RNA BIOLOGY
2016, VOL. 13, NO. 10, 1000–1010
http://dx.doi.org/10.1080/15476286.2016.1212153

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RESEARCH PAPER

OPEN ACCESS

The protein domains of the *Dictyostelium* microprocessor that are required for correct subcellular localization and for microRNA maturation

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ABSTRACT

The maturation pathways of microRNAs (miRNAs) have been delineated for plants and several animals, belonging to the evolutionary supergroups of Archaeplastida and Opisthokonta, respectively. Recently, we reported the discovery of the microprocessor complex in *Dictyostelium discoideum* of the Amoebozoa supergroup. The complex is composed of the Dicer DrnB and the dsRBD (double-stranded RNA binding domain) containing protein RbdB. Both proteins localize at nucleoli, where they physically interact, and both are required for miRNA maturation. Here we show that the miRNA phenotype of a Δ*drnB* gene deletion strain can be rescued by ectopic expression of a series of DrnB GFP fusion proteins, which consistently showed punctate perinucleolar localization in fluorescence microscopy. These punctate foci appear surprisingly stable, as they persist both disintegration of nucleoli and degradation of cellular nucleic acids. We observed that DrnB expression levels influence the number of microprocessor foci and alter RbdB accumulation. An investigation of DrnB variants revealed that its newly identified nuclear localization signal is necessary, but not sufficient for the perinucleolar localization. Biogenesis of miRNAs, which are RNA Pol II transcripts, is correlated with that localization. Besides its bidentate RNase III domains, DrnB contains only a dsRBD, which surprisingly is dispensable for miRNA maturation. This dsRBD can, however, functionally replace the homologous domain in RbdB. Based on the unique setup of the *Dictyostelium* microprocessor with a subcellular localization similar to plants, but a protein domain composition similar to animals, we propose a model for the evolutionary origin of RNase III proteins acting in miRNA maturation.

ARTICLE HISTORY
Received 10 June 2016
Revised 27 June 2016
Accepted 7 July 2016

KEYWORDS
Amoebae; double-stranded RNA binding domain; evolution; nuclear localization signal; RNase III

Introduction

MicroRNAs (miRNAs) are highly conserved small RNAs of 21–25 nt that act in endogenous gene regulation.[1] In the canonical miRNAs biogenesis pathway, 2 successive processing events take place, mediated by the ribonuclease (RNase) III proteins Dicer and/or Drosha.[2-5] Initially, primary miRNA (pri-miRNA) transcripts featuring characteristic secondary structures with a terminal loop and a bulge-rich stem are processed in the nucleus of animals by Drosha, or the Dicer-like protein DCL-1 in plants.[2-7] In plants, the resulting stem-loop structured precursor miRNAs (pre-miRNA) are further processed in the nucleus by DCL-1 to mature miRNAs.[8] In animals, this final processing step is carried out in the cytoplasm by a Dicer protein[6] after nuclear export by exportin-5.[9,10] In general, Dicer and Drosha proteins contain bidentate RNase III domains and a double-stranded RNA binding domain (dsRBD; reviewed in[11,12]). The RNase III domains are arranged in tandem and are thought to form the catalytic center by intramolecular pseudo-dimerization.[13] Dicer proteins additionally feature several other protein domains. Of these, their helicase and PAZ domains are of particular importance, as they have been implied in measuring the distance between the 3′-overhang and the terminal loop, a strict requirement for pre-miRNA cleavage at the correct position.[14-19] Conspicuously, Drosha enzymes do not feature these domains. A current model proposes that junctions between single stranded (ss) and double-stranded (ds) parts of the pri-miRNA might determine the cleavage sites of Drosha, with both structures in the lower and the upper stem critical for precise processing.[20] Additional determinants for Drosha cleavage sites were also identified in human pri-miRNAs sequences, such as basal UG or apical GUG motifs; however, those sequence patterns do not appear to be conserved between animals.[21,22]

Despite the differences between animals and plants in both subcellular localization and participating enzymes in the miRNA processing, a common theme is observed: each of the RNase III enzymes requires interaction with a dedicated dsRBD-containing protein for full functionality. The nuclear complex containing Drosha and DGCR8/Pasha in animals has been dubbed the microprocessor,[23,24] and deletion of either the

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Supplemental data for this article can be accessed on the publisher's website.

RNase III protein or the double stranded RNA binding protein (DsRBP) abolishes or significantly impairs the formation of pre-miRNAs. The exclusively nuclear miRNA processing in plants is carried out by DCL1 and interaction partners within so-called nuclear dicing bodies found in the periphery of, but not within nucleoli.[25] Recent data indicate that dicing bodies co-localize with Cajal-body markers,[26] where small RNAs in plants are 2'O-methylated by the Cajal-body associated methyltransferase Hen1.[27]

The species *Caenorhabditis elegans, Drosophila melanogaster* and *Homo sapiens* all belong to the Opisthokonta, one of 5 supergroups that have evolved from the last eukaryotic common ancestor (LECA).[28] *Arabidopsis thaliana* belongs to another supergroup, the Archaeplastida. The miRNA processing pathways and their key players thus appear to differ in these 2 supergroups, but are similar within the Opisthokonta. Adding information about the organization of miRNA maturation outside those supergroups, we recently have reported the identification of the microprocessor in the amoeba *Dictyostelium discoideum*,[29] a well established model organism of the Amoebozoa supergroup. In *D. discoideum*, the Dicer DrnB and its associated DsRBP RbdB form the microprocessor that displays similarities in localization to the plant Dicing bodies, however, the domain composition of DrnB is more comparable with that of animal Drosha proteins (Fig. 1). Curiously, the genome of the amoeba does not encode for any PAZ and helicase domain containing Dicer protein,[30] which are, with that domain composition, central enzymes in miRNA processing in both other supergroups.

Here we investigated the functional importance of the identified protein domains in both DrnB and RbdB for correct localization and miRNA maturation.

Results

N- and C- terminal GFP DrnB fusion proteins complement the drnB *null phenotype*

DrnB has been shown to be important for the generation of mature miRNAs in *Dictyostelium discoideum*.[31] Tagged versions of the protein were found to localize to nuclear foci,[32] and our recent study indicated that these foci represent nucleoli.[29] To detail the functional importance of the DrnB localization in miRNA biogenesis, we generated a series of N- and C-terminal GFP fusions of DrnB with varying expression levels. These were mediated by the use of the high copy extrachromosomal plasmid pDM317[33] or the integrating plasmid pDneo2a,[32] containing strong act15 or weak act6 promoters,[34] respectively. Upon transformation of these constructs in both the wild type AX2 and the Δ*drnB* gene deletion [35] strains, fluorescence microscopy consistently showed localization of the fusion proteins in the nucleolar foci,[29] independently of the localization of the tag, or the type of vector used (Fig. 2a). The number of these foci varied, however, from 1 to 7 between individual cell nuclei and appeared to be correlated with differing expression levels obtained from the different plasmids (Fig. 2a,b). Expression of the GFP-fusions was observed in the majority of Δ*drnB* cells, but only in about 1% of the AX2 wildtype, indicating that DrnB levels might be under tight control in the wild type.

