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Search for the Argonaute protein that governs miRNA regulation in *Dictyostelium discoideum*

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Abstract

MicroRNAs are small non-coding RNAs that regulate gene expression through RNA interference. These small RNAs enact gene silencing by forming a RNA-inducing silencing complex together with the effector protein Argonaute. The function of the Argonautes in the social amoeba *Dictyostelium discoideum* is not yet fully understood. In this study, we look closer at Argonaute B by investigating if it is possible to extract the protein from the cells by the addition of a polypeptide protein tag called 3xFlag. At the same time, we also look into if Argonaute B is important for cell growth. Sequences of the 3xFlag tag with or without the Argonaute B gene (*agnB*) attached had previously been cloned into a vector and transformed into *Dictyostelium discoideum* cell. The 3xFlag::*agnB* sequence was confirmed in wild type and *agnB* knock-out strains through polymerase chain reaction. We then verified the expression of the fusion protein in the cells by western blot. The cell growth was measured by how the number of cells changed over time. The experiment suggested that Argonaute B is important for growth. Our result show that the construct 3xFlag::agnB sequenced had correctly been transformed into the strains and is highly expressed under tested conditions. We could also see that Argonaute B is an important factor in cell growth.

Table of contents

Abstract 1
Introduction 3
Results 4
Verification of inserted fragments in plasmid through PCR 4
Confirmation of the *agnB* sequence through sequencing 6
The expression of the Flag-AgnB fusion protein in the *D. discoideum* cells 7
No clear indication of the AgnB effect on cell growth 8
Discussion 8
Methods and materials 9
Strains and growth conditions 9
Oligonucleotides 10
PCR 10
TA cloning 10
Sequencing 10
Western blot 10
Growth curve 11
Acknowledgments 11
References 11
Supplementary material 14

Introduction

MicroRNAs (miRNA) are short and non-coding RNAs that regulate gene expression in both plants and animals. These miRNAs are around 21 nucleotides (nt) long and derived from precursor stem-loop structures in newly transcribed RNA (Moran *et al.* 2017). It is estimated that miRNA takes part in the regulation of more than half of the human genes, which covers most cellular processes in eukaryotic cells (Friedman *et al.* 2009). Aberrant expression of these small RNAs in the cell can have severe consequences and lead to diseases such as cancer, neurological and cardiovascular disorders (Esteller 2011). The regulation by miRNA is mediated post-transcriptionally through the silencing of messenger RNA (mRNA) (Montgomery *et al.* 1998, Bartel 2004). This is a conserved process, shared by many eukaryotic organisms and mediated by the RNA interference (RNAi) machinery (Fire *et al.* 1998). The miRNA enacts gene silencing through a RNA-protein complex called RNA-induced silencing complex (RISC) (Hammond *et al.* 2000).

Beside miRNA, the other key components of the miRNA silencing pathway in eukaryotic cells are the RNase III enzyme Dicer and the effector protein Argonaute (Ipsaro & Joshua-Tor 2015). Dicer processes precursor miRNA (pre-miRNA) by cleaving it, creating mature miRNA in form of small double stranded RNAs (dsRNA) (Zamore *et al.* 2000, Bernstein *et al.* 2001). The mature miRNA is loaded onto RISC by binding to the Argonaute protein. With the guiding strand of the dsRNA molecule, it can guide the Argonaute to the target through base-pairing while the non-active strand, called the passenger strand, is degraded and discarded by the RISC complex. The Argonaute inhibits the expression of the mRNA through cleavage and/or translational inhibition depending on which Argonaute the miRNA is bound to. In RISC Argonaute acts as the effector molecule and is the central component of the whole RNAi pathway (Ipsaro & Joshua-Tor 2015).

Argonaute proteins were first discovered in *Arabidopsis thaliana*, where they play an important part in the regulation of the plant development (Bohmert *et al.* 1998). Since then, these proteins have been identified in numerous species, with the number of Argonaute genes varying between them. Most Argonaute proteins have four functional domains which are conserved, called the N-terminal, PAZ, Mid and PIWI domains. The PAZ domain recognizes 3´-overhangs of miRNA. This allows the small RNA to bind to the Argonaute and form RISC. Meanwhile, the PIWI domains have a conserved catalytic tetrad DEDH, which allows endonudeolytic cleavage of target (Hutvagner & Simard 2008).

The genome of the social amoeba *Dictyostelium discoideum* encodes for 2 Dicer (DrnA and DrnB) and 5 Argonaute proteins (AgnA-AgnE) (Cerutti & Casas-Mollano 2006, Avesson *et al.* 2013). *D. discoideum* is one of few unicellular organisms where miRNAs have been identified (Hinas *et al.* 2007, Moran *et al.* 2017). The presence of these components would enable gene regulation through the miRNA silencing pathway. Phylogenetically the family Amebozoa, to which *D. discoideum* belongs, is placed between plants and animals (Baldauf & Doolittle 1997, Eichinger *et al.* 2005). This placement gives an interesting opportunity to study the evaluation of the RNAi machinery in eukaryotic cells.

The function of the Argonaute in the RNAi pathway is not yet fully understood. All five Argonautes have the conserved PAZ and PIWI domains. However, in only four of them the catalytic tetrad DEDH has been identified, which indicates them as active slicers (figure 1).

The DEDH motif has not been found in AgnD and therefore it have been speculated that the protein have no active function in the RNAi pathway (Liao 2018).

