SahA or SamS between AgnAas15 and AgnAas16 shown in Northern blot (**Fig. 2-38 C, D**) might be due to the different silencing levels of the two mutants.

2.5.6 The influence of Argonaute A on DNA methylation in *Dictyostelium* cells

The previous results revealed that Argoanute A influences the expression of SahA and SamS, which are two enzymes involved in the SAM metabolism pathway. This indicates that Argonaute A might affect DNA methylation through the interaction with SahA and SamS. It is know that in *Dictyostelium* cells, DIRS-1 is methylated in the LTR and Skipper is methylated in the RT gene, both at asymmetric C-residues (Kuhlmann et al. 2005). Further, the endogenous mvpB and telA genes are found to be upregulated in a DnmA knock out mutant shown by the micorarray experiments. We therefore investigate the DNA methylation level of DIRS-1, Skipper, mvpB and telA in AgnA knock down mutants. In this experiment, the antisense RNA mediated AgnA knock down termed AgnAas15 and AgnAas16 are used for the study of DNA methylation.

2.5.6.1 Expression of retrotransposons in the AgnA knock down mutants

The expression of DIRS-1 and Skipper are detected by Northern blot with DIRS-LTR and Skipper-GAG probes.

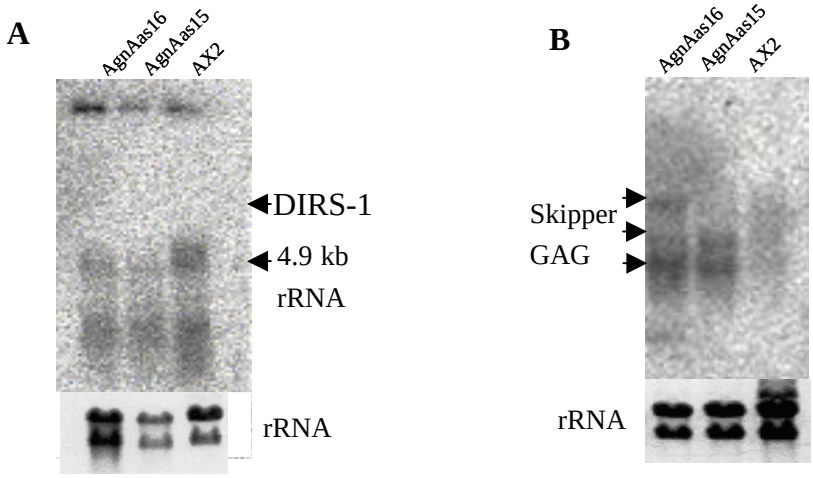


Fig. 2-39 Expression of DIRS-1 and Skipper in AgnA knock down cell lines.
(A) Northern blot analysis of expression of DIRS in AgnA knock down mutants probed with DIRS LTR. Arrows indicate the positions of DIRS-1 and rRNA. **(B)** Northern blot analysis of expression of Skipper in AgnA knock down mutants probed with Skipper-GAG. Ax2 is used as control. Arrows indicate the position of Skipper.

Northern blot analysis show that DIRS-1 and Skipper are rarely expressed in Ax2 wild type strain, and the AgnA does not affect the expression of DIRS-1, but the expression of Skipper is upregulated in AgnAas mutants (**Fig.2-39**).

2.5.6.2 The inhibition of DNA methylation in the AgnA knock down mutants

The DNA methylation in DIRS-1 LTR region, Skipper RT region, mvpB and telA are analyzed by bisulfite sequencing. The genomic DNA is extracted from 3 different cultures for each of these two mutants. These four targets are amplified with specific bisulfite primers in AgnAas15 and AgnAas16 mutants and wild type genomic DNA. The target of DIRS-1 LTR is amplified with Bi-DIRSltr F and Bi-DIRSltr R primers, and use 40 and 50 sec for annealing on the basis of standard PCR program. The target of Skipper RT is amplified with Bi-Skipperrt F and Bi-Skipperrt R primers using 46℃ for annealing, of mvpB is with Bi-mvp F and Bi-mvp R primers using 44 ℃ for annealing, and of telA is with Bi-Tel F and Bi-Tel R primers using 44℃ for annealing. Then the amplified fragments are cloned into pGem T-easy vector for sequencing. The sequencing results from AgnAas15 and AgnAas16 are grouped into AgnAas shown below.

DIRS-1

To analyze the DNA methylation of DIRS-1, 7 samples are selected randomly for bisulfite sequencing. The 128 bp DIRS- LTR region is sequenced, and the DNA methyaltion efficiency is compared with wild type (Kuhlmann et al., 2005). Among these 7 methylated asymmetric C-residues, two methylation sites are lost both in AgnAas15 and AgnAas16 mutants for unknown reasons, and only 2 out of 49 sequenced C-residues are methylated (**Fig. 2-40**).

Skipper

In this experiment, 8 samples succeeded in sequencing. There are 14 C-residues methylated in Skipper RT in wild type, and 112 C-residues are sequenced totally in AgnAas mutant. Out of these 112 C-residues, 14 C-residues are methylated.

Comparing this to the wild type, the DNA methylation level decreases to 12% in AgnA knock down mutants (**Fig. 2-41**).

mvpB

MvpB, the major vault protein, is necessary for optimal growth of *Dictyostelium* cell (Vaus and Rome 1995). Two methylated C-residues in mvpB gene are found in wild type, but the data have not been confirmed in DNA methyltransferase defect strain. As the first observation in *Dictyostelium* mutants, the bisulfite sequencing results from 9 AgnA mutants show that the methylation is inhibited 100% in the AgnA knock down mutants (**Fig. 2-42**).

telA (TelemeraseA)

The same case as mvpB, 3 methylated C-residues are found in telA gene in wild type stain by bisulfite sequencing. As the first observation of DNA methylation in *Dictyostelium* mutants, the results from 7 AgnA knock down mutants show that methylation on two C-residues sites are inhibited completely, while one methylation site is lost as shown in DIRS-1 (**Fig. 2-43**).

All results from the DIRS-1, Skipper, mvpB and telA show that DNA methylation in AgnA knock down mutants is inhibited, which means that AgnA involves in DNA methylation. This indicates a connection between RNAi and DNA methylation in *Dictyostelium* cells.

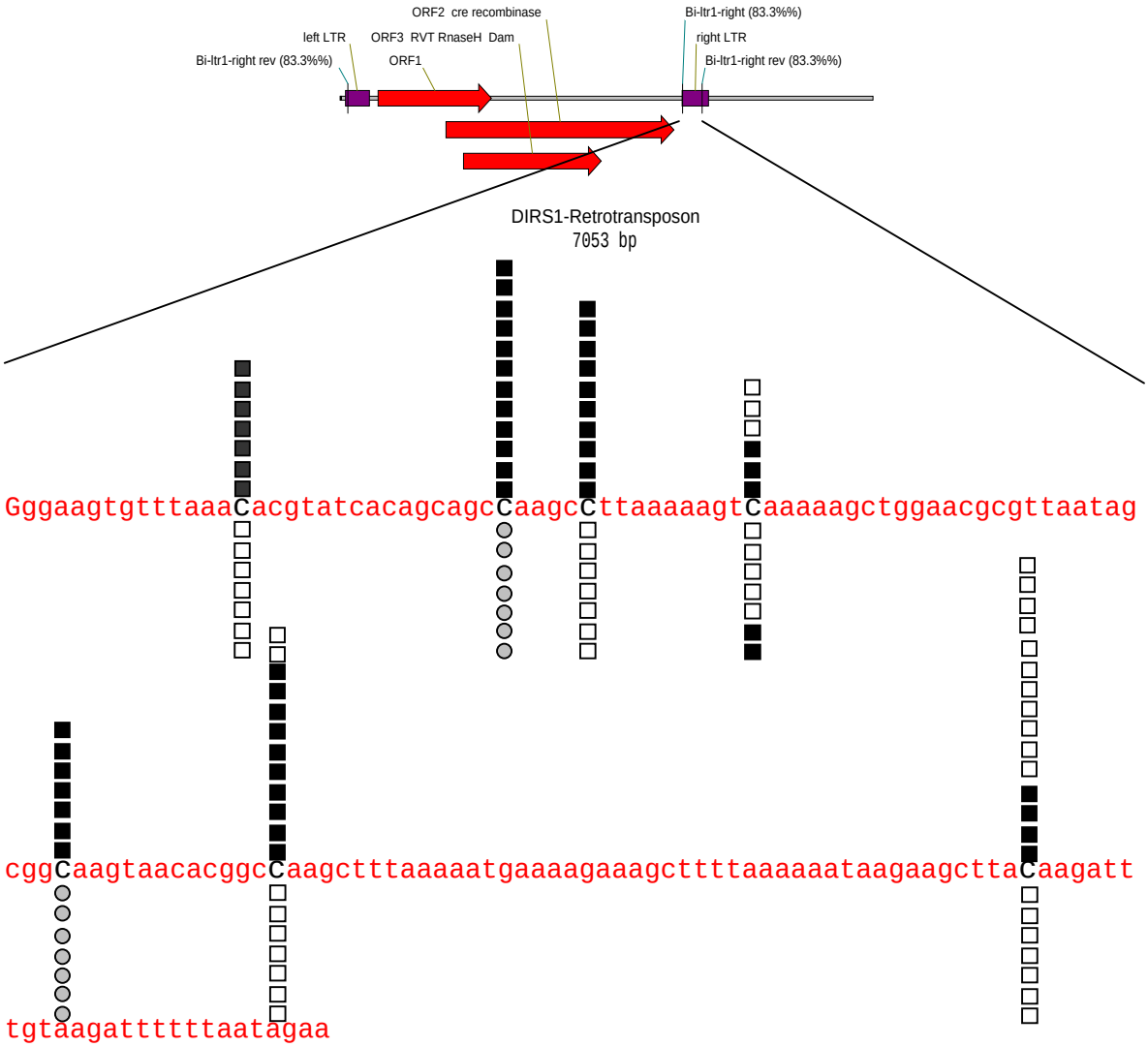


Fig. 2-40 The methylation pattern of DIRS-1 LTR gene in AgnA mutant by bisulfite sequencing. Symbols above the sequence are from Ax2, symbols below the sequence from AgnAas mutants.

■ methylated site

□ Not methylated site

○ not detected methylation site

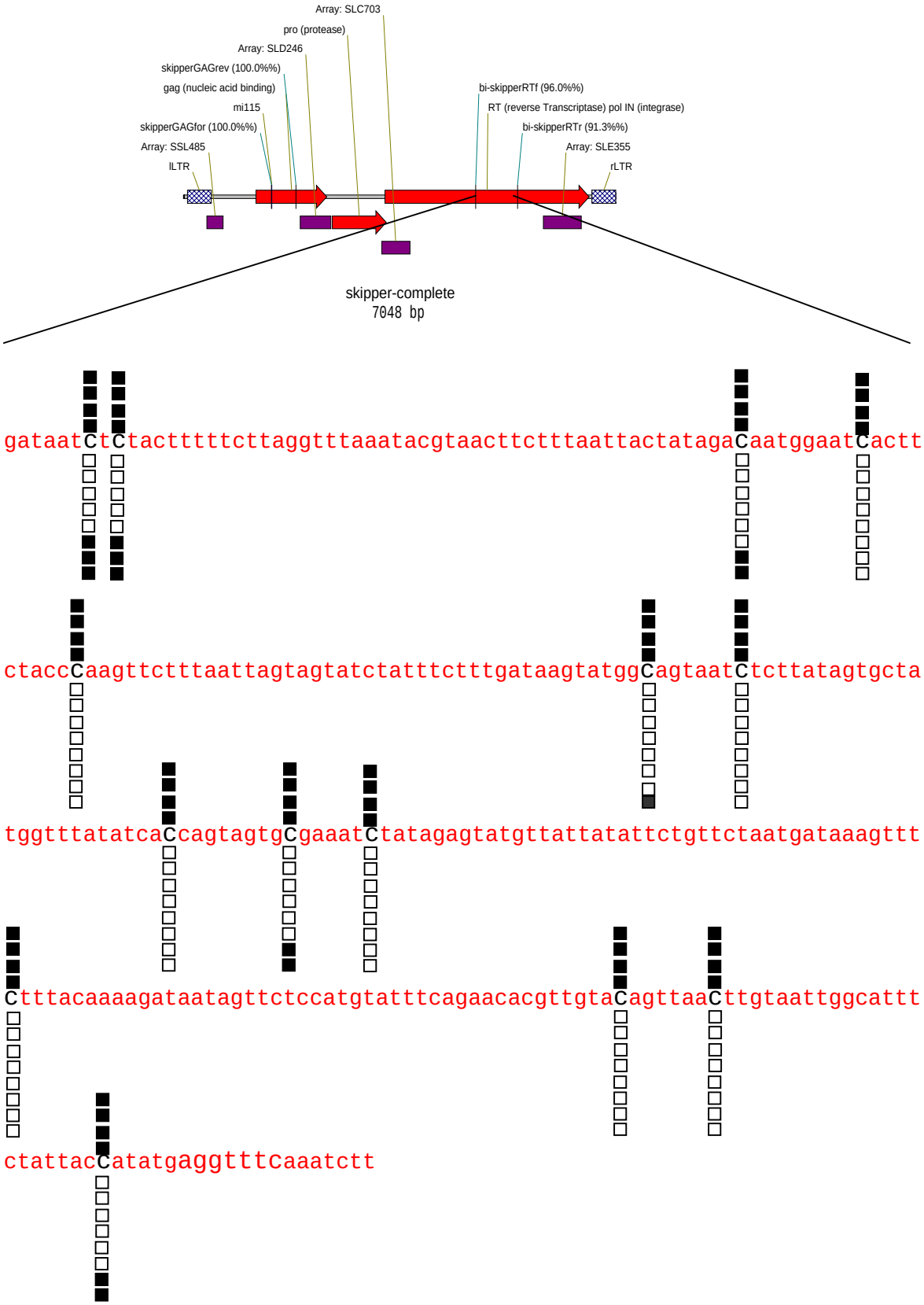


Fig. 2-41 The methylation pattern of Skipper RT in AgnA mutant by bisulfite sequencing. Symbols above the sequence are from Ax2, symbols below the sequence from AgnAas mutants.

■ methylated site □ Not methylated site

Fig. 2-42 The methylation pattern of mvpB in AgnA mutant by bisulfite sequencing. Symbols above the sequence are from Ax2, symbols below the sequence from AgnAas mutants

■ methylated site □ Not methylated site

Fig. 2-43 The methylation pattern of TelA in AgnA mutant by bisulfite sequencing. Symbols above the sequence are from Ax2, symbols below the sequence from AgnAas mutants.

■ methylated site □ Not methylated site

○ not detected methylation site

3. Discussion

3.1 The characterization of Argonaute A

The *Dictyostelium* genome encodes five Argonaute proteins, and all of them comprise conserved PAZ and Piwi domains. The PAZ-PiwiAgnA was expressed for first studies. We also attempted to study the full length Argonaute A protein, but the full length AgnA could not be induced in *E. coli* under different conditions, such as different temperatures, different concentration of IPTG or different induction time. This is might due to AgnA being toxic to *E. coli*.

A GFP fusion showed that the PAZ-PiwiAgnA is cytoplasmic, and appeared as green spots in the cytoplasm (**Fig. 2-3**). The subcellular localization of PAZ-PiwiAgnA to the cytoplasm is similar to the Argonaute proteins in other organisms (Liu J. et al. 2005). Recently, Argonaute proteins and their target mRNAs were shown to localize to cytoplasmic foci or mRNA decay foci, also known as P-bodies (mRNA processing bodies) (Liu 2005) or GW-bodies because of the accumulation of the RNA binding protein GW182 in these bodies (Eystathioy et al. 2002; Sheth and Parker 2003). Sen and Blua (2005) showed that human Ago1 and Ago2 co-localized to this mRNA decay center known as P-body. The images of fluorescent PPWa-GFP revealed that the protein localized to about 10-15 green spots in the cytoplasm, which could be interpreted as a localization of the PPWa-GFP fusion proteins to P-bodies.