We next set out to investigate whether the different GFP DrnB fusion proteins (Fig. 2a,b) can rescue the phenotype of Δ*drnB* cells, in which mature miRNAs disappear.[35] When these cells were transformed with the constructs encoding the different GFP DrnB fusions, mature miRNAs could readily be detected by Northern Blot analysis (Fig. 2c). Again, this observation was made independently of the location of the tag, and whether the fusion protein was expressed from extrachromosomal or integrating vectors. The observation that the miRNA phenotype of Δ*drnB* could be rescued by expression of DrnB fusion proteins suggests that their localization (Fig. 2a) corresponds to that of the endogenous protein. Nuclear run on experiments were carried out to detail which RNA polymerase transcribes the miRNA genes. In the presence of low concentrations of α-Amanitin, miRNAs disappeared (Fig. S1). This indicates that miRNAs in *D. discoideum* are transcripts generated by RNA polymerase II, similar to canonical miRNAs in animals, as summarized recently.[36]

DrnB localizes to the periphery of nucleoli

We recently reported the nucleolar localization of DrnB, which was concluded from its localization to regions on the nuclear periphery that are characterized by reduced DAPI staining.[29] Here, we set out to detail the specific nuclear localization, for which we employed laser scanning microscopy on paraformaldehyde (PFA) fixed cells. As no molecular marker for nucleoli in *Dictyostelium* was available for our purposes, we cloned mRFP-tagged fibrillarin. Upon expression in the amoeba, we observed the expected nucleolar localization of fibrillarin by fluorescence microscopy (Fig. 3a). Its fluorescence signal perfectly co-localized with the dark arcs in the nuclear periphery, which can be observed in PFA fixed cells by phase contrast microscopy. This data confirms the hypothesis that these dark arcs are the nucleoli, as has been suggested earlier.[37] In line with our recent report for GFP-DrnB,[29] also all other GFP DrnB fusion proteins were found to localize at these arcs, as shown for the

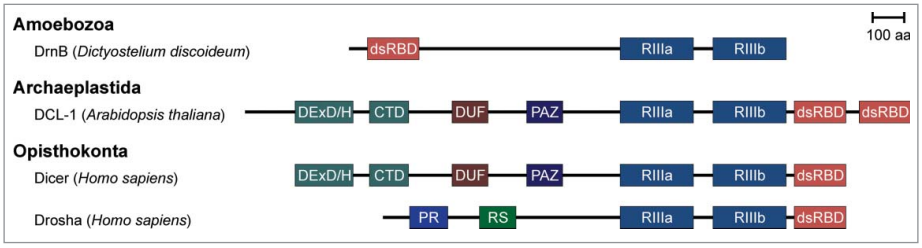

Figure 1. Domain organization of RNase III family proteins in the 3 eukaryotic supergroups Amoebozoa, Archaeplastida and Opisthokonta. Domains are shown for the RNase III proteins DrnB, DCL-1, Dicer and Drosha from the indicated model organisms. Proteins are drawn to scale with RIII (RNase III domain); dsRBD (double stranded RNA binding domain); DUF (domain of unknown function); DExD/H (RNA helicase/ATPase superfamily); CTD (C-terminal domain of helicase); PAZ (PIWI/Argonaute/Zwille); PR (Proline rich); RS (Arginine/Serine rich).

