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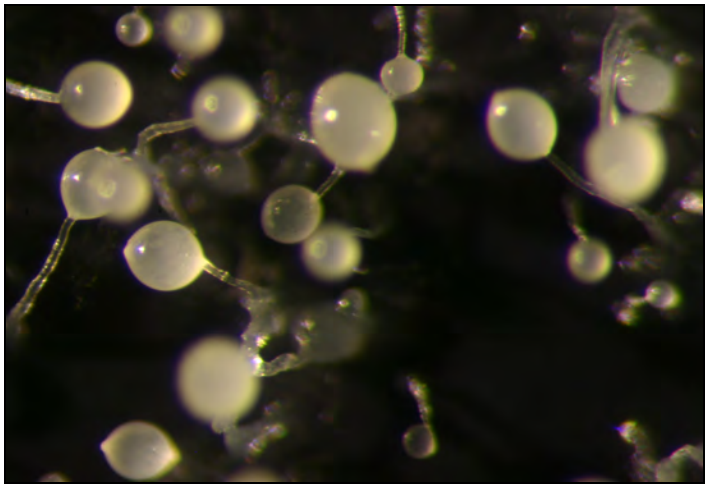
Argonautes in *Dictyostelium discoideum*: another slimy journey



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Degree Project in Evolutionary Biology, Uppsala University
30 ECTS Points, 2008
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Abstract

Small RNAs of 20-32 nucleotides have recently been discovered to be of enormous importance for gene regulation in most eukaryotes. These RNAs come in different classes and can broadly be divided into small interfering RNA (siRNAs), microRNAs (miRNAs), and Piwi interacting RNAs (piRNAs). Their function is diverse and they play important roles in a large number of biological processes such as development, stress responses, and protecting cells from virus infections and transposon mobilization. All these RNAs exert their function by binding to a class of effector proteins called Argonautes. These proteins are guided to their target RNA, e.g. mRNA, by the small RNAs. Hence, the small RNAs give specificity by interacting with the correct target RNA via antisense interaction while the Argonautes are exerting the function. Argonautes can either affect gene expression post-transcriptionally by inhibiting translation or by inducing cleavage of the targeted mRNA. In addition, Argonaute proteins are involved in transcriptional silencing; in fission yeast for example, centromeric repeats are methylated leading to silenced heterochromatin.

Dictyostelium discoideum has emerged as a model organism for the study of a plethora of biological phenomena such as cell motility, cell death, bacterial pathogenesis, social evolution and the transition to multicellularity. Furthermore, previous studies have disclosed the existence of a complex RNA silencing machinery with two Dicer, three RNA dependent RNA polymerases and five Argonautes. Remarkably, all five *D. discoideum* Argonautes belong to the Piwi clade of the Argonaute protein family. Their subcellular localization, their associated small RNAs and possible molecular functions remain largely unexplored.

In this study we used knock-out strains for two *D. discoideum* Argonautes, agnC and agnD, in order to disclose the molecular circuits they are involved in. We failed to detect aberrant phenotypes related to growth or development. Furthermore, the knock-out strains did not affect biogenesis of the tested miRNA. Other approaches, possible molecular functions and the nature of metabolic networks are discussed.

Cover picture (this study): fruiting bodies or the culmination of D. discoideum developmental life cycle

1. Introduction

1.1 The social amoebae

The social amoebae, or Dictyostelia, are organisms on the border between unicellular and multicellular life. Growing cells are unicellular amoebae; when starved though, amoebae aggregate to form a multicellular fruiting body [1]. Dictyostelia thus, during part of their life cycle, exhibit characteristics of true multicellular organisms such as inter-cellular communication, specialized cell types, and programmed cell death [2,3,4]. As a group (~100 known species), Dictyostelia are ecologically exceptionally successful, filling niches in a plethora of biomes [7] and include interesting evolutionary outcomes such as the consummate predator *Dictyostelium caveatum* [44]. The best studied species of Dictyostelia is the model organism *Dictyostelium discoideum*.

1.2 *Dictyostelium discoideum*

The species *Dictyostelium discoideum* was isolated in 1935 by Raper; the name *Dicty* means ‘’net-like’’ while -*stelium* means ‘’tower’’ and they describe the aggregation stage and the fruiting bodies respectively [5]. Since then, it has made an increasingly important contribution to many biological fields [6]. *D. discoideum* is a cosmopolitan species; it can be found in forest soils around the globe [7].

1.2.1 Life cycle of *D. discoideum*

D. discoideum amoebae are omnivorous predators of bacteria and yeast. This solitary life is led until their prey is consumed. The onset of starvation forces a major revision in their life cycle due to a dramatic change in the expression of more than 25% of the genes in the genome [46]. The amoebae, responding to cAMP relays, start collecting into aggregates which transform into an organism that undergoes cell differentiation and morphogenesis [47]. The final morph is a fruiting body consisting of a ball of spores suspended on a stalk at an 80:20 ratio. Spores will propagate the organism whereas the stalk consists of non viable cells which aid in the dispersal of the spores by small arthropods, nematodes and even birds [2]. The entire developmental process takes place in approximately 24 hours (Figure 1) and is systemically independent of vegetative growth; mutants for example with impaired development can still be isolated since their growth is not affected [12].

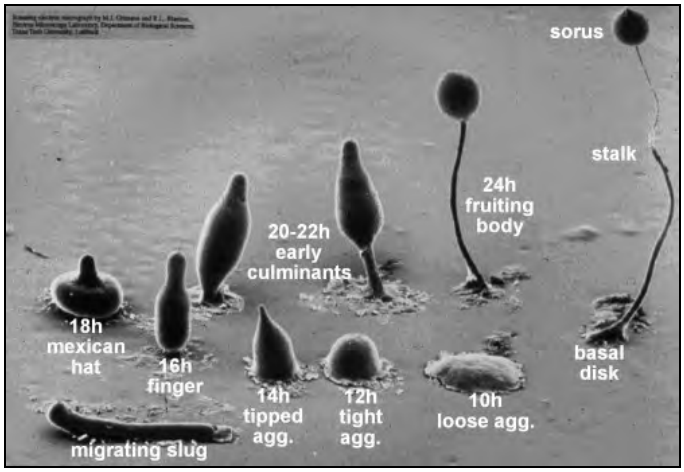


Figure 1. Life cycle of *D. discoideum* [52]

1.2.2 The genome of *D. discoideum*

The 34 Mb genome of *D. discoideum* was recently described [9]. It has a broadly uniform composition and is (A+T)-rich (~78%). The genome codes for approximately 12500 proteins in six chromosomes, among which are many polyketide synthases and ABC transporters, conceivably an adaptation for the noxious soil environment [11]. The genome is also rich in repeats, one class of which may serve as centromeres [9]. Other interesting genomic features include 17 potential instances of horizontal gene transfers from bacteria [9] and unusually abundant microsatellites [39].

1.2.3 *D. discoideum* in the tree of life

Amoebozoa represent one of the earliest branches from the last common ancestor of all eukaryotes [9]. A proteome-based phylogeny (Figure 2) shows that amoebozoa diverged from the animal-fungal lineage after the plant-animal split. However, *D. discoideum* seems to have retained more of the ancestral genomic arsenal than have plants, animals or fungi [9,36]. Moreover, the *D. discoideum* proteome suggests that the last common ancestor of eukaryotes had a greater number of genes than had been previously appreciated [9,10], another paradigm shift offered by the genomics era.

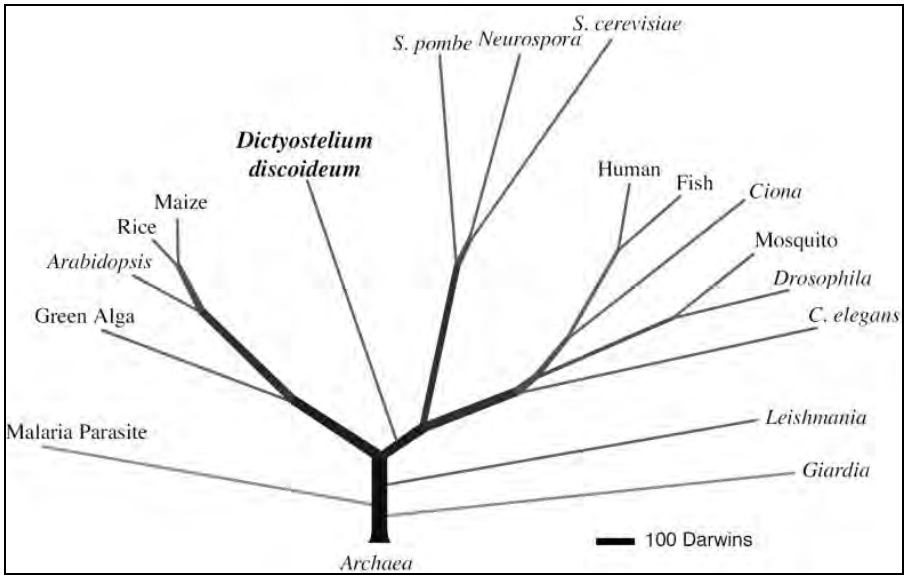


Figure 2. Proteome-based eukaryotic phylogeny [9]

D. discoideum has seemingly achieved multicellularity by using strategies similar to plants and Metazoa in a convergent evolution fashion [9]. This hypothesis is further supported by the radically different multicellular form of *D. discoideum*; it may consist of genetically different individual cells while plants and animals comprise clonal cell populations.

