

The Evolution of MicroRNA Pathway Protein Components in Cnidaria

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Abstract

In the last decade, it became evident that posttranscriptional regulation of gene expression by microRNAs is a central biological process in both plants and animals. Yet, our knowledge about microRNA biogenesis and utilization in animals stems mostly from the study of Bilateria. In this study, we identified genes encoding the protein components of different parts of the microRNA pathway in Cnidaria, the likely sister phylum of Bilateria. These genes originated from three cnidarian lineages (sea anemones, stony corals, and hydras) that are separated by at least 500 My from one another. We studied the expression and phylogeny of the cnidarian homologs of Droscha and Pasha (DGCR8) that compose the microprocessor, the RNase III enzyme Dicer and its partners, the HEN1 methyltransferase, the Argonaute protein effectors, as well as members of the GW182 protein family. We further reveal that whereas the bilaterian dicer partners Loquacious/TRBP and PACT are absent from Cnidaria, this phylum contains homologs of the double-stranded RNA-binding protein HYL1, the Dicer partner found in plants. We also identified HYL1 homologs in a sponge and a ctenophore. This finding raises questions regarding the independent evolution of the microRNA pathway in plants and animals, and together with the other results shed new light on the evolution of an important regulatory pathway.

Key words: microRNA, Cnidaria, posttranscriptional regulation, Nematostella.

Introduction

MicroRNAs (miRNAs) are 21–24 nucleotide long RNAs that serve as important posttranscriptional regulators in plants and animals (Flynt and Lai 2008; Bartel 2009; Axtell et al. 2011). In most cases, they are produced from long messenger RNA-like transcripts, known as primary miRNAs (pri-miRNA) that are processed in a stepwise manner by RNase III-containing protein complexes (Kim et al. 2009). In bilaterian animals, the first step, the cropping of the pri-miRNA, is carried out in the nucleus by the microprocessor complex. This complex is composed of the RNase III Droscha and the double-stranded RNA-binding protein Pasha (known in mammals as DGCR8). Its cropped product is the precursor miRNA (pre-miRNA), a hairpin-containing structure (fig. 1). After cropping, the pre-miRNA is exported out of the nucleus by Exportin-5 (known as Hasty in plants) and is further processed in the cytoplasm by a protein of the RNase III Dicer family assisted by various double-stranded RNA-binding protein partners such as Loquacious (Loqs) in flies or TRBP and PACT in mammals (Kim et al. 2009; Fukunaga et al. 2012). The resulting duplex consisting of the miRNA and miRNA* is then loaded into an Argonaute protein (Ago) that selects one of the strands, usually the miRNA (Czech and Hannon 2011) (fig. 1). The miRNA-loaded Ago is part of an active RNA-induced silencing complex (RISC). The active RISC is guided by the miRNA to its complementary target and can either directly cleave (slice) mRNAs or alternatively, induce indirect translational inhibition and transcript decay with the help of a protein of the

GW182 family (Huntzinger and Izaurralde 2011; Fabian and Sonenberg 2012). Slicing requires an extensive complementarity match between the small RNA and its target, whereas a short match restricted to the miRNA seed (nucleotides 2–8) is sufficient for translational inhibition and indirect target decay. In bilaterian animals, slicing of targets is a common mode of action for short interfering RNAs (siRNAs) but rare for miRNAs (Axtell et al. 2011; Huntzinger and Izaurralde 2011).

In plants, the miRNA biogenesis process differs from that in animals in that the Dicer homolog Dicer-like 1 (DCL-1) is responsible for the two processing steps and they both happen in the nucleus (fig. 1). DCL-1 is assisted by HYL1 (hyponastic leaves 1) and Serrate during miRNA processing (Voinnet 2009). Whereas it was recently found that Serrate has a homolog in animals called Ars2 (Gruber et al. 2009; Sabin et al. 2009), HYL1 is still considered a plant-specific protein and it was recently shown that paralogs of HYL1 in *Arabidopsis* such as DRB2, 3, and 5 also participate in miRNA biogenesis (Eamens et al. 2012). Moreover, neither Droscha nor Pasha homologs were detected in nonmetazoan species. Another difference between plant and animal miRNA pathways is their mode of action: Unlike in animals, miRNA-mediated target slicing in plants is very common, although in recent years, the role of miRNA-mediated translational inhibition was also demonstrated in plants (Voinnet 2009; Axtell et al. 2011). Those differences in miRNA processing and mode of action are among the main arguments supporting the

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claim that miRNAs have evolved independently in plants and animals. A third strong argument against a common origin of miRNAs in plants and animals is the fact that no significant sequence homology has yet been found between miRNAs of these two kingdoms. Similarly, no sequence homology has been found between miRNAs of plants and green algae or between miRNAs of sponges and other animals (Grimson et al. 2008; Tarver et al. 2012; Robinson et al. 2013). This raises the possibility that the miRNA pathway has evolved independently at least four times or, alternatively, that miRNA sequence turnover has been sufficiently high in certain early lineages to obscure any common origin.

To get deeper insight into the evolution of microRNA biogenesis and the effectors of microRNA-mediated posttranscriptional regulation, one needs to look into a wider repertoire of extant species, including nonbilaterian animals that represent lineages that diverged more than 600 Ma from the rest of the metazoa. These include the phyla Porifera (sponges), Ctenophora (comb jellies), Placozoa (*Trichoplax*), and Cnidaria (corals, sea anemones, hydroids, and jellyfish). Recent reports have shown that microRNAs and key components of their classical processing machinery are absent from Placozoa and Ctenophora, pointing to a secondary loss of this pathway, or, alternatively, this absence might represent an ancestral state (Grimson et al. 2008; Wheeler et al. 2009; Maxwell et al. 2012). Distinguishing these two scenarios is currently impossible due to an ongoing debate regarding the order of divergence of these two animal phyla (Hejnl et al. 2009; Philippe et al. 2011).

Cnidaria represent an ancient lineage that diverged more than 600 Ma from the rest of metazoa (Erwin et al. 2011). They share important features with Bilateria, such as muscles and neurons but are diploblastic and lack the mesodermal layer, a bilaterian hallmark. Several independent phylogenetic analyses robustly position them as the sister group of Bilateria (Hejnl et al. 2009; Philippe et al. 2011), highlighting their importance for comparative studies (Steele et al. 2011; Technau and Steele 2011). Interestingly, the model cnidarian *Nematostella vectensis* was shown to possess 40 miRNAs, but only one of them, miR-100, is shared with Bilateria (Grimson et al. 2008). A recent analysis of the cnidarian *Hydra magnipapillata* revealed 126 miRNAs, with none of them shared with bilaterians (Krishna et al. 2013). Some homologs of bilaterian miRNA biogenesis factors were sporadically described in Cnidaria (Grimson et al. 2008; de Jong et al. 2009; Kerner et al. 2011), but have not been studied extensively. In this study, we took advantage of the genomic and transcriptomic data sets that recently became available for the cnidarians *Acropora millepora* (stony coral of the Australian great barrier reef), *Acropora digitifera* (stony coral of Okinawa, Japan), *Hydra vulgaris* (a fresh water hydroid), and *N. vectensis* (starlet sea anemone) (Putnam et al. 2007; Chapman et al. 2010; Shinzato et al. 2011; Moya et al. 2012; Wenger and Galliot 2013) to comprehensively catalog and analyze cnidarian homologs of bilaterian miRNA biogenesis factors and protein effectors guided by miRNAs. With this approach, we aimed to gain further insight into the evolution of key components of microRNA-related pathways.