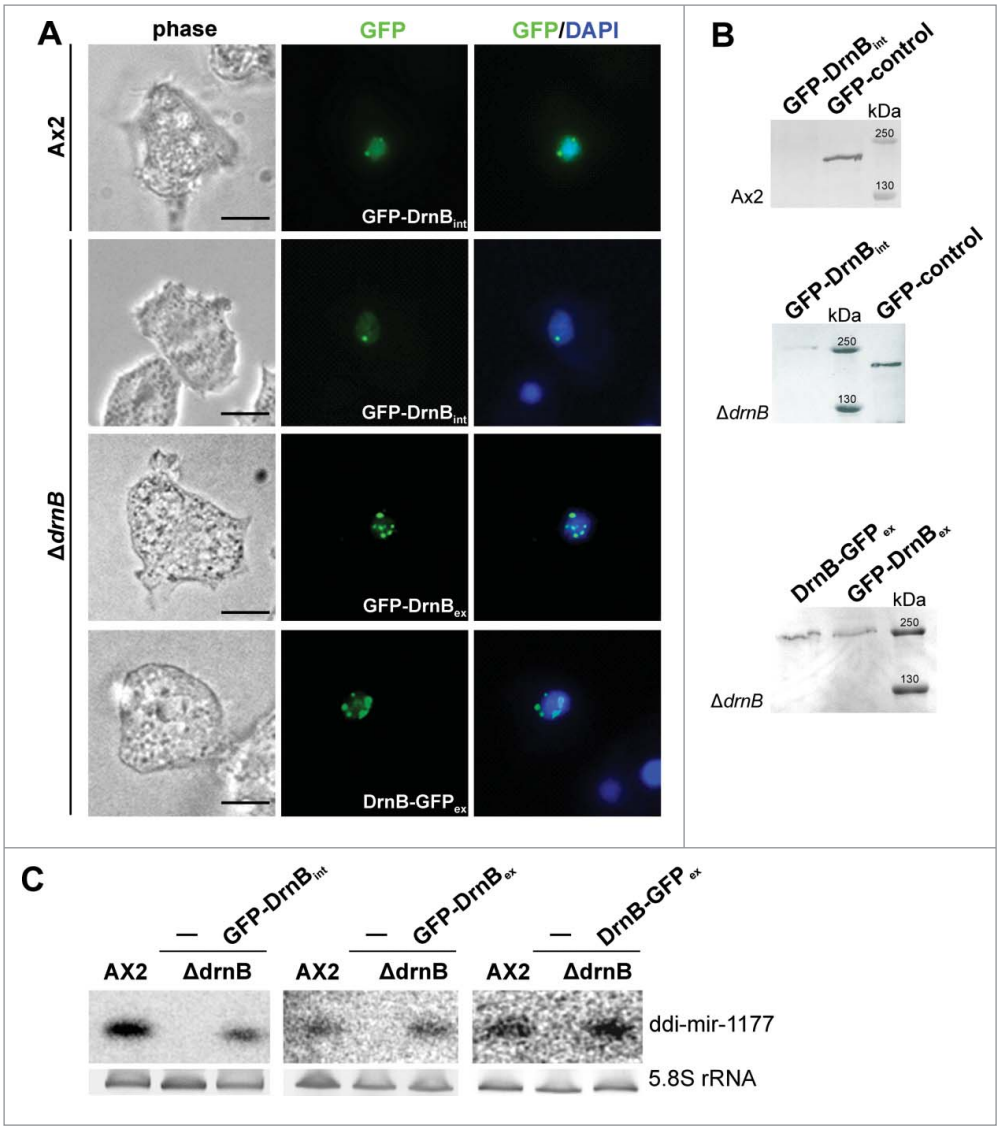

Figure 2. Subcellular localization of GFP DrnB fusions and phenotype complementation. (a) Subnuclear localization of GFP DrnB fusion proteins in methanol fixed *D. discoideum* AX2 and Δ*drnB* cells. Localization is shown for N- and C-terminal fusion proteins expressed from integrating (int) and low copy vector pDneo2a, or extrachromosomal (ex) and high copy number pDM317 and pDM323 plasmids. Nuclei are counter stained with DAPI and the size bar represents 5 μm. (b) Western blot analyses of GFP DrnB fusions. Expression of GFP fusions of DrnB (189 kDa) were analyzed using an α-GFP antibody, and using an unrelated GFP control fusion protein of 170 kDa in size. No signal was observed for the transformed AX2 strain, where GFP-DrnB was expressed in less than 1 % of the subcloned culture, as judged by fluorescence microscopy. Expression in the Δ*drnB* background led to detectable Western Blot signals, consistent with a higher fraction (~60%) of the transformed and subcloned cells displaying GFP fluorescence. Experiments were carried out with cells under G418 (10 mg/l) selection. (c) Northern blot analyses of ddi-miR-1177 in Δ*drnB* cells expressing N- or C-terminal GFP DrnB from integrating (int) or extrachromosomal (ex) plasmids, compared to AX2 wild type or untransformed Δ*drnB* cells. Ethidium bromide stained rRNA (bottom) served as loading control.

example of Δ*drnB*:DrnB-GFP cells (Fig. 3b). A closer inspection of the nucleolar localization of GFP-DrnB suggested, however, that the signals overlap, but do not perfectly coincide (Fig. 3b). To detail this, Z-stacks through a single nucleus were recorded (Fig. 3c). These images revealed an association of the fusion protein exclusively to the periphery of nucleoli, with no observable fluorescence from within these sub-nuclear bodies. This indicates that DrnB is a perinucleolar protein.

DrnB foci do not depend of the integrity of nucleoli

Having shown that GFP DrnB fusions localize in perinucleolar foci, we were interested to study whether this localization depends of intact nucleoli. To test this question, we employed Actinomycin D (AMD), which has been shown to cause disintegration of nucleoli in *Dictyostelium* at concentrations that specifically inhibit RNA polymerase I [37,38]. When we treated living cells with such AMD concentrations, the DrnB foci remained (Fig. 4a), while the dark arcs representing the nucleoli (Fig. 3a) vanished. This indicates that the clustering of DrnB in the focal structures does not depend on intact nucleoli. Since nucleoli are the sites of rDNA and rRNA transcription [39] we considered that the punctate localization of DrnB might require RNA or DNA as a scaffold. To test these possibilities, we digested nucleic acids *in situ* with RNase A or DNase I, either individually or together, and monitored nucleic acids with Draq5^{TM}, which stains both RNA and DNA in *Dictyostelium* nuclei (Fig. 4b). When this treatment was applied to living cells, the DrnB foci remained intact (Fig. 4b). While these experiments cannot rule out that the assembly of DrnB in the perinucleolar foci might require intact nucleoli, or the presence of nucleic acids, the

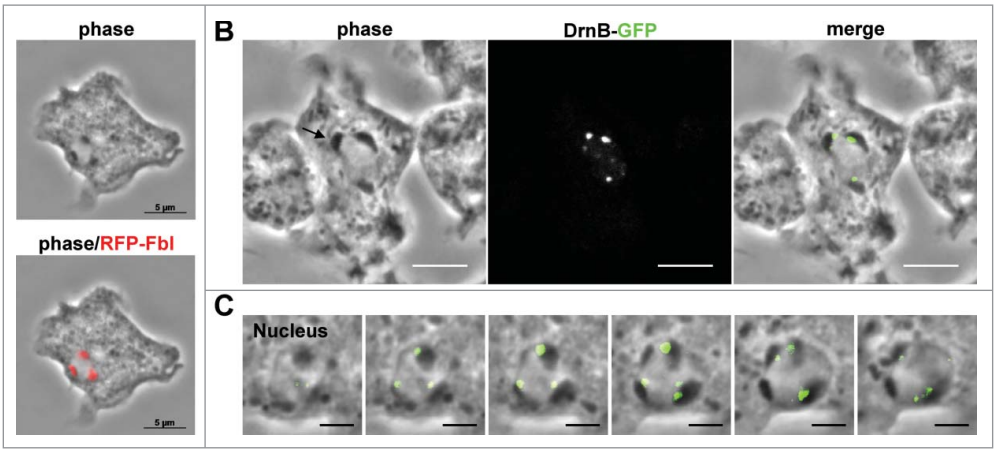

Figure 3. Perinucleolar localization of DrnB. (a) Localization of N-terminally mRFP-tagged Fbl, the *D. discoideum* homolog of the human rRNA 2′ O-methyltransferase Fibrillarin as nucleolar marker in AX2 cells. A phase contrast image is shown on top, which is superimposed with the mRFP fluorescence of the fusion protein (bottom). Scale bars represent 5 μm. (b) Localization of GFP-DrnB, relative to nucleoli (arrow). (c) Z-stack imaging of GFP-DrnB through a single nucleus (2 μm z-intervals). Scale bars in A, B represent 5 μm, and 2.5 μm in C. Cells in A-C were fixed with 4% PFA.

data indicate that once the punctate structures are formed, they do not require intact nucleoli, or the presence of nucleic acids.

The dsRBD of DrnB is dispensable for miRNA biogenesis

We next set out to explore the functional importance of the individual protein domains of DrnB for subcellular localization and miRNA biogenesis. At the N-terminus, DrnB features a dsRBD and, toward the C-terminus, 2 RNase III domains (Fig. 5a): the same domains are found also in the nuclear Drosha proteins in animals, albeit with a shuffled order [40]. Additionally to those protein domains, using PSORT [41], we identified a typical nuclear localization signal (NLS) consisting of SIRKRRRKR at amino acid position 493–501 (Fig. 5a). We generated a series of constructs that allowed us to overexpress protein variants, in which individual protein segments of DrnB were deleted or joined, and tested these constructs for subcellular localization and miRNA rescue in the Δ*drnB* background (Fig. 5b).