1.2.4 *D. discoideum* as a model organism

At a systems level, *D. discoideum* stands between yeast and animals or plants. Moreover, it is experimentally amenable making it the organism of choice for diverse fields. *D. discoideum* research may hold the key to various biological phenomena such as cell motility, the transition to multicellularity and the evolution of immunity [37].

Until now, *D. discoideum* has been the workhorse for cell biology-related issues such as chemotaxis, motility, cell death and morphogenesis [11,41,42,43]. Its future in research remains rosy. Social and evolutionary biologists are now studying its peculiar pathway to multicellularity with some tantalizing results already reported [12,38,40,45]. In addition, the molecular details of cell communication, warfare and secondary metabolism can be studied using *D. discoideum* [11]. Another area that has attracted interest is that of bacterial pathogenesis [48]. A number of human pathogens, like *Legionella pneumophila*, can be studied in relation with *D. discoideum.* Elucidating the molecular crosstalk between intracellular bacteria and amoebae may lead to novel therapeutic applications for life-threatening diseases.

1.3 RNA silencing

RNA-mediated silencing is a powerful mechanism for gene regulation at transcriptional and post-transcriptional level through which double-stranded RNA (dsRNA) induces the inactivation of cognate sequences. This evolutionarily conserved phenomenon was initially characterized in *Caenorhabditis elegans* and termed RNA interference (RNAi) [14].

At the heart of RNAi lie small RNAs of 20-32 nucleotides that can be broadly divided into miRNAs, siRNAs and piRNAs [13]. All these RNAs exert their function by binding to a class of effector proteins called Argonautes [20,21]. These proteins are guided to their target RNA by the small RNAs. Thus, the small RNAs provide specificity by interacting with the correct RNA target while the Argonautes exert the function. The sorting of small RNAs into specific Argonautes takes place partially through the nature of the 5' terminal nucleotide of the small RNA [49, reviewed in 71]. Argonautes can either affect gene expression post-transcriptionally by inhibiting translation or by inducing cleavage of the targeted mRNA. In addition, Argonautes have been shown to be involved in transcriptional silencing e.g. of centromeric repeats [50].

1.4 The Argonautes

1.4.1 Introduction

The term Argonaute was coined by Bohmert *et al*. to describe a mutant of *Arabidopsis thaliana* in which the leaves resembled *Argonauta argo*, a small squid [19]. In the past years, Argonaute proteins have been extensively studied in many different organisms, including fission yeast, fruit fly, *C. elegans* and various mammals. Argonaute proteins are key nodes in many RNA silencing pathways; gene inactivation studies have revealed that they are important for embryonic development, cell differentiation and stem cell maintenance [reviewed in 20].

Argonaute proteins are classified into three paralogous groups (Figure 3), [21]:

1) Argonaute-like proteins, similar to *Arabidopsis thaliana* AGO1
2) Piwi-like proteins, related to *Drosophila melanogaster* PIWI
3) and WAGO, the recently identified *C. elegans*-specific group 3 Argonautes

The base of this classification is sequence divergence which is coupled with functional divergence since different Argonautes bind different small RNAs. Argonaute-like and Piwi-like proteins are present in bacteria, archaea and eukaryotes while they are absent from *Saccharomyces cerevisiae*, *Trypanosoma cruzi* and *Leishmania major* [21].

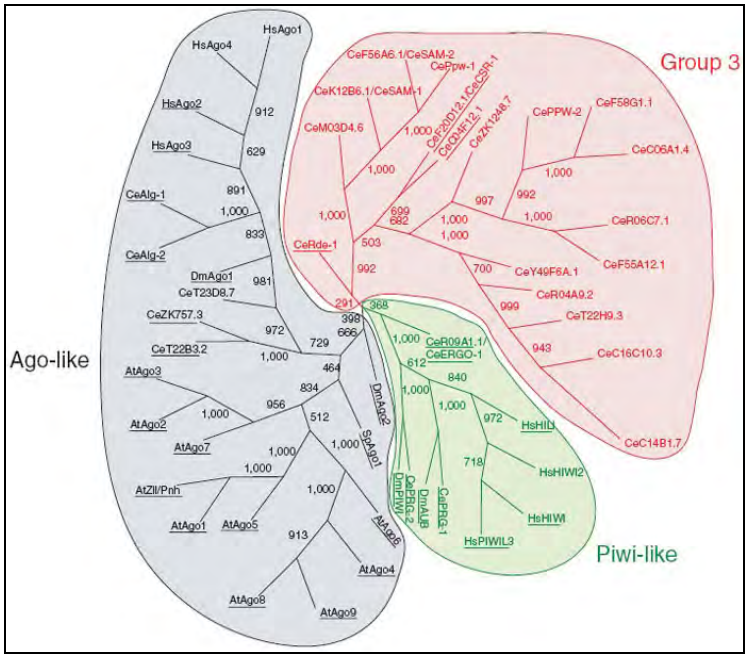


Figure 3. Phylogeny of Argonaute proteins [53]

Phylogenetic analysis suggests that both Argonaute-like and Piwi-like proteins were present in the last common ancestor of eukaryotes [13,65]. The Argonaute-like paralogue was probably targeting mRNAs, regulating thus translation in the cytoplasm while the ancestral Piwi-like protein would probably have effected transcriptional silencing, e.g. transposon control in the nucleus. The extant pattern is thought to have arise by lineage-specific losses and gene duplications followed by diversification. Animals for example exhibit both groups; in contrast, plants encode only the Argonaute-like and Amoebozoa (including *D. discoideum*) only the Piwi-like proteins [21].

Of particular interest are the Piwi-like Argonautes. These proteins have been found to exert fundamental cellular functions in various organisms; Piwi-like Argonautes bind piRNAs and are implicated in germ line regulation in mice [62] and stem cell maintenance in the flatworm planaria [64]. Their molecular mode of function entails transposon control [63].

1.4.2 Functional domains of Argonautes

Argonaute proteins are about 100 kDa and consist of three distinct domains (Figure 4): the PAZ, Mid and Piwi domains [21]. The PAZ domain is situated near the N'-terminus of the protein and is named after the proteins Piwi, Argonaut and Zwille [24]. This domain has been shown to bind to single stranded RNAs (ssRNAs) by recognizing their 3'-ends. Both miRNAs and siRNAs –in their dsRNA form- have two protruding nucleotides at the 3'-end and thus can be distinguished and bound by Argonautes, an interaction that excludes other non regulatory RNAs from binding [21].

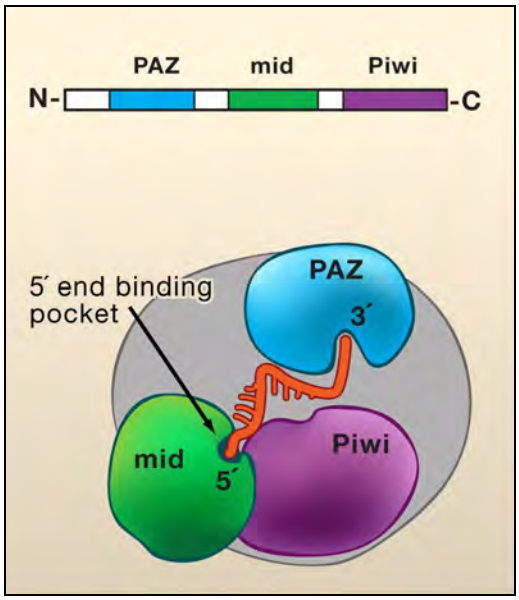


Figure 4. Functional domains of Argonaute proteins [55]

The Mid domain contains a highly basic pocket which functions as anchoring site for the 5' phosphate of small RNAs. The Piwi domain is found near the C'-terminus of the protein and it is similar to ribonuclease H domains. In some cases, Piwi domain exhibits endoribonuclease activity (slicing) and contains the catalytic residues DDH. Interestingly, we also know of Argonautes capable of slicing with other catalytic triads, such as DDK. Finally, Argonautes with no slicer activity and the DDH triad conserved have been reported, suggesting that other factors too may contribute to the slicing procedure [20,21]. Indeed, it was shown that alterations of amino acid residues in the Mid domain reduced slicing activity [72].