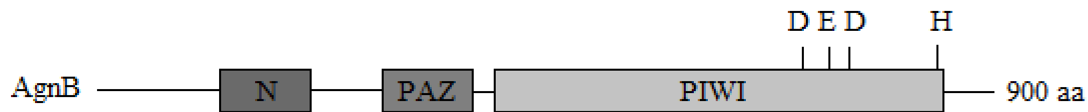

Figure 1. *Schematic picture showing the organization of domains in the AgnB. Shown is the relative position of N-terminal, PAZ and PIWI domains. In the PIWI domain the catalytic tetrad DEDH is marked. The size of the protein is indicated with the number of amino acids (aa).*

The AgnA has a role in both the production of small interfering RNA (siRNA) (another group of small RNAs similar to miRNA) during immobilization of the retrotransposon DIRS-1, and the inhibition of miRNA production by an unknown mechanism (Boesler *et al.* 2014, Meier *et al.* 2016). Meanwhile, AgnB, AgnC and AgnE, appear to play a big part in cell growth through the regulation of both genes encoding ribosomal proteins and genes involved in nucleotide synthesis (Liao 2018). AgnC and AgnE are also part of the regulation of another retrotransposon, TRE5-A (Schmith *et al.* 2015). Furthermore, results from previous studies are also pointing towards AgnB being a potent miRNA binder and having a role in miRNA mediated gene regulation (Liao 2018).

The aim of this study is to look closer at the Argonaute B in *D. discoideum*, where it is believed to bind miRNA and regulate genes through silencing of the targeted mRNA. Previously a plasmid had been constructed, consisting of the *agnB* gene tagged to a sequence expressing the Flag peptide. The vector had then been introduced into *D. discoideum* cells with the wild type (wt) and *agnB*$^-$ genotype. For this study the focus was on the verification of the strains and the construct of the 3xFlag::*agnB* by PCR and testing the expression of the sequence through western blot.

Results

In this study we seek to discover if Argonaute B is the argonaute responsible for the binding of miRNAs in *D. discoideum*. To characterize the RNAs that Argonaute B binds, we wish to pull down the protein with a 3xFlag tag. This marker was previously cloned into *D. discoideum* AX-2 cells with or without the *agnB* gene. The constructed plasmids were introduced into *agnB*$^-$ cells to see if the absence of the gene would have any effect on the cell and if that being the case, if it could be rescued. Also, the plasmid was introduced into wt cells for control.

Verification of inserted fragments in plasmid through PCR

We began our study by verifying if the transformation of the plasmid into the *D. discoideum* cells had been successful. This was done by PCR. Fragments of an ampicillin resistance cassette, present in the plasmids, were amplified. The result we received showed that the transformation into both wt and *agnB*$^-$ had been successful (figure S1). However, this could not be done for *agnB*$^-$ strain with the pDM-3xFlag, because these cells would not grow on agar plates.

Since the presence of plasmids was confirmed, we next wanted to verify the actual 3xFlag and 3xFlag::*agnB* sequences in the plasmids in the *D. discoideum* cells. The Flag tagged *agnB* was designed without introns by using GeneArt (ThermoFisher Scientific) and therefore the structure of the constructed gene was different from the genomic *agnB* (figure 2a). This meant that we could use primers that only base-paired with the cloned Flag *agnB* gene and expect to receive no genomic *agnB* in the PCR products. Because of the large size of the entire 3xFlag::*agnB* sequence, we choose to begin with amplifying smaller fragments of the cloned gene. The result of the PCR showed that the *agnB* tagged gene was only present in cells containing the 3xFlag::*agnB* plasmid (figure 2b and 2c).