3.2 Positive interaction partners of PAZ-PiwiAgnA

From screening by the yeast two-hybrid, the elongation factor 1-I, elongation factor 2, ifdA, SahA, SamS were found as positive interacting partners of PAZ-PiwiAgnA in confirmation experiments (**Table 2-5**). Elongation factor 1-I, has also been found as Argonaute-associated protein in human cells (Meister et al. 2005). Besides the elongation factor 1-I, the MOV10 (Meister et al. 2005), a putative DExD-box helicase is being found as well, which is similar in structure to the DExD-box of ifdA (**Fig. 2-13C**). PRMT5, an arginine methyltransferase, is identified as another

component (Meister et al. 2005), which indicates that Argonaute protein maybe related to histone methylation. Being involved in SAM metabolism, SahA and SamS were further studied. Presumably AgnA might be involved in DNA methylation because of the interaction with SahA and SamS.

3.3 The pLPBLP Cre-Loxp system to knock out genes in *Dictyostelium*

Faix et al (Faix et al. 2004) adapted the Cre-loxP recombination system to generate new targeted mutations continuously with the recycling of the BS-resistance gene. The *Bsr*-cassette is floxed by l*oxP* recombination sites, and the *Bsr* expression cassette can be removed by the transient expression of the Cre recombinase through intramolecular recombination. The construct facilitates the targeted disruption of multiple genes in a single cell.

This Cre-loxP system was used for disruptions of agnA, sahA, samS and ifdA genes in *Dictyostelium* cells. Among the agnA knock out mutants, most cells showed the homologous recombination of Bs^r cassette into the *Dictyostelium* genome. But the gene was not disrupted possibly due to the duplication of agnA in the *Dictyostelium* genome (**Fig. 2-31**). Similar to agnA gene, the drnA could not be disrupted either with this knock out system, and the same result was observed in ifdA knock out transformant. The disruptions of sahA, samS and ifdA genes failed after hundreds of transformants had been checked for each construct respectively. This indicated that the efficiency of Cre-loxP system was not high.

3.3.1 Duplication of AgnA and IfdA in *Dictyostelium* genome

Eichinger et al (Kuspa et al. 1992; Glöckner et al. 2002; Eichinger et al. 2005) showed a specific inverted 1.51Mb duplication on Chromosome 2 of *D. discoideum* Ax4 strain, but it is absent from the wild-type isolate NC4 and from one of its direct descendents Ax2 (Kuspa et al. 1992). But in my research, a duplication of Argonaute

A was found in Ax2, which is different from the report from Kuspa (Kuspa et al 1992). As well, the duplication of ifdA was shown on when the gene was attempted to be knocked out. Due to the result obtained from only one successful transformant, the putative duplication of ifdA needs to be confirmed.

3.3.2 AgnA knock down mutants by RNAi and antisense RNA constructs

RNAi and antisense transcripts are two tools to knock down genes. Due to the assumed duplication, the AgnA cannot be knocked out. We try to knock down AgnA by RNAi and antisense RNA constructs. Both constructs succeed in silencing AgnA gene partially or completely in *Dictyostelium* cells. The silencing efficiency mediated by RNAi (5/9 clones are silenced) (**Fig. 2-33**) is higher than by antisense RNA (3 out of 10 clones are silenced) (**Fig. 2-34**). Two mutants mediated by antisense RNA were shown silencing at high levels because no expression signals were detected after the PCR reaction was run for 47 cycles. These two mutants were selected for further study.

AgnA silencing did not affect the expression of AgnB/C/D/E (**Fig. 2-37**). This suggests that AgnA is not required for AgnB/C/D/E and that there appears to be no compensation by the other genes for a loss of the agnA gene product. But the AgnAas mutant down regulated the expression of SahA and SamS, especially strongly down regulated the expression of SamS as tested on the level of cDNA and by Northern blot (**Fig. 2-38**).

To understand the relationship between SamS and AgnA in more depth, the SamS was studied. The yeast-two-hybrid revealed that the SamS interacted with PPWa *in vivo*. Since the GFP fused SamS localized to the cytoplasm (**Fig. 2-16 B**), it might co-localize to P-body with PPWa. These two results give a hypothesis that PPWa and SamS reside in one complex, and that PPWa acts upstream of SamS and influences

its expression in the pathway from DNA to mRNA.

3.4 Study of protein-protein interaction in vitro by pull down assay and mass spectrometry

Because the Far Western blot and Swapping experiments failed (data not shown), we decided to use pull down and mass spectrometry to study protein-protein interactions *in vitro*. But these experiments were not successful. When the induced His-PPWa protein from BL21 strain was immobilized on the Ni-NTa column, SahA, elongation factor I/II were found in the co-eluates as the interaction partners. But SahA was also found in control with lower match score, and the elongation factors appeared frequently in control too. We cannot define whether these proteins are real candidates or not because it is impossible to determine if the binding is caused by specific protein interaction or unspecific association of the column, or both. There were no strongly specific interacting proteins in line with yeast-two-hybrid determined by mass spectrometry. This may be explained by the hypothesis that the proteins found in the two-hybrid system only interact with PPWa in *vivo*, or the interaction is too weak to be detected *in vitro*.

The unexpected binding of PPWa-GFP protein extract from *Dictyostelium* cells to the Ni-NTa column brought another technical problem when the cell lysate were tried to be co-elute with induced His-SamS (**Fig. 2-22, 23**). The prospect work can try to purify the PPWa-GFP protein from overexpressed stains through the Ni-NTa column, and analyze the eluate directly by mass spectrometry. It might be possible to find the binding proteins of PPWa from the eluate.

There were some specific proteins found in the co-eluates of His-SamS and wild type *Dictyostelium* extracts (**Fig 2-24**,**Table 2-6**), but the expected PPWa did not appear. This experiment needs to be repeated.

3.5 The effect of PAZ-PiwiAgnA on RNA interference and antisense RNA mediated gene silencing in *Dictyostelium*

Argonaute family genes have been isolated from several organisms in screens for mutants that are deficient in RNAi and related phenomena, including post-transcriptional gene silencing (PTGS) in plants and quelling in fungi. The *Dictyostelium* PAZ-PiwiAgnA was shown here to influence the RNAi mediated gene silencing. The overexpressed PPWa-GFP increased the RNAi efficiency to 100% compared to approximately 50% in the wild type (**Fig. 2-27**). Because it was not possible to clone the full length Argonaute A gene, we only studied the function of PAZ-Piwi domain here. The effect of PAZ-Piwi domain on RNAi in *Dictyostelium* is the same as Argonaute proteins in other organisms, for example, rde-1 in *Caenorhabditis elegans* (Tabara, 1999), AGO1 in *Arabidopsis* (Fagard et al. 2000) and QDE-2 in *Neurospora* (Cogoni and Macino 1997) that are related in RNAi. Thus this phenomenon implies that the PAZ-Piwi domain is the functional domain of Argonaute protein. The PAZ-Piwi domain comprises only 39% of the entire polypeptide chain and lacks the N-terminal part of the wild type protein. It thus appears that the other parts of the protein are not essential for its function in the RNAi pathway. Alternatively, AgnA has an inhibitory role in RNAi and overexpression of the truncated form has a dominant negative effect on an inhibitory complex. Assumed that AgnA resides in a complex which inhibits RNAi pathway, overexpression of PAZ-Piwi domain hold up the inhibition, thus leads to enhancement of RNAi. But the individual function of the PAZ and the Piwi in *Dictyostelium* is still unknown.

Our results also showed that the PAZ-Piwi domain specifically functioned in RNAi mediated gene silencing, but not in antisense RNA mediated gene silencing (**Fig. 2-28**). Until now, there is no report about the effect of Argonaute proteins on antisense RNA mediated gene silencing. This suggests that antisense RNA mediated gene silencing in *Dictyostelium* is affected by other factors even though it shares

some components with RNAi mediated gene silencing pathway. Antisense RNA most likely acts via providing a target for an RdRP that synthesizes the complementary strand (Martens et al. 2002b).

Currently, there is no data available to prove directly that the PAZ-Piwi domain influences RNAi by degrading the mRNA or inhibiting the translation in *Dictyostelium* as Argonaute proteins do in mammalian cells (Liu et al. 2004; Pillai et al. 2005). The interaction between the PAZ-Piwi domain and ifdA suggests that the PPWa may influence the gene silencing by translation inhibition. The ifdA might inhibit the translation initiation in PPWa overexpression strains. This is only a speculation because we did not check RNA levels in co-transformants only the protein level. If the mRNA levels still maintained but the protein level decreased in co-transformants, it is worthy to study the mechanism with ifdA.

3.6 The effect of Argonaute A on cell biological features of *Dictyostelium*

3.6.1 The effect of PAZ-PiwiAgnA overexpression on growth and development

In *Trypanosoma brucei*, the TbAGO1 is required for RNAi and the deletion mutant shows a dramatically reduced growth rate (Durand-Dubief and Bastin 2003). In *Dictyostelium*, the AgnA knock down mutants showed a decreased growth rate as well. The doubling time of knock down cell lines was about 14 hrs in average as opposed to 10.6 hrs of wild type.

In *Trypanosoma brucei*, the growth defects shown in TbAGO1 knock out mutants are caused by defects in mitosis and abnormal chromosome segregation (Durand-Dubief and Bastin 2003). The abnormal chromosome segregation, which appeared in TbAGO1 knock out mutants, such as delayed or unequal segregation

(Durand-Dubief and Bastin 2003) however was not found in AgnA knock down mutants. The phenotypes of shortened spindle with no recognizable spindle poles or largely bent spindle (Durand-Dubief and Bastin 2003) were also not observed in AgnA knock down mutants (data not shown).

Genetic studies showed that Argonaute proteins are important in developmental pathways in plants and animals (Bohmert et al. 1998; Coxet al. 1998; Moussian et al. 1998; Kataoka et al. 2001). To investigate a putative influence of AgnA on this, the development time course was performed on *Dictyostelium* PAZ-PiwiAgnA overexpression stains. The experiments showed delayed development in overexpression strains, and cells showed a thinner, smaller and less transparent phenotype of fruiting bodies compared with wild type. But this delayed development was not seen always, which might be explained by varying overexpression levels in individual clones.

3.6.2 The effect of AgnA on phagocytosis

To address the decreased growth rate, the phagocytosis process and the growth on bacterial lawn were investigated in two AgnA knock down cell lines. The defects of phagocytosis and growth on bacterial lawn were consistent.

Previous studies clearly indicated that mutant strains with impairment in fluid-phase endocytosis are correlated with an inability to proliferate in axenic culture (Bacon et al. 1994), while impairment of the phagocytosis system affects cell growth on the bacterial lawn (Cohen et al 1994). The impaired growth phenotypes of the AgnA knock down cells in the axenic culture and on the bacterial lawn are consistent with the observed defects in phagocytosis.

3.7 The effect of Argonaute A on DNA methylation

3.7.1 Expression of DIRS-1 and Skipper in AgnA knock down mutants

The transcription of the retrotransposon Skipper was activated when DNA methylation was inhibited in AgnA knock down cell lines, but the expression of DIRS-1 was not affected by a loss of DNA methylation (**Fig. 2-39**). These results were similar to those obtained for knock out mutants of the only DNA methyltransferase DnmA in *Dictyostelium* (Kuhlmann et al. 2005). Assumed that this discrepancy maybe caused by the different organization and the different strategies of transposition of the elements (Kuhlmann et al. 2005). The inactive transcription of DIRS-1 in AgnA mutant is maybe due to the transposition of the elements. DIRS-1 has methylated C-residues on the inverted terminal repeats, while Skipper has unmethylated direct terminal repeats. Furthermore, there are many incomplete copies of DIRS-1 in the genome and the element frequently transposes into its own copies, while Skipper has almost complete and separate copies in the genome (Glockner et al. 2001).

3.7.2 Decreased DNA methylation in AgnA knock down mutants

In *Arabidopsis*, AGO4 controls histone and non-CpG DNA methylation, and accumulation of locus-specific siRNA (Zilberman 2003). The deletion of AGO4 decreased CpNpG and asymmetric DNA methylation as well as histone H3 lysine-9 methylation. In addition, the mutant blocked the accumulation of siRNA that correspond to the retroelement AtSN1. In *Dictyostelium* a decreased DNA methylation was observed in AgnA knock down mutant as well. The bisulfite sequencing showed that the AgnA knock down mutants decreased the methylation of asymmetric C-residues in DIRS-1 loci and Skipper RT, and completely abolished C-methylation on both mvpB and telA genes. Interestingly, two C-residues on DIRS-1 and one on telA were consistently not detectable. The loss of C-residues

consistently appeared in every sequencing data for DIRS-1 and telA. We cannot explain the reason why these C-residues were lost in sequencing data. The difference of methylation pattern between AgnA knock down and DnmA knock out mutants might be explained by their different silencing level: the dnmA is interrupted in the knock out, which leads to the loss of DNA methylation, while the residual AgnA cannot be completely excluded in the knock down, which leads to the inhibition of DNA methylation not the complete loss.

All tested methylated genes showed reduced or no DNA methylation in AgnA knock down cell lines. This demonstrates that AgnA influences methylation as AGO4 does in *Arabidopsis* (Zilberman et al. 2003). However AGO4 in *Arabidopsis* affect symmetric DNA methyaltion that is mediated by CMT3, a different class of methyltransferase, than by the only Dnmt2-like enzyme that is present in the *Dictyostelium* genome. Moreover, DnmA functions on mostly asymmetric DNA methylation in *Dictyostelium.*

The loss of DNA methylation was observed in DnmA knock outs (Kuhlmann et al 2005). However, from the directed yeast two-hybrid experiments it could be concluded that there was no direct interaction between AgnA and DnmA. Therefore AgnA does not affect DNA methylation through DnmA directly, but rather through some other factors, possibly SahA and SamS, the two enzymes involved in the S-Adenosyl-methionin biosynthesis pathway that were identified as PPWa interaction partners in the Yeast two-hybrid screen.

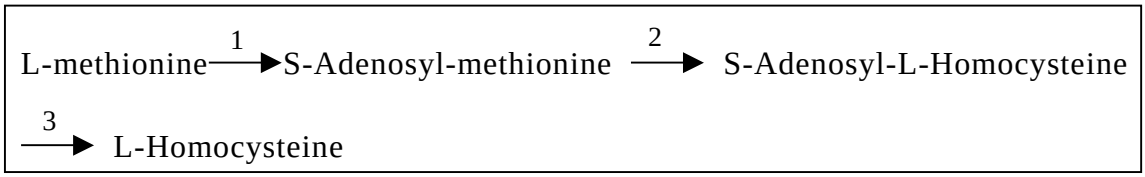


Fig. 3-1 Metabolism of SAM. 1. SamS, 2 methyltransferase, 3 SahA

SamS functions as methionine adenosyl transferase in the synthesis of S-Adenosyl-methionine. The second enzyme, SahA, hydrolyses the reaction product

of RNA and DNA methyl transferases, S-Adenosyl-L-Homocysteine, to L-Homocysteine (**Fig.3-1**).

The strong down regulation of SamS on both RNA and cDNA levels detected by Northern blot and RT-PCR might be the main reason for lack of DNA methylation in the AgnA knock down mutants. SamS functions in providing the methyl group donors for methyltransferase, the decreased SamS might result in less substrates for reaction, which, as a result, would reduce DNA methylation.

The slight down regulation of SahA in the AgnA knock down mutant may be the second reason for decreased DNA methylation. The *Arabidopsis* HOG1 gene coding for SahA is required for DNA methylation-dependent gene silencing (Rocha et al. 2005). It is shown in this paper that mutations of HOG1 relieve transcriptional gene silencing and result in genome-wide demethylation, and the mutated plants show reduced SAH hydrolase activity. Moffatt and Weretilnyk (2001) explained that SahA plays a role in the removal of SAH (S-Adenosyl-L-Homocysteine), the by-product of the transmethylation reactions where SAM (S-Adenosyl-methionine) is the group donor. SAH is a strong inhibitor of SAM-dependent methyltransferases, and it appears that the relative levels of SAH and SAM are critical in the methylation of DNA, proteins, pectins and other small molecules in the cell.

Then we speculate that AgnA may influence DNA methylation by its effects on expression of SamS and SahA not by its direct effects on DnmA. The knock down of AgnA downregulated the expression of SamS and SahA in the pathway from DNA to RNA (shown by RT-PCR and Northern blot), then this maybe lead to low expression levels of SamS and SahA in AgnA mutants. The decreased SamS may lead to less methyl donor for DNA methyltransferase, and the slightly decreased SahA might lead to accumulation of SAH, and consequently the accumulated SAH may inhibit the activity of methyltransferase. This speculation is different from AGO4 in *Arabidopsis*, which directly affects CMT3 (Zilberman 2003). However, AgnA also

could comply the similar mechanism as in *Arabidopsis* by which the protein is directly involved in DNA methylation machinery.