1.5 The small RNA repertoire of *D. discoideum*

Seminal studies [23] have shown that small RNAs are derived from different parts of the *D. discoideum* genome, such as retrotransposons, intergenic regions and antisense to mRNAs. Moreover, there seems to exist at least three different, but partly overlapping, pathways in the biogenesis of small RNAs in *D. discoideum.* These pathways employ two Dicer proteins (*drnA, drnB*) and three RNA dependent RNA polymerases (*rrpA, rrpB, rrpC*).

1.6 Argonautes in *D. discoideum*

The published genome, bioinformatical approaches and expression studies have revealed the existence of five functional and one truncated Argonaute genes in *D. discoideum* [22]. The functional genes are *agnA, agnB, agnC*, *agnD* and *agnE* while the truncated one is *agnF*. Microarrays expression data imply differential expression of *agnA* in axenically exponentially growing cells as compared to cells growing exponentially on bacteria [74] and of *agnB* during *L. pleumophila* infection as compared to uninfected cells [75]. Remarkably, all five *D. discoideum* Argonautes belong to the Piwi clade of the Argonaute protein family (Figure 5). Their subcellular localization, their associated small RNAs & proteins and possible molecular functions remain largely unexplored.

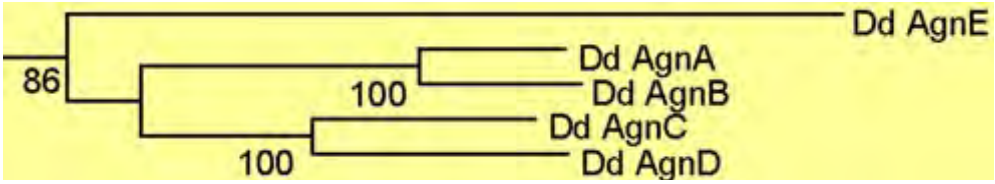


Figure 5. Phylogeny of Piwi-like Argonautes [54]. The five *D. discoideum* proteins cluster together forming two high-similarity pairs: agnA with agnB and agnC with agnD.

1.7 Project aims

The evolutionary significance of *D. discoideum* and its RNAi pathways beg for more research. Particularly intriguing appear the actual mediators of RNA silencing, the Argonautes. In order to understand the function of Argonautes in *D. discoideum*, the following approaches were used:

-Bioinformatical analysis of Argonautes sequences to identify functional important motifs and predict subcelllular localization

-Phenotypic investigations. Knock-out strains were studied in respect to growth, development and miRNA biogenesis in order to score putative phenotypic effects

- Subcellular localization studies. Argonaute genes were translationally fused to Red Fluorescent Protein (RFP) with the goal to be expressed in *D. discoideum* cells and study their subcellular localization.

- Identification of associated proteins and small RNAs. Argonaute genes were translationally fused to TAP (tandem affinity purification) tags with the goal to be expressed in *D. discoideum* cells. This would allow us to pull down and analyze associated proteins and RNAs.

2. Materials and methods

2.1 Cultures and handling of bacterial cells

2.1.1 Bacterial strains

The *Escherichia coli* strain used was DH5α. The *Klebsiella aerogenes* strain used was kindly provided by the Department of Genetics at Kassel University, Germany.

2.1.2 Bacterial cultures

E. coli cells were grown at 37 ºC either in LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, H_2O to 1000ml, adjust pH to 7 with NaOH) in shaking cultures or on LB-agar medium (LB+ 2% agar). The appropriate antibiotic (ampicillin) was added to a final concentration of 50 µg/ml as a selective marker. *K. aerogenes* cells were routinely grown in SM medium (10 g bacto-peptone, 1.6 g KH_2PO_4, 0.6 g K_2HPO_4, H_2O to 950ml, adjust pH to 6-6.4 with H_3 PO_4, autoclave, add glucose to 0.056 M and $MgSO_4$ to 8.3 mM) and SM-agar (SM+ 2% agar). After inoculation, liquid cultures and agar plated were incubated at 22ºC.

2.1.3 Preparation of permanent bacterial stocks

E.coli permanent stocks were prepared by harvesting cells at 10000 rpm for 15 min at 4ºC and resuspending them in 2 ml LB 7%DMSO. The cell mix was vortexed and kept at 4ºC for 30 minutes followed by additional vortexing before storage at -80ºC.

2.1.4 Preparation of chemically competent *E. coli* cells

One single colony of freshly streaked DH5α cells was transferred to a 250 ml shaking culture (18ºC, 250 rpm) of SOB medium (20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2.5 mM KCl, H_2O to 1000ml, adjust pH to 7 with NaOH, autoclave, add $MgCl_2$ to 10mM). Cells were grown to an optical density (OD_{600}) of 0.6 and then incubated on ice for 10 minutes. Cells were harvested at 2500 g for 10 minutes at 4 ºC and resuspended in 80 ml ice-cold TB (10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, adjust pH to 6.7 with KOH, add $MnCl_2$ to 55 mM, sterile filtered through 0.22 µm), incubated on ice for 10 minutes and harvested at 2500 g for 10 minutes at 4ºC. Cells were then resuspended in 20 ml of TB, 7% DMSO. After incubation on ice for 10 minutes, cells were dispersed as 50 µl aliquots and stored at -80ºC.

2.1.5 Preparation of electro-competent *E. coli* cells

One single colony of freshly streaked DH5α cells was transferred to 5 ml LB medium and incubated at 37ºC and 250 rpm for 16 hours. Then, 2 ml of the culture were transferred to 200 ml LB medium and incubated at 37ºC and 250

rpm to midlog phase (0.5-0.7 OD_{600}). Cells were then cooled on ice and harvested at 6000 rpm for 10 minutes at 4ºC followed by resuspension in 200 ml and centrifugation at 6000 rpm for 10 minutes at 4ºC. Cells were washed two times, first in 200 ml then 100 ml ice-cold H_2O (centrifuged at 6000 rpm for 10 minutes at 4ºC). Cells were resuspended in 5 ml ice-cold 10% glycerol and harvested at 10000 rpm for 5 minutes at 4ºC. Cells were finally resuspended in 0.75 ml ice-cold 10% glycerol, at an approximate concentration of 10^{10} cells/ml. Aliquots of 50 μl were then placed in 1.5 ml eppendorf tubes and stored at -80 ºC.

2.1.6 Heat-shock transformation of *E. coli* cells

DNA, commonly 10-100 ng, was added to 50 μl of chemically competent DH5α *E.coli* cells thawed on ice. The mix was incubated on ice for 5 min, then at 42ºC for 30 sec and again on ice for another 2 min. Subsequently, 200 μl LB were added and cells were incubated at 37ºC, 220 rpm for 30-60 min. Finally, cells were plated on a selective LB-agar plate and incubated at 37ºC for 12-16 hours.

2.1.7 Electroporation of *E. coli* cells

Prior to electroporation, DNA solutions were rendered salt-free by dialysis. Dialysis was performed by applying the DNA solution on a 0.025 μm Millipore membrane floating on distilled H_2O for one hour. Then, 1-50 ng DNA were added to 50 μl of electro-competent DH5α *E.coli* cells thawed on ice. The mix was incubated on ice for 5 min and transferred to a pre-cooled 0.1 mm electroporation cuvette. Electroporation was performed at 1.5 kV, 400 Ω, 25 μF whereafter 1 ml of pre-warmed (37ºC) LB was added to the electroporated cells followed by incubation at 37ºC, 250 rpm for 1 hour. Cells were finally plated on a selective LB-agar plate and incubated at 37ºC for 12-16 hours.

2.1.8 Plasmid isolation from *E. coli* cells

Plasmids were routinely isolated from *E. coli* cells using QIAprep Spin Miniprep or HiSpeed Plasmid Midi Kit, according to the manufacturer's instructions (QIAGEN).

2.2 Molecular Biology methods

2.2.1 Determination of nucleic acids concentrations

The NanoDrop instrument (ThermoScientific) was routinely used for determining nucleic acids concentrations.

2.2.2 Restriction digestions

All restriction digestions of dsDNA were performed according to the manufacturer's instructions (Fermentas). Routinely, 1 μg of DNA was digested with 1 unit of restriction enzyme at 37ºC for 1 hour.

2.2.3 Extraction of DNA fragments from agarose gels

DNA fragments were excised from agarose gels and extracted using the QIAquick Gel Extraction Kit according to the manufacturer's instructions (QIAGEN).

2.2.4 Plasmid vectors

The pCR 2.1 TOPO vector (Invitrogen) was used for standard cloning of PCR products. The mRFPmars in pBsrH vector [56] and the nTAP in pDneo2a vector [57,76] were used for cloning *agn* genes in-frame with the mentioned epitopes, whereas mRFPmars in pBsrH vector was also used to transform *D. discoideum* cells. Plasmids KO-agnC and KO-agnD were used to knock out *D. discoideum agnC* and *agnD* genes respectively [J. Chubb, personal communication].