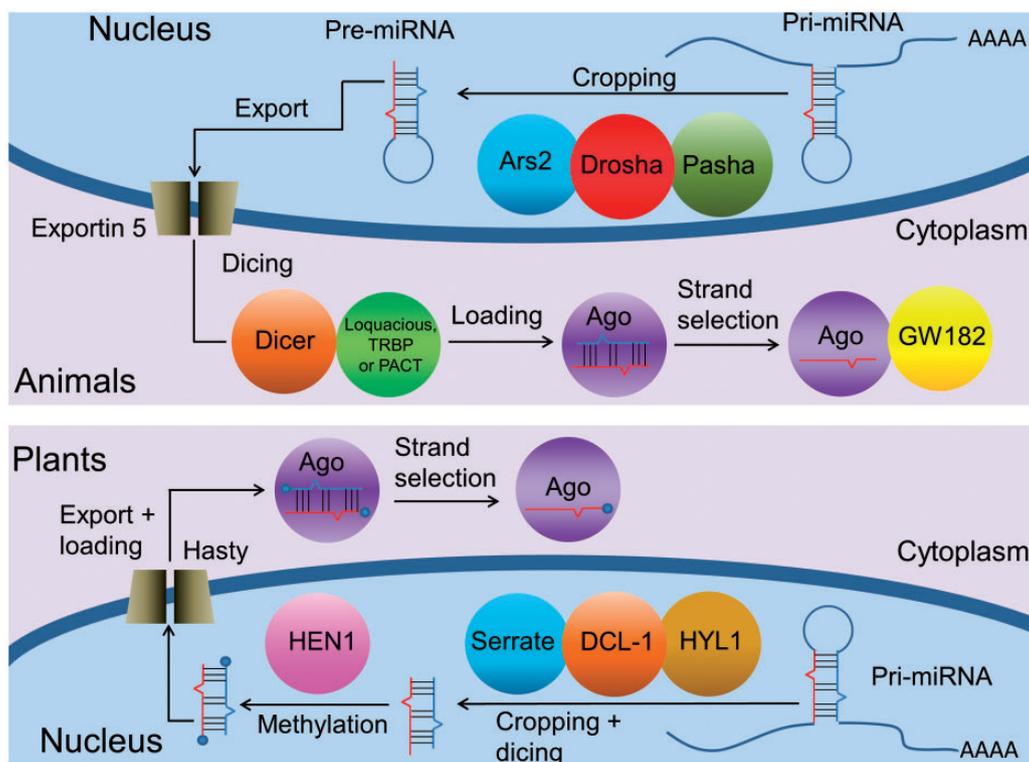


FIG. 1. A scheme describing the plant and animal canonical miRNA biogenesis pathways. Homologs carrying similar functions such as Dicer of animals and DCL-1 of plants are represented in the same color.

Results and Discussion

Cnidarian Microprocessor Components

Through sequence search against the collected transcriptome and genome data sets (see Materials and Methods), we detected one Droscha and one Pasha/DGCR8 homolog in each of the species *Nematostella*, *Acropora*, and *Hydra* (table 1). Because the *Nematostella* gene models appeared to represent only gene fragments, we used RACE (rapid amplification of cDNA ends) followed by sequencing to retrieve the full *Nematostella* transcripts (see Materials and Methods). The canonical domains were present and support a conserved role for both of the *Nematostella* proteins (data not shown). These observations are in agreement with previous reports (Grimson et al. 2008; Maxwell et al. 2012). The absence of both Droscha and Pasha in the ctenophore *Mnemiopsis leidyi*, and of Pasha in the placozoan *Trichoplax adhaerens*, could support the idea that animal miRNAs appeared only in the common ancestor of Cnidaria and Bilateria. However, the finding of miRNAs, Droscha and Pasha in several sponges (Grimson et al. 2008; Robinson et al. 2013) together with the highly debated phylogenetic position of all those basal phyla greatly complicates the evolutionary inference. Moreover, it is noteworthy that Droscha and Pasha were shown, in mammals, to carry additional roles that are independent of the miRNA pathway, and that Pasha/DGCR8 might interact with RNAses other than Droscha (Wu et al. 2000; Chong et al. 2010; Macias et al. 2012).

Cnidarian Dicers: Domain Loss and Possible Subfunctionalization

Dicer is a key enzyme in miRNA biogenesis, and there are only a few examples of dicer-independent pre-miRNA processing (Kim et al. 2009; Cheloufi et al. 2010; Cifuentes et al. 2010). However, Dicers are common in many eukaryotes and also carry out non-miRNA-related functions, such as antiviral defense (Ding 2010). A previous work reported two Dicers in *Nematostella* and two in *Hydra* (de Jong et al. 2009). However,

we could detect only fragments of Dicers in the *Hydra* genome and transcriptome, and as they cover different protein domains, it is possible that they constitute one gene. We found evidence for one Dicer transcript in the *A. millepora* transcriptome and recovered one complete Dicer gene and various additional short and fragmented models from the *A. digitifera* data (table 1). The *Nematostella* Dicer sequences previously published (de Jong et al. 2009) were only gene fragments, and we complemented them by RACE. Phylogenetic analysis positioned *Nematostella* Dicer 1 (NveDcr1) in a clade with the *Acropora* Dicer (AmiDcr1) that is a sister group to the bilaterian Dicers (fig. 2A). The *Hydra* Dicer was positioned as a sister group to all animal Dicers. Intriguingly, *Nematostella* Dicer 2 (NveDcr2) clusters with the Dicer 2 proteins from *Drosophila* and *Tribolium*. This result is in agreement with another recent phylogeny of Dicer proteins (Mukherjee et al. 2013). The insect Dicer 2 group was shown to specialize in processing of long double-stranded RNAs into siRNAs, and not to be involved in miRNA biogenesis (Lee et al. 2004; Tomoyasu et al. 2008). Thus, it is possible that NvDcr2 is also a Dicer specialized in non-miRNA-related functions. Indeed, this notion is supported by the fact that NvDcr2 lacks the Dead-like helicase and helicase-superfamily C-terminal domains typical of most animal Dicers (fig. 2B). These domains were shown to be crucial for miRNA processing by *Drosophila* Dicer-1 (Tsutsumi et al. 2011). We verified by 5' RACE that the lack of these two domains was not due to a truncated transcript. The presence of siRNAs in *Hydra* was recently reported (Krishna et al. 2013), and we have also detected siRNAs in *Nematostella* (Technau U, unpublished results). Thus, it is possible that in some cnidarians such as *Nematostella*, siRNAs and miRNAs are processed by different Dicers that underwent subfunctionalization following gene duplication, like in insects. When helicase domains were removed from the human Dicer through experimental domain deletion, this resulted in a hyperprocessive enzyme with more than 50-fold higher activity compared with other Dicers (Ma et al. 2008). Hence, NvDcr2 might possess

Table 1. Cnidarian Homologs of microRNA Pathway Components.