Removal of the NLS signal resulted in the loss of the punctate foci seen for full-length DrnB, and GFP-signals were observable all over the cell; although the ΔNLS fusion protein was still slightly accumulated in the nucleus, the miRNA phenotype of the Δ*drnB* cells was no longer rescued (Fig. 5). Previous studies on human Drosha have shown that its dsRBD is essential for processing of pri-miRNAs [42] leading us to assume a similar indispensable role for the dsRBD in the *Dictyostelium* DrnB protein. To our surprise deletion of the dsRBD containing N-terminus of DrnB did not affect complementation of the miRNA phenotype nor did it greatly alter the cellular localization of the resulting ΔdsRBD protein variant, as the GFP signal is also found in foci, although of smaller size (Fig. 5). This finding indicates that the dsRBD of DrnB is not required for the generation of mature miRNAs. Expression of the tandem RNase III domains alone resulted in ubiquitous distribution of the RNase III construct throughout the cell with enrichment in the nucleus, indicating that these domains might contain a non-canonical NLS (Fig. 5). This is supported, albeit indirectly, by a similar localization seen for the NLS-RNase III construct expressing

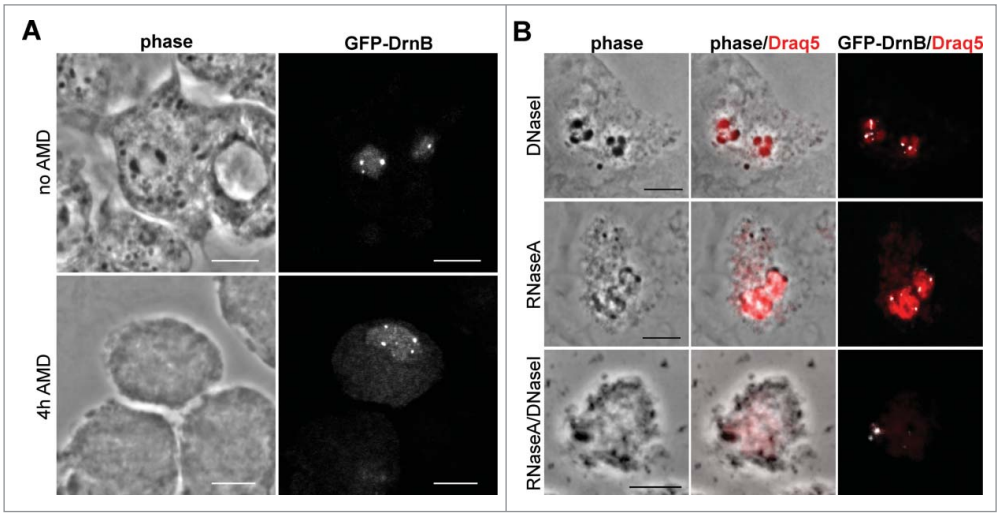

Figure 4. Stability of DrnB foci. (a) Localization of GFP-DrnB in untreated cells (top) and after disintegration of nucleoli by Actinomycin D (AMD) treatment (bottom). (b) Localization of GFP-DrnB after DNase I (top), RNase A (center) or combined DNase I/RNase A (bottom) treatment in living cells stained with Draq5™. After the respective treatment, cells were fixed with 4% PFA. Scale bars represent 5 μm.

