anecdotal evidence of the ability of green tea to remedy various amyloid diseases, such as light-chain amyloidosis, have begun to appear[24].

It remains unclear whether EGCG can remodel mature amyloid fibers in a similar way to other small molecules[7]. However, once again there is reason to be optimistic. EGCG may take advantage of Le Châtelier's principle to disassemble amyloids and prevent conformational conversion by shifting the equilibrium dramatically toward nonamyloid conformers. Amyloid fibers seem to dynamically exchange monomers from their ends by the spontaneous dissociation and reassociation of monomers over a biologically relevant timeframe (days)[25]. Given that EGCG inhibits seeded polymerization, it is possible, perhaps even probable, that it might inhibit the reassociation of dissociated monomers with fiber ends and drive the equilibrium toward soluble forms.

One must keep in mind, however, that EGCG binds to unfolded proteins in a nonselective fashion[8], which may have unanticipated pleiotropic consequences *in vivo*. Indeed, this property might explain the diverse activities attributed to EGCG[14]. EGCG might inhibit the amyloidogenesis of many polypeptides that begin in a natively unfolded state. However, such broad specificity may be undesirable, because mounting evidence suggests that amyloids and prions have also been captured during evolution for beneficial purposes[26]. Pmel17 amyloids mediate melanosome biogenesis and particular CPEB prions might promote synaptic changes associated with memory[26]. Nonetheless, the studies by Ehrnhoefer *et al.*[8] provide an important foundation to understand the interactions between small molecules and natively unfolded proteins, and may facilitate the design of more potent and selective compounds with activity against exclusively deleterious amyloids.

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1. Fink, A.L. *Curr. Opin. Struct. Biol.* **15**, 35–41 (2005).
2. Nelson, R. & Eisenberg, D. *Adv. Protein Chem.* **73**, 235–282 (2006).
3. Skovronsky, D.M., Lee, V.M.-Y. & Trojanowski, J.Q. *Annu. Rev. Pathol. Mech. Dis.* **1**, 151–170 (2006).
4. Dobson, C.M. *Nature* **432**, 824–828 (2004).
5. Ehrnhoefer, D.E. *et al. Hum. Mol. Genet.* **15**, 2743–2751 (2006).
6. Masuda, M. *et al. Biochemistry* **45**, 6085–6094 (2006).
7. Wang, H. *et al. Proc. Natl. Acad. Sci. USA* **105**, 7159–7164 (2008).
8. Ehrnhoefer, D.E. *et al. Nat. Struct. Mol. Biol.* **15**, 558–566 (2008).
9. Wells, J.A. & McClendon, C.L. *Nature* **450**, 1001–1009 (2007).
10. Krishnan, R. & Lindquist, S.L. *Nature* **435**, 765–772 (2005).
11. Petkova, A.T. *et al. Science* **307**, 262–265 (2005).
12. Kayed, R. *et al. Science* **300**, 486–489 (2003).
13. Pardridge, W.M. *NeuroRx* **2**, 3–14 (2005).
14. Yang, C.S., Maliakal, P. & Meng, X. *Annu. Rev. Pharmacol. Toxicol.* **42**, 25–54 (2002).
15. Hammarstrom, P., Wiseman, R.L., Powers, E.T. & Kelly, J.W. *Science* **299**, 713–716 (2003).
16. Bertoncini, C.W. *et al. Proc. Natl. Acad. Sci. USA* **102**, 1430–1435 (2005).
17. Del Mar, C., Greenbaum, E.A., Mayne, L., Englander, S.W. & Woods, V.L., Jr. *Proc. Natl. Acad. Sci. USA* **102**, 15477–15482 (2005).
18. Conway, K.A. *et al. Proc. Natl. Acad. Sci. USA* **97**, 571–576 (2000).
19. Bitan, G. *et al. Proc. Natl. Acad. Sci. USA* **100**, 330–335 (2003).
20. Cheon, M. *et al. PLoS Comput. Biol.* **3**, e173 (2007).
21. Behrends, C. *et al. Mol. Cell* **23**, 887–897 (2006).
22. Shorter, J. & Lindquist, S. *Mol. Cell* **23**, 425–438 (2006).
23. Evans, C.G., Wisen, S. & Gestwicki, J.E. *J. Biol. Chem.* **281**, 33182–33191 (2006).
24. Mereles, D., Wanker, E.E. & Katus, H.A. *Clin. Res. Cardiol.* published online, doi:10.1007/s00392-008-0649-6 (3 March 2008).
25. Carulla, N. *et al. Nature* **436**, 554–558 (2005).
26. Shorter, J. & Lindquist, S. *Nat. Rev. Genet.* **6**, 435–450 (2005).