Protein	<i>Acropora digitifera</i> ^a	<i>A. millepora</i> ^b	<i>Hydra vulgaris</i> Strain Basel ^c	<i>Nematostella vectensis</i> ^d
Argonaute	aug_v2a.00902; aug_v2a.00903	JR981312; JT022739	HAAC01042396	KF192061; KF192062
Dicer	aug_v2a.09093; aug_v2a.19226	JR973616	HAAC01029903; HAAC01044477	KF192063; KF192064
Droscha	aug_v2a.06281	JR987603	HAAC01022365	KF192065
GW182/TNRC6	aug_v2a.21715	JT003469	HAAC01028300	KF192071
HEN1	–	JR998175	HAAC01033586	KF192069
HYL1	aug_v2a.05493	JR983909	HAAC01028389	KF192067; KF192068
Loqs/TRBP	–	–	–	–
PACT	–	–	–	–
Pasha/DGCR8	aug_v2a.16054	JR992349	HAAC01022633	KF192066
Serrate/Ars2	aug_v2a.01424	JT022468	HAAC01032859	KF192070

^aGene models and their accession numbers are taken from a public database (Shinzato et al. 2011) of the Okinawa Institute for Science and Technology.

^bThe protein sequences were translated from assembled short-read sequences (Moya et al. 2012) of the following accession numbers, available at the Transcriptome Shotgun Assembly (TSA) section of GenBank.

^cThe protein sequences were translated from assembled short-read sequences (Wenger and Galliot 2013) of the following accession numbers available at the European Nucleotide Archive.

^dThe protein sequences were translated from cDNA products amplified by PCR and sequenced by the Sanger method. All sequences are novel and were deposited at the Nucleotide section of GenBank under the following accession numbers.

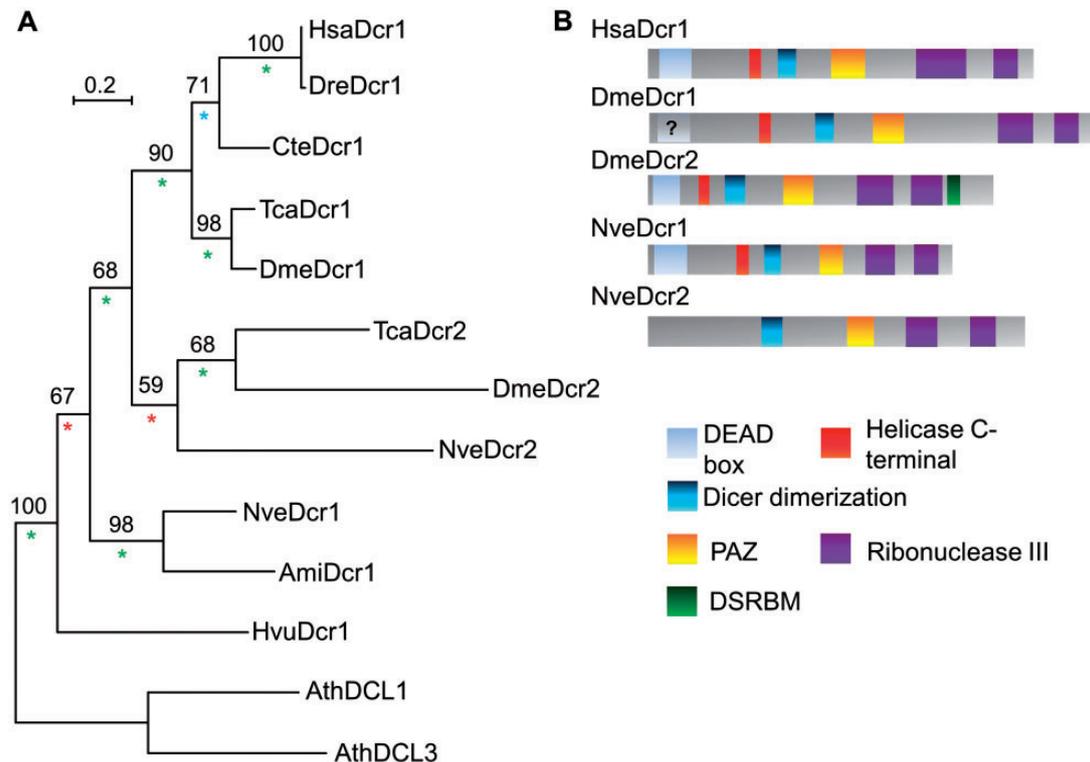


Fig. 2. Phylogeny of cnidarian Dicers based on their two Ribonuclease III domains. (A) An ML phylogenetic tree was constructed with the LG model (+ I, + G). Bootstrap support values above 50% are indicated above branches. Posterior probability (PP) values of a Bayesian tree constructed with the WAG model are indicated by a green (PP = 1.0), blue (0.95 ≤ PP < 1.0), or red (0.7 < PP < 0.95) asterisk. (B) Conserved domain composition of several Dicer proteins. Abbreviations of species names are: Ami, *Acropora millepora* (stony coral); Ath, *Arabidopsis thaliana* (thale cress); Cte, *Capitella teleta* (annelid); Dme, *Drosophila melanogaster* (fruit fly); Dre, *Danio rerio* (zebrafish); Hsa, *Homo sapiens* (human); Hvu, *Hydra vulgaris* (hydra); Nve, *Nematostella vectensis* (starlet anemone); Tca, *Tribolium castaneum* (red flour beetle).

unique thermodynamic properties that make it an intriguing candidate for future biochemical and substrate-specificity studies. A previous work on human and *Drosophila* Dicer 1 revealed a specialized pocket that harbors the 5' phosphate end of the double-stranded RNA precursor (Park et al. 2011). The pocket is composed of five Arginine residues that are conserved between human and fly Dicer 1 but are missing from fly Dicer 2 that specializes in siRNA biogenesis. These results led to the suggestion that the five Arginines are required for miRNA biogenesis by all animal Dicers. Surprisingly, this region is poorly conserved in cnidarian Dicers, and we detected four out of the five Arginines in NvDcr2, but only one out of five in NvDcr1 (supplementary fig. S1, Supplementary Material online). However, plant DCL-1 proteins, which efficiently process miRNAs, completely lack the five Arginines, and it was proposed that they may have other regions that contribute a function similar to that provided by the bilaterian 5' pocket (Mukherjee et al. 2013). Similarly, although cnidarian Dicers lack a 5' pocket identical to that found in bilaterian Dicers, miRNAs are efficiently processed also in Cnidaria (Grimson et al. 2008; Krishna et al. 2013), suggesting that there are other compensatory elements.