2.2.5 Polymerase chain reaction

In order to amplify DNA fragments with high fidelity for cloning procedures, PFU Ultra polymerase was used according to the manufacturer's instructions (Stratagene). For routine amplifications, Taq polymerase was used according to the manufacturer's instructions (Fermentas). Lower than optimal extension temperatures were used since the AT-rich *D. discoideum* genes can be difficult to amplify at 72ºC, the optimal temperature for the mentioned polymerases. Briefly, *D. discoideum agnA* gene was amplified using primers agnA forward/BamHI and agnA reverse/BamHI with cycling conditions as follows: 95°C for 5 min and 35 cycles of 95°C for 30 s, 56°C for 30 s, and 62°C for 8 min followed by 62°C for 8 min. *D. discoideum agnC* gene was amplified using primers agnC:5' for mRFPmars and agnC:3' for mRFPmars with cycling conditions as follows: 95°C for 5 min and 35 cycles of 95°C for 30 s, 55°C for 30 s, and 62°C for 10 min followed by 62°C for 10 min. Primers are listed in Table A, Appendix.

2.2.6 Cloning of DNA fragments in plasmid vectors

Standard cloning of PCR products took place using TOPO TA cloning Kit (vector pCR 2.1) according to the manufacturer's instructions (Invitrogen). During cloning strategies that entailed restriction cleavage by a single restriction enzyme, after cleavage the vectors' 5' termini were dephosphorylated to prevent self-ligation using Calf Intestinal Alkaline Phosphatase (CIAP) according to the manufacturer's instructions (Fermentas). Ligation of DNA fragments was carried out using T4 DNA ligase according to the manufacturer's instructions (Fermentas). Briefly, 100 ng of vector DNA were used per ligation reaction at a molar insert:vector ratio of 3:1. Ligation mixtures were incubated for 1 hour at 22ºC or 16 hours at 16ºC.

Briefly, the *agnA* gene was PCR-amplified and cloned in TOPO vector following the manufacturer's instructions (Invitrogen) to yield TOPO-agnA plasmid. The *agnC* gene was PCR-amplified, digested with BamHI and cloned

in mRFPmars in pBsrH vector and in nTAP in pDneo2a vector downstream of the epitopes to yield mRFPmars-agnC and nTAP-agnC plasmids respectively. Part of *agnC* (2067-3466 nt) was PCR-amplified using primer CF and primer CR, digested with HindIII and NotI, then cloned in pBluescript. Subsequently, the blasticidin resistance gene (bsr) was cloned inside the *agnC* gene (3023 nt) with EcoRI to yield KO-agnC plasmid [J. Chubb, personal communication]. Part of *agnD* (1677-3175 nt) was PCR-amplified using primer DF and primer DR, digested with HindIII and NotI, then cloned in pBluescript. Subsequently, the blasticidin resistance gene (bsr) was cloned inside the *agnD* gene (2316 nt) with BamHI to yield KO-agnD plasmid [J. Chubb, personal communication]. Plasmids are listed in Table B, Appendix.

2.2.7 DNA sequencing

Sequencing at Uppsala Genome Center (UGC) was performed to verify the fidelity of the cloned genes. Briefly, 200-400 ng of plasmid DNA were mixed with 4 pmol of the appropriate primer (Table A, Appendix) and H_2O in a total volume of 18 µl. Samples were then sequenced at UGC with BigDye Terminator v3.1 (Applied Biosystems) and ABI 3730 capillary electrophoresis (AME Bioscience).

2.2.8 Northern Blot analysis of small RNAs

Northern blots were performed as follows [23,51]: A 15% polyacrylamide/7M urea/20mM MOPS-NaOH gel was pre-run at 30 mA for 30 min. Total RNA samples (20 µg) were then separated at 60 mA for 3-4 hours. The electrophoresis buffer was 20mM MOPS/NaOH (pH 7). End-labeled pUC19/MspI DNA ladder and end-labeled Decade Marker RNA ladder (Ambion) served as markers. For blotting, gels were placed on a sheet of nylon hybridization membrane (Hybond-NX, Amersham), pre-wetted in distilled water. This was then put between pieces of 3MM Whatman filter paper (three layers on each side), pre-wetted in distilled water. A 'semidry' electroblotter (SciPlas) was used to transfer the RNA samples at 20V, 4°C for 40 min. The cross-linking was carried out with l-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Immediately prior to use, a solution of 0.16M EDC (Sigma) was prepared in 0.13M 1-methylimidazole at pH 8 (pH adjusted with HCl). The membrane was placed on an EDC-saturated 3 MM paper with the side onto which the RNA had been transferred facing up. This was then wrapped in SARAN wrap and incubated at 55°C for 2 hours. The membrane was subsequently washed with distilled H_2O. Prehybridization was performed with Church buffer (0.5 M $NaPO_4$ buffer pH 7.2, 7% SDS, 1 mM EDTA, 5 g BSA, H_2O to 500 ml) at 42°C for 1 hour. Probes were prepared by labeling oligos in the following fashion: 15 pmol oligo, 10 µl γ-ATP (100 µCi), 2.5 µl buffer, 0.5 µl T4 PNK (10 units) and H_2O to 25 µl were incubated at 37°C for 1 hour. Unincorporated nucleotides were removed by QIAquick nt removal kit, according to the manufacturer's instructions (QIAGEN). The probe was denaturated at 95°C for 5 min, put on ice for 5 min and added to the hybridization tube with 20-30 ml fresh pre-warmed (42°C) Church buffer. Hybridization was performed at 42°C for 16 hours whereafter the membrane was washed twice with 2xSSC/0.1% SDS for 5 min at 42°C, twice with

1xSSC/0.1% SDS for 10 min at 42ºC and twice with 0.5xSSC/0.1% SDS for 5 min at 42ºC. The damp membrane was then placed into a plastic hybridization bag which was sealed, exposed, and analyzed by a PhosphorImager (Molecular Dynamics).

2.3 Cultures and handling of *D. discoideum* cells

D. discoideum cells were cultured in association with *K. aerogenes* as nutrient source on SM-agar plates or axenically in HL5 medium (10 g peptic peptone, 5 g yeast extract, 0.35 g Na_2HPO_4, 0.34 g KH_2PO_4, H_2O to 950 ml, adjust pH to 6.4 with H_3PO_4, autoclave, add glucose to 0.056 M).

2.3.1 *D. discoideum* cultures on SM-agar plates

In order to retrieve *D. discoideum* cells from frozen stocks, 350 µl of a *K. aerogenes* overnight culture were plated on a SM-agar plate followed by plating ~0.1 ml frozen permanent *D. discoideum* stock. Single-cell clones from transformed cells were isolated by plating 5-200 *D. discoideum* cells together with 350 µl of a *K. aerogenes* overnight culture on a SM-agar plate. In both cases, plates were then incubated at 22ºC.

2.3.2 *D. discoideum* axenic cultures

Shaking cultures were incubated at 22ºC, 150 rpm and contained 100 units of penicillin and 100 µg of streptomycin per ml (Gibco). Blasticidin S (Invitrogen) was added as a selective marker when appropriate at a final concentration of 5 µg per ml. Cell densities were monitored and always kept between $5x10^4$ and $4x10^6$ per ml. Cultivation of *D. discoideum* cells in 10 cm Petri dishes was employed for newly transformed cells. Petri dishes were filled with 10 ml HL5 containing 100 units of penicillin and 100 µg of streptomycin per ml. Blasticidin S was added as a selective marker when appropriate at a final concentration of 5 µg per ml. Medium was changed every three days (see 2.3.6). Cultivation of *D. discoideum* cells in 24-well plates was employed for growing transformant colonies. Briefly, 1 ml HL5 containing 100 units of penicillin and 100 µg of streptomycin per ml was added per well. Blasticidin S was added as a selective marker when appropriate at a final concentration of 5 µg per ml. Medium was changed every three days (see transformation).

2.3.3 DNA extraction from *D. discoideum* cells

1-$2x10^8$ cells were harvested at 300g, 5 min at 4°C. Cells were resuspended in 1.5 ml sterile filtered nuclei buffer (40 mM Tris-Cl pH 7.8, 1.5% sucrose, 0.1 mM EDTA, 6 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 0.4% NP40) and transferred to an eppendorf tube. Cells were then incubated on ice for 5 min and checked under microscope for complete lysis. Cell lysates were centrifuged at 16000 g for 5 min at 4°C and resuspended again in 1.5 ml nuclei buffer. Subsequently, lysates were incubated on ice for 5 min and centrifuged at 16000 g for 5 min at 4°C. The nuclei pellet was resuspended by adding 20 µl 0.5M EDTA and H_2O to a final volume of 100 µl. Then, 100 µl 10% SDS were added, mixed gently and incubated at 55°C for 15 min.