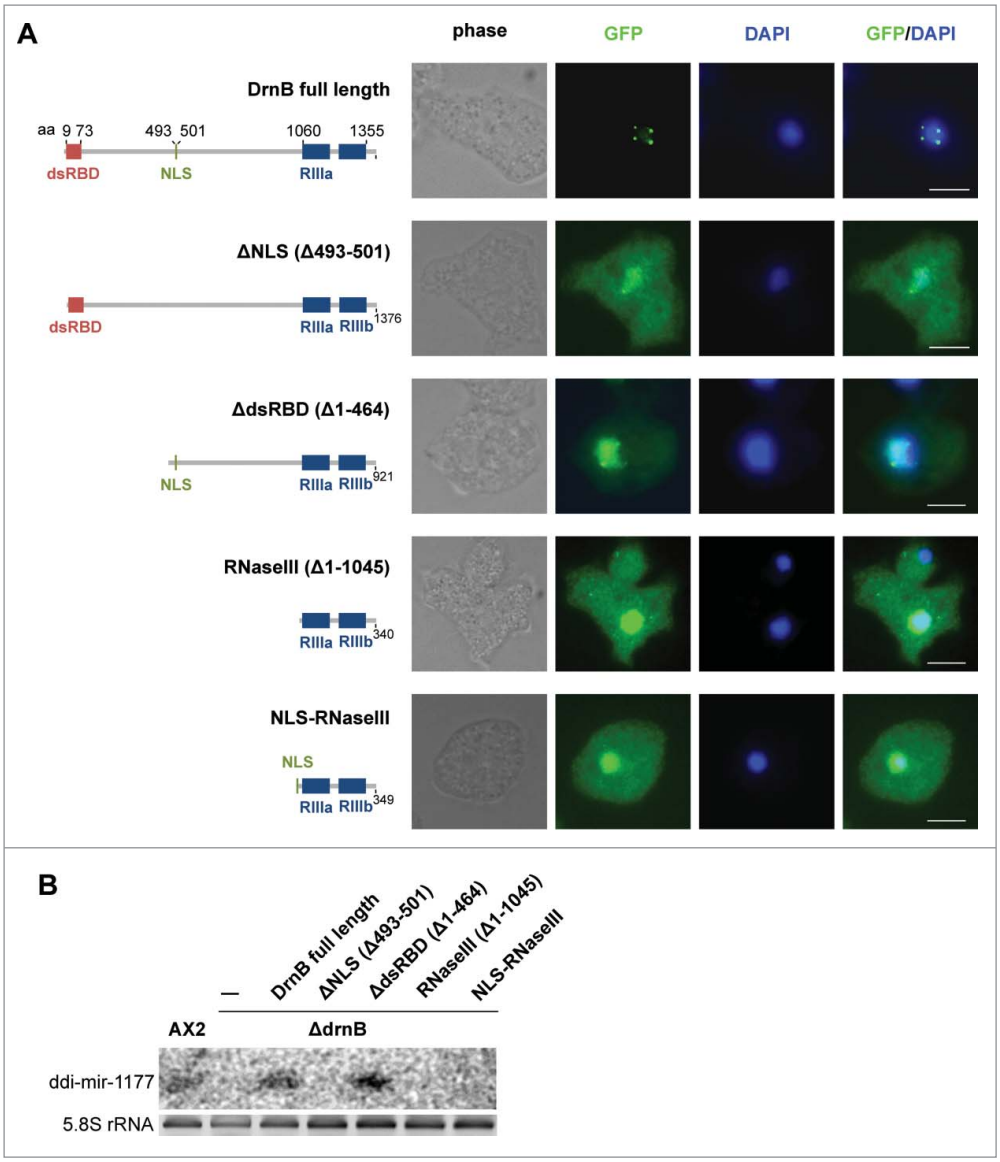

Figure 5. Subcellular localization and miRNA phenotype complementation of DrnB-GFP variants. (a) Domain structures of DrnB full length protein with double-stranded RNA binding domain (dsRBD), putative nuclear localization signal (NLS) and 2 RNase III domains, and the indicated variants thereof (left). To the right, the corresponding subcellular localization is shown for methanol fixed cells expressing the respective C-terminal GFP fusion constructs of DrnB from extrachromosomal plasmids, with phase contrast, GFP fluorescence microscopy, DAPI stain and GFP/DAPI merge. The scale bar represents 5 μm. (b) Northern blot of miRNA ddi-miR-1177 in the strains shown (a), next to AX2 wild type and Δ*drnB* cells as controls. Ethidium bromide stained rRNA served as loading control.

the NLS fused to the tandem RNase III domains (Fig. 5). Although both, RNase III and NLS-RNaseIII were still present in the nucleus, neither of the constructs could revert the miRNA phenotype of Δ*drnB* cells and neither appeared accumulated in the perinucleolar foci. In summary, these data indicate that the identified NLS is required, but not sufficient for the punctate perinucleolar localization of DrnB. This localization to perinucleolar foci appears to be correlated with a function of DrnB in miRNA biogenesis, for which DrnB's dsRBD, however, is dispensable.

RbdB accumulation in perinucleolar foci depends on DrnB levels

We have recently shown that RbdB, a protein solely featuring an N-terminal dsRBD, co-localizes with DrnB. Furthermore, Δ*rbdB* cells display the same molecular phenotype in miRNA biogenesis as Δ*drnB* cells.[29] In that study, we have also shown that the localization of the 2 proteins in the nucleolar foci is independent of each other. For the microprocessor complex in animals, a recent study has shown crossregulation of the levels of Drosha and its DsRBP DGCR8.[43] When we expressed GFP-DrnB in Δ*drnB* cells either from a low or from a high copy vector, we observe increased numbers of DrnB foci in the nucleus (Fig. 6). Notably, this was observed despite the fact that RbdB-mRFP was co-expressed in either case from the same plasmid, indicating that its steady state levels seemed to follow that of its interaction partner DrnB (Fig. 6).

The two dsRBDs within the Dictyostelium *microprocessor are functionally exchangeable*

We noticed that the dsRBDs of the 2 proteins, which in both cases cover the amino acid residues 9–75 in Pfam,[44] display significant sequence conservation with 92.4% identical or similar amino acids within the typical $\alpha\beta\beta\beta\alpha$ fold of dsRBDs [45,46] (Fig. 7a). This high

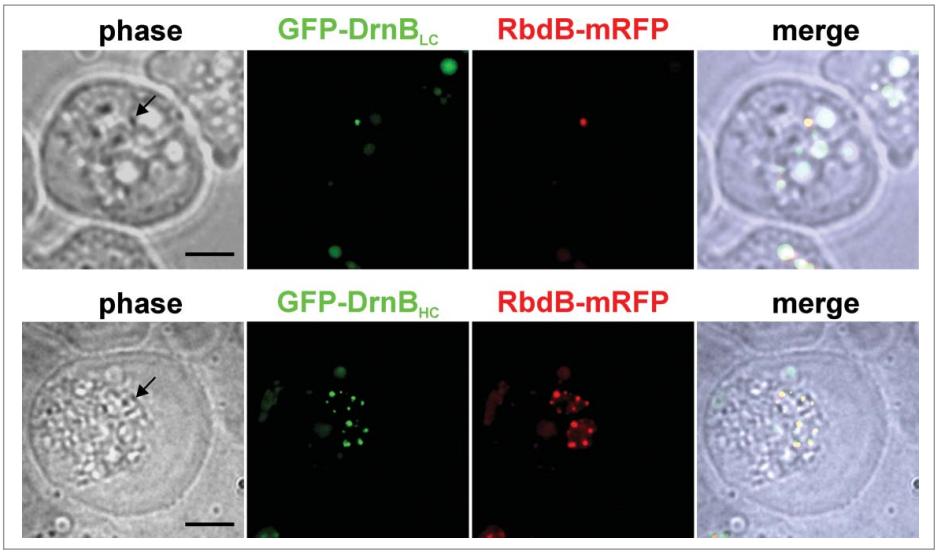

Figure 6. DrnB-dependent foci accumulation of RbdB in *D. discoideum* Δ*drnB* cells. Live cell microscopy using the indicated channels of cells co-expressing RbdB-mRFP from the same low copy number vector, and GFP-DrnB from a low copy (LC, top), or a high copy (HC, bottom) vector. In phase contrast images, arrows mark nucleoli. Scale bars represent 5 μm.

sequence identity of the dsRBDs of DrnB and RbdB prompted us to investigate whether they are interchangeable. To address this, we generated chimeric proteins, in which the endogenous dsRBD of RbdB was replaced by that of DrnB, and vice versa. Since the dsRBD of DrnB is not required for miRNA maturation in its native context (Fig. 5), it was not surprising that also the chimeric protein DrnB [dsRBD$_{RbdB}$] rescued the miRNA phenotype of Δ*drnB* cells (Fig. S2). The other chimeric protein RbdB [dsRBD$_{DrnB}$], however can fully rescue the miRNA phenotype of Δ*rbdB* cells (Fig. 7b). These data indicate that the DrnB dsRBD is functional in the context of RbdB, albeit being dispensable for DrnB.