Dicer Partner Proteins in Cnidaria: A Possible Link to miRNA Biogenesis in Plants

Dicer proteins in plants and animals highly depend on partner proteins to perform miRNA processing in an accurate

manner (Kim et al. 2009; Voinnet 2009). We could not detect distinct homologs of the bilaterian Dicer partner proteins Loqs, TRBP, and PACT in Cnidaria. The best hits in the Cnidaria nucleotide data for these partners were all more similar to the double-stranded RNA-binding protein Staufen, which is involved in mRNA localization and decay in Bilateria (Roegiers and Jan 2000; Park and Maquat 2013) (data not shown). Driven by this result, we decided to look for cnidarian homologs of HYL1, the plant double-stranded RNA-binding protein that serves as a Dicer partner crucial for processing accuracy (Han et al. 2004; Vazquez et al. 2004). Surprisingly, we could detect homologs of HYL1 in all cnidarian species tested (table 1). Blasting those proteins against the nr data set of GenBank always retrieved best hits to plant HYL1 proteins. Next, we searched for similar proteins in other members of basally branching phyla. We could not detect potential HYL1 homologs in Placozoa (*T. adhaerens*); however, we identified potential homologs in the sponge *Amphimedon queenslandica* and in the ctenophore *M. leidyi*. Phylogeny reconstruction of the double-stranded RNA-binding motifs (DSRBM) of these proteins supported the notion that they are indeed HYL1 homologs, as they cluster with *Arabidopsis* and rice HYL1 DSRBMs (fig. 3A). However, most of these animal proteins have an additional DSRBM domain, and one of the *Nematostella* proteins also possesses a NUDIX domain (fig. 3A). Thus, we decided to name those proteins HYL1-Like (HYL1L). As the support for

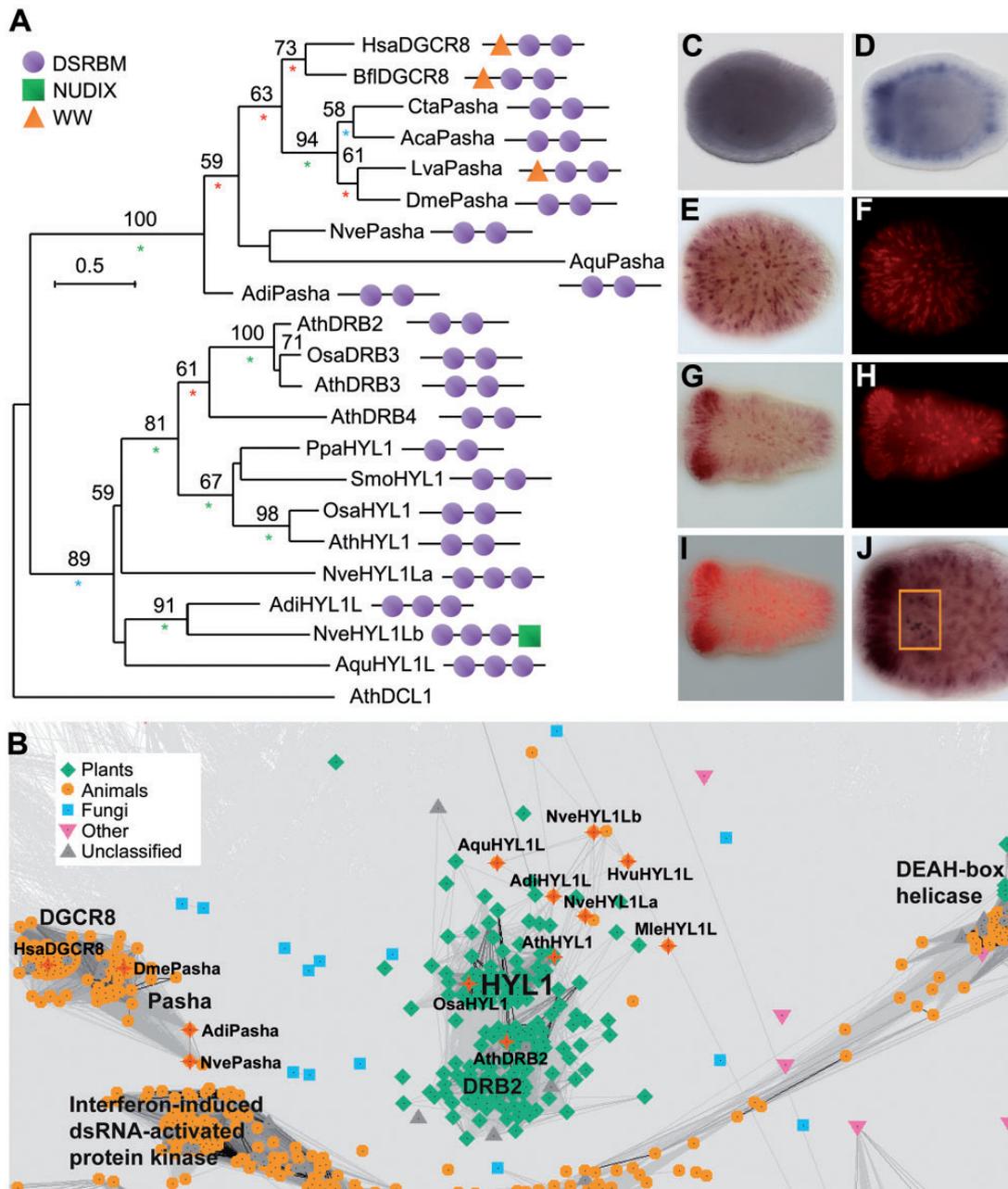


Fig. 3. Phylogeny and spatial expression of HYL1 homologs of Cnidaria. (A) Phylogeny of cnidarian homologs of HYL1 and Pasha (also known as DGCR8) based on their DSRBMs. An ML phylogenetic tree was constructed with the LG model (+I, +G). Bootstrap support values above 50% are indicated above branches. PP values of a Bayesian tree constructed with the RtRev model (+I, +G) are indicated by a green (PP = 1.0), blue (0.95 ≤ PP < 1.0), or red (0.7 < PP < 0.95) asterisk. The tree was rooted by the DSRBMs of the *Arabidopsis thaliana* Dicer-like 1 (AthDCL1). (B) Partial view of the CLANS cluster analysis of DSRBM domain-containing proteins classified by kingdom (see graphic legend) centered on the HYL1 cluster. Connecting gray lines between proteins represent pairwise Blast homology, with darker lines indicating significant hits. Named sequences are highlighted (vermillion star). The HYL1L proteins in basal metazoa cluster with plant HYL1 proteins. The closest neighbor cluster is of plant DRB2 proteins, paralog of HYL1. Distant neighbor clusters include animal Pasha (DGCR8), interferon-induced dsRNA-activated protein kinase and DEAH-box helicases. Abbreviations of species names in (A) and (B) are as follows: Aca, *Aplysia californica* (sea slug); Adi, *Acropora digitifera* (stony coral); Aqu, *Amphimedon queenslandica* (sponge); Ath, *Arabidopsis thaliana* (thale cress); Bfl, *Branchiostoma floridae* (lancelet); Cte, *Capitella teleta* (annelid); Dme, *Drosophila melanogaster* (fruit fly); Hsa, *Homo sapiens* (human); Hvu, *Hydra vulgaris* (hydra); Lva, *Litopenaeus vannamei* (white shrimp); Mle, *Mnemiopsis leidyi* (ctenophore); Nve, *N. vectensis* (starlet anemone); Osa, *Oryza sativa* (rice); Ppa, *Physcomitrella patens* (moss), Smo, *Selaginella moellendorffii* (spikemoss). Expression of *NveHYL1La* (C) occurs throughout the cells of the late planula of *Nematostella* as detected by ISH. In contrast, the expression of *NveHYL1Lb* (D) is specific mostly to elongated ectodermal cells. These cells are nematocytes as determined by double-ISH of *NveHYL1Lb* (purple signal) and the nematocyte marker *Ncol-3* (red fluorescent signal). The signals co-localize in both the early planula (E, F) and primary polyp (G, H; I is a merged picture) stages. In the late planula stage, some small round cells in the pharynx expressing *NveHYL1Lb* do not express *Ncol-3* and therefore are not nematocytes (J, their region is indicated by yellow frame).