Subsequently, 250 µl of 4M NH_4Ac were added, mixed gently and the solution was centrifuged at 16000 g for 15 min at 4°C. Supernatant was transferred to a new eppendorf tube and DNA was precipitated by adding 1 ml ice-cold 99% EtOH and spun down at 16000 g for 10 min. The pellet was washed once with 1.5 ml ice-cold 70% EtOH and centrifuged at 16000 g for 10 min whereafter the pellet was air-dried for 5 min and resuspended in 40 µl of 10 mM Tris-Cl pH 8.5. Finally, RNase A to a final concentration of 10 µg/ml was added to the DNA solution and incubated at 37°C for 1 h. DNA integrity was analyzed in a 0.7% agarose/0.5xTBE gel.

2.3.4 RNA extraction from *D. discoideum* cells

Approximately 10^8 growing cells were harvested by centrifugation at 1000 rpm, 5 min, 4°C, resuspended in sterile filtered cold PDF (20 mM KCl, 5 mM $MgCl_2$, 20 mM KPO_4, adjust pH 6.2,), centrifuged at 1000 rpm for 5 min at 4°C and resuspended in 1 ml TRIzol reagent (Invitrogen). If RNA was extracted from developed cells, 10^8 cells of synchronous developed cells were collected in a 1.5 ml eppendorf tube and resuspended in 1 ml TRIzol. Resuspended cells were then vortexed and incubated 5 min at room temperature (RT). Subsequently, 200 µl chloroform were added, the solution was vortexed and incubated 3 min at RT. The solution was centrifuged at 12000 rpm for 15 min at 4°C and the supernatant was transferred to a new eppendorf tube to which 500 µl isopropanol were added. The solution was incubated for 10 min at RT and centrifuged at 13000 rpm,10 min, 4°C. The pellet was washed with 1 ml 70% EtOH and centrifuged at 13000 rpm, 5 min, 4°C. The pellet was air-dried for 5 min and resuspended in RNase-free H_2O. RNA integrity was monitored in a 0.7% agarose/0.5xTBE gel.

2.3.5 Development of *D. discoideum* cells

One filter pad was placed in a 15 mm Petri dish to which 1 ml PDF was added. A nitrocellulose filter was put atop the wet filter pad. *D. discoideum* cells were grown to a concentration of 1-3x10^6 cells/ml whereafter 5x10^7 cells (for one filter) were collected by centrifugation at 300 g for 5 min. Cells were resuspended in 20 ml PDF and centrifuged at 300 g for 5 min. After repeating the wash, cells were resuspended in 0.5 ml PDF (10^8 cells/ml). Cells were then spread on the nitrocellulose filter evenly and another 0.5 ml PDF was added to the filter pad. Cells were incubated in a moist chamber at 22°C.

2.3.6 Transformation of *D. discoideum* cells

Electroporation was the method of choice to transform *D. discoideum* cells. Transformation of AX2 wild type (Wt) strain with the ApaI-NotI fragment of KO-agnC plasmid resulted in AX2 C4 strain which had the *agnC* gene disrupted. Transformation of AX2 Wt with the ApaI-NotI fragment of KO-agnD plasmid resulted in AX2 D23 strain which had the *agnD* gene disrupted. Transformation of AX2 Wt with the ScaI-linearized mRFPmars-pBsrH plasmid resulted in AX2 mRFPmars strain which expressed mRFPmars. *D. discoideum* strains are listed in Table C, Appendix.

Briefly, 10^7 cells (from cultures with cell density 1-4x10^6 cells/ml) were transferred to 12 ml tubes (10^7 cells/tube) and chilled on ice for 15 min. Cells were then harvested at 300 g, 5 min, 4°C and the pellet was resuspended in ice-cold sterile filtered Dicty Zap buffer (10 mM $NaPO_4$, 50 mM Sucrose, pH 6.1) to a concentration of 10^7 cells/ml. Then, 800 µl cells were mixed with 10-30 µg transformation construct (Table D, Appendix for details) in a pre-chilled 0.4 cm cuvette and incubated on ice for 3 min. Electroporation was performed at 3 µF, 1 kV using a hot-wired electroporator. The resulting time constant was 0.9-1.4. Then, 100 µl of transformed cells were transferred to two Petri dishes and 200 µl to three Petri dishes which each contained 10 ml HL5 with100 units of penicillin plus 100 µg of streptomycin per ml. After 16-24 hours, medium was removed and replaced by 10 ml HL5, 100 units of penicillin plus 100 µg of streptomycin per ml and 5 µg/ml blasticidin S. Medium was changed in similar fashion every three days. Positive control were cells with neither DNA nor blasticidin added while as negative control served cells with no DNA but with blasticidin added. Transformant colonies were harvested after 7 days and transferred to 24-well plates. Single colonies were harvested from each well and cultured on SM-agar with *K. aerogenes*. Single plaques, representing single transformants, were then harvested and transferred to 24-well plates.

2.3.7 Counting *D. discoideum* cells

Cell densities of *D. discoideum* liquid cultures were determined using a hemocytometer. Briefly, 10 µl of a *D. discoideum* culture were introduced into the counting chamber and cells were counted under the microscope. The number of cells in a square of 1 mm^2 multiplied by 10^4 equaled the number of cells per ml.

2.3.8 Preparation of permanent *D. discoideum* stocks

D. discoideum cells were harvested by centrifuging 25 ml cultures (1-4x10^6 cells/ml) at 300 g, 10 minutes, 4°C. Cells were resuspended in 5 ml ice-cold HL5 and centrifuged once more at 300 g, 10 minutes, 4°C. Cells were then resuspended in 1.5 ml ice-cold HL5 containing 10% DMSO, transferred to sterile cryo-vials and stored at -80°C.

2.3.9 Fixation, permeabilization and DAPI staining of *D. discoideum* cells

D. discoideum cells were let to settle on cover slips for 1 hour in a Petri dish. Cells were then fixed for 30 minutes in 3.7% formaldehyde in PDF at room temperature with gentle rocking. Cells were washed three times for 5 minutes with PDF and permeabilized with ice-cold 0.5% Triton X-100 in PBS for 5 min at room temperature. Cells were washed three more times for 5 minutes with PDF at room temperature and then rinsed twice for 10 minutes in 2xSSC at room temperature. Cells were stained with DAPI (0.3 mM stock solution in DMSO diluted 1:1000 in PDF) for 5 min at room temperature and then rinsed three times in PDF for 5 min at room temperature. Cover slips were finally drained from excess liquid and put onto a slide with 8 µl mounting medium. Edges were sealed with nail polish and the cover slip was cleaned with water

to remove salts. Slides were kept in opaque boxes till the samples were observed under the microscope.

2.3.10 Microscopy

Fluorescence and phase-contrast microscopy [66] was performed using an Axioplan II imaging fluorescence microscope equipped with appropriate filter sets, an Axiocam charge-coupled device camera and Axiovision software (Carl Zeiss Light Microscopy). Digital images were processed using Adobe Photoshop CS version 8.0 software.

2.4 Bioinformatical analyses

Protein domain predictions were performed with Pfam 23.0 [33] and ScanProsite [34] online tools. Domain figures were generated with MyDomains-Image Creator at http://expasy.org/tools/mydomains/.

Localization predictions were made using pTarget (against animal proteins) [28], SherLoc (against animal proteins) [29], SubLoc v1.0 (against eukaryotic proteins) [30] and ESLpred (against eukaryotic proteins) [32].

3. Results

3.1 Bioinformatical analyses

Bioinformatical analyses have become indispensable tools in discerning the biological world's complexity. We performed domain and subcellular localization predictions for *D.discoideum* Argonautes. Domain predictions are listed in Table 1 and depicted in Figure 6.