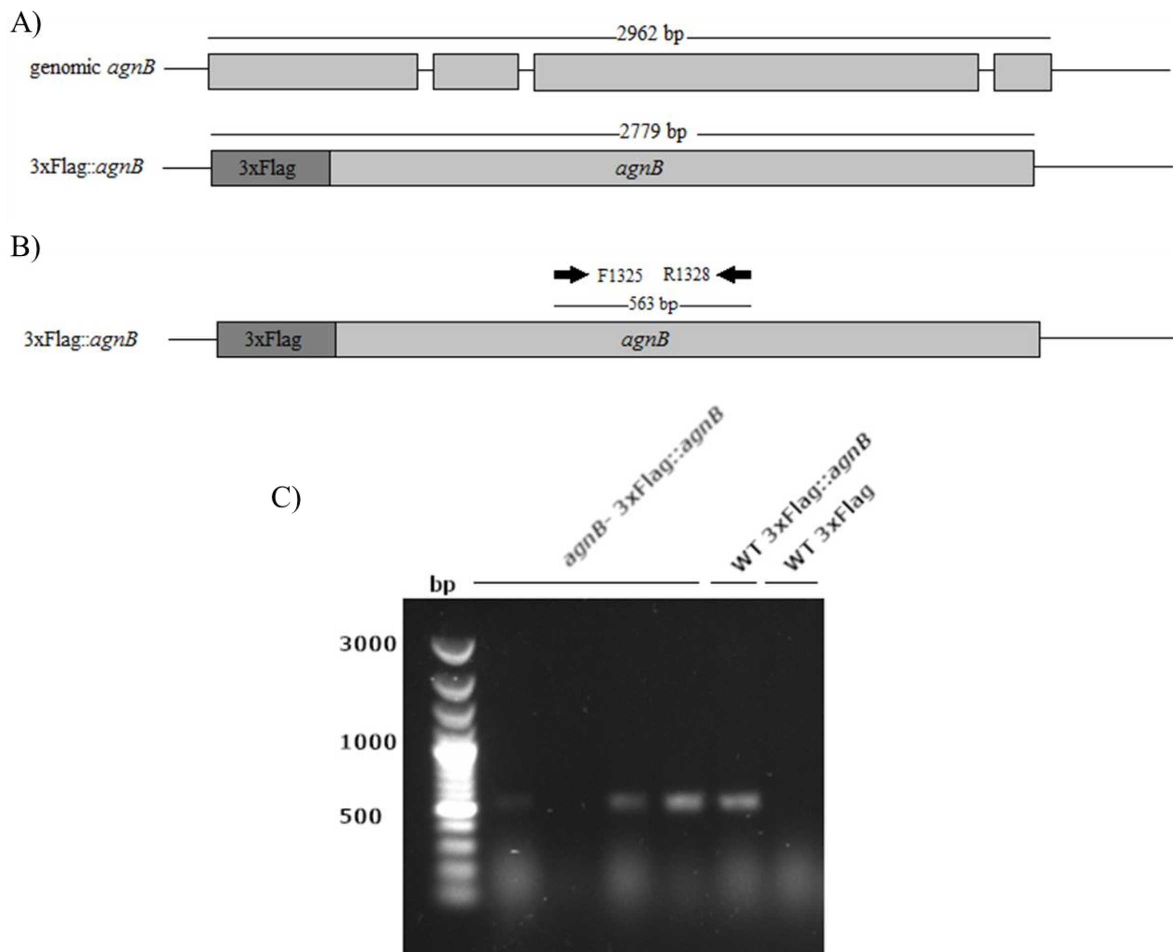

Figure 2. *Construction and verification through PCR of part of the agnB gene of the 3xFlag::agnB sequence. The size of the genes are indicated with the number of bp (A) Schematic picture showing the differences between the genomic agnB normally found in D. discoideum (top), and the 3xFlag tagged agnB gene (bottom). (B) Schematic picture of 3xFlag::agnB. The oligonucleotides used in the PCR are indicated with arrows. The prefix F and R stands for forward and reverse primer respectively. (C) Visualization of the PCR products on agarose gel indictaes strains with 3xFlag::agnB present in the plasmid. Strains with only the 3xFlag gave no band.*

Moving forward, the goal was to amplify the entire 3xFlag::*agnB* sequence in order to verify the presence of the full-length of the cloned sequence. We used a primer pair where the forward primer based-paired with the 3xFlag sequence and the reverse primer based-paired

downstream of the 3´end of the coding sequence (figure 3a). With an extension time of 1 min, we only received PCR products from cells with the 3xFlag plasmid (figure S2). Because of the large size of the entire 3xFlag::*agnB* sequence compared to only the 3xFlag, we had to optimize the PCR to receive the full fragment. We did this by increasing the extension time from 1 min to 5 min. At the same time, we also opted to change the DNA polymerase for one that was less error prone to minimize changes in the sequence. When the PCR products were analyzed by agarose gel electrophoresis, the size of the fragments strongly indicated that we had succeeded with amplifying the entire 3xFlag::*agnB* fragment (figure 3b).

The full 3xFlag::*agnB* that we had amplified was sent off to be sequenced. Here, we also decided to do a TA cloning of the PCR fragments. By doing this we could ensure that the sequencing result we received also included the 5´and 3´ends of the PCR product.

