Fly Antiviral RNA Silencing and miRNA Biogenesis Claim ARS2

Olivier Voinnet[1,*]
[1]Institut de Biologie Moléculaire des Plantes, Université de Strasbourg, CNRS UPR 2357, 67084 Strasbourg Cedex, France
*Correspondence: olivier.voinnet@ibmp-ulp.u-strasbg.fr
DOI 10.1016/j.chom.2009.08.002

In plants and invertebrates, small silencing RNAs function in antiviral defense and developmental patterning through pathways that were so far considered genetically distinct. In a recent issue of *Cell*, Sabin and colleagues report the identification of *Drosophila* Ars2, a protein required for both these small RNA-mediated functions.

Viruses are obligate intracellular pathogens of all life forms, and organisms have evolved diverse mechanisms to combat their aggressions. One mechanism, RNA silencing, involves small RNAs, 19–30 nucleotides in length, that repress gene expression by annealing to target RNA or DNA. Silencing small RNAs include small-interfering RNAs (siRNAs) and microRNAs (miRNAs), which are both processed from double-stranded (ds)RNA by the RNaseIII Dicer. siRNAs derive from perfectly-to-near-perfectly base-paired dsRNAs of endogenous or exogenous origin, including viruses. miRNAs originate from primary miRNA transcripts (pri-miRNA) produced from independent transcription units found in intergenic or intronic nuclear DNA. pri-miRNAs contain imperfect intramolecular stem loops, which, in animals, are first excised in the nucleus by the Microprocessor complex, primarily composed of the Dicer-like enzyme Drosha and binding partner, Pasha. Dicer then cytoplasmically converts the resulting precursor miRNA (pre-miRNA) into a single, mature miRNA (Figure 1A). miRNAs are important cell-fate determinants; consequently, miRNA-deficient organisms display severe-to-lethal developmental defects.

miRNAs and siRNAs incorporate RNA-induced silencing complexes (RISCs), assembled upon loading of one selected small RNA strand into an Argonaute (Ago) protein family member. siRNA- and miRNA-programmed RISCs then repress target gene expression through endonucleolysis or translational repression of fully or partially complementary mRNAs (Figures 1A and 1B). In *Drosophila*, miRNAs and siRNAs are produced by two separate Dicers, Dcr1 and Dcr2—and also distinctly effected by Ago1 and Ago2, respectively. The fact that many viruses infect and are transmitted by insects was probably an important driving force leading to the separation of siRNA and miRNA pathways in arthropods (Obbard et al., 2006). Several studies have now established that Dcr2 generates siRNAs from viral dsRNA, a common replication intermediate of most viruses. siRNA-loaded Ago2 then executes antiviral silencing by targeting viral RNAs with sequences complementary to the siRNAs (Figure 1B). Accordingly, *Drosophila dcr2* and *ago2* mutants are developmentally normal, but hypersusceptible to viruses (reviewed in Ding and Voinnet, 2007).

Sabin and colleagues now report the isolation of Ars2, a novel, general antiviral silencing factor in *Drosophila*. Ars2 was isolated in a small-throughput RNAi-based genetic screen aimed at identifying virus-restricting host factors in cultured S2 cells (Sabin et al., 2009). The screen employed Vesicular Stomatitis virus, modified to contain a GFP reporter gene. Among ~100 individual gene knockdowns tested, Ars2 depletion consistently caused a 5-fold increase in the number of GFP-positive cells. Several additional viruses displayed enhanced infection rates that did not result from increased internalization of viral antigens (measuring viral entry into cells), but rather from improved viral RNA replication possibly inherent to defects in the siRNA pathway, because the viruses tested were previously characterized as being susceptible to RNA silencing. Ars2-depleted cells were indeed less competent to silence expression of sensor transgenes reporting the activity of Dcr2-dependent siRNAs produced from exogenous or endogenous dsRNA. The effect was, however, bypassed if siRNAs were delivered directly into cells, suggesting that Ars2 acts upstream of RISC. Accordingly, Ars2 was required for endogenous siRNA accumulation and was coimmunoprecipitated with Dcr2. Moreover, extracts of Ars2-depleted cells produced less siRNAs from labeled dsRNA compared to extracts of control cells. Thus, Ars2 apparently facilitates Dcr2 action, possibly through direct protein-protein interactions. These findings are relevant to authentic infections because adult flies in which Ars2 expression was conditionally suppressed succumbed to viruses more rapidly than control flies.