The slightly down regulated SahA maybe another reason for slow growth of the AgnA knock down mutants, which is similar to the result shown by Moffatt and Weretilnyk (2001). They reported that an *Arabidopsis* line with a point mutation of SAH hydrolase 1 showed reduced growth. It is unclear whether this problem is caused by the SAH hydrolase deficiency directly or mediated by genome hypomethylation indirectly (Moffatt and Weretilnyk 2001). To investigate these possibilities, a knock down of SahA in *Dictyostelium* cells might be used to analyze the growth behavior

The involvement of AgnA in DNA methylation and RNAi indicated a connection between RNAi and DNA methylation, i.e. gene regulatory processes on the transcriptional and post-transcriptional level in *Dictyostelium*. While accumulation of siRNA in the AgnA knock down mutants was not investigated, the role of AGO4 in siRNA accumulation is known in *Arabidopsis* (Zilberman et al., 2003). Since siRNAs against DIRS-1 were found to cover essentially the entire DIRS-1 sequence, all methylation sites in the short segment that were sequenced by the bisulfite method had a corresponding siRNA (Kuhlmann et al. 2005). It is worthy to investigate if AgnA has a function in accumulation of siRNA derived from DIRS-1, which are produced by the Dicers and RNA dependent RNA polymerases (Martens et al. 2002b). The produced siRNA might direct DNA methylation in cells.

In *Dictyostelium* cells, the coding sequencing rather than the LTR are methylated in Skipper. This may indicate that DNA methylation causes chromatin remodeling over the entire retroelement and thus blocks the accessibility for the transcription machinery (Kuhlmann et al. 2005). As known in yeast, Ago1 is required for silencing of pericentric chromatin (Ekwall 2004); the AGO4 in *Arabidopsis* is linked to transposon siRNAs as well as DNA and histone methylation (Zilberman et al.

2003) The inhibition of DNA methylation of Skipper in AgnA knock down mutants indicates that AgnA in *Dictyostelium* is linked to chromatin remodeling as Ago1 in yeast and AGO4 in *Arabidopsis*. This suggests that RNAi, DNA methylation and histone modification regulate gene expression simultaneously in *Dictyostelium*.

4. Materials and methods

4.1 Materials

Antibiotics

Ampicillin	Sigma, Deisenhofen
Amphotericin	Serva, Heidelberg
Blasticidin	ICN, Arora
Chloramphenicol	Sigma, Deisenhofen
Geneticin (G418)	Sigma, Deisenhofen
Kanamycin	Sigma, Deisenhofen
Penicillin/ Streptomycin	Gibco BRL, Eggenstein

Antibodies

Monoclonal antibodies	
discoidin antibody	Department of Cell Biology, Uni. of Kassel
coronin antibody	Department of Cell Biology, Uni. of Kassel
Cmyc antibody QE10	Department of Cell Biology, Uni. of Kassel
GFP antibody 264-171-12	Department of Cell Biology, Uni. of Kassel
His antibody 32-470-5	Department of Cell Biology, Uni. of Kassel
IgG, goat-anti-mouse, alkaline phosphatase-coupled	Bianova, Hamburg
IgG, goat-anti-mouse, Cy3 (Cyanine 3.18) coupled	BioTrend

Biological Materials

Bacterial strains

Escherichia coli DH5 α	Promega, USA
Escherichia coli JM 109	Promega, USA
Escherichia coli XL-1 Blue	Promega, USA
Escherichia coli BL21	Novagen, USA
Escherichia coli BNN132	Promega, USA
Klebsiella aerogenes	Department of Genetice, Uni. of Kassel

Yeast strain

Y109	Clontech, Heidelberg
Dictyostelium discoideum	Department of Genetice, Uni. of Kassel
Ax2	

Chemicals and reagents

Acetic acid	Fluka, Deisenhofen
Aceton	Fluka, Deisenhofen
(19:1 acrylamide: bisacrylmide) acrylamide Protogel 30%	National Diagnostics, USA
Agarose SeaKem	FMC Bioproducts, USA
Ammonium peroxodisulfate (APS)	Merck, Darmstadt
3-AT (3-Amino-1,2,3-Trizol)	Roth, Kalsruhe
Bacto-peptone	Difco, Augsburg
Bacto-tryptone	Difco, Augsburg
Bacto-yeast extract	Roth, Kalsruhe
β -mercaptoethanaol	Fluka, Deisenhofen
Boric acid	Roth, Kalsruhe
Bromophenol blue	Fluka, Deisenhofen
BSA	Roth, Kalsruhe
Calcium chloride (CaCl2)	Roth, Kalsruhe
Chloroform	Fluka, Deisenhofen
Coomaasie brilliant blue R-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
DMF (N,N-dimethylformamide)	Merk, Darmstadt
DMSO	Serva, Heidelberg
DTT	Roth, Kalsruhe
EDTA	Roth, Kalsruhe
Ethanol 99.8%	Roth, Kalsruhe
Ethidium bromide	Fluka, Deisenhofen
Formaldehyde 37%	Riedel-de-Haen, Seelze
Formamide	Roth, Kalsruhe
D (+) glucose	Fluka, Deisenhofen
Glycerol, 86%	Roth, Kalsruhe
DIFCO (bacto-yeast nitrogen base, without AA)	Roth, Kalsruhe
Gylcine	Roth, Kalsruhe
Guanidine thiocyanate	Roth, Kalsruhe
HEPES	Fluka, Deisenhofen
IPTG	Bts, ST. Leon-Rot
Isoproponal	Fluka, Deisenhofen
Liquid nitrogen	Messer Griesheim, Krefeld
Lithium acetate (LiAc)	Roth, Kalsruhe
Lithium chloride (LiCl)	Roth, Kalsruhe
Magnesium chloride (MgCl2)	Roth, Kalsruhe
Magnesium sulphate (MgSO4)	Fluka, Deisenhofen

Methanol	Fluka, Deisenhofen
Methylene blue	Roth, Kalsruhe
MOPS	Fluka, Deisenhofen
N-lauroylsarcosine	Roth, Kalsruhe
NP-40	Fluka, Deisenhofen
PEG 4000	Roth, Kalsruhe
Phenol	Roth, Kalsruhe
Phenol/ chloroform	Roth, Kalsruhe
Phosphor acid (H3PO4)	Fluka, Deisenhofen
PMSF	Fluka, Deisenhofen
Potassium acetate (KAc)	Riedel-de-Haen, Seelze
Potassium chloride (KCl)	Roth, Kalsruhe
Potassium hydrogenphosphate (KH2PO4)	Fluka, Deisenhofen
Potassium dihydrogenphosphate (K2HPO4)	Roth, Kalsruhe
Rotiphorese gel (acrylamide)	Roth, Kalsruhe
Sucrose	Roth, Kalsruhe
SDS (sodium dodecyl (lauryl) sulfate)	Riedel-de-Haen, Seelze
Sephadex (G25, G50)	Pharmacia, Freiburg
Sodium acetate (NaAc)	Fluka, Deisenhofen
Sodium azid (NaN3)	Merck, Darmstadt
Sodium carbonate (NA2CO3)	Roth, Kalsruhe
Sodium citrate	Roth, Kalsruhe
Sodium dihydrogenphosphate (NaH2PO4)	Fluka, Deisenhofen
Sodium hydrogenphosphate (Na2HPO4)	Fluka, Deisenhofen
Sodium hydroxide	Fluka, Deisenhofen
ss-carrier DNA (deoxyribonucleic acid, sodium salt, Salmon sperm)	MP (ICN), Germany
TEMED	Biomol, Hamburg
Tris	Riedel-de-Haen, Seelze
Triton-x-100	Serva, Heidelberg
Tween 20	Roth, Kalsruhe
Urea	Merck, Darmstadt
X-gal	Roth, Kalsruhe

Devices

Autoclave	Zirbus, Bad Grund
Binocular	Olympus, Hamburg
Centrifuges:	
Microfuge®Lite	Beckmann, Munich
Avanti™ 30	Beckmann, Munich
C0650, C1015, F2404 rotors	Beckmann, Munich
Centrifuge 5417 C	Eppendorf, Hamburg
Rotina 48R	Hettich, Tuttlingen

E.A.S.Y. gel documenting system	Herolab, Wiesloch
Fluorescence microscope	Leitz, Wetzlar
Gel dryer	Bachofer, Reutingen
Gene pulser®	Bio-Rad, Canada
GeneQuant®	Pharmacia, Freiburg
Gradi Frac®	Pharmacia, Freiburg
Glasspipette	Hirschmann, Germany
Heating block	Workshop, University of kassel
Hybridization oven	Bachofer, Reutlingen
Imager: Fuji X Bas 1500	Raytest, Straubenhart
Bio Imaging Analyzer (BAS cassette 2025)	Raytest, Straubenhart
LI-COR DNA sequencer 4000 and 4200	MWG-Biotech
Magnetic stirring plate	Bachofer, Reutlingen
Microscope	Zeiss, Jena
Microwave oven	Panasonic
PCR-Mastercycler personal	Eppendorf, Hamburg
PH-meter 320	Bachofer, Werthem/Main
Pipette boy	Integra bioscience, Fernwald
Pipettes	Eppendorf, Hamburg
Scales	Satorius, Goettingen
Semidry blotting apparatus	Von Kreuz, Reiskirchen
Speed Vacuum concentrator	Savant, USA
Ultra-sonicator UP 200S	Dr. Hielscher GmbH, Standsdorf
UV/visible spectrophotometer (Ultrospec 2000)	Pharmacia Biotech.
Vortex Genie	Bender Hohbein AG, Germany
Water bath Julabo F25	Schuett, Goettingen

DNA and protein markers

100bp DNA ladder	MBI Fermentas, ST. Leon-Rot
100bp plus DNA ladder	MBI Fermentas, ST. Leon-Rot
1kb DNA ladder	MBI Fermentas, ST. Leon-Rot
Protein marker	Carl Roth GmbH, Karlsruhe
SeeBlue pre-stained protein marker	Novex, USA

Enzymes and proteins

Protease inhibitor	MBI Fermentas, ST. Leon-Rot
Proteinase K	Boehringer, Mannheim
Restriction endonucleases	Boehringer, Mannheim
	Gibo BRL, Eggenstein
	MBI Fermentas, ST. Leon-Rot
	New England Biolabs

Reverse transcriptase (Mu-MLV)	MBI Fermentas, ST. Leon-Rot
RNaseA	Boehringer, Mannheim
Shrimp alkaline phosphatase (SAP)	USB, USA
T4 DNA ligase	MBI Fermentas, ST. Leon-Rot
Taq DNA polymerase	Homemade, Department of Genetics, University of Kassel
Klenow fragment	MBI Fermentas, ST. Leon-Rot

General buffer and solutions

6x DNA loading dye	40%(w/v) sucrose 0.25% bromophenol blue 0.25% xylene cyanol FF
100x Denhardt	2% Ficoll 400 2% polyvinylpyrollidone 2% BSA
Ethidium bromide solution	10 mg/ml
2 X HBS buffer (Hepes buffered saline)	4.0 g NaCl 0.18 g KCl 0.05 g Na_2HPO_4 or 0.062 g $Na_2HPO_4 \cdot 2H_2O$ 2.50 g Hepes 0.50 g Destrose dissolved in 250ml water, pH 7.05 sterilized by filter
2X Laemmli	25 ml 4X Tris/ SDS, pH 6.8 (upper buffer) 4 g SDS 20 ml Glycerin 3.1 g DTT (or 2ml B-mecaptoethanol) 0.05% (w/v) Bromphenoblue Add H_2O to 100ml
9X Laemmli buffer	3.3 ml Glycerin 1.5 ml β-mercaptoethanol 0.69 g SDS 0.22 g Tris 0.30 g Bromphenol blue H_2O to 10ml (pH 6.8)
10x NCP buffer	10 mM Tris-HCl, pH 8.0 150 mM NaCl 0.05% Tween 20
Phosphate buffer, pH 6.7	56.5 ml 1M KH_2PO_4 43.5 ml 1M K_2HPO_4

20x SSC	3 M NaCl 0.3 M sodium citrate
TAE buffer	40 mM Tris-acetate 2 mM EDTA
TBE buffer	90 mM Tris 90 mM Boric acid 2 mM EDTA
TE buffer	10mM Tris-HCl 1mM EDTA pH7.4 or 8.0
Z buffer	16.1 g/l $Na_2HPO4 \cdot 7H_2O$ 5.50 g/l $NaH_2PO4 \cdot H_2O$ 0.75 g/l KCl 0.246 g/l $MgSO_4 \cdot 7H_2O$ pH 7.0

Kits and reagents sets

Nucleotrap	Macherey-Nagel, Dueren
Nuclespin ExtractII	Macherey-Nagel, Dueren
Nucleobond AX100	Macherey-Nagel, Dueren
ThermoSequenase labeled primer cycle sequencing kit	Amersham pharmacia, Freiburg
Bisulfite	Qiagen, Hilden

Media

Dictyostelium general media

Hl-5 medium, pH 6.7	14.3 g Bacto-peptone 18.0 g glucose 0.616 g $Na_2HPO_4 \cdot 2H2O$ 0.486 g KH_2PO_4 7.15 g yeast extract add H_2O to 1000ml and autoclave for 20minutes at 121˚C
DD20 medium	20 g/l proteose peptone 7 g/l yeast extrat 8 g/l glucose 0.35 g/l KH_2PO_4 0.47 g/l $Na_2HPO_4 \cdot 12H_2O$ or 0.33g $Na_2HPO_4 \cdot 7H_2O$ adjust pH to 6.5 and autoclave for 20 minute at 121˚C
G0 medium	Hl-5 medium plus 50 µg/ml ampicillin 100 µg/ml streptomycin/penicillin 0.25 µg/ml amphotericin

MES-Hl-5 medium	5 g/l yeast extract 10 g/l glucose 10 g proteose peptone 1.3 g MES 0.05 g Dihydrostreptomycin-sulfate adjust pH to 7.1 and autoclave for 20 minute at 121˚C
SM plate, pH 6.5	15 g bacto-agar 10 g peptone 10 g glucose 1 g yeast-extract 1 g $MgSO_4 \cdot 7H_2O$ 2.2 g KH_2PO_4 1 g K_2HPO_4 add H_2O to 1000ml, autoclave for 20minutes at 121˚C 20ml/petridish
Bacterial media	
LB medium	10 g Bacto-Tryptone 5 g yeast-extract 5 g NaCl add H_2O to 1000ml, autoclave for 20minutes at 121˚C
LB agar	10 g Bacto-Tryptone 5 g yeast-extract 5 g NaCl 9g agar add H_2O to 1000ml, autoclave for 20minutes at 121˚C
LBamp plate	LB agar is chilled to 55˚C, and Ampicillin is added to a final concentration of 50μg/ml
Yeast medium	
YPD medium pH5.8	20 g/l DIFO peptone 10 g/l yeast extract 20 g/l Agar (for plate only) add H_2O to 950ml, adjust pH 5.8, autoclave, and cool to 50 ˚C, add dextrose(glucose) to 2%
SD medium	6.7 g yeast nitrogen base without amino acids 20 g agar (for plate only) 850 ml H_2O 100 ml of the appropriate steriled 10x dropout amino acid solutions