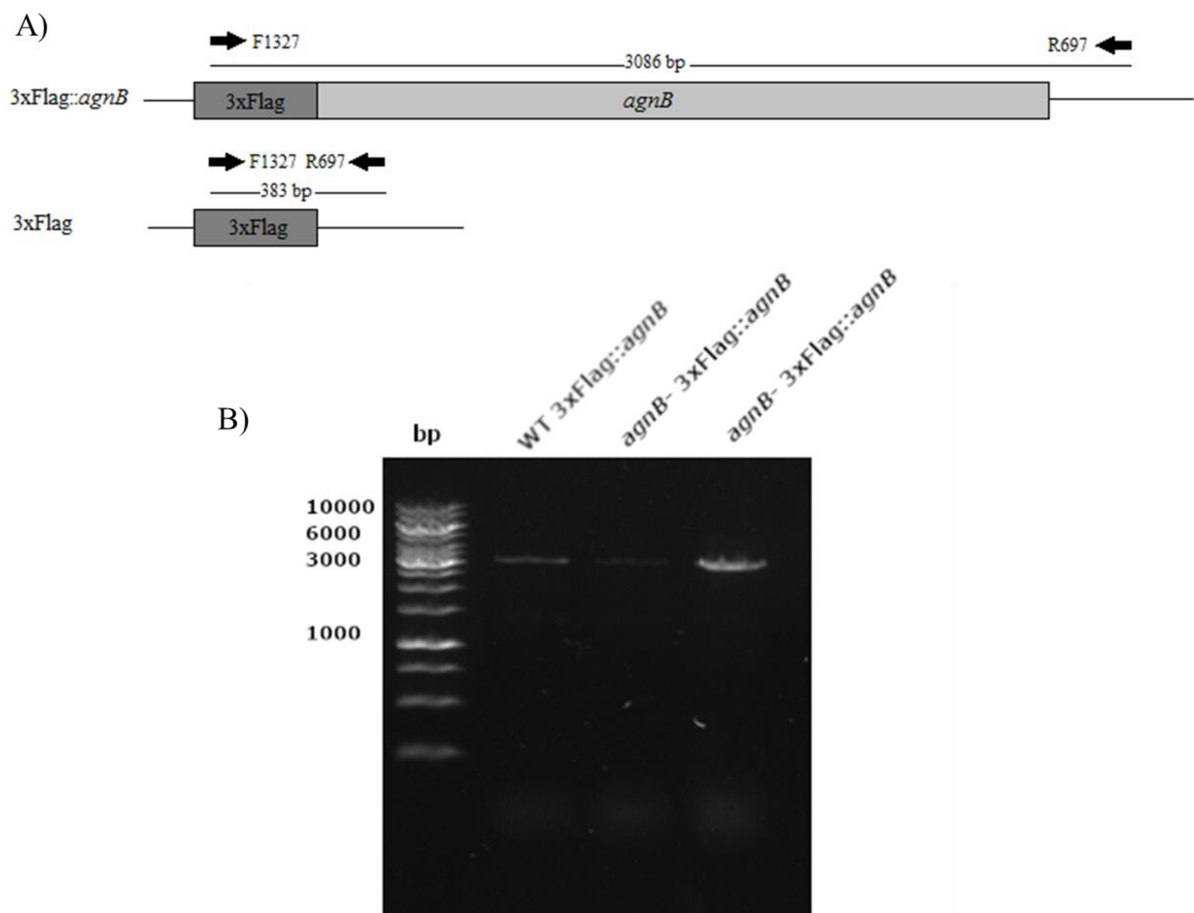

Figure 3. *Construction and verification of full 3xFlag::agnB sequence by PCR. (A) Schematic picture of 3xFlag::agnB (top) and 3xFlag (bottom) inserted in the plasmids. The size of the PCR fragments is indicated with the number of bp. The oligonucleotides used in the PCR are indicated with arrows. The prefix F and R stands for forward and reverse primer respectively. (B) Visualization of the PCR products on agarose gel of strains shows the amplification of the full fragment.*

Confirmation of the *agnB* sequence through sequencing

From the PCR, we had received full fragments of the 3xFlag::*agnB* which could be sent off for sequencing. The sequencing result we received of the PCR products verified most of the

3xFlag::*agnB* (figure S3), although it left out parts of the 5´and 3´ends. In order to sequence the 5´and 3´ends we opted to do a TA cloning. This meant that we again amplified the full 3xFlag::*agnB* sequence and cloned it into a new vector framed by TA repeats. We could then purify and sequence the vector. This time we received results verifying the 5´and 3´ ends of the sequence (figure S4).

By compiling the sequencing result, we got a sequence of the whole gene. Any conflicts, such as base substitution or deletion of bases, in one sequence could in most cases be dismissed as an error by DNA polymerase in the PCR reaction, because these errors were not found in all sequences we received. The result showed that no mutation had occurred during the cloning of the 3xFlag::*agnB* gene.

The expression of the Flag-AgnB fusion protein in the *D. discoideum* cells
The results from the PCR verified that the expected 3xFlag::*agnB* sequence was present in both wt and *agnB*$^-$ cells. Next, we wanted to look into if these cells did in fact express the Flag-AgnB fusion protein. The protein expression was studied by western blot using anti-Flag antibodies (figure 4). For every strain, half of the samples were treated with dithiothreitol (DTT), which reduces disulfide bonds, letting the protein unfold and move through the SDS-gel.

For *D. discoideum* cells with the 3xFlag::*agnB* we received a band between 100 and 140 kDa. The expected size of the fusion protein was around 120 kDa, which indicates that the bands shown on the gel likely is the expressed Flag-AgnB protein. For the wt strain with only the 3xFlag sequences, we received no band on the gel. Instead we observed in all the strains the expression of a protein around 50 and 70 kDa, most likely a native protein that also binds the anti-Flag antibodies.

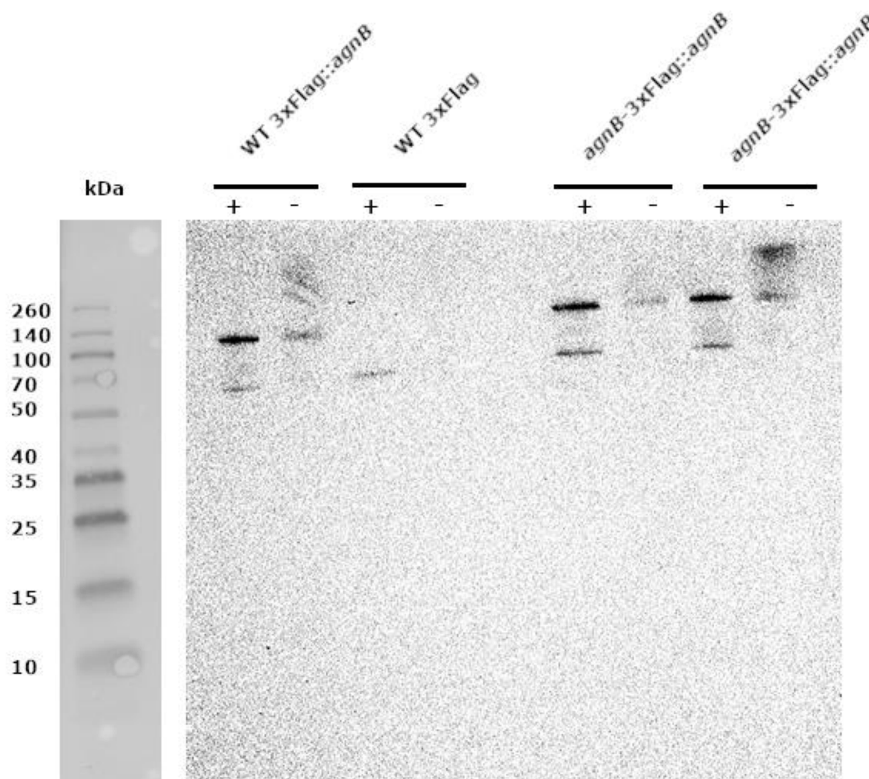

Figure 4. Verification of expressed Flag-AgnB fusion protein in D. discoideum cells. Western blot showing the expression of Flag tagged AgnB using anti-Flag antibodies. The size of the protein is indicated in kilo Dalton (kDa). The symbols + and - represent with and without DTT. The result was visualized on a SDS-gel.