Ars2-depleted flies were not easily engineered, though: transposon insertions or constitutive RNAi were embryonic lethal. This was unexpected, since *ago2* or *dcr2* knockouts are innocuous to *Drosophila*, unlike miRNA pathway mutations. The closest Ars2 homolog, the *Arabidopsis* C2H2-Zinc-finger protein SERRATE (SE), is required for pri-to-pre-miRNA processing, and this hinted at the possibility that Ars2 could be similarly required in the fly miRNA pathway, in addition to its antiviral function. Ars2-depleted cells indeed exhibited compromised miRNA functions, owing to reduced pri-miRNA accumulation. This suggested that Ars2 might stabilize pri-miRNAs to facilitate their Microprocessor-dependent nuclear conversion into pre-miRNAs (Figure 1A). Coimmunoprecipitation of Ars2 and Pasha in S2 cells further supported this idea. A report from Gruber et al., in the same issue of *Cell*, directly implicates human Ars2 in pri-miRNA processing, and documents its interaction with CBP80, a subunit of the nuclear Cap-binding complex (CBC)

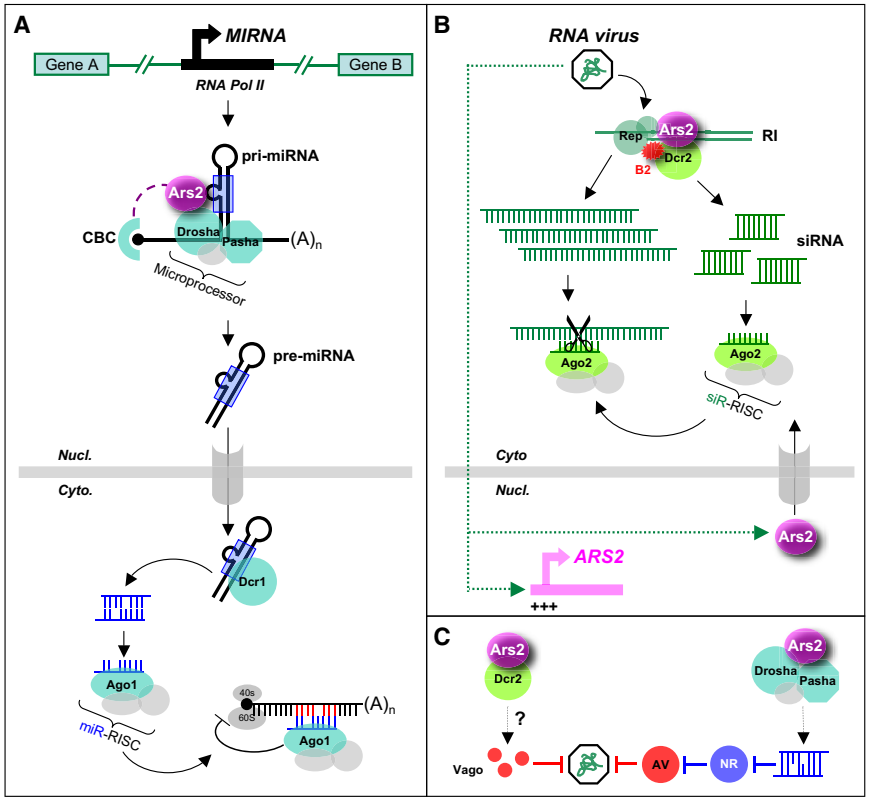

Figure 1. Possible Scenarios Accounting for the Roles of Ars2 in miRNA Biogenesis and in Antiviral Defense in *Drosophila*

(A) Most primary miRNA transcripts (pri-miRNA) are produced by RNA polII and thus have a polyA tail and a 5′ cap strucure (dark dot) which is protected from degradation by the nuclear Cap binding complex (CBC) whose subunits CBP80 and CBP20 interact with Ars2 (dotted line). Cointeraction of Ars2 with the CBC on one hand, and Drosha/Pasha on the other, would thus naturally bring the Microprocessor complex into contact with the pri-miRNA and allow pre-miRNA processing. Alternatively, and as favored by the authors, Ars2 could merely act as an enhancer or add precision to the action of the Microprocessor that would bind to its pri-miRNA template independently of Ars2/CBC. Both models account for the faithful excision of the pre-miRNA stem-loop structure, which, upon transport to the cytoplasm, is then processed by Dcr1 into a single, mature miRNA species. One selected miRNA strand engages into Ago1 and allows assembly of the miRNA-programmed RNA-induced silencing complex (miR-RISC) that scans the cell for partially or fully complementary target mRNAs, shown here as undergoing translational repression.