Oligonucleotide primers

Table 4-1 Oligonucletoide primers used in experiments

Name	Sequence (5'---3')
PPW 5'	ATTCTGCAGATCATATGGCTGGTACAATGTTATTATGTG
PPW 3'	ATTGTCGACTTAGGATCCATCTTTATCAACATTACGACC
PPW-GFP 3'	ATTGTCGACTTAGGATCCATCTTTATCAACATTACGACC
PPW-GFP5'	ATTGAATTCAAAATGGCTGGTACAATGTTATTATGTG
cmyc-PPw 5'	ATTGTCGACGCATGGAGGAGCAGAAGCTGATC
AgnAi sense 5'	ATTCTGCACGTTGAAGGTTGTGTATTAC
AgnAi sense3'	ATTGGATCCTGAATTGGATCAATGACTTC
AgnAi antis 5'	ATTGGATCCAACCACTGAATGACCTTT
AgnAi antis 3'	ATTGAATTCGTTGAAGGTTGTGTATTAC
AgnA Ko RA 3'	AATCCGGAGAAGACTGAATGACCTTTCATATCTGA
Ago1 RA 3'	AAGGATCCGAAGACTGAATGACCTTTCATATCTGA
Ago1 RA 5'	AAAAAGCTTAGCAGAAGAATCAAATGTTGAACCAT
Ago1 LA 3'	AAAAGCATGCCCAGTTAAATAAGTTAATTCTGGAAC
Ago1 LA 5'	AAGGGCCCGAAGACGAGATCAATGGGATATTCATTAATGG
AgnA outer 5'	CAAGAGATAGAAGATTCAAGG
BSrG1 5′ #385	CGCTACTTCTACTAATTCTAGA
BSr G1 3' #492	TCTAGAATTAGTAGAAGTAGCG
AgnA-GFP 5'	ATTCTGCAGAAAATGGGTCCTAAAAAACCAAACATA,
AgnA-GFP 3'	GTCGGATCCTTTATCAGATAAATCTTTATCAACATTACG,
ifdA 5'	ATGTCCGCAAGTAATAAAG
ifdA 3'	TTAGAGGAAACTTCCAATG
ifdA 3 BamHI	ATTGGATCCTTAGAGGAAACTTCCAATG,
Saha 5'	TAATATCTGTAGTGATCAAC
Saha 3'	ATGACTAAATTACACTACAAAG
Saha 3' BamHI	ATTGGATCCTTAATATCTGTAGTGATCAAC
SAMS 5'	ATGTCATCTTATTTATTCACTAG
SAMS 3'	TTACATCTTGAGTTCTTTGAC
SAMS 5'XhoI	ATTCTCGAGATGTCATCTTATTTATTCACTAG
SAMS 3'BamHI	ATTGGATCCTTACATCTTGAGTTCTTTGAC
Saha LA 5'	ATTGGATCCGAAGACAGATATTTCACTTGCCGC
Saha LA 3'	ATTCATATGGAACATTTTGTAGAGGTTGT
Saha RA 5'	ATTAAGCTTTGGGTGCTCGTGTTTTA
Saha RA 3'	ATTGTCGACGTCTTCTATCTGTAGTGATCAACTTTG,
SAMS LA5'	ATTGGATCCGAAGACTCACTAGTGAATCCGTCA
SAMS LA3'	ATTCTGCTAACCACAAGCAACTTTTGA
SAMS RA5'	ATTAAGCTTAGAGTACACACCATCGTTA
SAMS RA3'	ATTGTCGACGTCTTCTCTTGAGTTCTTTGACGG
ifdA LA 5'	ATTGGATCCGAAGACATGTCCGCAAGGTAAATAGA
ifdA LA3'	ATTCATATGCAGTACCAGATTGAGCTT

ifdA RA 5’	ATTCCATGGTATTCTCTGCCACCATGA
ifdA RA 3’	ATTGTCGACGTCTCCGAGGAAACTTCCAATGTC
Saha 5’outer	AATAGAATGACTAAATTACACTACA
Saha 3’outer	ATAATTCAACCTAATGACAACCAG,
SAMS 5’outer	GTAGTTATTTAAGTAATATTTCACAC
SAMS 3’outer	CCAATGATACCTTATCAAAATAGTC,
IfdA 5’outer	ATTAGAAATAAAACATGTCCGCAAG
IfdA 3’outer	CGCCTACTTTATACAACAACAC
SAMSi sense5’	CTGCAGCAACTTTAATGCCACTTACTC
SAMSi sense3’	GGATCCAAACCAGAATCACCCATTGG
SAMSi As5’	GGATCCGGTATCAACAAAGACACTGAG
SAMSi As3’	GAATTCCAACTTTAATGCCACTTACTC
pACT2 SEQ/PCR primer 263	GAGATGGTGCACGATGC
pACT2 sequence/pcr primer 262	CGCGTTTGGAATCACTAC
Act6-Seq-P	GAGGTTTCTGAGATTATAAAATG
DISC-AS-P-ML1	GCTACTCTCCCAAAGGTTGCCC
Coronin-run-on 5	GTGAATTCAAATCTGCCACCCCAC
SkipperGAG F	TGAAGCTAAAACCATTGACGC
SkipperGAG R	CTAATTGAACTTCAGCAGTACC
Bi-Skipperrt F	AAATCTTACATATATTATCAATAAA
Bi-Skipperrt R	AATAATTGAGTAGTATGTTGGGT
DIRS 2F	GTATGCCCTGTTCGCCACCTTGC
DIRS 1R	CGTAGAAGGTATCTACAGTATC
Bi-DIRSltr F	ATCAAATTATTTTAATTTTTAATA
Bi-DIRSltr R	AATATTTATTTATTTGAATTTTTT
Bi-mvp F	ATCTTACAACTAACACTTTAAA
Bi-mvp R	GTAATTTTAATGGTTAATTGAAT
Bi-Tel F	ATCATACTCAAAATATTCTTCA
Bi-Tel R	TTAGTTAGAATTGTTAATAAATT
#790 agnb2	GGTTGATATGTAATTCCCC
#791 agnb3	GGTCAAGTGGAGTTAATG
#798 agnd2	ATGTAAAGAAACCTGGCC
#799 agnd3	ATCAGGATTACAAGGTGC
#802 agne2	ACCGCTTTCAACTGTATG
#803 agne3	CATTAGGATTTGTAAAATTGGG
#688 Trx-1F	GAACGAGCTCCATGGCCAATAGAGTAATTCATG
#689 Trx-1R	CGCGGATCCTTATTTGTTTGCTTCTAGAGTACTTC
HP1_5'_Y2H	GTCCGATGTGTCCATGGGAAAAAGAGATAAGAGAATAATAG
HP1_3'_Y2H	CACTAGTGATTGGATCCTTTTAACTTTGTTGACCC
T7 promoter Primer	TGTAATACGACTCACTATAGGG
Sp6 promoter Primer	ATTTAGGTGACACTATAGAATAC

plasmids

pACT2	Department of Genetic, University of Kassel
pet-15b	Department of Genetic, University of Kassel
pDd-GFP	Department of Genetic, University of Kassel
pDex-RH	Department of Genetic, University of Kassel
pdneo2	Department of Genetic, University of Kassel
pdneo2-GFP	Department of Genetic, University of Kassel
pGBKT7	Clontech, Heidelberg
pGem T-easy	Promega, USA
pLPBLP-cre-lox	Department of Genetic, University of Kassel
#979 K.O	Department of Genetic, University of Kassel

Additional materials

Charged (Hybond ^{TM}) nylon membranes	Pharmacia, Freiburg
Costar plates	Schuett, Goettingen
Eppendorf tubes	Eppendorf, Germany
Falcon tubes	SARSTEDT Aktiengeeilschaft & Co
Glass pipettes	Hirschmann, Germany
Injection needles	B.Braum, Melsungen
Injection syringes	B.Braum, Melsungen
Nitrocellulose membranes	Macherey Nagel
Parafilm M	American Can ^{TM}, USA
Petri dishes	Sarstedt, Nuembrecht
PCR tubes	New Englands, Schwalbach
Sterile-filter (0.22µm, 1.45µm pores)	Millipore, Eschborn
Transfer membranes Parablot NCP	Mecherey & Nagel, Dueren
Tips	Eppendorf, Germany
Scalpels	C.Bruno Bayha GmbH, Tuttligen
Whatman paper 3MM	Whatman, Goettingen

4.2 Methods

4.2.1 Cell biological methods

4.2.1.1Cell growth of *Dictyostelium discoideum*

Dictyostelium discoideum Ax2 and the transformants are incubated either in axenic medium or selective medium with Geneticin (G418) or Blasticidin, or on SM plates with *Klebsiella aerogenes* (KA) as a food source.

4.2.1.2Development time course of *Dictyostelium discoideum*

Vegetative cells are harvested at the density of $1x10^6$ / ml to get $1\text{-}5x10^7$ cells for one filter. The cells are centrifuged at 1600 rpm for 10 min at 4℃. Cell pellets are washed with 1x cold phosphate buffer by centrifuging at 1600 rpm for 10 min at 4℃. Then pellets are resuspended with proper amount of 1x phosphate buffer, and $1\text{-}5x10^7$ cells are applied on one filter.

Before applying the suspension on the filter, the filters (HABP 45mm diameter) are boiled in water for 3-5 min, and placed in a plastic container that contains two layers of 3mm Whatman paper soaked in 1x Phosphate buffer. The container is covered with a lid that contains a piece of Whatman paper soaked with 50x Phosphate buffer. Cells are incubated in the culture room and can be collected at different time points.

1 x Phosphate buffer	17 mM Na-phosphate, pH 6.4

4.2.1.3*Dictyostelium discoideum* transformation

Axenic Ax2 cells are harvested at the density of 1-2 x 10^6 cells/ml and then transformed either by the classical method (Nellen et al, 1984) or electroporation (Howard et al, 1988). All gene disruption mutants are done by electroporation, and the overexpressoin transformants with Geneticine (G418) resistance are generated by classical methods.

(i) Classical transformation (Calcium method)

The cell culture is prepared by shaking in proper medium till the cell density reaches $1x10^6$ cell/ml. 10ml axenically grown cells are plated on the petri dish, after half an hour the medium is changed with MES-HL5, and cells are incubate for 30 minutes. Meanwhile, the DNA mixture for transformation is prepared according to the protocol below. The MES-HL5 medium is gently removed without destroying the cell layer, then the DNA mixture is dropped on cells. After 20 min incubation, 10ml MES-HL5 medium is added to the petri dish, and cells are incubated for 3 hours at room temperature. At the end, all medium is removed carefully, 2ml glycerol/HBS solution is added and cells are incubated for 5 min. Subsequently, the glycerol/HBS solution is removed completely, replaced by 10ml DD20 medium and cells are incubated overnight. The next day, the DD20medium can be changed with selective medium. The medium should be changed regularly every three days. The successful transformation should give colonies within a week.

DNA mixture preparation

Transformation	**Co-Transformation**
300µl 2x HBS	300µl 2x HBS
280µl MP H_2O	260µl MP H2O
20µl DNA	20µl + 20µl DNA

38µl 2M $CaCl_2$ are dropped into the mixtures, the mixtures are vortexed and incubated for maximum 25 min at room temperature.

Glycerol/ HBS solution	3 ml 60% glycerol (18% final) 2 ml H_2O 5ml 2X HBS

(ii) Electroporation

Axenically grown cells ($2x\ 10^7$) are harvested in a falcon tube, centrifuged at the speed of 1600 rpm for 10 minutes, at 4˚C. The pellets are washed with ice cold phosphate buffer once, and twice with ice cold electroporation (EP) buffer. Then the pellets are resuspended in 800µl EP buffer and 10-20µg DNA. The mixture is pipetted into an ice cold cuvette (0.4cm), and incubated on ice for 10 min. The electroporation is performed at 1 KV, 25µF with the time constant between 2 and 3

sec. Cells are plated on a petri dish, mixed with two drops (8µl) of each 100mM $CaCl_2$ and 100mM $MgCl_2$, and left at room temperature for 15minutes. The transformed cells are incubated overnight with 10ml DD20 or G0 medium. The next day the medium is changed with appropriate selective medium.

EP buffer	10 mM Na_2HPO_4 50 mM sucrose sterilized by filter

4.2.1.4 Subcloning of *Dictyostelium discoideum* on SM plates

To obtain single *Dictyostelium discoideum* clones, 50-200 cells are resuspended in 100µl KA suspension, and plated out on SM plates. Plates are incubated at 22˚C for several days until colony plaques appeared on the bacterial lawn. Single clones can be picked with tooth sticks, and transferred to both new KA plates and Costar plates with selective medium to eliminate KA contamination.

4.2.1.5 Fluorescence assay by DAPI staining

Exponentially growing *Dictyostelium* cells at the density of 1 x 10^6 cell/ml are dropped (30µl) on a HCL-treated clean coverslip, after the cells settle down, the medium is removed and replaced by one drop of phosphate buffer, then cells can be checked under fluorescent microscope.

Alternatively, 300µl exponentially grown cells are dropped on a HCL-treated clean coverslip, after 45min when the cells settle down, the medium is removed and washed with cold phosphate buffer twice. The cells are fixed with cold methanol at -20˚C for 20min. After that methanol is washed out twice with 1x PBS buffer. To stain the cell nuclei, 300µl DAPI (4',6-diamidino-2-phenylindole)solution is added for 20min at room temperature and washed out with 1x PBS buffer twice. A drop of antiquenching reagents, such as DABCO (1,4-diazabicyclo 2.2.2. octane) is added onto the cell and the coverslip is put on a glass slide and leave overnight in the

refrigerator. The images can be taken by the fluorescence microscope.

10x PBS	80 g/l NaCl 2 g/l KCl 11.5 g/l Na_2HPO_4 or 14.4 g Na_2HPO_4*$2H_2O$ 2.04 g/l KH_2PO_4 pH 7.4

4.2.1.6 Fluorimetric analysis of Phagocytosis of *Dictyostelium* cells using TRITC-labeled yeast cells

Dictyostelium cells are harvested and resuspended at 2 x 10^6 cell/ml in fresh nutrient medium. 100µl of cell suspension is dispensed in an Eppendorf tube, spun down for 10 sec at maximum speed. The supernatant is removed and the pellets are frozen at -20°C. 10ml of cell suspension is dispensed in a 30ml Erlenmeyer flask and incubated for 15 min on a rotary shaker to allow cells to recuperate. This sample is used to determine protein content. An aliquot of fluorescent yeast cells are thawed, sonicated briefly and vortexed vigorously to dissociate aggregates. 120µl of fluorescent yeast cells are added into *Dictyostelium* cell suspension and incubated on a rotary shaker. The amount of added yeast cells corresponds to a 6-fold excess relative to *Dictyostelium* cells. The experiment starts immediately (time point 0), at the same time 1ml sample is removed to an Eppendorf tube containing 100µl of Trypan blue solution. Different time points (every 15 min) are selected to continue the experiment. Samples are incubated for 3 min with agitation, and pelleted for 2 min at 800g. The supernatant is carefully and completely removed. Cell pellets are resuspended carefully in 1ml of Soerensen buffer and measured immediately in a fluorimeter (excitation 544nm; emission 574nm). The data is analyzed through the relative fluorescence against time after subtraction of fluorescence at time 0 and correction for differences in the protein content.

4.2.2Molecular Biology methods

4.2.2.1 Preparation of Plasmid DNA from *E. coli*

(i) Plasmid Mini-preparation by alkaline lysis

The alkaline lysis method (Birnboim and Doley 1979) is used to extract the plasmid DNA from small cultures (1ml) of *E. coli* transformants. The culture is harvested by centrifuging at the speed of 4000rpm for 5 minutes at room temperature. The pellets are resuspended with 100µl Solution I, then with 200µl Solution II by vortexing. The mixture is incubated for 5 min at room temperature. After incubation, 150µl Solution III is added and the mixture is incubated for 10minutes on ice. The sample is centrifuged at maximum speed for 15min to precipitate protein and cell debris. The supernatant is mixed with 800µl 100% ethanol, and centrifuged at maximum speed for 20 minutes to precipitate DNA. The DNA pellets are washed with 70% ethanol through centrifuging 1min at maximum speed, vacuum dried and dissolved in 30µl UV water or 1X TE buffer.

Solution I	25 mM Tris pH 7.4
	10 mM EDTA pH 7.4
	15% succrose
Solution II	0.2 M NaOH
	1% SDS
SolutionIII	3M NaAc pH 4.7
1X TE buffer	90 mM Tris
	2 mM EDTA

(ii) Plasmid maxi-preparation

100ml culture is harvested by centrifuging at the speed of 3500rpm for 10 minutes at 4 ˚C, cell pellets are resuspended in 4ml solution I by vortexing, then the suspension in mixed with 8ml Solution II by vortexing again, and incubated for 5 min at room temperature. With the addition of 6ml Solution III, the mixture is inverted 2 times by hands and incubated for 10 minutes on ice. The supernatant is collected after the sample is centrifuged for 20 min at 10000rpm, 4˚C, and mixed with 0.6 Volume isoproponal. The mixture is centrifuged at maximum speed, 4˚C for 25 min to

precipitate the DNA pellets. The pellets are dissolved in 100-200µl UV water, and mixed with equal amount of 10M LiCl to precipitate proteins by centrifugation for 10 minutes at maximum speed. The supernatant is mixed with 3 v/v of ethanol, centrifuged for 25 min at maximum speed, 4˚C. The DNA pellets are washed with 1ml 70% ethanol twice, vacuum dried and dissolved in 100µl UV water or 1X TE buffer.