Discussion

All GFP DrnB full length fusion proteins studied here were capable of reverting the miRNA processing defect of the *drnB* deletion strain,[31] indicating that their observed localization in perinucleolar foci corresponds to that of the endogenous protein (Figs 2, 3). To our knowledge, such perinucleolar bodies have not yet been described for *Dictyostelium*, and they appear to not co-localize to putative Cajal body markers or gem body markers in the amoeba (Fig. S3). We show that they are stable after cellular digestion of nucleic acids, and are retained after the disintegration of nucleoli (Fig. 4). DrnB localization clearly depends on the presence of the identified NLS (residues 493–501), as deletion of this sequence results in disintegration of the foci, which is concurrent with the mutant miRNA phenotype (Fig. 5). Cellular localization signals in *Dictyostelium* have been analyzed before,[47] and it appears that the newly identified signal sequence is necessary for proper perinucleolar localization of DrnB. The NLS is, however, not a sufficient perinucleolar localization signal, since typical DrnB foci were not restored in cells expressing a fusion of the NLS with the RNase III domains (Fig. 5). The observation that both RNase III and NLS-RNase III localize within the nucleus but are

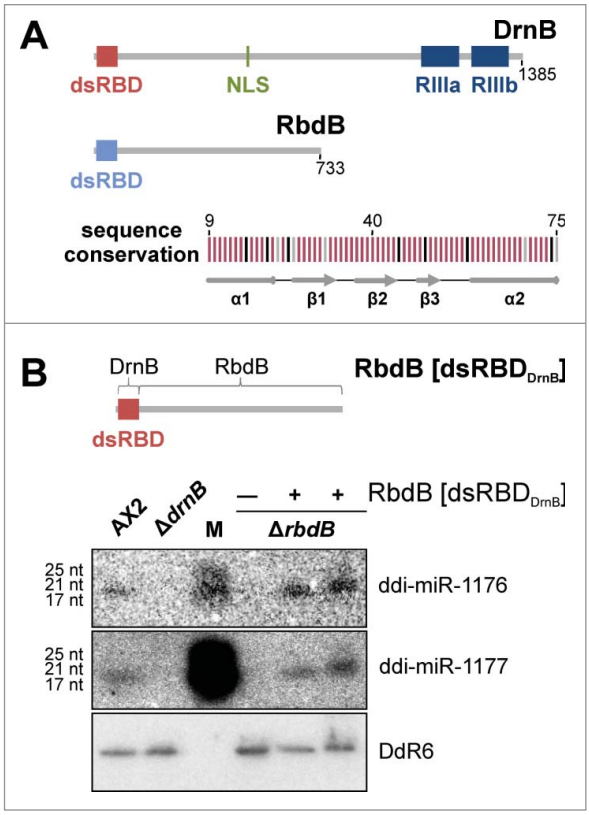

Figure 7. Replacement of RbdB's dsRBD against that of DrnB. (a) Domain structure of DrnB and RbdB drawn to scale with abbreviations as in Fig. 4. The sequence conservation of the 2 N-terminal dsRBDs (residues 9–75) is shown with red, black and gray, indicating identical, similar and dissimilar amino acid residues, respectively. The position of the predicted typical $\alpha\beta\beta\beta\alpha$ fold of dsRBDs is shown below. Exchange of the dsRBD of RbdB with that of DrnB results in the chimeric proteins RbdB [dsRBD$_{DrnB}$] (b, top). Northern blots of ddi-miR-1176 and -1177 and the snoRNA DdR6 (loading control) in the indicated strains (b, bottom) with AX2 and the untransformed Δ*rbdB* strains serving as controls. Sizes of the marker (M) are indicated on the left.

not functional in generation of mature miRNAs indicates a strong connection between specific perinucleolar localization of DrnB and its function in miRNA maturation.

The dsRBD of DrnB does not appear to influence the subcellular localization, and in general is dispensable for miRNA processing (Fig. 5). This observation was particularly surprising as previous studies on human Drosha have shown that its own dsRBD is essential for miRNA processing.[42] Further, with the exception of the *Giardia intestinalis* Dicer, which has lost its dsRBD,[48] eukaryotic Dicer and Drosha proteins generally contain this domain, as summarized recently.[11,12] Contrary to what their name suggests, dsRBDs are not necessarily involved in the binding of perfectly double-stranded RNA,[49,50] but are, as parts of Drosha and Dicer, essential for precise processing of miRNA precursors.[51] The interaction partners of eukaryotic Dicer and Drosha proteins are typically DsRBPs,[11] and we have recently identified RbdB as the cognate interaction partner of DrnB, which displays the same localization and phenotype upon deletion.[29] While we have shown in that study a physical interaction of these proteins, we could show here additionally a DrnB dependent number of foci (Fig. 6). This might point toward a genetic interaction between the 2 proteins, additionally to the previously shown physical interaction.[29] Such interactions have previously been reported also for the microprocessor complex in animals, in which Drosha regulates the expression level of its DsRBP interaction partner.[43]

The highly conserved N-terminal dsRBDs of DrnB and RbdB can be functionally exchanged (Figs 7 and S2), and this implies that the dsRBD of DrnB is a functional protein domain, raising the questions of why it is not used, and why it is not lost. While experimental answers are lacking at present, it is conceivable that by outsourcing the dsRBD functionality to RbdB, DrnB attains more flexibility in additional processes that it might be involved in and, in such presumed processes, it might make use of its own dsRBD. In this context, it is worth noting that rRNA processing appears altered in Δ*drnB* cells (Kruse and Hammann, unpublished). This lends support to the idea of additional possible cellular functions of DrnB, and similar functions have also been implied for a Dicer protein from *Candida albicans*.[52] Furthermore, a recent study identified a large number of Dicer-bound RNA sequences beyond the miRNA pathways in human and *C. elegans* cells.[53]

During evolution, distinct solutions were found for the implementation of the nuclear miRNA processing. Plants feature Dicing- or Cajal-bodies, which were shown to accommodate several components of the si- and miRNA pathway such as DCL, dsRBPs, Argonautes and Hen1,[8,26,54-56] and the organization of the *Dictyostelium* microprocessor seems similar to that observed in plants (Fig. 3 and [29]). In terms of the domain structures of the involved RNase III proteins, however, clear differences can be discerned, as plants employ DCL1, a genuine Dicer protein, while DrnB features the protein domains of Drosha, albeit in a shuffled version. Drosha proteins act in nuclear complexes in the RNA regulation and miRNA processing of opisthokonts,[2,23,24,51,57] which include *C. elegans, D. melanogaster* or mammals. Droshas have been suggested to have evolved as an independent protein family in animal lineages, based on the observation that their polypeptides outgroup with respect to plant and animal Dicer proteins.[58] The *Dictyostelium* RNase III proteins DrnA and DrnB feature a shuffled Drosha domain structure (Fig. 1), but cluster with the Dicer proteins.[58]

The Amoebozoa, including *Dictyostelium*, branched off during evolution after the plants (Archaeplastida) and form a sister group of the Opisthokonta.[28] It thus seems plausible that *Dictyostelium* may have retained the nuclear organization of the plant-like miRNA processing pathway. This would imply that their Dicers have lost those protein domains, which are absent in the animals' Drosha proteins. This scenario of convergent evolution with respect to the Ophisthokonts, would explain the unique setting of miRNA processing in this amoeba, and beyond: also the other social amoebae encode exclusively RNase III proteins with a domain structure like DrnB (G. Glöckner, personal communication), indicating this to be a general characteristic of the Amoebozoa supergroup.