Table 1. Domain predictions for *D. discoideum* Argonaute*s. NID* stands for Not Identified

Protein	**Length** (aa)	**PAZ domain** (aa)		**Piwi domain** (aa)		**Catalytic triad** (aa)
		Pfam prediction	**PROSITE** prediction	**Pfam** prediction	**PROSITE** prediction	
agnA	979	419-545	438-522	669-965	669-965	**DDH** (748,819,954)
agnB	900	344-467	*NID*	590-886	590-886	**DDH** (670,741,875)
agnC	1208	664-780	674-757	896-1194	896-1194	**DDH** (977,1048,1183)
agnD	1295	772-904	796-881	1017-1281	1062-1281	**NDH** (1063,1135,1270)
agnE	1200	578-622 671-718	*NID*	867-1166	867-1166	**DDH** (949,1022,1155)

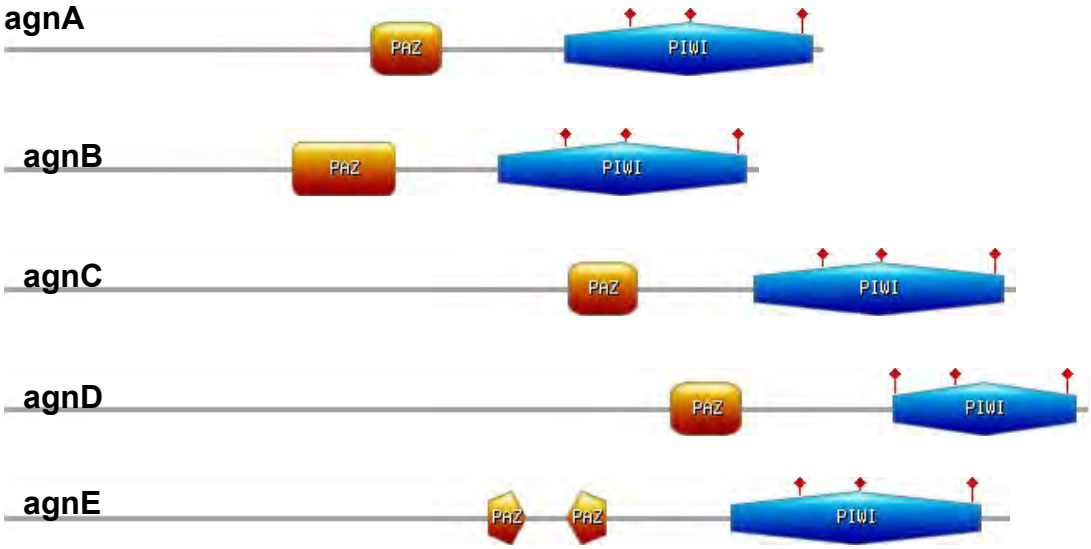


Figure 6. Domain (orange & blue shapes) and catalytic triad (red rhombs) predictions for *D. discoideum* Argonautes. Depictions are based on Table 1 and the concurrence of Pfam-PROSITE predictions

Pfam 23.0 predicted PAZ domains in all five *D. discoideum* Argonautes. Apart from agnE where the 91aa domain consists of two bits, agnA to agnD were predicted to harbor unique ~125aa domains. ScanProsite failed to identify PAZ domains in agnB and agnE. In the rest Argonautes, the predicted PAZ is ~85aa. Both Pfam 23.0 and ScanProsite predicted ~300aa PIWI domains in all Argonautes, except for agnD where the prediction is 264 and 219aa respectively. A putative DDH catalytic triad [31,67] was found in agnA, agnB, agnC and agnE. Interestingly, a NDH triad was found in agnD, similar to *Mus musculus* Ago5 [reviewed in 20].

Subcellular localization predictions for *D. discoideum* Argonautes are listed in Table 2.

Table 2. Subcellular localization predictions for *D. discoideum* Argonautes

Protein	**Subcellular localization prediction** (% probability)			
	pTarget	**SherLoc**	**SubLoc**	**ESLpred**
agnA	Nucleus (100%)	Cytoplasm (55%)	Nucleus (97%)	Nucleus (94%)
agnB	Nucleus (100%)	Peroxisome (30%)	Nucleus (97%)	Nucleus (94%)
agnC	Nucleus (100%)	Nucleus (96%)	Nucleus (97%)	Extracellular (54%)
agnD	Nucleus (100%)	Nucleus (99%)	Nucleus (95%)	Nucleus (94%)
agnE	Nucleus (100%)	Nucleus (80%)	Nucleus (84%)	Nucleus (75%)

Both agnD and agnE are predicted by all four algorithms to localize in the nucleus. The rest Argonautes-agnA, agnB, agnC- are predicted as nuclear

proteins by three algorithms at a high probability while the fourth non-nuclear prediction- cytoplasm for agnA, peroxisome for agnB and extracellular milieu for agnC- is substantially less probable.

3.2.1 Growth of Argonaute knock-out strains

We studied two knock-out strains, derived from the wild type strain AX2. Both strains were kindly provided by Dr. Jonathan R. Chubb (University of Dundee, UK). The first strain had the *agnC* gene disrupted (agnC4) and the second strain had the *agnD* gene disrupted (agnD23). We first asked if agnC and agnD are implicated in vegetative growth of *D. discoideum* amoebae. Liquid cultures were thus monitored for over 100 hours. Cell counting (see 2.3.7) was performed in the following fashion: 0, 4, 8, 24, 30, 34, 50, 54, 58 etc. The resulting growth curves are shown in Figure 7.

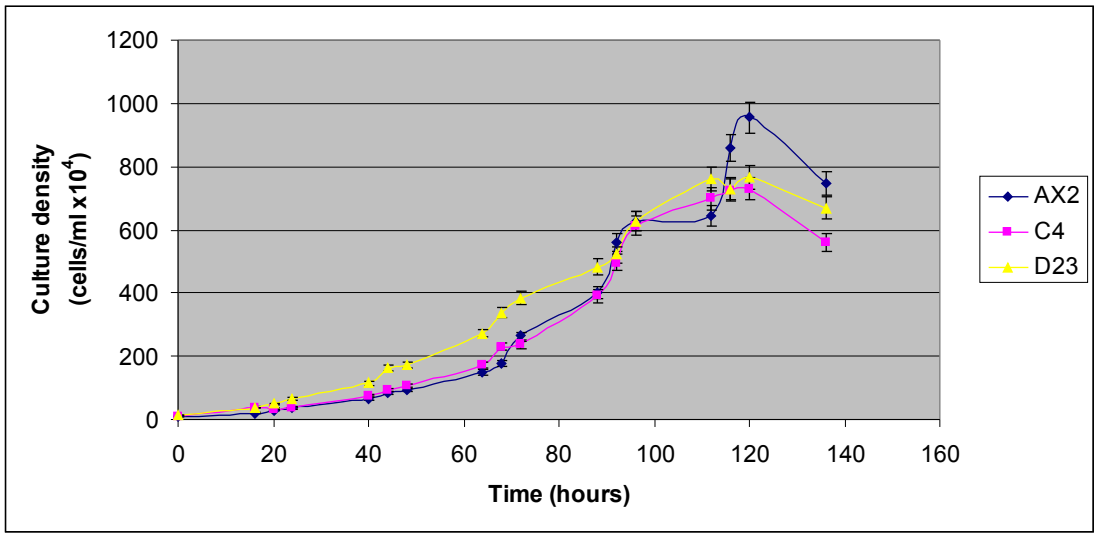


Figure 7. Growth curves of *D. discoideum* strains. AX2 is the wild-type, C4 is the *agnC* knock-out and D23 is the *agnD* knock-out strain

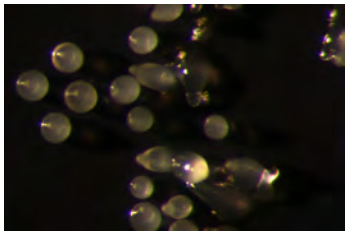
Both knock-out strains and the wild type strain showed similar growth patterns. However, there seems to be a minor difference in how high cell density strains can reach at stationary phase, at ~ 120 hours; both knock-out strains reached lower densities than the wild type strain. Apart from that, the disruption of *agnC* or *agnD* had no other discernible impact on the vegetative growth of unicellular amoebae.

3.2.2 Development of Argonaute knock-out strains

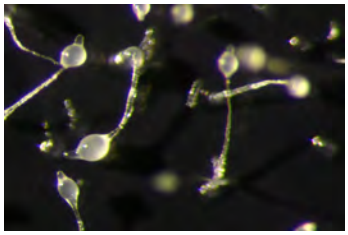
We then asked if the Argonaute-disrupted strains are impaired in their development despite exhibiting wild-type vegetative growth. This is possible since development is gratuitous and is orchestrated by the differential expression of ~3000 genes (see Introduction). We thus chose to analyze *D. discoideum* strains at 16 and 24 hours after onset of development - at 16

hours wild type strains have reached the (standing or migrating) slug phase and after 24 hours they have produced fruiting bodies.

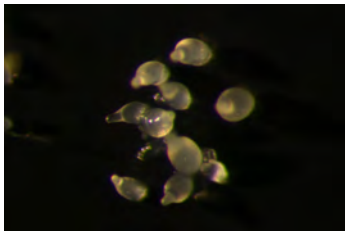
We failed to observe any developmental defects in the knock-out strains. All three strains, wild type and the two knock-outs, proceeded normally throughout development, forming standing slugs at 16 hours and fruiting bodies at 24 hours (Figure 8).