No clear indication of the AgnB effect on cell growth

The last thing we looked into was if the cell growth was affected by AgnB depletion and if that being the case, if it could be rescued by introducing a new *agnB* gene. As previously mentioned, *agnB⁻* with the pDM-3xFlag would not grow at all and therefore no cultures could be started from this strain. The result we received showed that the wt strain grew better than the *agnB⁻* strain, as has been observed previously. We could also see that it was possible for the 3xFlag::*agnB* to help recover the growth in the *agnB⁻* strain. However, our data also showed that in some cases the cell growth in the *agnB⁻* strain could not be rescued with the 3xFlag::*agnB* sequence (figure 5).

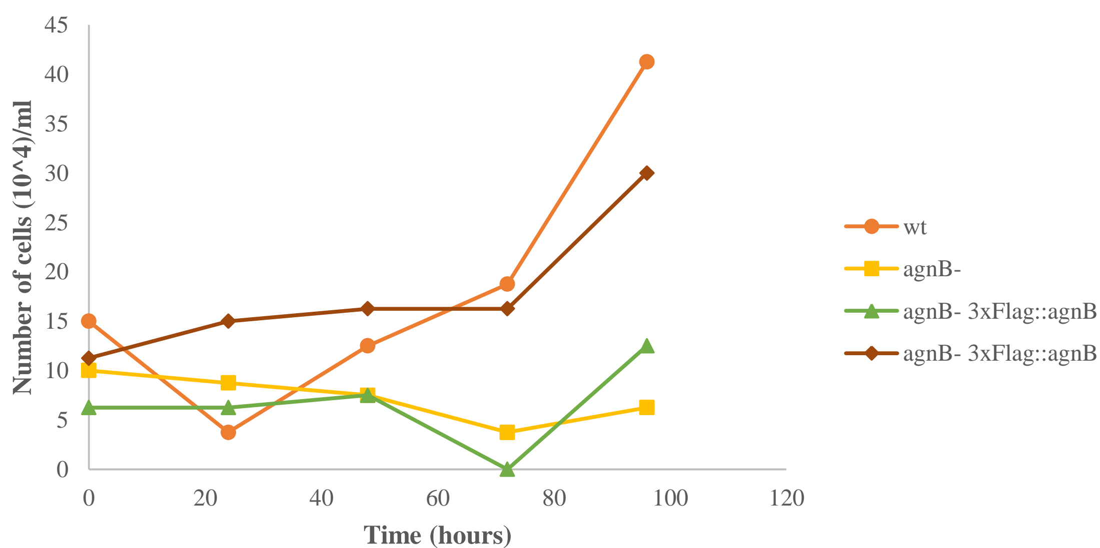

Figure 5. Cell growth for D. discoideum. The growth curve shows the cell growth for wt and agnB⁻ strains over 96 h. It also includes the cell growth for the agnB⁻ rescue strains. The number of cells are indicated in 10^4/ml.

Discussion

The function of Argonaute proteins in *D. discoideum* is not fully understood. In this study we look closer at the Argonaute B which is speculated to bind miRNAs in the miRNA pathway. Previous studies have shown that a depletion of AgnB in the cells leads to a depletion of miRNAs as well (Liao 2018). We wanted to investigate if a 3xFlag tag could be used to pull down AgnB and from there be able to characterize which RNAs it binds. Previously the 3xFlag marker, with or without the *agnB* gene, had been cloned and transformed into wt and *agnB⁻* cells. By using PCR we could confirm that this procedure had been successful and that no mutation had occurred.

To see if these transformed cells did express the Flag-AgnB fusion protein, we performed western blot analysis. From the result we could not only see that Flag-AgnB was expressed,

but also deduct that the 3xFlag marker may be used to extract the AgnB from the cells. This means that in the future, this could potentially be used to pull down the Argonaute B and characterize which RNAs it binds. In addition to the Flag-AgnB protein, we could also see another fragment in the blot. This protein was expressed in all strains, with or without the 3xFlag::*agnB*. Most likely this is a native protein that also binds the anti-Flag antibodies. Interestingly it only appeared in DTT treated cells. DTT is a reducing agent that breaks disulfide-bonds in proteins leading to the disruption of the tertiary and quaternary structure. Knowing this we could speculate that the reason for the protein not to shown in the untreated cells was that either the antibodies could not bind the untreated protein or that it could not run through the gel, although then we would probably have seen it on top of the western still in the wells.

In addition to this, we also decided to investigate if single knock-outs of the genomic *agnB* affect the growth in *D. discoideum* and if this was the case, if it could be rescued. All the strains we studied grew poorly in the shaker. The doubling time for wt cells is stated to be around 12 h *in vitro* (Soll *et al.* 1976). For our cells, the doubling time was much longer than that, in fact most strains did not double in number during the days we studied them. Many of the wt cells seem to have died off during the first day when they were introduced into a new growth medium. Growth only picked up again after a couple of days. An explanation for this could be that there was something wrong with the growth medium, which would prevent the cells from growing. However, when the same strains were allowed to grow in petri dishes outside of the shaker in the same medium, they grew much better. Noticing this we started to speculate if this was a result from a trivial error, either with the shaker or if there were some remnants of detergent in the glassware which killed the cells. The plastic dishes we used were made of plastic which would support that it was a detergent which caused the cells to grow poorly.