(B) Most fly-infecting viruses such as those tested in Sabin et al. have a single-stranded RNA genome replicated through the action of a viral-encoded replicase (Rep) thought to generate partially double-stranded replication intermediates (RI). RIs may serve as direct templates for Dcr2, which, in association to Ars2, might generate antiviral siRNAs that engage into Ago2. Note, however, that the contribution of Ars2 to the production of viral-derived siRNAs was not documented in the study of Sabin et al. Viral silencing suppressors, such as the B2 protein of FHV (red star), which is known to inhibit viral siRNA production, might inhibit Ars2 function. The assembled siRNA-loaded RISC (siR-RISC) then suppresses viral RNA accumulation through mechanisms that include endonucleolytic cleavage. Viral infections might stimulate transcription of the *Ars2* gene and also promote cytoplasmic relocalization of nuclear pools of Ars2.

(C) At least two nonmutually exclusive models could account for indirect antiviral effects of Ars2 in fly. Ars2 might be required for the documented, albeit mechanistically ill-defined, action of Dcr2 in promoting production of the antiviral peptide Vago (left). Ars2 may also be required for production of miRNA-regulating nonsilencing-based antiviral innate immune responses (e.g., JAK-STAT pathway) as has been found in mammals (right). In the model, the miRNA repress a negative regulator (NR) of a host factor with intrinsic antiviral (AV) activity. In both cases (right and left), depletion of Ars2 would result in enhanced virus accumulation.

(Gruber et al., 2009). Remarkably, *Arabidopsis* with mutations in *ABH1/CBP80* and *CBP20* also show impaired pri-to-pre-miRNA processing and exhibit developmental defects overlapping with those of *se* mutants (Gregory et al., 2008; Laubinger et al., 2008). Sabin and colleagues confirmed that the fly Cbp20 immunoprecipitates overexpressed Ars2, and that Cbp20 or Cbp80 depletion compromises silencing by miRNAs, and by exogenous and endogenous siRNAs. Also as in Ars2-depleted cells, pri-miRNA levels were reduced in CBC-depleted S2 cells, which, furthermore, supported greater replication of at least two viruses tested in the siRNA pathway experiments described above.

The work of Sabin et al. put forward a tangible link between two fly silencing pathways so far considered as largely distinct: Ars2 could well represent a key factor that permitted the evolutionary transition from a gene-regulatory pathway operated by miRNAs to an innate immune pathway operated by siRNAs, (or vice versa, as it is presently impossible to state confidently which arose first). Unlike *Dcr1* and *Ago1*, *Dcr2*, and *Ago2* are among the fastest evolving 3% of all *Drosophila* genes (Obbard et al., 2006), and it will be interesting to investigate the extent to which this signature of host pathogens arms race is found in natural Ars2 variants among arthropods. Besides, the study raises many questions. The most pressing point is to clarify whether the roles of Ars2 in antiviral defense and pri-to-pre-miRNA processing entail similar mechanisms. The first uncertainty pertains to Ars2 expression and subcellular localization. Hence, Gruber et al. show that human Ars2 is nearly exclusively expressed in dividing cells, where it facilitates the differential accumulation of miRNAs specifying totipotency. Likewise, the role of SERRATE was discovered through the defects exhibited by *se* mutants in meristems, the stem cell niches of plant apexes. However, the fat body cells that normally accumulate viruses in infected adult flies are nonproliferating. Human Ars2 and *Arabidopsis* SERRATE are also very predominantly nuclear localized, yet many viruses tested by Sabin et al. are exclusively cytoplasmic. One testable way to reconcile the data is to propose that Ars2 is not only induced, but also cytoplasmically relocalized upon infection (Figure 1B). Second, plant and animal pri-miRNA are produced by PolII and, thus, are 5′-capped. This, evidently, provides an ideal scenario as to how Ars2 might recruit the Microprocessor to pri-miRNA through its interaction with nuclear CBC components (Figure 1A). A similar scenario cannot, however, apply to the antiviral Dcr2 complex, because at least one virus tested by Sabin et al. replicates via uncapped RNAs. The Sabin and Gruber studies therefore propose that Ars2 is a general, direct facilitator of cytoplasmic and nuclear RNase III activities across species.