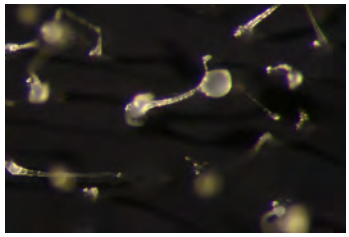
A. AX2 at 16 hours



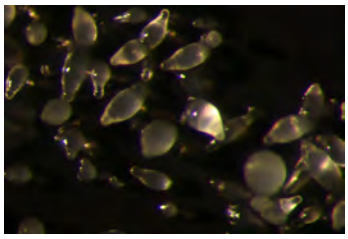
B. AX2 at 24 hours



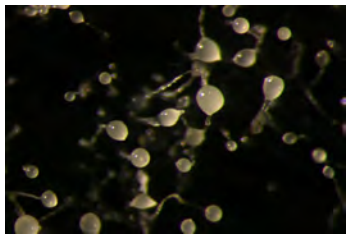
C. C4 at 16 hours



D. C4 at 24 hours



E. D23 at 16 hours



F. D23 at 24 hours

Figure 8. Developmental stages of *D. discoideum* strains, AX2 is the wild-type, C4 is the *agnC* knock-out and D23 is the *agnD* knock-out strain

3.2.3 miRNA biogenesis in Argonaute knock-out strains

We investigated if *D. discoideum* Argonautes are involved in the biogenesis of a specific miRNA, mica1198. Previous studies have shown that mica1198 is up-regulated during development, but can be observed already at vegetative growth phase [23]. We thus performed a Northern blot probing for mica1198 using total RNA samples from vegetative unicellular amoebae of AX2 (Wt), *agnC* and *agnD* knock-out strains (Figure 9).

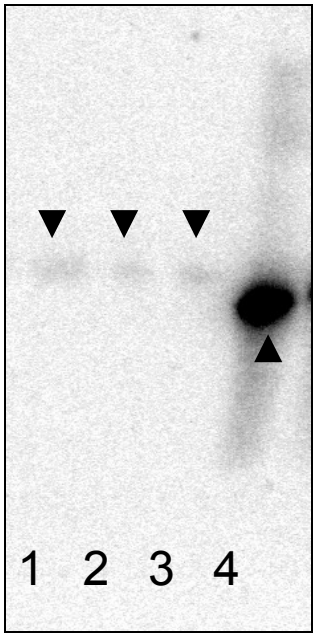
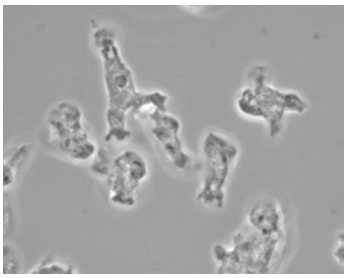


Figure 9. Northern blot for mica1198. All total RNA samples are from vegetative unicellular amoebae. Lane 1: RNA from AX2, wild type strain, Lane 2: RNA from C4, the *agnC* knock-out strain, Lane 3: RNA from D23, the *agnD* knock-out strain, Lane 4: 21 nt ss DNA marker. Arrows in lanes 1-3 point at mica1198, in lane 4 at 21nt marker

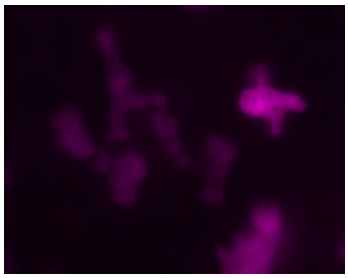
We failed to observe any significant up-regulation or down-regulation of the mica1198 in the *agnC* and *agnD* knock-out strains in growing cells.

3.3 Localization studies

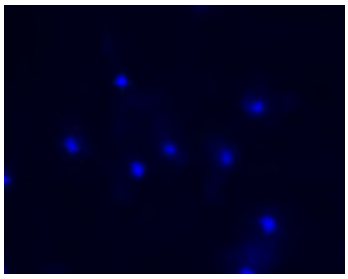
As a first step and internal control for the localization studies, we transformed AX2 wild-type cells with mRFPmars in pBsrH vector [35]. This plasmid expresses monomeric brilliant RFP (mRFPmars) under the constitutive promoter of actin-15. Successful transformation resulted in strain AX2 mRFPmars (Figure 10) and was a necessary step before transformations with translational fusions of mRFPmars-Argonaute genes to exclude possible artifacts.



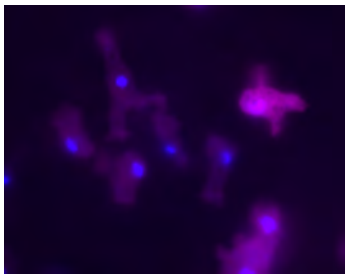
A. Phase image



B. mRFPmars



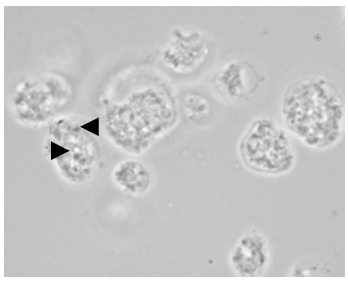
C. DAPI staining



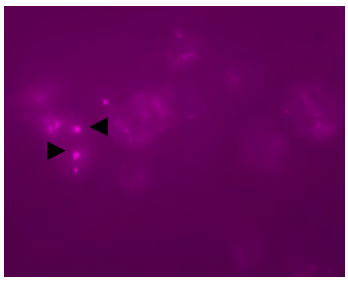
D. mRFPmars + DAPI

Figure 10. Phase image of *D. discoideum* (AX2) expressing mRFPmars (A), mRFPmars filter (B), stained with DAPI for nuclei (C), merged image of figures B and C (D)

Transformation and expression of mRFPmars were successful. As shown in previous studies [56], mRFPmars is not toxic for *D. discoideum* cells and gets properly folded. Moreover, it seems to localize mainly in the cytoplasm (Fig. 10, B). However, optical sections will be needed to see if mRFPmars also localizes to the nucleus. Interestingly, the anticipated high expression of mRFPmars (due to the actin-15 promoter) was stable only in cells treated with the selection marker used for transformation, the antibiotic blasticidin; cells grown without blasticidin were less fluorescent while the mRFPmars protein was mainly found in discrete foci, conceivably cellular vesicles (Figure 11). Nevertheless, in some samples the vesicles appeared to be outside of the cell periphery, indicating secretion of mRFPmars-rich vesicles (data not shown).



A. Phase image



B. mRFPmars filter

Figure 11. Phase image of mRFPmars *D. discoideum* cells (AX2) grown without blasticidin (A), mRFPmars filter (B). Arrows point at mRFPmars-rich foci

Moreover, we have cloned *agnC* in-frame with mRFPmars in pBsrH plasmid [56]. Successful transformation of this construct in *D. discoideum* cells will allow us to identify the subcellular localization of agnC protein.

3.4 Identification of associated proteins and small RNAs

We have cloned *D. discoideum agnC* in-frame with TAP in pDneo2a plasmid [57,76]. Successful transformation of this construct in *D. discoideum* cells and subsequent biochemical assays will allow us to characterize Argonaute-associated proteins and small RNAs. The TAP tag allows for a double-step purification scheme through column chromatography since it comprises the calmodulin binding peptide tag, a TEV protease cleavage site and a protein A tag. The purified protein complexes can subsequently be separated by gel electrophoresis and identified by mass spectrometry. In parallel, any associated small RNAs can be cloned to create cDNA libraries representing *D. discoideum* Argonaute-associated small RNAs.

4. Discussion and future challenges

4.1 Gene disruption studies

Gene knock-out has been a key genetics approach since it can be very informative about the normal gene function. Due to time constraints, we have studied only the *agnC* and *agnD* knock-out strains. No obvious growth or developmental defect was observed in either strain. Moreover, the deletion of either gene did not affect the biogenesis of the miRNA mica1198 during vegetative growth. Nevertheless, agnC and/or agnD might be implicated in the up-regulation of mica1198 during development, reminiscent of tasiRNA biogenesis [reviewed in 72,73]. This hypothesis could be tested with a simple Northern blot. Furthermore, the implication of agnC and agnD in other *D. discoideum* RNAi pathways can be tested in similar fashion.

We have thus tested only three aspects of the possibly vast ecological-phenotypic landscape. Could the deletion of *agnC* or *agnD* result in an aberrant phenotype in conditions other than the ones tested? Previous studies [58] have revealed this possibility in yeast, where 97% of gene deletions exhibited a measurable growth phenotype, suggesting that nearly all genes are essential for optimal growth in at least one condition. In standard lab conditions however, the redundancy of metabolic circuits masks any possible aberrant phenotype. In this light, the lack of phenotypic defects in transposon-up-regulating Dicer minus *D. discoideum* strains comes as little surprise [23]. Unfortunately, there is no known virus infecting *D. discoideum* to test for another possible environmental pressure on knock-out strains.