(iii) Maxi-preparation with Macherey & Nagel Kit

Nucleobond ^{TM} AX100 is used according to the manual of the supplier.

4.2.2.2 Preparation of genomic DNA from *Dictyostelium discoideum*

(i) Maxi-preparation

Genomic DNA is prepared from isolated nuclei as described by Nellen *et al*., (1987). 50ml-100ml culture is harvested at a density of 1-2 x 10^6 cell/ml by centrifuging for 10 min at 2000rpm, 4˚C. Cell pellets are washed twice with ice cold H_2O by centrifuging for 10 min at 2000rpm, 4˚C, then resuspended with 45ml cell lysis buffer and maximum 5ml 10% NP-40 to make the concentration of NP-40 lower than 1% in the mixture. The mixture is shaken by hands until the mixture turns clear. The nuclei fraction is obtained by centrifuging the mixture for 15 min at 4000rpm, 4˚C. The nuclear pellet is then carefully resuspended in 5ml SDS lysis buffer and incubated with 100µl proteinaseK (25mg/ml) at 60˚C for 1-3 hours. The genomic DNA is extracted with equal amount of phenol/chloroform, then precipitated with 2vol. of 100% ethanol, 1/10 vol. of NaAc (pH 5.2) by centrifugation for 25 min at maximum speed, 4˚C. The DNA pellets are washed with 70% ethanol, and dissolved in 100µl UV H_2O and kept at 4˚C.

Cell lysis buffer	50 mM Hepes pH 7.5 40 mM $MgCl_2$ 20 mM KCl 5% Sucrose (50 g/l) H_2O to 1L., sterilized by filter
SDS lysis buffer	0.7 % SDS in 1x TE buffer

(ii) Fast mini-preparation (Barth et al. 1998)

Dictyostelium discoideum cells grown on Costar plates until a monolayer of cells is formed. Approximately 1 x 10^6 cells are collected, washed once with phosphate buffer and resuspend in 300µl TES buffer, then snap frozen in liquid nitrogen. Thaw Cells are thawed slowly, then incubated for 1hr at 60℃ with 25µg proteinase K. The genomic DNA is extracted with phenol/ chloroform and precipitated with ethanol. The resulting genomic DNA can be used for PCR reactions or single restriction digestion.

TES buffer	10 mM Tris/ HCl 1 mM EDTA 0.1% SDS 30µg/ml RNaseA

4.2.2.3 Preparation of total RNA from *Dictyostelium discoideum*

Dictyostelium discoideum cells are grown to a density of 1-2 x 10^6 cell/ml, 10^7 cells are harvested and dissolved in 400µl solution D by pipetting. 50µl 3M NaAc (pH 4.7) and 400µl phenol/chloroform are added to cell suspension, and mixed by strongly vortexing. After centrifugation for 15 min at 14000rpm, the upper phase is collected and the total RNA is precipitated with 900µl isopropanol. The RNA pellet is washed twice with 70% ethanol, dried and dissolved in DEPC water or formamide. .

Solution D	4 mM guanidium thiocyanate 25 mM sodium citrate 0.1 M β -mercaptoethanol 0.5% sarcosyl
DEPC water	Millipore water is treated with 0.1% DEPC overnight, then autoclave

4.2.2.4 Nucleic acid electrophoresis on agarose gel

(i) Agarose gel for DNA

The DNA agarose gel electrophoresis is used to separate DNA according to the size. In general, 1% agarose gel is recommended for most use. Agarose powder is melted

in 1X TBE buffer, after cooling down to 60˚C the ethidium bromide is added to a final concentration of 0.5µg/ ml. then the agarose is poured into a horizontal gel chamber. Agarose gels are run in 1x TBE buffer at 10V/cm. The DNA in the gel is visualized by UV light (256nm) directly on a transiluminator or in the E.A.S.Y. system. The ethidium bromide is potentially mutagenic and must be handled carefully, contaminated tips and used TBE buffer should be disposed of into a dedicated waster container.

(ii) GTC agarose gel for RNA

The isolated RNA needs fully denatured to get fractionation based on the size. To perform RNA electrophoresis, the RNA sample is denatured in RNA loading buffer by heating for 2min at 80˚C or for 10min at 70˚C, then put on ice prior to loading. The agarose is boiled in 1X TBE buffer, after cooling down to about 60˚C, freshly prepared GTC (20mM finally) is added as a denaturant. The gel is run in 1x TBE buffer at 5 V/cm in the cold room. The RNA in the gel can be visualized by UV light (256nm) directly on a transiluminator or in the E.A.S.Y. system.

RNA loading buffer	950 µl Formamide
	50 µl 0.5M EDTA pH 8.0
	traces of bromophenol-blue
	traces of Xylene cyanole
	10 µl ethidium bromide (10mg/ml)
1X TBE buffer	90 mM Tris
	90 mM Boric acid
	2 mM EDTA pH 8.3

4.2.2.5 Cloning of DNA fragments

(i) Restriction digestion

Restriction enzymes from different companies are used. Restriction digestions are performed by using appropriate buffers and temperatures according to the manual of the supplier.

(ii) Dephosphorylation of vectors

To prevent religation of linearized plasmid, the 5'-phosphate of the vector is dephosphorylated by SAP (shrimp alkaline phosphatase, from USB). 1U SAP is added to a 20µl digestion mixture and incubated for 30 minutes at 37˚C, then SAP is inactivated by heating 10 minutes at 80˚C.

(iii) DNA purification from agarose gel

The desired band is cut from the agarose gel under the UV light (366nm). DNA is purified using the Nucleotrap™ (Macherey & Nagel) purification kit according to the manual of the supplier.

(iv) Polymerase Chain Reaction (PCR)

Polymerase chain reaction (Saiki et al. 1988; Mullis and Faloona 1987) is used to amplify DNA in vitro. The following protocol is used.

Reaction mixture	1ng DNA 1µl 5pmol/µl forward and reverse primers each 5µl 2mM dNTPs mix. 5µl 10x PCR buffer (MBI) 1µl Taq polymerase add H2O to 50µl
Standard program	
1st step (initial denaturation)	T=95˚C, 3min
2nd step (denaturation)	T=95˚C, 30sec
(annealing)	T=50˚C, 30sec → 30 cycles
(elongation)	T=72˚C, 1min
3rd step (final elongation)	T=72˚C, 4min
	T=4˚C

(v) Reverse Transcription polymerase chain reaction (RT-PCR)

The reverse transcription is performed on total RNA. To synthesis the first strand (cDNA), the following protocol is used:

Total RNA	2µl (10ng—5µg)
oligodT_{18} or sequence-specific primer (5 pmol/µl)	2µl
UV H_2O	8.6µl

The above mixture is incubated at for 5min 70˚C, chilled on ice immediately, then the following reagents are added in the order indicated:

10x M-MuLV reaction buffer	5µl
100mM DTT or UV water,	2.4µl
dNTPs (2 mM),	5µl

The mixture is incubated for 2min at 42˚C, immediately 1µl M-MuLV reverse transcriptase (200U) is added, and incubated further for 50min at 42˚C. The reaction can be stopped by heating at for 5--10min 70˚C. The cDNA can be used as template for PCR.

(vi) Ligation

The purified DNA insert fragment obtained from restriction digestion or PCR is mixed with the vector in different ratios (insert : vector =1:1, 3:1, 1:3), ligation is performed at 16˚C or 4˚C overnight with 10x ligation buffer and T4 DNA ligase..

2X ligation buffer can be used for quick ligation of the T-easy vector by incubating at for 2 hours room temperature.

4.2.2.6 Transformation of *E. coli*

(i) Preparation of competent cells

An *E. coli* preculture (2ml) is used to inoculate 100ml LB medium, which is grown at 37˚C till the OD600 reaches 0.5. Cells are pelleted and washed once with ice-cold 100mM CaCl2, and incubated for 30 minutes in 50ml ice-cold 100mM CaCl2. Afterwards, cells are pelleted by centrifuging for 15 minutes at a speed of 4000rpm at 4˚C. Pellets are resuspended carefully with 5ml ice-cold 100mM CaCl2, aliquoted in 200µl and frozen immediately at -80˚C

(ii) Classical transformation

Competent cells (100µl) are melted on ice, DNA is added and the tube put on ice for 30 min. The mixture is heat shocked for 90 seconds at 42˚C, and chilled by placing

on ice for 2 min. After addition of 400µl LB medium, the cells are shaken for one hour at 37˚C, then plated on selective plates and incubated at 37˚C overnight .

4.2.2.7 Hybridization

(i) Northern blot analysis (Goda and Minto 1995)

A total amount of 10 µg RNA is prepared on a 1% GTC agarose gel at 4˚C. The RNA is photographed under UV light and the two rRNA bands are positioned by a ruler, which are used as molecular size marker for subsequent analysis. The RNA is transferred to nitrocellulose membrane with 20x SSC buffer overnight. The nitrocellulose membrane is air dried and cross-linked under UV light (314nm, 0.12J/cm^2).

Before the hybridization, the hybridization buffer is pre-warmed at 42˚C. Pre-hybridized for 1hour at 48˚C in the hybridization oven, the membrane is incubated with the labeled probe at 48˚C overnight. On the next day, the blot is washed with washing buffer A for 30minutes, then with washing buffer A/2 for 30minutes subsequently. It can be washed with washing buffer B if the membrane is not clean. The membrane then is exposed on a imaging plate for analysis by the Fuji X Bas 1500 bioimaging analyzer.

The membrane can be reused after stripping. For this purpose, the membrane is washed twice by adding 500ml of boiling stripping buffer followed by shaking for 20 minutes at 42˚C in a water bath. Then the membrane is ready for reuse.

(ii) Radioactive labeling of nucleic acid (probe)---Olio-labeling method

The probes used for Northern blot are prepared by the Oligo-labeling method. The oligo mixture is prepared according to the protocol below, denatured by heating at 100˚C for 5 minutes, then chilled immediately on ice for 5 minutes. Then the mixture is incubated for 1 hour at 37˚C with 3µl ^{32}P α dATP and 1µl Klenow fragment. The

labeled oligo is separated from unincorporated nucleotides by gel filtration. For this, a G50-150 sephadex column is settled first by centrifuging for 5 min at 1000 rpm.

After one hour reaction, the labeled oligo is mixed with 80µl H_2O and placed into the 1ml G50-150 sephadex column. The labeled oligo is collected by centrifugation for 5 min at 1000 rpm, while the free labeled nucleotides remain in the column. The purified radioactive probe is denatured for 5minutes at 95°C and used for hybridization.

20X SSC	3 M NaCl
	0.3 M Na Citrate
Oligo mixture	3µl DNA PCR product
	2µl 10mM dGTP, dCTP, dTTP
	2µl 10X klenow buffer
	3µl OLB mix
	6µl UV H_2O
OLB mix	100µl 1M Tris-HCl pH 7.5
	12.5µl 1M $MgCl_2$
	1.7µl 2-Mercaptoethanol
	2.5µl 50mM dCTP
	2.5µl 50mM dGTP
	2.5µl 50mM dTTP
	250µl 2M Hepes pH 6.6
	150µl 90units/ml (A260) oligo
Hybridizing buffer	50% formamide
	5X Denhard solution
	120 mM phosphate buffer, pH6.7
	1% SDS
	5X SSC
Washing buffer A	2X SSC
	0.1% SDS
Washing solution A/2	0.1% SSC
	0.1% SDS
100X Denhard	2% Ficoll 400
	2% Polyvinylpyrrolidone
	2% BSA

4.2.2.8 Bisulfite sequencing to detect DNA methylation

Bisulfite sequencing is carried out according to (Kuhlmann et al. 2005). 5 µg RNase-treated genomic DNA is denatured with 1/10 volume of 3 M NaOH for 20 min at room temperature, then 3 min at 100°C in a total volume of 60 µl. Aliquots containing 600 µl of freshly prepared bisulfite solution (3.1 M $Na_2S_2O_5$, 5 mM hydroquinone, pH 5) are added and the samples are overlayed with mineral oil. Reactions are carried out for 3 h at 55°C; then briefly heated at 95°C for 1 min and incubated for 12–16 h at 55°C again. After the reaction, the DNA is purified with Geneclean® or glass milk and dissolved in 100 µl H_2O. Desulfonation is carried out for 20 min at 37°C by addition of 11 µl of 3 M NaOH. The converted DNA is precipitated with 35 µl NH_4Ac and 3 vol of 100% ethanol, washed with 70% ethanol and dissolved in 20 µl H_2O; 2–4 µl are used for PCRs with control primers (complementary to non-converted DNA) and bisulfite primers (complementary to converted DNA).

Alternatively, the genomic DNA can be treated with the QIAGEN Bisulfite Field-Test Kit according to the manual for complete bisulfite conversion and cleanup of methylated DNA.
The Sequencing is carried out in MWG Company.

4.2.3 Protein Analytical methods

4.2.3.1 Preparation of proteins from *Dictyostelium discoideum*

(i) Quick preparation

Cells are harvested from Costar plates when grown to monolayer, pelleted by centrifugation for 3 min at1600rpm, at 4˚C. The cell pellets are washed once with phosphate buffer and lysed with 2x Laemmli buffer ($5x10^4$ cell/ml) by heating for 5—10 min at 95˚C. The samples are ready for SDS-PAGE protein gel.

(ii) Preparation of *Dictyostelium* cell extracts

A total of $8x10^7$cells are harvested at a density of 1-2 x 10^6 cell/ml by centrifuging for 3min at 1600rpm, 4˚C. The cell pellets are washed twice with cold phosphate buffer and resuspended in 10ml of lysis buffer. The cells are then lysed by sonifying four times (15 sec every time) on ice with 30sec intervals. The setting of the sonifier is 100% intensity with 50% duty cycle. The cell lysate is centrifuged at 10000rpm, 4 ˚C for 10min. The supernatant is collected and one aliquot is kept as the input fraction. The rest materials can be used for protein purification or pull down assay.

Lysis buffer	20 mM Tris pH 7.5 300 mM NaCl 0.5% NP40 0.5 mM PMSF (keep as a stock solution in 100% ethanol or isopropanol, freshly add before use) 10 mM imidazole

4.2.3.2 Preparation of recombinant protein from *E. coli* strain

E. coli BL21 is commonly used for recombinant protein expression, it encodes the T7 polymerase, which is inducible with IPTG, allowing transcription of the recombinant protein.

An *E. coli* preculture is prepared in 5-10ml LB_{amp} medium by shaking at 37˚C overnight. The next day cells are inoculated in optimum volume of LB medium,

shaken at 37˚C until the OD600 value reaches 0.5-0.6. 1ml cell culture is kept as the start point, and OD600 value is used as the start point for later comparison. Meanwhile, 20ml culture is shaken at 30˚C with 1mM IPTG to induce the recombinant protein. During 4 h, the OD600 value is measured every hour, and the same amount of cells as the start point is harvested according to the comparison of the OD600 value. The cells are pelleted by centrifuging for 10min at 4000rpm, room temperature, resuspended with 200µl TE buffer. The cell suspensions can be frozen at -20˚C or some aliquots can be pelleted again and lysed with 20µl 2X Lammli buffer for SDS-PAGE protein gel.

Table4-2 The recipe of SDS-PAGE gel

	Separate gel (10%) ml	Separate gel (12%) ml	Stack gel ml
Acrylamid (30%)	3.65	4.42	0.50
Lower buffer	2.68	2.64	
Upper buffer			1
H_2O	4.15	3.4	2.5
250mM EDTA (pH8.0)	43.2µl	43.2µl	16µl
TEMED	2.68µl	2.7µl	2µl
20%APS (Ammonium persulate)	120µl	120µl	60µl

Lower Buffer	1.5M Tris 14mM SDS 900ml H_2O, adjust pH 8.8 with HCl add H_2O to 1L
Upper buffer	0.5M Tris 14mM SDS 80ml H_2O, adjust pH 6.8 with HCl, add H_2O to 100ml

4.2.3.3 Purification of protein through Ni-NTa column

A total amount of 50ml BL21 *E. coli* culture expressing His-tagged protein is induced for 3 h, cells then are pelleted by centrifugation for 10minutes at 4000rpm, 4˚C. Cell pellets are resuspended with 10ml lysis buffer, and the cell suspension is

lysed by sonifying 3 times on ice for 20sec each with 20sec intervals. The setting of the sonifier is 100% intensity with 50% duty cycle. Cell lysate is centrifuged at 10000rpm for 30min at 4˚C, the supernatant is collected, and one aliquot is kept as the input fraction.