the clustering was modest (fig. 3A) and weakened when the long-branched ctenophore and hydra sequences were included (supplementary fig. S2, Supplementary Material online), we decided to carry out additional complementary analysis of the relationship between the detected HYL1-like sequences and other DSRBM-containing proteins using a different approach. To this end, we used the CLANS software (Frickey and Lupas 2004) to perform cluster analysis of all DSRBM-containing proteins available in UniProt as well as the full-length HYL1L protein sequences identified earlier, based on their pairwise protein sequence similarity. This analysis revealed a close relationship between the HYL1L proteins from basal metazoa and bona fide HYL1 homologs from plants (fig. 3B). In addition, a handful of fungal putative proteins are located in the vicinity of the HYL1 cluster. However, the fungal proteins lack the strong connectivity that is evident between the animal and plant proteins in the cluster (fig. 3B). A likely evolutionary scenario emerging from this analysis is that HYL1-like proteins were already present in the common ancestor of plants, animals, and fungi and were independently lost in multiple lineages, including Bilateria. Moreover, the finding of HYL1 homologs in Cnidaria, a sponge and a ctenophore suggests that the roots of miRNA processing are much deeper than previously thought (Axtell et al. 2011) and may precede the split of the metazoan and plant lineages. It is tempting to assume that HYL1L proteins act as Dicer partners in Cnidaria and regulate small RNA processing, especially in light of the absence of homologs of bilaterian Dicer partners, but investigating this idea would require thorough functional studies. As *Mnemiopsis* carries a HYL1 homolog, but lacks bona fide miRNAs (Maxwell et al. 2012), it is possible that at least some of the HYL1-like proteins are involved in production of siRNAs rather than miRNAs.

We used in situ hybridization (ISH) to localize the spatial expression of the two *Nematostella* HYL1 homologs, *NveHYL1La* and *NveHYL1Lb*. While *NveHYL1La* seems to be expressed throughout the animal, as would be expected of a ubiquitous Dicer partner, *NveHYL1Lb* is expressed in distinct elongated cells in the ectoderm of the animal (fig. 3C and D). The shape and location of these cells is reminiscent of nematocytes, the stinging cells which typify cnidarians (David et al. 2008). To test whether these cells are nematocytes, we performed double ISH with probes for the combined detection of *NveHYL1Lb* and *NvNcol3*, a marker for stinging cells in *Nematostella* (Zenkert et al. 2011; Moran et al. 2013). Indeed, the vast majority of cells expressing *NveHYL1Lb* also express *NvNcol3*, proving they are nematocytes (fig. 3E–I). The only exception is a group of small round cells in the developing pharynx of the animal that seem to express *NveHYL1Lb* but lack expression of *NvNcol3* (fig. 3J). These pharyngeal cells appear only in the late planula developmental stage.

Another plant Dicer partner is the zinc finger domain protein Serrate (Voinnet 2009), and we have detected its homolog in all the cnidarian species we tested (table 1). However, a Serrate homolog, *Ars2*, is also found in bilaterians and can serve as a partner of both the microprocessor and Dicer (Sabin et al. 2009).

The HEN1 Methyltransferase of Cnidaria Is Similar to Those of Other Animals but Is Not Restricted to the Germline

2'-O-methylation on the 3'-terminal ribose of all categories of small RNAs is common in plants, but, in bilaterians, this kind of methylation is mostly restricted to siRNAs and Piwi-interacting RNAs (piRNAs), a group of animal-specific small RNAs that serves in germ line defense (Voinnet 2009; Axtell et al. 2011). This modification is carried out by the methyltransferase HEN1 (HUA Enhancer 1) and has an important role in providing stability to the small RNA and controlling the small RNA metabolism (Ji and Chen 2012). We detected a single HEN1 homolog in each of the cnidarian species tested (table 1). Phylogenetic analysis of the methyltransferase domains grouped the cnidarian HEN1 homologs with HEN1 proteins of the protostomes *Drosophila* and *Capitella* (fig. 4A). Although HEN1 exists both in plants and animals, it functions quite differently in these two kingdoms: in plants, it recognizes RNA duplexes and methylates the ends of both strands (fig. 1); whereas in animals, it methylates single-stranded small RNAs after they are loaded into Ago or Piwi proteins (Ji and Chen 2012). Indeed, plant and animal HEN1 proteins occupy well-separated phylogenetic clades (fig. 4A), and we noticed a few differences in their domain structure. Whereas plant HEN1 proteins have their methyltransferase domain located in their C-terminus, animal HEN1 proteins usually carry this domain at the N-terminus (fig. 4B). Moreover, when using PFAM or CDD to annotate the domain structure (see Materials and Methods), we could detect a DSRBM domain in *Arabidopsis* and rice HEN1, but the annotation confidence score was insignificant (data not shown). However, X-ray crystallography demonstrated that *Arabidopsis* HEN1 has not one, but two DSRBMs, which are probably too divergent to be accurately detected by comparison with domain models in current databases (Huang et al. 2009). The region containing these domains is absent from cnidarian and other animal HEN1 proteins. In vertebrates, HEN1 is responsible mainly for methylation of piRNAs, and its expression pattern is restricted to the nuage of germ cells, where piRNAs are produced (Kamminga et al. 2010). However, when we localized HEN1 in *Nematostella* by ISH, it became clear that it is expressed throughout the animal, with possible enrichment in the endoderm and the oral ectoderm (fig. 4C and D). This observation fits nicely to a previous study reporting that a substantial fraction of the miRNAs of *Nematostella* is methylated (Grimson et al. 2008). For example, *Nematostella* miR-100 was detected in oxidized libraries that retain only methylated small RNAs but is expressed at the oral ectoderm of the planula (Christodoulou et al. 2010), a body region in *Nematostella* that lacks most germ line markers (Extavour et al. 2005).