Even if the *D. discoideum* cells grew poorly, we can conclude from the results that wt cells grew better than *agnB*$^-$ as previously observed (Liao 2018). We could also see that in some cases it was possible for the introduced *agnB* gene to help recover the growth in the *agnB*$^-$ cells. However, nothing can be said with certainty because our result for this varied. In general, it seems that AgnB is somewhat needed in *D. discoideum* for the cells to be able to grow. This was also observed in the *agnB*$^-$ strains with only the 3xFlag, the strain would not grow on agar plate while the other strains did.

Our result show that the 3xFlag::*agnB* strains was correctly cloned and expressed under the tested conditions, and that it can be recognized by the anti-Flag antibody on western blot. This means that in the future the 3xFlag could potentially be used to pull down AgnB, which will allow characterization of which species of RNAs it binds. We could also see that AgnB in part is important for cell growth. Taken together, this helps to somewhat extend our knowledge of the Argonaute proteins and their part in the miRNA silencing pathway in *D. discoideum.*

Methods and materials

Strains and growth conditions

D. discoideum cells (Table S1) were grown in HL5c medium (Formedium) in the presence of 100 U/ml Penicillin Streptomycin (ThermoFisher Scientific) at 22 ^oC in both shaking and

petri dish cultures. Cells transformed with the pDM304 (Veltman *et al.* 2009) plasmid (harboring the cloned genes) were selected with G418 (10 µg/ml) (ThermoFisher Scientific).

Oligonucleotides
All DNA oligonucleotides used were synthesized by Eurofins (Table S2).

PCR
Cells were lysed with lysis buffer (10 mM Tris pH 8.3, 50 mM KCl, 24 mM MgCl2, 0.45 % Triton-X100, 0.45 % Tween-20) and proteinase K (ThermoFisher Scientific) at 95 ^{o}C for 2 min. Of the lysed cells, 1 µl was used as template for the PCR reaction that was carried out in PCR 2X Master Mix (ThermoFisher Scientific). If the PCR product was sent off to be sequenced, the PCR reaction was instead carried out in Taq 2X Master Mix (New England Biolabs). PCR cycles for PCR products less than 1500 bp long: 95 ^{o}C, 1 min; [95 ^{o}C, 15 s; 49 ^{o}C, 30 s; 65 ^{o}C, 1 min] for 35 cycles, 65 ^{o}C 7 min for final extension. PCR cycles for PCR products longer than 1500 bp: 95 ^{o}C, 1 min; [95 ^{o}C, 15 s; 42 ^{o}C, 30 s; 65 ^{o}C, 5 min] for 35 cycles, 65 ^{o}C 7 min for final extension. The products were visualized by 1 % agarose gel electrophoresis. The gel was pre-stained with sybr-safe (ThermoFisher Scientific).

TA cloning
PCR fragments of the 3xFlag::*agnB* sequence were ligated into plasmids using InsTA clone PCR cloning kit (ThermoFisher Scientific) and transformed into competent *Escherichia coli* cells (DH5α). Cells were plated on ampicillin LA plates and incubated at 37 ^{o}C overnight. Colony PCR was performed using one colony as template for the PCR and the PCR reaction was done by Taq 2X Master Mix (New England Biolabs). PCR cycles: 95 ^{o}C, 1 min; [95 ^{o}C, 15 s; 42 ^{o}C, 30 s; 65 ^{o}C, 5 min] for 35 cycles, 65 ^{o}C 10 min for final extension. The products were visualized by 1 % agarose gel electrophoresis. Cells containing the plasmid with cloned 3xFlag::*agnB* were harvested and the plasmid was prepared using GeneJet Plasmid Miniprep kit (ThermoFisher Scientific).

Sequencing
The PCR products and plasmids were sequenced by Macrogen Europe.

Western blot
About $2*10^{7}$ cells were harvested by centrifugation for 5 min at 400 xg at 4 ^{o}C. The cells were washed with cold PBS twice and then lysed with cold Flag-IP lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Triton X-100, and 1 mM EDTA) together with HALT 100x protease inhibitor cocktail (ThermoFisher Scientific). The cells were incubated on ice for 30 min and after every 10 min the samples were mixed. The cells were centrifuged for 10 min at 20000 rpm at 4 ^{o}C. To 300 µl from each sample 300 µl 2xLaemmli buffer (4% SDS, 20% glycerol, 0.004 % bromophenol blue, 0.125 M Tris pH 6.8) was added. To another 300 µl of each sample 300 µl 2xLaemmli buffer together with DTT was added. The cells were denatured for 3 min at 95 ^{o}C. Protein concentration was determined by Brandford Protein determination (BioRad). 1 µg of protein was loaded onto a Mini-PROTEAN TGX Stain Free precast gel (BioRad). After the electrophoresis, the proteins were transferred to a membrane using the BioRad Trans-Blot Turbo system. The membrane was blocked with Tris-buffered aline (TBST) (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Tween-20) and 3 % BSA by shaking at room temperature for 1 h. The membrane was incubated with an anti-Flag antibody solution (1:10000) by shaking at 4 ^{o}C for 1 h and washed three times for 5 min each with

TBST. The antibodies were visualized using Enhanced Chemiluminescent Substrate (ThermoFisher Scientific)

Growth curve
The different *D. discoideum* strains were grown in shaking cultures at 22 °C. Every 24 h the number of cells were counted using a hemocytometer and from that the number of cells per ml was calculated.