An aliquot of 300µl-500µl Ni-NTa sepharose is centrifuged for 1-2min at 5000rpm at the room temperature, the sepharose then is resuspended with lysis buffer and placed into the column. Before use, the column (sepharose beads volume is 300-500µl) is equilibrated with several column volumes of lysis buffer

The supernatant can be poured into the column directly, or incubated with the sepharose for 20-30min on a rotatory wheel in the cold room, and then poured into .the column. The flow through is collected and a small aliquot is kept for further analysis. The column is washed intensively with 20 column volumes of washing buffer, and finally the protein is eluted from the column in four steps with 400µl elution buffer for each step.

An aliquot of the eluates is denatured with 9X Laemmli buffer, and loaded on an SDS-PAGE gel to check the protein. The remainder can be kept at -20˚C for further use or dialyzed.

Lysis buffer	10 mM Tris pH 7.5 300 mM NaCl 10% Glycerin 0.1% Triton-100 1 mM PMSF (keep as a stock solution in 100% ethanol or isopropanol, add before use) 10 mM Imidazole
Washing Buffer	lysis buffer with 20mM Imidazole
Elution buffer	lysis buffer with 500mM Imidazole

4.2.3.4 Pull down assay

The principle of this method is to immobilize a known 6x His-tagged protein on Ni-NTa agarose beads. The preincubated beads are then challenged with proteins from *Dictyostelium* cells or nuclear extract. After washing, the His-tagged protein on the beads can be eluted with imidazole, and co-eluted proteins are analyzed by SDS-PAGE and / or western blot. Subsequently, bands cut from the gel can be analyzed by mass spectrometry.

50ml induced BL21 *E. coli* culture expressing His-tagged protein are harvested by centrifuging for 10minutes at 4000rpm at 4˚C. Cell pellets are resuspended in 10ml lysis buffer and lysed by sonifying 3 times on ice for 20sec each with 20sec intervals. The setting of the sonifier is 100% intensity with 50% duty cycle.
The cell lysate is spun for 30min at 10000rpm at 4˚C to separate soluble proteins (supernatant) from insoluble inclusion bodies proteins and other cell debris (pellets). The supernatant is collected, and a sample of 1ml is kept as input fraction.
The column (300-500µl agarose slurry) is equilibrated as mentioned before (4.2.2.3). The supernatant is loaded onto the Ni-NTa column, and the fraction is collected as flow through. The column then is washed intensively with at least 20 column volumes of washing buffer. The optimal imidazole concentration in the washing buffer is to eliminate unspecific binding of protein to the column, but without washing out the targeted protein.

The His-tagged protein can either be eluted, dialyzed and reloaded on fresh Ni-NTa sepharose beads, or the preincubated beads can be directly challenged with *Dictyostelium* extracts (prey).

The *Dictyostelium* extracts are divided into two parts for control and experiment individually, transferred together either with the preincubated or empty beads in 15ml falcon tubes. The two mixtures are incubated for 1-2 h or overnight at 4˚C by

rotating on a rotatory wheel. The beads-cell lysate mixture is transferred into empty column. After the beads sediment, the flow through fraction is collected. The beads are washed intensively with 20 column volumes of washing buffer. The bound proteins are eluted with elution buffer stepwise in a 400µl volume for each step. The first fraction may contain no or very few amount of protein. Every aliquot is kept for further analysis (e.g. SDS-PAGE, western blot, Mass spectrometry)..

Lysis buffer	20 mM Tris pH 7.5 300 mM NaCl 0.5% NP40 0.5 mM PMSF 10 mM Imidazole
Washing Buffer	lysis buffer with 20mM imidazole
Elution buffer	lysis buffer with 500mM imidazole

4.2.3.5 Recharging Ni-NTa

1. Wash beads with 2Vol. water
2. Wash with 3Vol. 2% SDS in water
3. Wash with 1Vol. 25% ethanol
4. Wash with 1Vol. 50% ethanol
5. Wash with 1Vol. 75% ethanol
6. Wash with 3Vol. 100% ethanol
7. Wash with 1Vol. 75% ethanol
8. Wash with 1Vol. 50% ethanol
9. Wash with 1Vol. 25% ethanol
10. Wash with 1Vol. water
11. Wash with 5Vol. 100mM EDTA pH 8.0
12. Wash with 2Vol. water
13. Equilibrate with 1Vol. lysis buffer

4.2.3.6 Western blot

Proteins can be separated by SDS-PAGE gel based on size and charge. The smaller proteins migrate through the gel faster than larger ones. The sufficiently separated proteins can be transferred to a nitrocellulose membrane for western blot. The antibody will specifically bind to the blotted protein, against which it is directed. The second antibody, an antibody-enzyme conjugate, which is directed against the

primary antibody allows to visualize the reaction between blotted protein and the primary antibody.

(i) Discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The 12% or 10% polyacrylamide gel is used for separating proteins by electrophoresis generally. Protein samples are denatured in 2x or 9x Laemmli buffer by heating for 5-10min at 95℃, 10-20µl proteins are loaded on the SDS-PAGE gel with prestained protein molecular weight marker. The electrophoresis is carried out in 1x protein running buffer at 20mA/gel for 1 h. The separating gel then can be used for immunoblotting or immersed directly in coomassie blue staining solution. Destaining is performed by shaking in H_2O

5x protein running buffer	151 g Tris 72 g Glycin 5 g SDS H_2O to 1L
Coomassie blue	20ml H_3PO_4 in 600ml H_2O 1g coomassie blue in 10ml H_2O 60g $(NH_4)_2SO_4$ in 50ml H_2O mix together and add H_2O to 1L

(ii) Electroblotting of proteins

Proteins separated by SDS-PAGE are transferred to nitrocellulose membranes by the semi-dry blotting system (Bjerrum and Schafer-Nielsen 1986). To blot the protein to the nitrocellulose membrane, two layers of Whatman paper are placed on the Semidry Blot apparatus, wet with blotting buffer, followed by the protein gel, the nitrocellulose membrane and two layers of Whatman paper on top. During every step, the membrane and Whatman paper are wetted with blotting buffer, and air bubbles are removed to get sufficient blotting. The blotting is performed with the Semidry Blot apparatus at 2mA/cm^2 membrane for 1hour.

Semi-dry blotting buffer	5.8g Tris 2.92g Glycin 0.38g SDS 200ml C_2H_5OH, H_2O to 1L

(iii) Immunodetection with enzyme conjugated secondary antibodies

The membranes transferred with protein are immersed in 10ml blocking buffer (3-5% milk powder in 1x NCP or PBST buffer),and then incubated with the first primary antibody at 4°C° for overnight. The blots are washed with 1x NCP buffer for several times, and then incubated with 3µl alkaline phosphatase (AP) conjugated secondary antibody (goat-anti mouse) directed against the primary antibody. After 2hours, the membrane is washed with NCP (or PBST) several times. Antigens are identified by chromogenic visualization of BCIP (37µl) substrate in 10ml AP buffer.

NCP buffer	10mM Tris-HCl, pH 8.0 150mM NaCl 0.05% Tween 20
AP buffer	100mM NaCl 100mM Tris-HCl, pH 9.5 5mM MgCl2
BCIP	50mg/ml BCIP in DMF (dimethyfomamide)

4.2.4 Protein-protein interaction analysis by the Yeast-two-hybrid system

The MATCHMARKER GAL4 yeast-two-hybrid system by Clontech is used to detect protein-protein interactions in vivo.

4.2.4.1 Culturing and handling yeast

The S.cerevisiae strain Y190 has the following genotype:

MATa, ura3-52, his3-200, ade-2-101, lys2-801, leu2-2, 112, Gal4Δ, Gal80Δ, cyh^r2

LYS2::$GAL1_{UAS}$-$HIS3_{TATA}$-HIS3

URA3::$GAL1_{UAS}$-$HIS3_{TATA}$-LacZ

Reporter: HIS3, LacZ

Transformation marker: trp1, leu2, cyh^r

Yeast strains can be stored in YPD medium with 25% glycerol at -80˚C. The transformed yeast strains are best stored in the appropriate SD dropout medium to keep selective pressure on the plasmid.

The frozen strains must be recovered before use by streaking a small portion of the frozen glycerol stock onto a YPD (or appropriate SD) agar plate, which are incubated at 30˚C until the yeast colonies reach ~2mm in diameter. A single colony is used as working stock.

4.2.4.2 Conversion of a library in λ YES-R into a plasmid library

Total amount of 10^8 phage from the amplified library is mixed with 2ml of logarithmically growing BNN132 bacteria ($3x10^8$ cells/ml OD=0.45) and 10nM $MgCl_2$, The mixture is incubated at 30˚C without shaking for 30 minutes. After addition of 2ml of LB medium is added into the culture and shaken for 1 hour at 30 ˚C, after which cells can be concentrated or plated directly. 200µl cells are spread on each of the 10 fresh LB plates (150mm) containing 50µg/ml ampicillin and 0.2%

glucose. The plates are incubate at 37℃ overnight. A lawn of cells can be seen the next day, occasionally small plaques are seen in the lawn. Dilutions of the original infection should be plated to determine the excision efficiency. Poor efficiency is usually due to using stationary phase BNN132 cells, using too much ampicillin in the plates, or adding no $MgCl_2$ during absorption. Cells on every plate are resuspended with 10ml LB medium. All bacteria from the plates are pooled together and used to inoculate 3 L terrific broth culture with 50μg/ml ampicillin. After the cells have grown to stationary phase, plasmid DNA is made by CsCl gradients or the Nucleobond AX100 Kit.

The library is made from poly(A)+RNAs which is isolated from vegetative cells.

Table 4-3 cDNA library from *Dictyostelium* (Lu)

Veg. cDNA Lib.	Size of insert (kb)	Nr. of independ clones	pfu/ml
V1/1	0.5-1	$8x10^6$	$1.5x10^{10}$
V2/1	1-1.6	$2.8x10^7$	$1.5x10^{10}$
V3/1	1.6-5kb	$8x10^6$	$3x10^{10}$

4.2.4.3 Preparation of single-stranded Carrier DNA (ssDNA)

To increase the transformation efficiency (Manivasakam and Schiestl 1993), an amount of 2g of high molecular weight DNA (ssDNA) is dissolved in 100ml TE buffer. The DNA is dispersed into solution by sucking it up and down repeatedly in a 10ml pipette, and mixed vigorously with a magnetic stirrer for 2-3hours or until fully dissolved. Alternatively, the covered solution can be mixed at the stage overnight in a cold room. The ready DNA is store at -20℃ as aliquots. Prior to use, an aliquot is boiled for at least 5min and quickly chilled on ice water slurry. The carrier DNA can be frozen after boiling and used for 3 or 4 times.

4.2.4.4 Transformation of *S.cerevisiae*

(i) Quick and easy plasmid transformation

Quick and easy plasmid transformation of yeast is developed by Gietz and Woods (Gietz and Woods 1994).

The yeast strain is inoculated on a YPD or SD plate and incubated overnight at 30˚C. Alternatively, the yeast strain can be inoculated into 5ml of liquid medium and shaken overnight at 30˚C, 200rpm. The next day 50µl of blob is scraped from plates, or 2ml of YPD shaking culture or 5ml of SD dropout culture is harvested. Cell pellets are resuspended in 1ml sterile water, and pelleted again at maximum speed in a microcentrifuge for 5sec and the supernatant is discarded. Cell pellets are then resuspended in 1ml 100mM LiAc, and incubated for 5min at 30˚C. Cells are pelleted again, following the addition of:

240µl PEG (50% W/V)

36µl 1M LiAc

10µl ssDNA (10mg/ml)

5µl plasmid DNA (10ng-5µg)

60µl sterile water

The mixture then is vortexed for 1min and incubated for 20-60min at 42˚C. The mixture is spun down by centrifuging 10sec at maximum speed, and the pellets resuspended in 150µl sterile water by careful pipetting. Cells are plated on appropriate selective medium.

(ii) Big scale transformation for two-hybrid system screens

The yeast-two-hybrid system involves the use of two different plasmids in a single yeast cell. One plasmid contains a cloned gene or DNA sequence while the other plasmid contains a library of genomic or cDNA. While both plasmids can be co-transformed into a single yeast cell, it is often more efficient to transform the library pool into a strain already contain the first plasmid (Gietz, unpublished data). To prepare a plasmid-carrying strain for further transformation, the initial growth phase should use SD dropout medium until the cell titer reaches 1-2 x 10^7 cells/ml. The following protocol is used to produce highly competent yeast cells containing a selectable plasmid.

The yeast strain containing the first plasmid is inoculated into the appropriate volume of the SD dropout medium in a flask and shaken at 30˚C overnight. The cell titer is determined to calculate the volume of the cells that yields $2.5x10^8$ cells for each 50ml YPD culture needed. Then the pre-culture is pelleted for 5 min at 3000g, and the cell pellets are resuspended in the appropriate volume of pre-warmed (30˚C) YPD medium and incubated at 30˚C by shaking for 3-4 hours at 200rpm until the cell titer reaches 2 x 10^7 cells/ml (OD600=2).

Then the cells are harvested by centrifuging for 5 min at 3000g, the cell pellets are washed via resuspension with 1/2 volume of sterile water and collected by centrifugation as above. The cell pellets are resuspended in the appropriate volume of 100mM sterile LiAc and incubated for 15min at 30˚C. Cells are pelleted again by centrifugation. The components of transformation mix are added in the order as below, and mixed thoroughly by vortexing vigorously to resuspend the cell pellet until it is totally resuspended. The mixture is incubated for 30min at 30˚C, and heat shocked at 42˚C for time indicated below. The mixture should be inverted for 15sec every 5 min to equilibrate the temperature. The large scale needs more frequent inversion.

Cells are collected by centrifugation as above, pellets are resuspended gently in an appropriate volume of sterile water and plated onto SD dropout medium. The plates are incubated for 3-5 days at 30˚C until colonies appear (http://www.umanitoba.ca).

In this study, 60x transformation scale is chosen, and several times transformations are performed to get as many positive colonies as possible. The yeast transformed with the bait vector termed pGBKT7-PPWa grows on SD without Trp (trp-) medium, with prey vector termed pACT2-cDNA library from Dictyostelium grows on SD without Leu (leu^-) medium, the strain containing both $pGBKT_7$ and $pACT_2$-cDNA library grows on SD without Trp, Leu, His (trp^-, leu^-, his^- triple dropout medium).

Table 4-4 Different transformation scales (http://www.umanitoba.ca)

Transformation scale	**10x**	**30x**	**60x**
SD culture size (ml)	25	50	100
YPD culture size (50ml)	50	100	300
Number of cells needed	$2.5x10^8$	$7.5\ x10^8$	$1.5\ x10^9$
100mM LiAc (ml)	3	3	6
Transformation mix			
50% PEG (ml)	2.4	7.2	14.4
1.0M LiAc (ml)	360µl	1.08	2.16
ss-DNA (2mg/ml) (ml)	500µl	1.50	3.00
Library plasmid DNA (µl)	A	B	C
Sterile H_2O (ml)	340-A (µl)	1.02-B	2.04-C
Heat shock time (min)	30	40	45-60
Resuspension volume (ml)	10	40	40

4.2.4.5 Preparation of plasmid DNA from *S.cerevisiae*

This method (Hoffman and Winston 1987) is used for quick preparation of plasmids from yeast cells.

The yeast carrying plasmid is inoculated in 2ml liquid medium, and incubated at 30 ℃ by shaking overnight. Cells are pelleted by centrifugation for 5 sec at maximum speed at room temperature, and loosened by brief vortexing. The sediment is resuspended with 200µl breaking buffer and transferred to a 1.5ml Eppendorf tube. The mixture is vortexed with 200µl glass beads (450-520µm diameter, acid washed) and 200µl phenol/chloroform for 2 min. The suspension is centrifuged for about 5 min at maximum speed at room temperature. The aqueous supernatant containing the plasmid can be used to transform to *E. coli*. Alternatively, plasmids can be precipitated by ethanol to get the desired purity and concentration before being transformed to *E. coli*

Breaking buffer
10 mM Tris/ HCl, pH 8.0
100 mM NaCl
1 mM EDTA
1% (v/v) SDS
2% (v/v) Triton-X-100

4.2.4.6 Screening for positive interaction partners

The selective media lacking Trp and Leu are used for selection of transformants. Positive interaction is shown by the two reporter system (β-galactosidase, and growth on His^- medium). In order to exclude as many false positives as possible, two reporter systems are being used.