Cnidarian Homologs of miRNA-Utilizing Effector Proteins

We detected two Ago-encoding genes in *Nematostella*, two in *A. millepora* and one each in *A. digitifera* and *Hydra* (table 1). A previous work reported three Ago genes in *Nematostella*

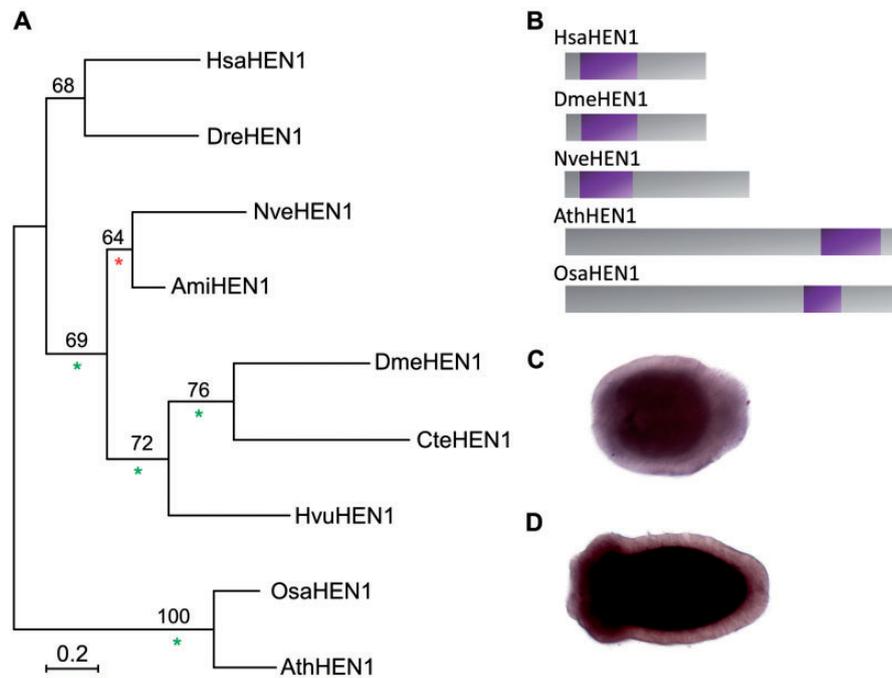


Fig. 4. Phylogeny and expression of cnidarian HEN1 proteins based on their methyltransferase domains. (A) An ML phylogenetic tree was constructed with the LG model (+ I, + G). Bootstrap support values above 50% are indicated above branches. PP values of a Bayesian tree constructed with the WAG model are indicated by a green (PP = 1.0), blue (0.95 ≤ PP < 1.0), or red (0.7 < PP < 0.95) asterisk. (B) Position of the methyltransferase domain within several HEN1 proteins. Abbreviations of species names are as follows: Adi, *Acropora digitifera* (stony coral); Ami, *Acropora millepora* (stony coral); Ath, *Arabidopsis thaliana* (thale cress); Cte, *Capitella teleta* (annelid); Dme, *Drosophila melanogaster* (fruit fly); Dre, *Danio rerio* (zebrafish); Hsa, *Homo sapiens* (human); Hvu, *Hydra vulgaris* (hydra); Nve, *Nematostella vectensis* (starlet anemone); *Oryza sativa* (rice). ISH suggests that expression of HEN1 in *Nematostella* is ubiquitous as seen in early (C) and metamorphosing (D) planulae.

(Grimson et al. 2008), but based on cloning experiments, we concluded that the third *Nematostella* gene is the result of an erroneous genome assembly because its model is only a short fragment that is nearly identical in nucleotide sequence to a part of *Nematostella Ago 2* (*NveAgo2*). Phylogenetic analysis of the encoded proteins using the domain of unknown function (DUF) 1785, as well as PAZ and Piwi domains, reveals that the two *Nematostella* Ago proteins cluster in pairs with those of the two *Acropora* species (*NveAgo1* with the Ago of *A. millepora* and *NveAgo2* with the Ago of *A. digitifera* [fig. 5A]). We detected homologs of the two *Nematostella* Ago transcripts in the transcriptome data from *A. millepora* (table 1), but because the transcript encoding *AmiAgo2* was incomplete and lacked one of the domains, we did not include it in the phylogeny. These findings suggest that two Ago genes in Hexacorallia are the result of a gene duplication that preceded the split of sea anemones and stony corals estimated to have occurred 500 Ma (Shinzato et al. 2011). The co-existence of the two Ago genes in both *Nematostella* and *Acropora* over such a long time suggests that their functions have diverged. Such functional divergence is known in insects, where miRNAs and siRNAs are preferentially loaded into two different Ago proteins in an apparent example of subfunctionalization. In addition, the catalytic characteristics of each of the two proteins are vastly different, making them more suitable for carrying their diverged functions (Tomoyasu et al. 2008; Wee et al. 2012). The two *Nematostella* Agos carry the three amino acids (two aspartates and a histidine, referred

to as the DDH triad) that constitute the Ago active site (fig. 5B), raising the possibility that both of them are capable of target slicing (Rivas et al. 2005). Several recent studies reported that an additional glutamate and a phenylalanine residue play an important role in slicing (Nakanishi et al. 2012; Faehnle et al. 2013). Both of these residues are conserved in *NveAgo1* and *NveAgo2* (fig. 5B). Moreover, all five residues are spatially positioned almost exactly the same way as their counterparts in the human slicer *HsaAgo2* (fig. 5C), supporting their possible role in small RNA-mediated target slicing in *Nematostella* (Rivas et al. 2005; Elkayam et al. 2012; Faehnle et al. 2013).

In bilaterians, the ability of the RISC to efficiently repress translation highly depends on proteins of the GW182 family (Huntzinger and Izaurralde 2011; Fabian and Sonenberg 2012). However, despite their conserved functional role the members of this protein family from vertebrates, flies and nematodes exhibit unusual diversity in their length, sequence composition, and domain structure (Eulalio et al. 2009). This diversity is probably the result of the fact that the interaction domains of GW182 proteins are based on many multiple linear motifs that were lost and gained along their evolution (Kuzuoglu-Ozturk et al. 2012). We could detect a single GW182 homolog in each of the cnidarian species tested (table 1). Just like in bilaterians, the sequence diversity of these putative proteins was remarkable even within Cnidaria and this fact prevented us from reconstructing a reliable phylogeny. Nevertheless, we could detect the