Acknowledgments

I would like to thank my supervisor Fredrik Söderbom for giving me the opportunity to work on this project and his overall support. I would also like to thank my other supervisor Bart Edelbroek for helping me with the lab work and always being willing to answer my questions and give his input as well as support. Last I would like to thank the department of ICM for making me feel welcomed during my time there.

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Supplementary material

A)

B)

Figure S1. *Verification of transformed plasmid in D. discoideum. (A) Schematic picture of ampicillin resistance cassette in the plasmid. Of the expected PCR fragment is indicated with the number of bp. The oligonucleotides used in the PCR are indicated with arrows. The prefix F and R stands for forward and reverse primer respectively. (B) Visualization of the PCR products on agarose gel, showing the present of the ampicillin resistance cassette.*

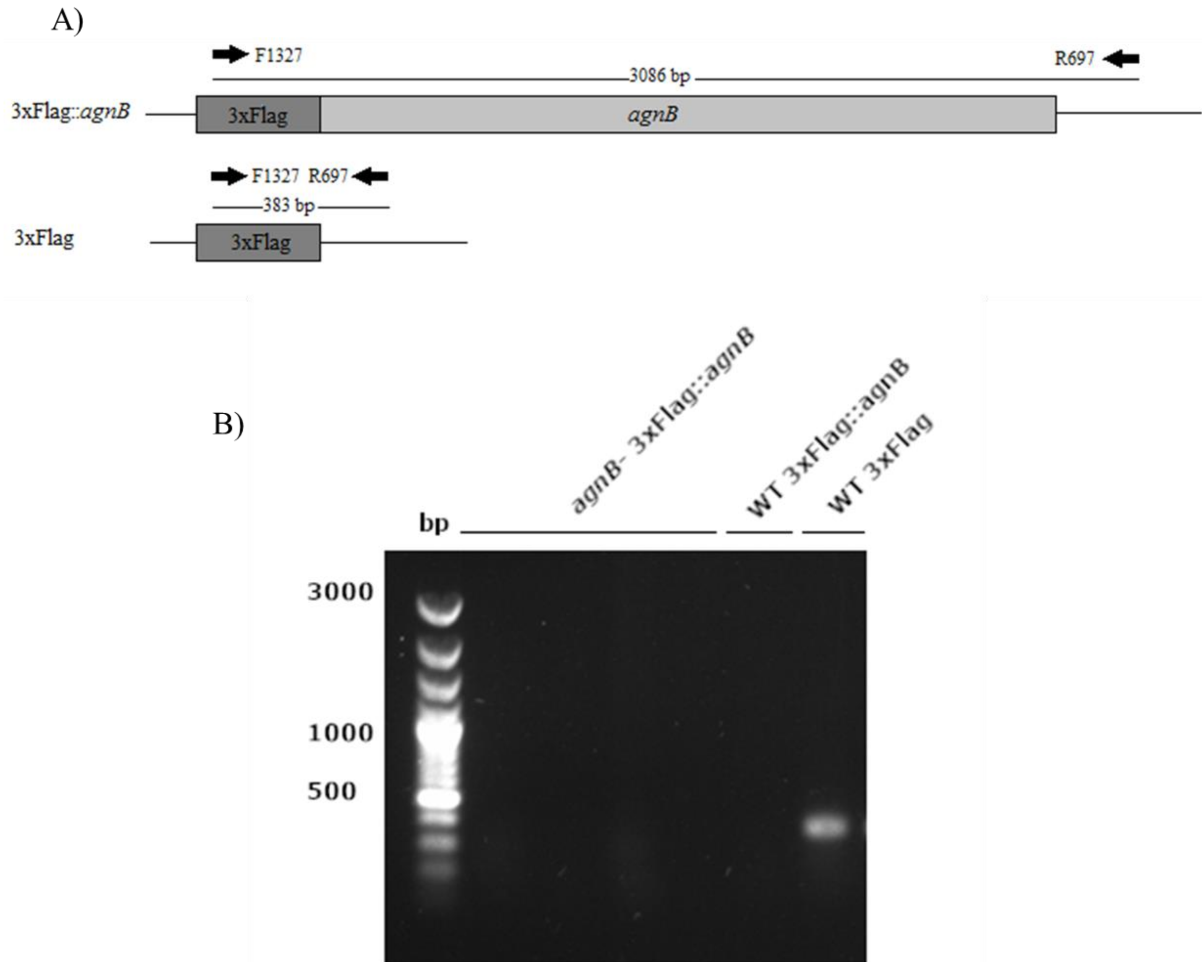

Figure S2. *Verification of 3xFlag sequence. (A) Schematic picture of 3xFlag::agnB (top) and 3xFlag (bottom) inserted in the plasmid. Of the expected PCR fragment is indicated with the number of bp. The oligonucleotides used in the PCR are indicated with arrows. The prefix F and R stands for forward and reverse primer respectively. (B) Visualization of the PCR products on agarose gel, showing the present of the 3xFlag sequence.*