(i) Growth on SD selective medium

The yeast transformed with $pGBKT_7$ vector grows on SD without Trp (trp^-) medium, with $pACT_2$-cDNA library grows on SD without Leu (leu^-) medium, the strain contains both $pGBKT_7$ and $pACT_2$-cDNA library grows on SD without Trp, Leu, His (trp^-, leu^-, his^-) triple dropout medium. The 3-AT functions on some reporter yeast strains as competitive inhibitor of the yeast HIS3 gene product (Bartel et al., 1993). This leads to a further reduction of the already weak expression of the His3 gene via the TATA-Box (TC). By this competition, the growth of the non-interaction colonies is slowed down.

(ii) β-Galactosidase assay (colony-life filter assay)

The colony-life filter assay is primarily used to screen large numbers of cotranfromants that survive the *HIS3* growth selection in a GAL4 two-hybrid or one-hybrid library screen. It also can be used to assay for an interaction between two known proteins in the GAL4 two-hybrid system.

Fresh colonies grow for 2-4 days at 30℃ until 1-3mm in diameter. A clean, dry nitrocellulose filter is placed over the surface of colonies to be assayed, and oriented to the agar by marker or by poking holes through the filter into the agar. When the filter has been evenly wetted, it is lifted off carefully the agar plate with forceps and transferred (colony facing up) to a pool of liquid nitrogen, and completely submerged for 10sec. After the filter has frozen completely, it is removed from the liquid nitrogen and thawed at room temperature. For each plate of transformants to

be assayed, a sterile Whatman #5 filter is presoaked in 2.5-5ml of Z-buffer/ X-gal solution in a clean petri dish. Then the filter is carefully placed, colony side up, on the presoaked Whatman paper, avoiding trapped air bubbles under or between the filters. The β-Galactosidase reaction can be carried out at 30˚C or room temperature (dark) and checked periodically for the appearance of blue colonies.

The β-Galactosidase-producing colonies are identified by aligning the filter to the agar plate with the orienting marks. The corresponding positive colonies are picked up to a new fresh master plate.

Z buffer	16.1 g/l $Na_2HPO_4 \cdot 7H_2O$ 5.50 g/l $NaH_2PO_4 \cdot H_2O$ 0.75 g/l KCl 0.246 g/l $MgSO_4 \cdot 7H_2O$ pH 7.0
X-gal stock solution 5-bromo-4-chloro-3-indolyl-β-D-galactosidase	Dissolve (x-gal) in DMF of 20mg/ml. Stock in the dark at -20˚C
Z-buffer/ X-gal solution	100ml Z buffer 0.27ml β-mercaptoethanol 1.67ml X-gal stock solution

4.2.4.7 Preparation of protein from *S.cerevisiae*

(i) Urea/SDS method *(clontech)*

Yeast cells are inoculated in 5ml appropriate medium, shaken at 30˚C overnight. The next day, the cell culture is vortexed for 0.5-1min to disperse cell clumps, then used to inoculate 50ml aliquot of YPD medium. The culture is shaken at 30˚C until the OD_{600} reaches 0.4-0.6, which is used for OD600 unit (culture volume x OD600 value). The culture then is quickly chilled by being poured into a prechilled centrifuge tube and immediately centrifuged at 1000x g for 5min at 4˚C. The pellets are collected and resuspended in 50ml ice cold H_2O, and recovered by centrifugation at 1000x g for 5min at 4˚C. Cell pellets are immediately frozen in liquid nitrogen, then can be stored at -80˚C or used for experiments.

The yeast proteins are extracted with freshly prepared complete Cracking buffer. The Cracking buffer is pre-warmed at 60˚C, 100µl are used per $7.5OD_{600}$ units. Cell pellets are thawed quickly in the pre-warmed Cracking buffer by pipetting. The sample can be briefly put at 60˚C if cell pellets are not thawed immediately. Cell suspension then is transferred to a 1.5ml screw-cap tubes containing 80µl of glass beads/ $7.5OD_{600}$ units. Samples are heated for 10min at 70˚C, vortexed vigorously for 1min, then centrifuged for 5min at 14,000rpm to spin down cell debris and unbroken cells. The supernatant is transferred to a fresh 1.5ml screw-cap tube and placed on ice. The samples are boiled for3-5min, vortex vigorously for 1min,then the pellet debris and unbroken cell are spun down again. The supernatant is collected and boiled briefly prior to loading on SDS-PAGE gels or stored at -80˚C. During these processes, an additional aliquot of 100X PMSF stock solution is added to the sample approximately every 7min for the first time 15 min after the first cracking buffer was added.

Cracking buffer (stock solution)	8M Urea	48g
	5% (w/v) SDS	5g
	40mM Tris-HCl pH6.8	4ml of 1M sock
	0.1mM EDTA	20µl of 0.5M stock
	0.4mg/ml Bromophenol blue	40mg
	H_2O	to 100ml
Complete cracking buffer	1ml cracking buffer stock solution	
	10µl β-mercaptoethanol	
	70µl protease inhibitor solution	
	50µl PMSF 100x stock solution	
PMSF 100x stock	0.1742g in 10ml isopropanol	

(ii) Rapid lysis for yeast protein extracts

Cell cultures are incubated for overnight, the next day the equivalent of $5OD_{600}$ (10^8) cells are spun down. Cell pellets are resuspended in ice cold 2M NaOH with 5% mercaptoethanol, and left on ice for 10min. With the addition of 50µl 20% TCA (Trichloroacetic acid), the cell suspension is left on ice for 10 min again. Cells are spun down by centrifugation for 5-10min at 4˚C with maximum speed. Cell pellets

are rinsed with 1M Tris-base, spun down briefly. The cell pellets are resuspended in 2x 50-100µl Laemmli buffer and 20µl is loaded on an SDS-PAGE gel.

4.2.4.8 Sequencing of plasmids by LI-COR DNA sequencer 4000 and 4200 series

For sequencing of the prey vectors, the ThermoSequenase labeled primer cycle sequencing kit from Amersham pharmacia is used.

The PCR reaction mixture is prepared as follows:

DNA from miniprepping or PCR	1-2µl
fluorescent dyed forward primer (20ppm)	1µl
fluorescent dyed reverse primer (20ppm)	1µl
H_2O	to 21µl

Then an aliquot of 4.5µl from the above mixture is loaded into 4 wells of a sequencing plate, 1.5µl 50mM dATP, dTTP, dCTP, dGTP reagents are added into each well. The PCR reaction is performed using the program below. 6µl stopping solution is used for every reaction when the PCR is over, 0.5µl of each reaction is loaded on the sequencing gel.

The PCR program

1st step	T = 95˚C, 2min
2nd step	T = 95˚C, 45sec T = 55˚C, 30sec T = 70˚C, 1min → 25 cycles
3rd step	T = 4˚C, hold

Long PA sequencing gel (3.75% , 66cm long)	25.2g urea 4.5ml long run ranger (50%) 830611 6.0 ml long run 10X TBE 40µl TEMED 400µl APS(10%) bidesty H_2O to 60ml

5 References

Bacon R.A., Cohen C.J., Lewin D.A. and Mellman I. (1994). Dictyostelium discoideum mutants with temperature-sensitive defects in endocytosis. J Cell Biol **127**: 387-399.

Bartel P., Chien C.T., Sternglanz R. and Fiedls S. (1993). Elimination of false positives that arise in using the two-hybrid system. Biotechniques **14 (6)**: 920-924.

Barth C., Fraser D.J. and Fisher P.R. (1998). A rapid, small scale method for characterization of plasmid insertion in the Dictyostelium genome. Nucl. Acids Res. **26**: 3317-3318.

Beclin C., Boutet S., Waterhouse P. and Vaucheret H. (2002). A branched pathway for transgene-induced RNA silencing in plants. Curr. Biol. **12**: 684-688.

Bird A.P. and Wolffe A.P. (1999). Methylation-induced repression – belts, braces, and chromatin. Cell **99**: 451--454.

Birnboim H.C. and Doley J.A. (1979). Rapid alkaline extraction procedure for screening recombinant plasmid-DNA. Nucleic Acid Res. **7**: 1513-1523.

Bjerrum O.J. and Schafer-Nielsen C. (1986). Buffer systems and transfer parameters for semidry electroblotting with a horizontal apparatus. Electrophoresis 86, ed. M. J. Dunn, Deerfield Beach, FL: VCH Publishers. 315-327.

Bjornsson H.T., Fallin M.D. and Feinberg A.P. (2004). An integrated epigenetic and genetic approach to common human disease. Trends Genet **20**: 350-358.

Bohmert K., Camus I., Bellini C., Bouchez D., Caboche M. and Benning C. (1998). AGO1 defines a novel locus of Arabidopsis controlling leaf development. EMBO J **17**: 170-180.

Boutet S., Vazquez F., Liu J., Beclin C., Fagard M., Gratias A., Morel J.B., Crete P., Chen X. and Vaucheret H. (2003). Arabidopsis HEN1: A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. Curr. Biol **13**: 843-848.

Boyes J. and Bird A. (1991). DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell **64**: 1123--1134.

Buchon N. and Vaury C. (2006). RNAi: a defensive RNA-silencing against viruses and transposable elements. Heredity **96**: 195-202.

Buhler M., Mohn F., Stalder L. and Muhlemann O. (2005). Transcriptional silencing of nonsense codon-containing immunoglobulin minigenes. Mol. Cell **18**: 307- 317.

Caplen N.J., Zheng Z., Falgout B. and Morgan R. A. (2002). Inhibition of viral gene expression and replication in mosquito cells by dsRNA-triggered RNA interference. Mol Ther **6**: 243-251.

Cappello J., Handelsman K. and Lodish H.F. (1985). Sequence of Dictyostelium DIRS-1: an apparent retrotransposon with inverted terminal repeats and an internal circle junction sequence. Cell **43 (1)**: 105–115.

Carmell M. A., Xuan Z., Zhang M. Q. and Hannon G. J. (2002). The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev **16**: 2733-2742.

Carrington J.C. and Ambros V. (2003). Role of microRNA in plants and animals development. Science **132**: 336-338.

Castanotto D., Tommasi S., Li M., Li H., Yanow S. and Pfeifer G. P. (2005). Short hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in HeLa cells. Mol. Ther. **12**: 179–183.

Cogoni C. and Macino G. (1997). Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in Neurospora crassa. Proc. Natl. Acad. Sci. U.S.A. **94**: 10233-10238.

Cohen C.J., Bacon R., Clarke M., Joiner K. and Mellman I. (1994). Dictyostelium discoideum mutants with conditional defects in phagocytosis. J Cell Biol **126**: 955-966.

Cox D.N., Chao A., Baker J., Chang L., Qiao D. and Lin H. (1998). A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. **12**: 3715–3727.

Cox D.N., Chao A. and Lin H. (2001). piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. Development **127**: 503–514.

Dernburg A.F., Zalevsky J., Colaiacovo M.P. and Villeneuve A.M. (2000). Transgene-mediated cosuppression in the C. elegans germ line. Genes Dev. **14**: 1578–1583.

Durand-Dubief M. and Bastin P. (2003). TbAGO1, an argonaute protein required for RNA interference, is involved in mitosis and chromosome segregation in

Trypanosoma brucei. BMC Biol. **1 (1)**: 2.

Edmondson D.G. and Dent S.Y.R. (2001). Current Protocols in Protein Science **19 (7)**: 1-10.

Eggar G., Liang G., Aparicio A. and Jones P.A. (2004). Epigenetics in humman disease and prespects for epigenetic therapy. Nature **429**: 457-463.

Eichinger L, Lee SS and S. M (1999). Dictyostelium as model system for studies of the actin cytoskeleton by molecular genetics. Microsc Res Tech **47**: 124–134.

Eichinger L., Pachebat J.A., Glöckner G., Rajandream M.A. and Sucgang R. (2005). The genome of the social amoeba Dictyostelium discoideum. Nature **435 (7038)**: 43-57.

Ekwall K. (2004). The RITS complex – a direct link between small RNA and heterochromatin. Mol. Cell. **13**: 304–305.

Eystathioy T., Chan E.K., Tenenbaum S.A., Keene J.D., Griffith K. and Fritzler M.J. (2002). A phosphorylated cytoplasmic auto-antigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles. Mol. Biol. Cell **13**: 1338–1351.

Fagard M., Boutet S., Morel J.B., Bellini C. and Vaucheret H. (2000). AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc. Natl. Acad. Sci. U.S.A. **97**: 11650–11654.

Faix J., Kreppel L., Shaulsky G., Schleicher M. and Kimmel A.R. (2004). A rapid and efficient method to generate multiple gene disruptions in Dictyostelium discoideum using a single selectable marker and the Cre-loxP system. Nucleic Acids Res. **32 (19)**: e143.

Fields S. and Song O.K. (1989). A novel genetic system to detect protein-protein interactions. Nature **340**: 245-246.

Fire A., Xu S. and Montgomery M.K. (1998). Potent and specific genetic interference by double stranded RNA in Caenorhabditis elegance. Nature **391**: 806-811.

Gerisch G. and Weber I. (2000). Cytokinesis without myosin II. 1. Curr Opin Cell Biol **12**: 26–132.

Gietz R.D., Triggs-Raine B., Anne Robbins A., Graham K.C. and Woods R.A.

(1997). Identification of proteins that interact with a protein of interest: Applications of the yeast twohybrid system. Molecular and Cellular Biochemistry **172**: 67–79.

Gietz R.D. and Woods R.A. (1994). High efficiency transformation in yeast. Invited Book Chapter in: Molecular Genetics of Yeast: Practical Approaches. J.A. Johnston, Oxford University Press: 121-134.

Glöckner G., Eichinger L., Szafranski K., Pachebat J.A., Dear P.H., Bankier A.T., Lehmann R., Baumgart C., Parra G., Abril J.F., Guigo R., Kumpf K., Tunggal B., Cox E., Platzer M., Rosenthal A., Noegel A.A. and Dictyostelium Genome Sequencing Consortium (2002). Sequence and analysis of chromosome 2 of Dictyostelium discoideum. Nature **418**: 79–85.

Glockner G., Szafranski K., Winckler T., Dingermann T., Quail M.A., Cox E., Eichinger L., Noegel A.A. and Rosenthal A. (2001). The complex repeats of Dictyostelium discoideum. Genome Res. **11**: 585-594.

Goda S. K. and Minto N. P. (1995). A simple procedure for gel electropheresis and northern blotting of RNA. Nucleic Acids Res. **23**: 3357-3358.

Grishok A., Pasquinelli A.E. and Conte D. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell **106**: 23–34.

Hall I., Noma K. and Grewal S.I. (2003). RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. Proc. Natl. Acad. Sci. U.S.A. **100**: 193–198.

Hall I.M., Shankaranarayana G.D., Noma K., Ayoub N., Cohen A. and Grewal S.I. (2002). Establishment and maintenance of a heterochromatin domain. Science **297**: 2232-2237.

Hamilton A., Voinnet O., Chappell L. and Baulcombe D. (2002). Two classes of short interfering RNA in RNA silencing. EMBO J. **21**: 4671–4679.

Hammond S. M., Boettcher S., Caudy A. A., Kobayashi R. and Hannon G. J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. Science **293**: 1146–1150.

Hammond S.M., Bernstein E., Beach D. and Hannon G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature (London) **404**: 293–296.

Hannon G. J. (2002). RNA interference. Nature **418 (6894)**: 244-251.

Hermann A., Schmitt S. and Jeltsch A. (2003). The human Dnmt2 has residual DNA-(cytosine-C5) methyltransferase activity. J. Biol. Chem. **278**: 31717--31721.

Hoffman C.S. and Winston F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. Gene **57 (2-3)**: 267-272.

Holliday R. (1987). The inheritance of epigenetic defects. Science **238**: 163-170.

Howard P.K., Ahern K.G. and Firtel R.A. (1988). Establishment of a transient expression system for *Dictyostelium discoideum*. Nucleic Acids Res **16**: 2613-2623.

Hunter C., Sun H. and Poethig R.S. (2003). The Arabidopsis heterochronic gene ZIPPY is an ARGONAUTE family member. Curr. Biol. **13**: 1734–1739.