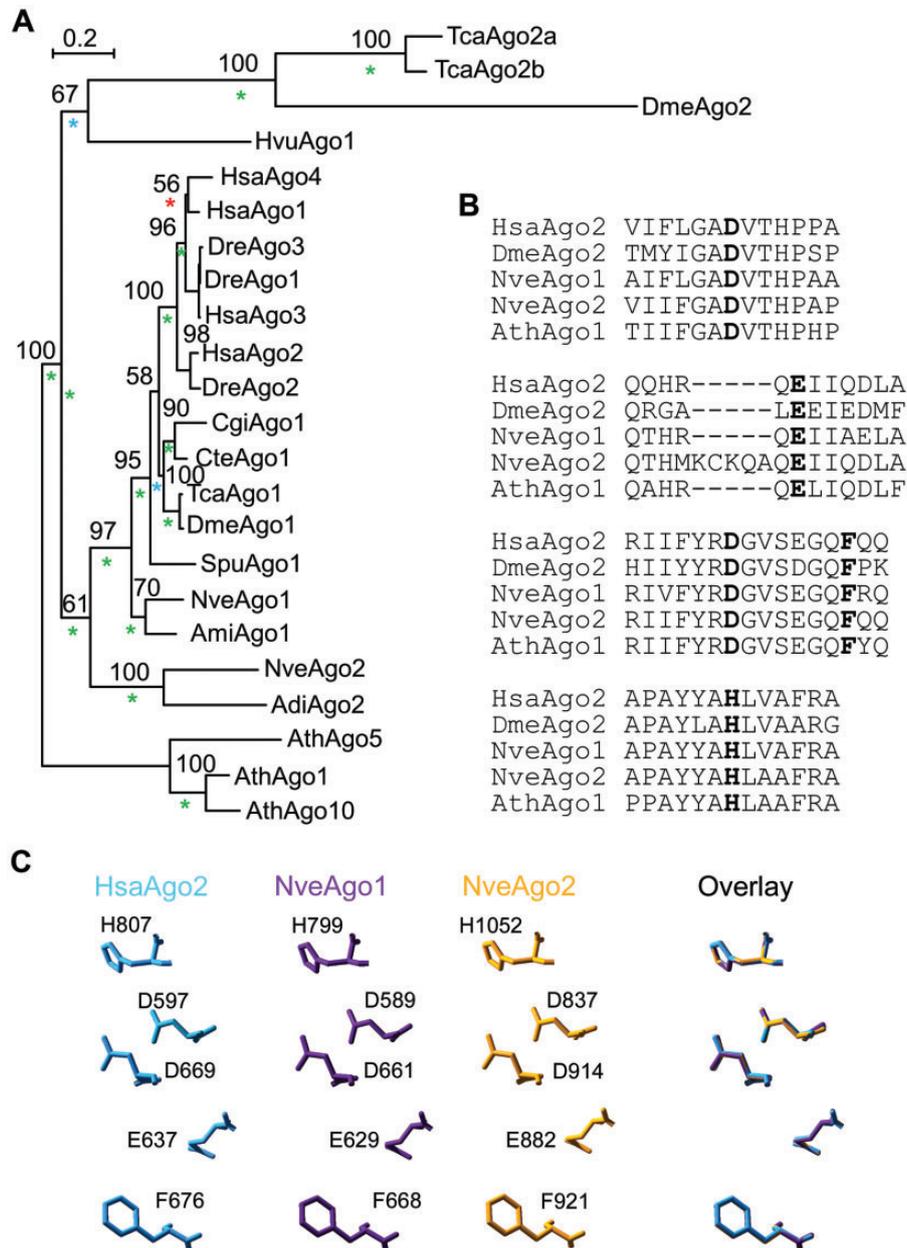


FIG. 5. Phylogeny of cnidarian Argonaute proteins based on their domain of unknown function (DUF) 1785, PAZ and Piwi domains. (A) An ML phylogenetic tree was constructed with the LG model (+ G). Bootstrap support values above 50% are indicated above branches. PP values of a Bayesian tree constructed with the RtREV model are indicated by a green (PP = 1.0), blue ($0.95 \leq PP < 1.0$), or red ($0.7 < PP < 0.95$) asterisk. (B) Multiple sequence alignment of the Argonaute active site. Residues shown to affect slicing appear in bold. (C) Spatial arrangement of the five residues shown to affect HsaAgo2 slicing activity, and their homologous residues in NveAgo1 and NveAgo2. Abbreviations are as follows: Adi, *Acropora digitifera* (stony coral); Ami, *Acropora millepora* (stony coral); Ath, *Arabidopsis thaliana* (thale cress); Cgi, *Crassostrea gigas* (Oyster), Cte, *Capitella teleta* (annelid), Dme, *Drosophila melanogaster* (fruit fly); Dre, *Danio rerio* (zebrafish); Hsa, *Homo sapiens* (human); Hvu, *Hydra vulgaris* (hydra); Nve, *Nematostella vectensis* (starlet anemone); Spu, *Strongylocentrotus purpuratus* (sea urchin); Tca, *Tribolium castaneum* (red flour beetle).

GW182 hallmarks such as stretches of glutamines and GW repeats in these cnidarian homologs (fig. 6A). Strikingly, despite the generally very poor sequence conservation, specific motifs as well as residues shown experimentally to carry bioactive roles in bilaterian GW182 proteins (Zipprich et al. 2009; Mishima et al. 2012) are conserved in the *Nematostella* homolog (fig. 6B). This suggests that the common ancestor of cnidarians and bilaterians probably carried a GW182 protein capable of interaction with Ago and is able to promote

translational inhibition and target decay. However, all the cnidarian homologs we detected carry a DnaJ domain, which is absent from bilaterian GW182 proteins (fig. 6A). DnaJ domains are also known as HSP40 (heat shock protein 40) domains and usually interact with HSP70 and enhance its chaperone capabilities by stimulating its ATPase activity (Qiu et al. 2006). The role of this domain in the cnidarian GW182 homologs is currently unknown, but one intriguing possibility we put forward here is that it is involved in loading small RNA

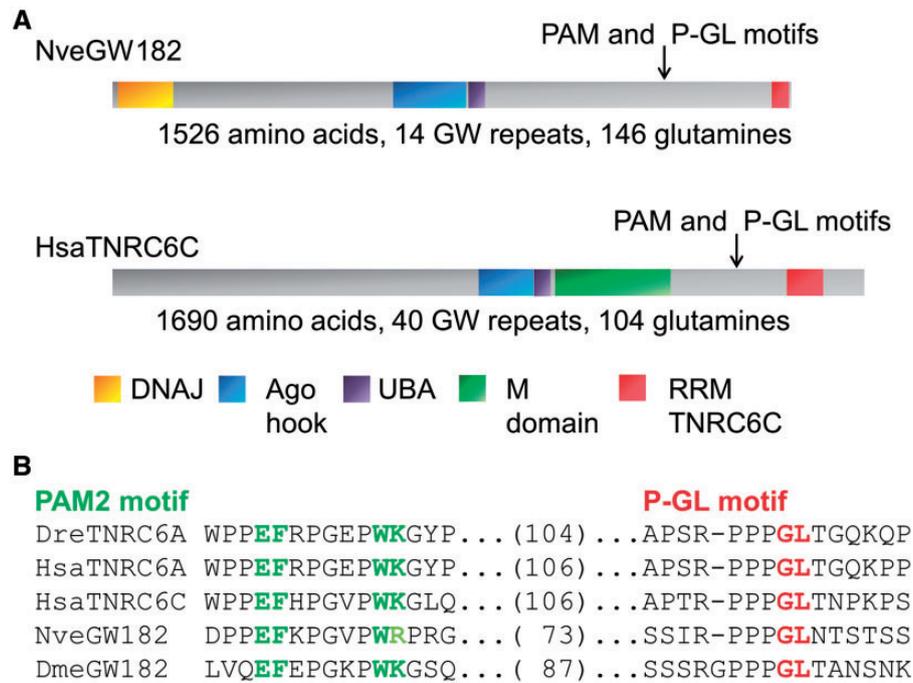


Fig. 6. Primary structure of *Nematostella* GW182. (A) Conserved domains of *Nematostella* and human GW182 proteins. (B) Alignment of the PAM2 and P-GL motifs that were indicated to participate in miRNA-mediated translational inhibition by vertebrate GW182 proteins. Sites in these motifs that were previously shown by mutagenesis to be crucial for translational inhibition are highlighted (Zipprich et al. 2009; Mishima et al. 2012).

duplexes into Ago proteins, as this process highly relies on the ATP-dependent chaperone activity of the HSC70/HSP90 chaperone complex and other proteins containing DNAJ domains were shown to regulate HSC70 proteins in bilaterians (Qiu et al. 2006; Czech and Hannon 2011).