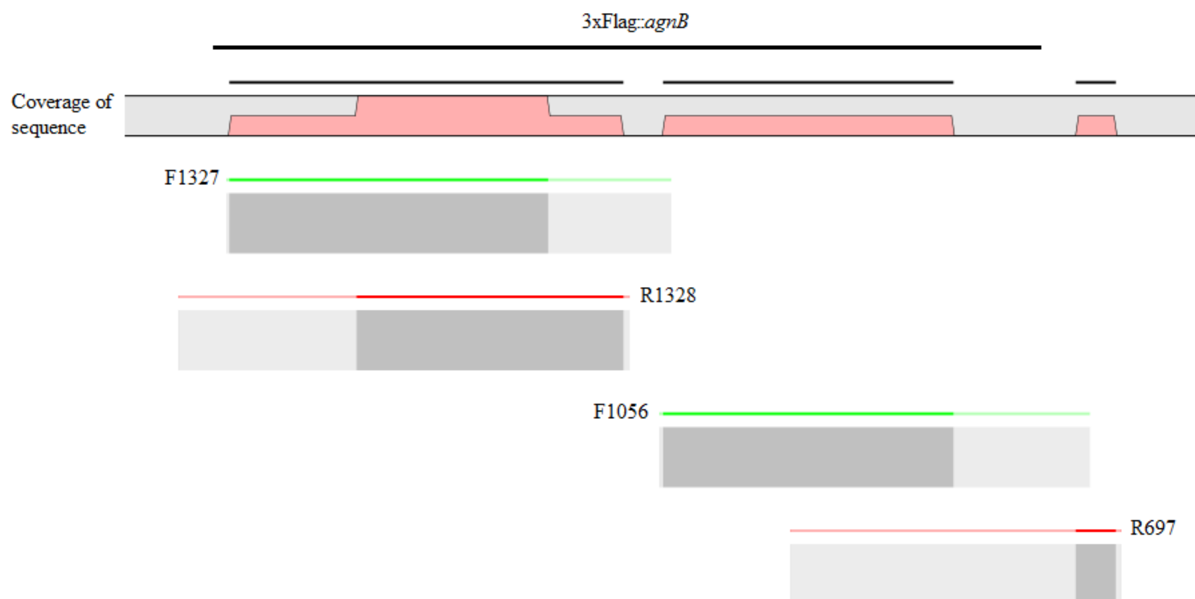

Figure S3. *Overview of sequencing results of 3xFlag::agnB PCR fragments from the plasmid. The green lines represent the trace data from the forward primers used in the sequencing. Meanwhile, the red lines represent the reverse primers. In coverage of sequence, the pink area shows the compilation of the results, it represent which parts of the gene have been successfully sequenced.*

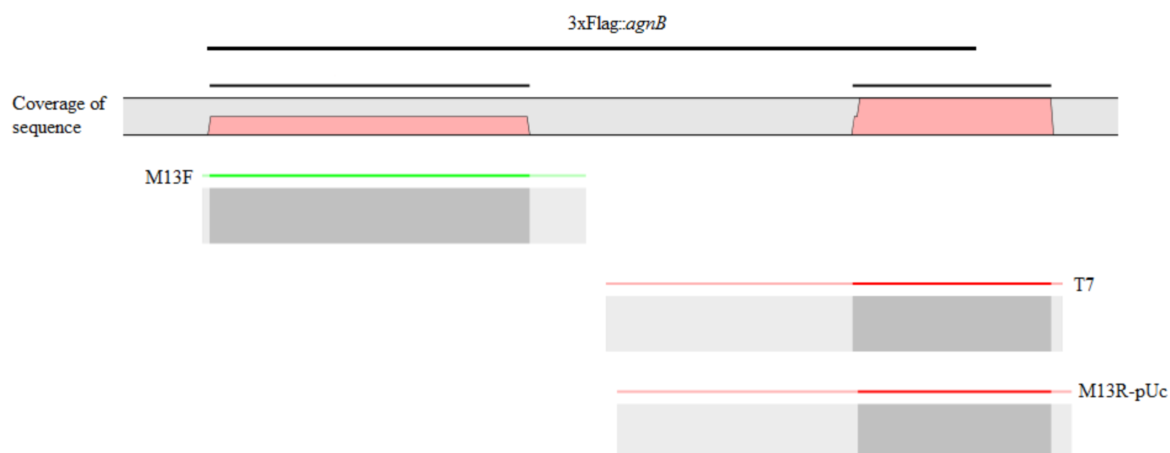

Figure S4. *Overview of sequencing results 3xFlag::agnB of PCR fragments from the TA cloning. The green line represents the trace data from the forward primer used in the sequencing. Meanwhile, the red lines represent the reverse primers. In coverage of sequence, the pink area shows the compilation of the results, it represent which parts of the gene have been successfully sequenced.*

Table S1. The genotypes of the *D. discoideum* strains used.

Strain	Genotype of plasmid
WT	pDM-3xFlag::*agnB*
WT	pDM-3xFlag
agnB^{-}	pDM-3xFlag::*agnB*
agnB^{-}	pDM-3xFlag

Table S2. Oligomers used in the PCR reaction and their sequences. All primers were synthesized by Eurofins with the exception of T7, M13F and M13R-pUc. These primers are from Macrogen Europe universal database.

Primer	Sequence
R697	CTATTTACTTTTTCGAAATC
F1327	GGATTATAAAGATCATGATGG
F1012	GCAGTGTTATCACTCATGGTTAT
R1013	ACCCTGATAAATGCTTCAATAATA
F1325	AGAAGTTAACCATAGGGA
R1328	TTTTACGCATATGATCAC
F1056	TGATCCAAGTCAAAGATATCAAACA
T7	AATACGACTCACTATAG
M13F	GTAAAACGACGGCCAGT
M13R-pUc	CAGGAAACAGCTATGAC