Hutvagner G. and Zamore P. D. (2002). RNAi: nature abhors a double-strand. Curr. Opin. Genet. Dev. **12**: 225–232.

Jablonka E. and Lamn M.J. (2003). Epigenetic heredity in evolution. Tsitologiia **45**: 1057-1072.

Jaronczyk K., Carmichael J.B. and Hobman T.C. (2005). Exploring the functions of RNA interference pathway proteins: some functions are more RISCy than others? Biochem J. **87 (Pt 3)**: 561–571.

Kaller M., Euteneuer U. and Nellen W. (2006). Differential effects of HP1 isoforms on mitotic chromosome distribution and growth in Dictyostelium. Eukaryot Cell **5 (3)**: 530-534.

Kataoka Y., Takeichi M. and Uemura T. (2001). Developmental roles and molecular characterization of a Drosophila homologue of Arabidopsis Argonaute1, the founder of a novel gene superfamily. Genes Cells **6**: 313–325.

Ketting R.F. and Plasterk R.H. (2000). A genetic link between co-suppression and RNA interference in *C. elegans*. Nature **404**: 296–298.

Kuhlmann M., Borisova B.E., Kaller M., Larsson P., Stach D., Na J.B., Eichinger L., Lyko F., Ambros V., Söderbom F., Hammann C. and Nellen W. (2005). Silencing of retrotransposons in *Dictyostelium* by DNA methylation and RNAi. Nucleic Acids Res **33 (19)**: 6405-6417.

Kuspa A., Maghakian D., Bergesch P. and Loomis W.F. (1992). Physical mapping of genes to specific chromosomes in Dictyostelium discoideum. Genomics **13**: 49–61.

Lee R.C., Feinbaum R.L. and Ambros V. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell **75**: 843–854.

Lee R.C., Feinbaum R.L. and Ambros V. (2004). A short history of a short RNA. Cell **116**: S89–S92.

Lee, Y., K. Jeon, J. T. Lee, S. Kim and V. N. Kim (2002). MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. **21**: 4663–4670.

Lee Y., Ahn C., Han J., Choi H., Kim J., Yim J., Lee J., Provost P., Rådmark O. and Kim S. (2003). The nuclear RNase III drosha initiates microRNA processing. Nature **425**: 415–419

Leng P., Klatte D. H., Schumann G., Boeke J. D. and Steck T. L. (1998). Skipper, an LTR retrotransposon of Dictyostelium. Nucleic Acids Res. **26 (8)**: 2008-2015.

Leuschner P.J., Ameres S.L., Kueng S. and Martinez J. (2006). Cleavage of the siRNA passenger strand during RISC assembly in human cells. EMBO Rep. **7 (3)**: 314-320.

Lippman Z., May B., Yordan C., Singer T. and Martienssen R. A. (2003). Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification. PLoS Biol. **1**: E67.

Liu J, Rivas F.V., Wohlschlegel J., Yates J.R. 3rd, Parker R. and Hannon G.J. (2005). A role for the P-body component GW182 in microRNA function. Nature Cell Biology **7**: 1261-1266.

Liu J., Carmell M.A., Rivas F.V. and et al. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science **305**: 1437–1441.

Liu J., Valencia-Sanchez M.A., Hannon G.J. and Parker R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat. Cell. Biol. **7**: 719–723.

Lu S.J. "Department of Biochemistry, Baylor College of Medicine. Houston."

Lund E., Güttinger S., Calado A., Dahlberg J.E. and Kutay U. (2004). Nuclear export of microRNA precursors. Science **303**: 95–98.

Lynn K., Fernandez A., Aida M., Sedbrook J., Tasaka M., Masson P. and Barton M.K. (1999). The PINHEAD/ZWILLE gene acts pleiotropically in Arabidopsis development and has overlapping functions with the ARGONAUTE1 gene.

Development **126**: 469–481.

Ma J.B., Ye K. and Patel D.J. (2004). Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature (London) **429**: 318–322.
Mallory A. C. and Vaucheret H. (2004). MicroRNAs: something important between the genes. Current opinion in plant biology **7**: 120-125.

Manivasakam P. and Schiestl R. H. (1993). High efficiency transformation of Saccharomyces cerevisiae by electroporation. Nucleic Acids Res. **21 (18)**: 4414-4415.

Martens H. and Nelle W. (2002a). Genesilencing durch RNAi und antisense RNA. Biospektrum **4**: 351-354.

Martens H., Novotny J., Oberstrass J., Steck T.L., Postlethwait P. and Nellen W. (2002b). RNAi in Dictyostelium: the role of RNA-directed RNA polymerases and double-stranded Rnase. Mol. Biol. Cell **13**: 445–453.

Martinez J., Patkaniowska A. and Urlaub H. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell **110**: 563–574.

Martinez J. and Tuschl T. (2004). RISC is a 5′ phosphomonoester-producing RNA endonuclease. Genes Dev. **18**: 975–980.

Matzke M.A., Priming M. and Trnovsky J. (1989). Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plant. EMBO J. **8**: 643-649.

Matzs M., Aufsatz W. and Kanno T. (2004). Genetic analysis of RNA-mediated transcriptional gene silencing. Biochim. Biophys. Acta. **1677**: 129-141.

Meister G., Landthaler M., Patkaniowska A., Dorsett Y., Teng G. and Tuschl T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol. Cell **15**: 185–197.

Meister G., Landthaler M., Peters R., Chen P.Y., Urlaub H., Luehrmann R. and Tuschl T. (2005). Identification of Novel Argonaute-Associated proteins. Current Biology **15**: 2149-2155.

Mello C.C. and Conte D. Jr. (2004). Revealing the world of RNA interference. Nature **431**: 338-342.

Mette M. F., Aufsatz W., Van der Winden J., Matzke A. J. M. and Matzke M.A. (2000). Transcriptional silencing and promoter methylation triggered by

double-stranded RNA. EMBOJ. **19**: 5194–5201.

Miyoshi K., Tsuhumo H., Nagami T., Siomi H. and Siomi M.C. (2006). Slicer function of Drosophila Argonautes and its involvement in RISC formation. GENES & DEVELOPMENT **19**: 2837-2848.

Moffatt B.A. and Weretilnyk E.A. (2001). Sustaining S-adenosyl-L-methionine-dependent methyltransferase activity in plant cells. Physiol. Plant **113**: 435–442.

Morris K.V., Chan S.W., Jacobsen S.E. and Looney D.J. (2004). Small interfering RNA-induced transcriptional gene silencing in human cells. Science **305**: 1289–1292.

Motamedi M. R., Verdel A., Colmenares S. U., Gerber S. A., Gygi S. P. and Moazed D. (2004). Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. Cell **119**: 789–802.

Moussian B., Haecker A. and Laux T. (2003). ZWILLE buffers meristem stability in *Arabidopsis thaliana*. Dev. Genes Evol. **213**: 534–540.

Moussian B., Schoof H., Haecker A., Jurgens G. and Laux T. (1998). Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during Arabidopsis embryogenesis. EMBO J. **17**: 1799–1809.

Mullis K.B. and Faloona F.A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol **155**: 335-350.

Napoli C., Lemieux C. and Jorgensen R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous gene in trans. Plant Cell **2**: 279-289.

Nellen W., Datta S. and Crowley T. (1987). Molecular biology in *Dictyostelium*: tools and applications. Methods Cell Boil. **28**: 67-100.

Nellen W., Silan C. and Fietel R. (1984). DNA-mediated transformation in Dictyostelium discoideum: regulated expression of an actin gene fusion. Mol. Cell. Biol. **4**: 2890-2898.

Okamura K., Ishizuka A., Siomi H. and Siomi M.C (2004). Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev. **18**: 1655–1666.

Pal-Bhadra M., Bhadra U. and Birchler J.A. (2002). RNAi related mechanisms affect

both transcriptional and posttranscriptional transgene silencing in Drosophila. Mol. Cell **9**: 315–327.

Parent C.A. and Devreotes P.N. (1999). A cell s sense of direction. Science **284**: 765-770.

Park W., Li J. and Song R. (2002). CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana.* Curr. Biol. **12**: 1484–1495.

Peedicayil J. (2006). Epigenetic therapy - a new development in pharmacology. Indian J. Med Res **123**: 17-24.

Pillai R.S., Bhattacharyya S.N., Artus C.G., Zoller T., Cougot N., B. E. Basyuk E. and Filipowicz W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science **309 (5740)**: 1573-1576.

Popova B., Kuhlmann M., Hinas A., Söderbom F. and Nellen W. (2006). HelF, a putative RNA helicase acts as a nuclear suppressor of RNAi but not antisense mediated gene silencing. Nucleic Acids Res. **34**: 773-784.

Reinhart B.J. and Bartel D.P. (2002a). Small RNAs correspond to centromere heterochromatic repeats. Science **297**: 1831.

Reinhart B.J. and Weinstein E.G. (2002). MicroRNAs in plants. Genes Dev. **16**: 1616–1626.

Rocha P.S.C.F., Sheikh M., Melchiorre R., Fagard M., Boutet S., Loach R., Moffatt B., Wagner C., Vaucheret H. and Ian Furner L. (2005). The Arabidopsis HOMOLOGY-DEPENDENT GENE SILENCING1 Gene Codes for an S-Adenosyl-l-Homocysteine Hydrolase Required for DNA Methylation-Dependent Gene Silencing. Plant Cell **17 (2)**: 404-417.

Saiki K.K., Gelfand D.H., Stoffel S., Scharf S.J., Higuchi R., Horn G.T., Mullis K.B. and Erlich H.A. (1988). Primer-directed enzyme amplicstion of DNA with a thermostabile DNA polymerase. Science **239**: 487-491.

Schwarz D.S., Tomari Y. and Zamore P.D. (2004). The RNA-induced silencing complex is a Mg2+-dependent endonuclease. Curr. Biol. **14**: 787–791.

Sen G.L. and Blau H.M. (2005). Argonaute2/RISC resides in site of mammalian mRNA decay known as cytoplasmic bodies. Nat. Cell. Biol. **7**: 633-636.

Sheth U. and Parker R. (2003). Decapping and decay of messenger RNA occur in

cytoplasmic processing bodies. Science **300**: 805-808.

Sigova A., Rhind N. and Zamore P.D. (2004). A single Argonaute protein mediates both transcriptional and posttranscriptional silencing in *Schizosaccharomyces pombe*. Genes Dev. **18**: 2359–2367.

Skriwan C., Fajardo M., Hagele S. and Horn M. (2002). Various bacterial pathogens and symbionts infect the amoeba *Dictyostelium discoideum*. Int J Med Microbiol. **291 (8)**: 615-624.

Soifer H. S., Zaragoza A. and Peyvan M. (2005). A potential role for RNA interference in controlling the activity of the human LINE-1 retrotransposon. Nucleic Acids Res. **33**: 846-856.

Song J.J., Smith S.K., Hannon G.J. and Joshua-Tor L. (2004). Crystal structure of Argonaute and its implications for RISC slicer activity. Science **305**: 1434–1437.

Tabara H., Sarkissian M., Kelly W.G., Fleenor J., Grishok A., Timmons L., Fire A. and Mello C.C. (1999). The rde-1 gene, RNA interference, and transposon silencing in C. elegans. Cell **99**: 123-132.

Tahbaz N., Kolb F.A., Zhang H., Joranczyk K., Filipowicz W., and Hobman T.C. (2004). Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. EMBO Rep. **5**: 189–194.

Thomason P., Traynor D. and Kay R. (1999). Taking the plunge. Terminal differentiation in *Dictyostelium*. Trends Genet **15**: 15-19.

Tijsterman M., Ketting R.F. and Plasterk R.H. (2002). The genetics of RNA silencing. Annu. Rev. Genet. **36**: 489-519.

Tijsterman M., Okihara K.L., Thijssen K. and Plasterk R.H. (2000). PPW-1, a PAZ/PIWI protein required for efficient germline RNAi, is defective in a natural isolate of *C. elegans*. Curr. Biol. **12**: 1535–1540.

Ting A.H., Schuebel K.E., Herman J.G. and Baylin S. B. (2005). Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. Nat. Genet. **37**: 906–910.

Tuschl T., Zamore P.D. and Lehmann R. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. **13**: 3191–3197.

Uetz P. (2001). Two-hybrid arrays. Current Opinion in Chemical Biology **6**: 57-62.

Urushihara, H. (1996). Choice of partners: sexual cell interactions in *Dictyostelium discoideum*. Cell Struct Funct **21**: 231-236.

Vastenhouw N.L., Fischer S.E., Robert V.J., Thijssen K.L., Fraser A.G., Kamath R.S., Ahringer J. and Plaster R.H. (2003). A genome-wide screen identifies 27 genes involved in transposon silencing in C.elegans. Curr. Biol. **13**: 1311–1316.

Vaucheret H., Vazquez F., Crete P. and Bartel D.P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Genes Dev.**18** 1187-1197.

Vaus S.K. and Rome L.H. (1995). Dictyostelium Vaults: Disruption of the Major Proteins Reveals Growth and Morphological Defects and Uncovers a New Associated Protein. J. Biol. Chem. **270 (28)**: 16588-16594.

Verdel A., Jia S., Gerber S., Sujiyama T., Gygi S., Grewal S.H.S., and Moazed D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science **303**: 672-676.

Volpe T., Schramke V., Hamilton G.L., White S.A., Teng G., Martienssen R.A. and Allshire R.C. (2003). RNA interference is required for normal centromere function in fission yeast. Chromosome Res. **11**: 137–146.

Volpe T. A., Kidner C., Hall I. M., Teng G., Grewal S.I.S. and Martienssen R.A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science **297**: 1833–1837.

Wassenegger M., Graham M.W. and Wang M.D. (1994). RNA-directed de novo methylation of genomic sequence in plants. Cell **76**: 567-576.

Watt F. and Molloy P.L. (1988). Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. Genes Dev. **2**: 1136--1143.

Weinberg M.S., Villeneuve L.M., Ehsani A., Amarzguioui M., Aagaard L., Chen Z.X., Riggs A.D., Rossi J.J. and Morris K.V. (2006). The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. RNA **12 (2)**: 256-262.

Wilson G.G. and Murray N.E. (1991). Restriction and modification systems. Annu. Rev. Genet. **25**: 585--627.

Wolffe A.P. and Matzke M.A. (1999). Epigenetics: Regulation through repression. Science **286**: 481-486.

Yi R., Qin Y., Macara I.G. and Cullen B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes & Dev. **17**: 3011–3016.

Young K.H. (1998). Yeast two-hybrid: so many interactions, (in) so little time. Biology of Reproduction **58**: 302-311.

Zammore P.D. (2002). Ancient pathways programmed by small RNAs. Science **296 (5571)**: 1265-1269.

Zamore P.D., Tuschl T., Sharp P.A. and Bartel D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101**: 25–33.

Zeng Y. and Cullen B.R. (2003a). Sequence requirements for micro RNA processing and function in human cells. RNA **9**: 112–123.

Zeng Y., Yi R. and Cullen B.R. (2003b). MicroRNA and small interfering RNA can inhibit mRNA expression by similar mechanism. PNAS **100 (17)**: 9779-9784.

Zilberman D., Cao X. and Jacobsen S.E. (2003). ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science **299**: 716–719.

Acknowledgement

I would like to thank my supervisor Prof. Dr. Wolfgang Nellen for his support during my work, and for his patient, paternal supervision and encouragement during my work and living in Kassel. I receive an excellent education in this department with his contribution to training and teaching.

I thank my co-supervisor Prof. Dr. Markus Maniak, who always answers my questions very patiently and detailedly, gives me a lot of suggestions on cell biological experiments, and kindly provides antibodies and some enzymes for my experiments.

I acknowledge my mentor Dr. Christian Hammann, who also supports and supervises me, and especially he spends a lot of his time on checking my thesis carefully and seriously.

I thank all colleagues for accompanying with me during these three years and sharing my success and stressed failure.

I own my acknowledgement to Iris Mueller, who did the phagocytosis experiment, and Oliver Bertinetti who did the mass spectrometry.

This work would not have been possible without the love and endless support from my parents. I thank them for encouraging me to pursue my goals, for financial support for the last few months during writing and for giving me great freedom in doing so.

This work was supported mainly by Otto Braun Fonds (OBF) and also Deutsche Forschungsgemeinschaft (DFG) grant.