Conclusions

We used phylogenetic analysis and ISH-based localization to detect and study the protein complement involved in the miRNA biogenesis and RISC machinery in four cnidarian species, providing novel insight into the evolution of these proteins and the genes encoding them. We find that those components have ancient origins and diverse roles in Cnidaria. The finding of the presence of HYL1L in cnidarians, sponges, and ctenophores might change our general view on the evolution of miRNA biogenesis. As Cnidaria are the likely sister group of Bilateria, and in light of the findings presented here, we believe that future functional studies of these proteins and the genes encoding them will be pivotal for understanding the evolution of the microRNA pathway in animals.

Materials and Methods

Detection of Homologs

Human, *Drosophila*, and *Arabidopsis* proteins known to take roles in biogenesis of miRNA, as well as the RISC components Ago and GW182, were used for Blast queries. The *N. vectensis* protein models were retrieved by BLASTP queries vs. the Cnidaria section of the nonredundant protein sequences data set (nr) of the National Center for Biotechnology Information (NCBI; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>, last accessed September 27, 2013). *Acropora*

millepora transcriptome data were searched by TBLASTN queries vs. the TSA (transcriptome shotgun assembly) of the same portal. The *H. vulgaris* strain Basel transcriptome data were downloaded from European Nucleotide Archive (ENA) and searched locally by TBLASTN. The *A. digitifera* protein model data set was searched by BLASTP queries through the publicly available genome browser (<http://marinegenomics.oist.jp/genomes/gallery>, last accessed September 27, 2013). Retrieved cnidarian protein models and translated transcripts were reciprocally matched against the NCBI protein data sets for Human, *Drosophila*, and *Arabidopsis* to further establish their relationship. Conserved domains were detected using the CDD and PFAM tool (Marchler-Bauer et al. 2011; Punta et al. 2012). Searches for HYL1 homologs in other basal animal phyla were done in a similar fashion against NCBI nr and UniProt (*Amp. queenslandica* and *T. adhaerens*) or against other online data sets (<http://research.nhgri.nih.gov/mnemiopsis/> [last accessed September 27, 2013] for *M. leidy*).

RNA Isolation and RACE

Total RNA was isolated from mixed developmental stages of *N. vectensis* using Trizol (Life technologies, USA) according to the manufacturer's instructions. The poly-A RNA was selected using the PolyAtract mRNA isolation system III (Promega, USA). The isolated poly-A RNA was used as template for all reverse transcription reactions performed. 5' and 3' RACE experiments were conducted using the RACE SMARTer kit (Clontech, USA) according to the manufacturer's instructions. Advantage2 DNA polymerase mix (Clontech) was used for polymerase chain reaction (PCR)

under the touchdown conditions suggested in the RACE SMARTer kit manual. The product of each initial PCR reaction in a final dilution of 1:1,000 served as template for a nested PCR. The PCR products were ligated into pGEM-T (Promega) and sequenced from both sides using T7 and SP6 universal primers. Sequencing was performed by MacroGen (the Netherlands) and Microsynth (Switzerland). The full novel transcripts encoding *Nematostella* proteins were deposited in GenBank (Accession numbers KF192061–KF192071).

Phylogenetic Analysis

The boundaries of conserved domains were determined according to PFAM (Punta et al. 2012). The domains were aligned using MUSCLE and low-quality alignment regions were removed by TrimAl (Edgar 2004; Capella-Gutierrez et al. 2009). ProtTest was used to find the most suitable model for phylogeny reconstruction (Abascal et al. 2005). The maximum-likelihood (ML) phylogenetic trees were constructed using PhyML, and the support values were calculated using 100 bootstrap replicates (Guindon et al. 2010). A Bayesian tree was constructed using MrBayes v3.2.1 (<http://mrbayes.sourceforge.net>, last accessed September 27, 2013) and the run lasted 5,000,000 generations. Every 100th generation was sampled. We estimated that the Bayesian analysis reached convergence when the potential scale reduction factor reached 1.0.

Clustering Analysis

We retrieved all 2,508 Uniprot proteins that harbored at least one DSRBM protein domain (PF00035) from PFAM v27.0 (Punta et al. 2012), retrieved the corresponding NCBI taxonomy identifier for each protein from Uniprot release 2013_08 (<http://www.uniprot.org/>, last accessed September 27, 2013), and used the NCBI Taxonomy database (accessed August 14, 2013; Federhen 2012) to assign each taxa to the corresponding kingdom (Metazoa, 1,699; Viridiplantae, 341; Fungi, 230; Other eukaryotes, 54; and Unclassified, 184). We then manually added the identified HYL1L sequences to this set and used CLANS v2.0 (Frickey and Lupas 2004) with Blast (blastp) to cluster the protein sequences based on their pairwise sequence similarities with an *e*-value cutoff of 10 for the initial Blast and a *P*-value cutoff of $1E-5$ in the subsequent clustering step, which we ran for 2,500 iterations. We verified that the cluster topology, in particular with regards to the HYL1-cluster, was robust to different *P*-value cutoffs and initial random protein locations by multiple runs of the clustering algorithm following random initialization. We also carried out this clustering analysis using concatenated DSRBM domains, rather than full-length proteins. Although the topology was similar, it appeared that some information on relatedness was lost through the removal of other protein regions, resulting in less well-defined clusters (data not shown). The cluster analysis based on full-length proteins was used for all analyses.

Structural Modeling

The published crystal structure of HsaAgo2 (Elkayam et al. 2012) was used as a template for modeling the structure of

NveAgo1 and NveAgo2 by the Swiss-Model online tool (Arnold et al. 2006). The model was visualized using DeepView/Swiss-PDB Viewer v4.1.0 and rendered by POV-Ray v3.6.2 (Persistence of Vision Ray Tracer, USA).

Single and Double ISH

For ISH experiments, *N. vectensis* larvae were fixed at 48–168 h postfertilization in 3.7% ice-cold formaldehyde in 1/3 seawater with 0.2% glutaraldehyde for 90 s and then in 3.7% formaldehyde in 1/3 seawater without glutaraldehyde for additional 60 min. Transcript fragments were amplified by PCR and cloned into pGEM-T (Promega). Antisense RNA probes for ISH were generated and labeled by using the T7 or SP6 MEGAscript kits (Life Technologies) and an RNA labeling mix with either digoxigenin (DIG) or fluorescein (FITC) (Roche, Germany). The ISH procedure for single probes was performed as described previously using DIG-labeled probes (Genikhovich and Technau 2009). Double ISH combining a DIG-labeled and a FITC-labeled probe was performed according to an established protocol (Moran et al. 2013). Samples were mounted either in SlowFade Gold medium (Life Technologies) or 85% glycerol and photographed in a Nikon Eclipse 80i fluorescent microscope connected to a Nikon Digital Sight DS-U2 camera.

Supplementary Material

Supplementary figures S1 and S2 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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