The deletion of the remaining Argonautes (*agnA*, *agnB*, *agnE*) should also be performed and tested for growth or developmental defects and for aberrations in RNAi pathways. Preliminary results have suggested that the agnA knock-out strain has no developmental defects (data not shown). Furthermore, the advent of the Cre-loxP system allows for multiple deletions with the use of

only one selectable marker [59]. Strains with two or more Argonaute genes simultaneously deleted could untangle the underlying genetic redundancy and provide valuable insights in their function. A challenging aspect of *D. discoideum* Argonautes is that they all belong to the Piwi-like group, proteins that function in specialized cell types such as germ or stem cells. Does the same apply for *D. discoideum*? Interestingly, a panoply of cell types have been reported for *D. discoideum,* e.g. stalk cells, spores, sentinel cells [37], aspidocytes [43] and the sexual-cycle giant cells. It remains to be seen whether *D. discoideum* Argonautes are expressed in any of them or if they have evolved to occupy the (Ago-like) translation-regulation molecular niche.

4.2 Localization studies

Live cell imaging with fusion fluorescence proteins can reveal the subcellular localization of the studied protein and its dynamic properties. We decided to study the subcellular localization of agnA and agnC. We have already cloned agnC -but not agnA- in frame with mRFPmars. The brilliant monomeric RFP has proven to be a suitable tag for protein subcellular localizations in *D. discoideum* [56, this study]. Transformation of *D.discoideum* cells and fluorescence microscopy are bound to reveal the subcellular localization of the studied Argonautes. Possible artifacts due to erroneous localization of the fusion protein could be ruled out by differentially tagging the RFP epitope at the N- and C-terminus of the studied protein. Nonetheless, the subcellular predictions (see 3.1) and the Piwi-like nature of the *D. discoideum* Argonautes point towards nuclear localization. Another intriguing possibility is dual localization, as it was recently shown for a *C. elegans* Argonaute which transports siRNAs from the cytoplasm to the nucleus [70].

4.3 Identification of associated proteins and small RNAs

In line with the localization studies, we decided to identify agnA and agnC associated proteins and small RNAs. We succeeded in cloning agnC -but not agnA- in frame with TAP. Transformation of *D.discoideum* cells and biochemical assays will disclose the nature of any Argonaute-associated proteins and small RNAs. It will be of utmost interest to identify the associated small RNAs- they may be piRNAs, siRNAs, miRNAs or even hitherto unidentified classes of small RNAs. Their nature could further elucidate the functions of *D. discoideum* Argonautes. In the same fashion, the protein sociology for Argonautes may be revealed. Do *D. discoideum* Argonautes interact in space with the other members of the RNAi pathways e.g. Dicer, RNA dependent RNA polymerases (RdRPs) or other regulatory proteins? Interestingly, a recent study [69] showed that serine-387 phosphorylation mediates human Ago2 localization to cytoplasmic processing bodies. What also remains to be discovered is the slicing (endoribonuclease) activity, if any, of *D. discoideum* Argonautes. Argonautes with DDH as catalytic triad (agnA, agnB, agnC, agnE) are not always Slicers while the activity of the NDH triad (agnD) has not been investigated yet [reviewed in 20].

4.4 Expression analysis

As another future challenge, the developmental expression of Argonaute mRNAs should be analyzed by Northern blot. If expression is below detection levels, then quantitative RT-PCR could be performed. Apart from development-specific Argonaute functions, this approach combined with the knock-out strains collection can bring to light any possible Argonaute-Argonaute interactions in time (sequential steps of a metabolic pathway) or space (regulation of expression or enzymatic synergy).

5. Conclusions

D. discoideum is at an evolutionary crossroad, exhibiting similarities to all three, mainly multicellular, crown-group eukaryotes. However, it represents the road to multicellularity not taken; it leads both a unicellular life and a multicellular one that is subject to genetic conflicts. The elucidation of its RNA silencing pathways could provide valuable insights into the evolution of this gene-regulation machinery and possibly novel systems biology subterfuges.

D. discoideum five Argonautes are thought to comprise key players of its RNAi pathways. Our study failed to identify a major and indispensable role for two Argonautes, namely agnC and agnD, in growth of unicellular amoebae, development of the multicellular fruiting bodies and in the biogenesis step of a miRNA pathway. What lies ahead? Knock-out strains, subcellular localization studies, expression studies and identification of associated proteins and small RNAs that will shed light on the *D. discoideum* Argonautes' mechanistic properties and thus on the entire RNA silencing phenomenon of this intriguing organism.

Acknowledgements

First of all, I would like to thank my supervisor Fredrik Söderbom for providing me with the opportunity to study the exciting Dicty. Fredrik is a *sui generis* amalgam of scientific rigorousness and teenage enthusiasm and thus every day in the lab was a rewarding one.

Thanks to Lotta Avesson for being the RNA lady and for running impeccably the lab. Lotta shared her remarkable experience with lab techniques and moreover her unique research zeal.

Thanks to Anna Åsman for contributing to the scientific feel-good atmosphere of the lab.

Thanks to Jonas Vasur for being the man behind the legend.

Thanks to Gelareh Askarieh for being the synchrotron of good vibes.

Thanks to all the SLU people for making the department a stimulating and enjoyable place to work.

Last but not least, I would also like to thank Jacob Höglund for being my *de facto* mentor. His guidance and help, in paving the way for my academic journey to come, have been invaluable.

Appendix

Table A. Primers used in this study

Primer	Sequence (5'-3')	Use
agnA Forw/BamHI	GACGGATCCATGGGTCCTAAAAAACCAAACATACAA	to clone agnA in TOPO vector
agnA reverse/BamHI	GACGGATCCTTAAAGGAAATATAATTTATCAGATAAAT	to clone agnA in TOPO vector
M13 forward	GTAAAACGACGGCAG	to sequence agnA in TOPO vector
M13 reverse	CAGGAAACAGCTATGAC	to sequence agnA in TOPO vector
Forw nTAP-Seq	GAAAAAGAATTTCATAGCCGTCTCA	to sequence agnC in nTAP-pDneo2a vector
Rev nTAP-Seq	GACCCGAGCTCGAGTTATGTCGA	to sequence agnC in nTAP-pDneo2a vector
AgnC:5' for mRFPmars	GCAGGATCCAGTGGGGAAAGGAAAAGAGATCTCCTT	to clone agnC in nTAP-pDneo2a and mRFPmars-pBsrH vectors and to sequence agnC in mRFPmars-pBsrH vector
AgnC:3' for mRFPmars	TACGGATCCTTATAAAAAGAATAAATGATTACTCA	to clone agnC in nTAP-pDneo2a and mRFPmars-pBsrH vectors and to sequence agnC in mRFPmars-pBsrH vector
AgnC:5' middle AccI	TTGGTAGACAATATTATAATAGTCAAT	to sequence agnC in mRFPmars-pBsrH vector
AgnC:3' middle AccI	ATTGTCTACCAACTACTGAATAACC	to sequence agnC in mRFPmars-pBsrH vector
Forw mRFPmarsSeq	CAAGTTCAATTACCAGGTGCTTATA	to sequence agnC in mRFPmars-pBsrH vector
Rev mRFPmarsSeq	CTGATATCATCGATGAATTCGGATC	to sequence agnC in mRFPmars-pBsrH vector
CF	GGAAAGCTTGGTGGACATAGTGGTTATGGAGGTAGTGAACG	To clone part of agnC in pBluescript [J. Chubb,pers. com]
CR	GGAGCGGCCGCCCTTGATCAGGATTACCAAGATCTCTTGAC	To clone part of agnC in pBluescript [J. Chubb,pers. com]
DF	GGAAAGCTTGGGTTCATCAAGTAATAGTGTCCCACAGAG	To clone part of agnD in pBluescript [J. Chubb,pers. com.]
DR	GGAGCGGCCGCATGGACGTTCCAAGCAATAGTTTTTCATCTCC	To clone part of agnD in pBluescript [J. Chubb,pers.com.]

Table B. Plasmids used in this study

Plasmid	Cloned gene	Vector	Restriction enzyme for cloning	Source
TOPO-agnA	agnA	TOPO (Invitrogen)	None	This study
nTAP-agnC	agnC	nTAP-pDneo2a [57,76]	BamHI	This study
mRFPmars-agnC	agnC	mRFPmars-pBsrH [56]	BamHI	This study
KO-agnC	Part of agnC	BlueScript	HindIII and NotI	J. Chubb, personal communication
	bsr	agnC-pBlueScript	EcoRI	
KO-agnD	Part of agnD	BlueScript	HindIII and NotI	J. Chubb, personal communication
	bsr	agnD-pBlueScript	BamHI	

Table C. *D. discoideum* strains used in this study

Strain	Comment	Source
AX2 Wt	Wild type	J. Chubb, personal communication
AX2 C4	*agnC* disrupted	J. Chubb, personal communication
AX2 D23	*agnD* disrupted	J. Chubb, personal communication
AX2 mRFPmars	Expresses mRFPmars	This study

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