While work with SERRATE in *Arabidopsis* extracts supports the above hypothesis (Dong et al., 2008), it remains possible that the mechanisms of Ars2-mediated antiviral defense and Ars2-mediated miRNA biogenesis overlap only partially. First, quantitative and/or qualitative profiling of viral small RNAs in wild-type (wt) versus Ars2-depleted S2 cells should provide stronger ground to the proposed role of Ars2 as a direct modulator of Dcr2-mediated processing of viral dsRNA (Figure 1B). Such analyses might also unravel specific viral RNA features resembling those of pri-miRNAs, which could preferentially attract Ars2 and Dicer-like enzymes on both types of templates. Limited changes in viral siRNA profiles between the two cell types, on the other hand, could point to more indirect contributions of Ars2 to antiviral silencing. Noteworthy, the induction of Vago, a cysteine-rich polypeptide with antiviral activity in adult flies, requires the function of Dcr2 through as-yet-unspecified mechanisms (Deddouche et al., 2008) (Figure 1C, left). Possible alterations in the levels of Vago (or other innate immune factors unrelated to silencing) in Ars2-depeleted cells thus warrant consideration. miRNAs also regulate innate and adaptive immunity across kingdoms, and their frequent dismissal as possible contributors to fly antiviral defense is mostly attributable to the unavailability of true *dcr1* or *ago1* hypomorphic mutations in *Drosophila* (Ding and Voinnet, 2007). Based on these premises, mysregulation of miRNA biogenesis in Ars2-deficient cells might also contribute indirectly to altered antiviral immunity.

An important role for Ars2 in antiviral silencing also predicts that it will be targeted by one or several viral suppressors of RNA silencing (VSR), which are commonly deployed by insect and plant viruses against critical steps of the machinery (Ding and Voinnet, 2007). The B2 protein of flock-house virus (FHV) is a strong candidate because it binds FHV replication complexes to directly inhibit viral siRNA production by Dcr2 (Aliyari et al., 2008) (Figure 1B, right). Targeting of Ars2 by B2 might actually explain the relatively modest effects of Ars2 depletion observed by Sabin et al. on FHV replication. However, it is of note that expression of B2, or indeed of any VSR studied so far in *Drosophila*, incurs no apparent defects in the miRNA pathway, yet RNAi is usually strongly suppressed in S2 cells and adult flies (Berry et al., 2009).

Ultimately, genetic studies might clarify this question, particularly in flies, which are highly amenable to forward and reverse approaches. Use of available sensor transgene reporting miRNA as well as siRNA activities should facilitate the identification of point mutation alleles of Ars2 that could possibly uncouple its involvements in the two pathways. Meanwhile, the study of Sabin and colleagues prompts an evaluation of the antiviral role of SERRATE in plants, which has eluded characterization so far. Based on the findings of Gruber and colleagues, use of meristem-infecting viruses or geminiviruses known to induce plant cell proliferation might constitute a reasonable starting point in this endeavor.

REFERENCES

Aliyari, R., Wu, Q., Li, H.W., Wang, X.H., Li, F., Green, L.D., Han, C.S., Li, W.X., and Ding, S.W. (2008). Cell Host Microbe *4*, 387–397.

Berry, B., Deddouche, S., Kirschner, D., Imler, J.L., and Antoniewski, C. (2009). PLoS ONE *4*, e5866.

Deddouche, S., Matt, N., Budd, A., Mueller, S., Kemp, C., Galiana-Arnoux, D., Dostert, C., Antoniewski, C., Hoffmann, J.A., and Imler, J.L. (2008). Nat. Immunol. *9*, 1425–1432.

Ding, S.W., and Voinnet, O. (2007). Cell *130*, 413–426.

Dong, Z., Han, M.H., and Fedoroff, N. (2008). Proc. Natl. Acad. Sci. USA *105*, 9970–9975.

Gregory, B.D., O'Malley, R.C., Lister, R., Urich, M.A., Tonti-Filippini, J., Chen, H., Millar, A.H., and Ecker, J.R. (2008). Dev. Cell *14*, 854–866.

Gruber, J.J., Zatechka, D.S., Sabin, L.R., Yong, J., Lum, J.J., Kong, M., Zong, W.X., Zhang, Z., Lau, C.K., Rawlings, J., et al. (2009). Cell *138*, 328–339.

Laubinger, S., Sachsenberg, T., Zeller, G., Busch, W., Lohmann, J.U., Ratsch, G., and Weigel, D. (2008). Proc. Natl. Acad. Sci. USA *105*, 8795–8800.

Obbard, D.J., Jiggins, F.M., Halligan, D.L., and Little, T.J. (2006). Curr. Biol. *16*, 580–585.

Sabin, L.R., Zhou, R., Gruber, J.J., Lukinova, N., Bambina, S., Berman, A., Lau, C.K., Thompson, C.B., and Cherry, S. (2009). Cell *138*, 340–351.