In LECA, the last eukaryotic common ancestor, one Argonaute-like protein, one Piwi-like protein, one RNA dependent RNA polymerase and one Dicer were suggested to have constituted the basic RNAi machinery.[58] Based on this, we suggest a model for the evolution of RNase III proteins (Fig. 8). Possibly, LECA featured next to a nuclear Dicer already a cytoplasmatic homolog, a scenario that would still be mirrored by contemporary plants. After the split of the Amorphea in the Opisthokonta and Amoebozoa supergroups, the Dicer proteins in the latter would have lost all but their dsRBD and RNase III domains. Alternatively, the 2 domain-poor Dicers in the Amoebozoa might be derived from another by gene duplication after domain loss. Only after the split of the Amoebozoa, the tasks of miRNA maturation were spatially separated in the Opisthokonts. They appear to be the sole organisms, in which Drosha proteins evolved to process pri-miRNA transcripts in the nucleus. After export of resulting pre-miRNA into the cytoplasm, their processing by Dicer yields mature miRNAs.

Material and methods

Cultivation and transformation of Dictyostelium

Dictyostelium discoideum AX2 and Δ*drnB* (rox) [35] cells were grown axenically in HL5 medium (Formedium) at 22 °C under constant light. Antibiotics against prokaryotic growth (10 μg/ml Penicillin, 10 μg/μl Streptomycin, 250 ng/ml Amphotericin, 50 μg/ml Ampicillin) were supplemented by default. Transformation of *D. discoideum* cells was performed by electroporation as described previously.[59] 12–16 hours after transformation cells were selected by 10 μg/ml G418 or Blasticidin S in HL5 for 7–10 d. Success of transformation and protein expression was verified by Western blot analysis.

Cloning of DrnB variants and RbdB

The genomic sequence of *drnB* was amplified and cloned into the integrating vector pDneo2a-GFP previously.[32] Full length DrnB, ΔdsRBD and (NLS-) RNase III domains were re-amplified by PCR for cloning into extrachromosomally replicating vectors pDM317 and pDM323 for N- and C-terminal fusion to GFP, respectively.[33] The NLS (residues 493–501) was deleted by using the StarGate® Mutagenesis kit (IBA GmbH, Göttingen). All *drnB* containing vectors contained G418-

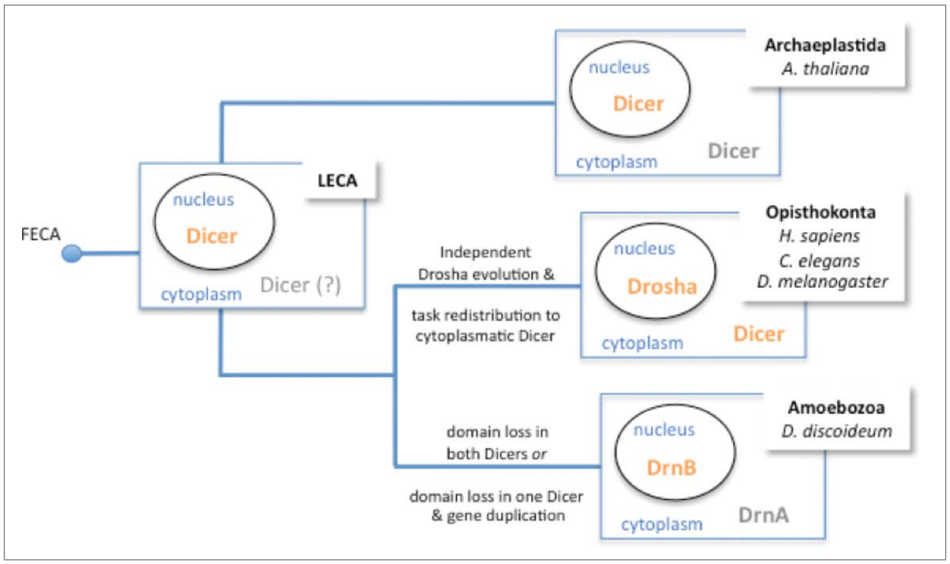

Figure 8. A model for the evolution of RNase III proteins acting in miRNA maturation. Shown are for species of the 3 indicated evolutionary supergroups in orange the RNase III proteins Dicer and Drosha that contemporarily act in miRNA maturation, and in gray those that are currently present, but that do not contribute to miRNA maturation. FECA/LECA denote the first and the last eukaryotic common ancestor, respectively. In this model, LECA featured at least one Dicer protein in the nucleus (orange bold) that acted in miRNA processing, and possibly an additional one in the cytoplasm (gray), which was not involved in that process. After the split of the Amorphea into Opisthokonta and Amoebozoa, either domain loss happened in both Dicers in Amoebozoa, or the proteins were derived by gene duplication after domain loss in one Dicer. In Opisthokonta only did Drosha proteins evolve and the final step of the miRNA maturation was redistributed to the cytoplasmatic Dicer protein. Note that this simplified scheme only includes those evolutionary supergroups that have been analyzed with respect to miRNA maturation.

resistance genes. The gene for RbdB (DDB_G0269426, dictybase.org) was amplified on genomic DNA by PCR and cloned into the extrachromosomal plasmid pDM326-C-RFP featuring a Blasticidin S resistance marker.[33] All genes were sequenced after amplification by PCR and cloning. Oligonucleotides used in this study are listed in Table S1.

Shuffling of dsRBDs

The endogenous dsRBDs of DrnB and RbdB are present at the N-terminus of each gene (residues 9–75 in both cases). The first 8 encoded amino acids are the same except for position 7, where DrnB features a leucine, and RbdB a threonine. The chimeric cDNA encoding the dsRBD of DrnB in the context of RbdB was cloned as follows: The dsRBD of DrnB was amplified from cDNA using the primers FZ009 and FZ010. The forward primer introduced the mutation L7T such that the sequence upstream of the $dsRBD_{DrnB}$ corresponded to that of RbdB and a BamHI restriction site was added. The reverse primer introduced a silent mutation downstream of the $dsRBD_{DrnB}$ coding sequence (G to C transition at position 228), yielding an XbaI recognition site. The PCR-fragment was cloned in the pGEM®-T Easy vector (Promega). The C-terminal part of RbdB was amplified with the primers FZ011 and DM053, adding N-terminal XbaI and C-terminal SpeI restriction recognition sites, respectively. The C-terminal *rbdB* fragment was then ligated into the pGEM®-T Easy vector adjacent to the N-terminal part of the gene encoding the dsRBD of DrnB. The chimeric cDNA was cut with BamHI and SpeI and ligated into the extrachromosomal expression vector pDM323. The gene was in frame with a C-terminal GFP-tag. The chimeric cDNA encoding the dsRBD of RbdB in the context of DrnB was cloned by amplifying the N-terminal part of RbdB including its dsRBD from cDNA using primers FZ005 and FZ006. The forward primer introduced the mutation T7L. By the reverse primer, a silent mutation was introduced downstream of the dsRBD coding region (A to T transition at position 231), yielding a HindIII site. The PCR-fragment was cloned in the pGEM®-T Easy vector (Promega). The C-terminal part of DrnB was amplified from cDNA using the primers FZ007 and FZ008. During PCR reaction, an N-terminal HindIII and a C-terminal SpeI site were added. The C-terminal *drnB* fragment was ligated adjacent to the N-terminal part of the rbdB gene, mainly encompassing the dsRBD of RbdB. The chimeric cDNA was cut with BglII and SpeI and then ligated into the extrachromosomal vector pDM323.

Cloning and expression of D. discoideum *Fibrillarin*

The genomic sequence of fbl (gene accession number DDB_G0269878, www.Dictybase.org) was amplified by PCR using the Primer pair fbl BglII forward and fbl BcuI reverse. The product was cloned into pJET1.2 (Thermo Scientific) and sequenced. Recloning of fbl into the destination vector pDM326-mRFP was conducted by restriction digestion with BglII and BcuI and ligation by using T4-DNA Ligase (Thermo Scientific). After verification of the correct insertion by restriction digestion, the plasmid was used for transformation of AX2 cells. Laser scanning microscopy was performed on fixed cells with 4 % PFA.

Cloning and expression of TC 1 and Gemin 1

Genes coding for Telomerase Cajal body protein 1 (TCAB 1) (dictybase.org accession DDB_G0269858) and Gemin 1 (dictybase.org accession DDB_G0276357) were amplified from *D. discoideum* AX2 genomic DNA by using primer pairs TC1 1 N BglII forward, TC 1 N BcuI reverse and Gemin 1 C BglII forward, Gemin 1 C BcuI reverse, respectively.

After verification of the correct insert by sequencing (Seqlab, Göttingen) genes were recloned into pDM326-mRFP between BglII/BcuI restriction sites and expressed in the *D. discoideum* GFP-DrnB strain (Low copy, pDneo2a-GFP-drnB). *Dictyostelium* transformants were selected by G418 (DrnB) and Blasticidine (TC 1 and Gemin 1) prior fixation with 4% PFA for fluorescence microscopy.

Nuclear run on assay

Nuclei of AX2 cells were isolated by sucrose gradient centrifugation as described before [60] with modification in cell lysis. Here, 5 x 10^8 cells were resuspended in 20 ml lysis buffer (50 mM Tris pH 7, 5 mM $MgCl_2$, 20 mM KCl, 10 % sucrose, 1 mM dithiothreitol, 0.2 mM phenylmethylsulphonyl chloride) and disrupted mechanically by the use of a cell homogenizer (Isobiotec, Heidelberg) with a clearance of 10 μm.

Nuclear run on reactions were carried out as described previously.[61] For inhibition of RNA Polymerase II, α-Amanitin (Applichem) was added to a concentration of 330 ng/ml for 15 minutes or 30 minutes, before addition of [^{32}P]UTP (Hartmann). RNA was extracted with 1 ml Trizol® (Life technologies)/0.2 ml chloroform. The aqueous phase was filtered through Sephadex G50 and RNA was denatured at 95°C prior to slot blot hybridization. For the slot blot, 100 pmol of each DNA oligonucleotide probe (Sigma Aldrich) were transferred to nitrocellulose filters by using a vacuum slot blot apparatus. Oligonucleotides were then chemically crosslinked as described[62] and hybridized with radioactively labeled run-on RNA at 42°C over night. After three washing steps with 0,1%SDS/0,1xSSC, filters were exposed to Phosphoimager plates for 24 h.

Fixation of Dictyostelium *and microscopy*

GFP or RFP expressing cells were grown on coverslips in 24-well plates for 1–12 hours prior fixation with 4 % PFA in Soerensen phosphate buffer (2 mM Na_2HPO_4, 15 mM KH_2PO_4; pH 6,0) for 1 hour at 22°C. Fixed cells were stored protected from light in 4 % PFA at 4°C until use. Alternatively, cells were fixed with methanol at -20°C for 20 min. After three wash steps with 1 x PBS cover slips were covered with embedding medium Mowiol 4–88/DABCO (Carl Roth GmbH) and mounted on a microscopy slide. Imaging of cells was conducted with a fluorescence microscope (Axioplan 2, Carl Zeiss Jena GmbH) or a confocal laser scanning microscope (LSM 510 Meta; Carl Zeiss Jena GmbH, Jena, Germany) and analyzed with the LSM 510 software, Release 3.2 (Carl Zeiss Jena GmbH).

Actinomycin D treatment

Live cells grown on coverslips were treated with 0.05 mg/ml Actinomycin D (1 mg/ml in DMSO) in HL5 for 4 hours at 21°C prior fixation with 4 % PFA.

RNase A and DNase I treatment of live cells

Live *D. discoideum* cells were permeabilized on coverslips by 3 washes with 0.05 % (v/v) Tween®20 / 2.5 mM $MgCl_2$ in 1 x PBS. RNase A (Sigma Aldrich) and DNase I (Thermo Scientific) were added at concentrations of 0.1 mg/ml and 50 u/ml in 2.5 mM $MgCl_2$/ 1 x PBS, either separately or together, and incubated at 37°C for 1 hour in the dark, as described before.[63] Cells were subsequently fixed with 4 % PFA, and nucleic acids were stained with 5 μM Draq5 (Biostatus) in 1xPBS for 20 min at 21°C and visualized by excitation at 647 nm wavelength.

Northern blotting of miRNA

Small RNAs were blotted and chemically crosslinked as described previously.[62,64] For detection of miRNAs, DNA oligonucleotides were 5′-radio-labeled by T4 Polynucleotide kinase and purified by Sephadex-G50 filtration. Hybridized filters were exposed to phosphor imager plates (Fujifilm) for 5–7 d. Plates were read by the Phosphoimager FLA3000 (Fujifilm, Düsseldorf) and images analyzed with the Multigauge Software (release 3.2).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Maro Iliopoulou and Julie Trolle for generating some of the cloning intermediates, and Markus Maniak for generously providing antibodies. We thank all our colleagues who discussed the manuscript and made helpful suggestions.

Funding

This work was in part supported by grants of the Deutsche Forschungsgemeinschaft (HA3459/5) to CH and (Ne285/14) to WN. DM acknowledges support by a Graduiertenstipendium desLandes Hessen.

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