Endo-siRNAs: yet another layer of complexity in RNA silencing

Timothy W Nilsen

Organisms possessing RNA-dependent RNA polymerase activity are known to produce endogenous small interfering RNAs (esiRNAs). It had been thought that organisms such as flies and mammals lacking this activity would not produce esiRNAs. However, it has now been shown that a functional esiRNA pathway is present in such animals; the esiRNAs are derived from a variety of endogenous double-stranded RNA substrates.

The world of small-RNA silencing phenomena has just become markedly larger with the recent publication of seven complementary papers (five in *Nature*[1–5], one in *Science*[6] and one in this issue of *Nature Structural & Molecular Biology*[7]) that collectively document an additional silencing pathway in *Drosophila melanogaster* and mammals. This pathway involves the synthesis and processing of endogenous double-stranded RNAs (dsRNAs) to yield functional small interfering RNAs (siRNAs) that serve to silence transposable elements in both germ cells and somatic tissues, and some specific mRNAs. It is also possible that these endo-siRNAs, esiRNAs, have a role in heterochromatin formation analogous to the function of some endogenous siRNAs in plants and fission yeast.

To date, esiRNAs have been detected only in organisms that possess RNA-dependent RNA polymerases (RDRPs): plants, *Caenorhabditis elegans* and fission yeast. Because these polymerases transcribe single-stranded RNA (ssRNA) to make dsRNA and are essential for the production of esiRNAs, it was thought that organisms that did not have RDRPs would not use an esiRNA pathway owing to the lack of endogenous dsRNA. However, it is well known that there are other sources of dsRNAs besides those generated by RDRPs (**Fig. 1**). These include long hairpin structures generated by the transcription of palindromic sequences and dsRNAs generated by the annealing of complementary RNAs synthesized by convergent transcription units. Indeed, these dsRNAs have now been shown to be the source of esiRNAs in both *D. melanogaster* and mice[1–7].

In both organisms, extensive studies in many laboratories have revealed three distinct RNA silencing pathways, each using distinct small RNAs and a distinct set of protein factors. The Piwi-interacting RNA (piRNA) pathway is involved in silencing transposons in the germ line, and piRNAs are bound to the Piwi class of argonaute proteins; it is not yet clear how these RNAs and proteins exert their silencing

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effects (reviewed in ref. 8). The microRNA (miRNA) pathway is responsible for the post-transcriptional regulation of many mRNAs via translational repression or enhancement of miRNA turnover. In *D. melanogaster,* miRNAs function in a ribonucleoprotein complex (RNP) containing Argonaute-1 (AGO1) and recognize their mRNA targets via imperfect base-pairing. The biogenesis of miRNAs is well-understood; long primary transcripts are processed by the RNase III–like dsRNA nuclease Drosha in collaboration with the dsRNA binding protein Pasha (reviewed in ref. 9). The Drosha-processed pre-miRNA is then exported to the cytoplasm as a pre-miRNA, where it is further processed by another dsRNase, Dicer-1, in collaboration with another dsRNA binding protein known as Loquacious. The processed miRNA is then loaded into AGO1 (reviewed in ref. 10). The third RNA silencing pathway in *D. melanogaster* is the small interfering RNA (siRNA) pathway, which is responsible for detecting and destroying exogenous dsRNA and any transcripts derived from the invading dsRNA. This pathway involves the direct cleavage of the dsRNA by a distinct dsRNase, Dicer-2, with a distinct partner, r2d2. Processed dsRNA is then loaded into a distinct argonaute protein, AGO2. The siRNA pathway works via perfect homology between the siRNA and target, resulting in cleavage (slicing) of the target by AGO2 (reviewed in ref. 11). It is this pathway in both flies and mammalian cells that is responsible for targeted knockdowns of your favorite gene. Until now, it was not known whether Dicer-2 and AGO2 were simply lying in wait for an invading dsRNA or had a more active role in organismal physiology.

To address this question, Czech *et al.*[2] and Kawamura *et al.*[3] used immunoprecipitation of AGO2 in the hope of identifying endogenous small RNAs associated with it. Both groups obtained strikingly similar results. Each identified populations of small RNAs that are clearly distinct from previously characterized piRNAs or miRNAs, and both found that most of these small RNAs, dubbed esiRNAs, were derived from mobile genetic elements. Other esiRNAs were processed from either hairpin structures or from overlapping RNAs formed by convergent transcription.

Three other papers addressed the same question via three different routes. Okamura *et al.*[1] used an informatics approach to predict long hairpin structures that might give rise to esiRNAs. They were able to identify many loci that could encode such RNAs, and they were then able to verify the expression of small RNAs (which they called hpRNAs) that were processed from the long hairpins. These hpRNAs comprise a subset of the esiRNAs described in Czech *et al.*[2] and Kawamura *et al*[3]. Instead of looking for inverted repeats, Okamura *et al.*[7] focused on bidirectional transcription units in *D. melanogaster*. Intriguingly, they found several such loci, and some but not all yielded esiRNAs. The reasons for the selectivity in processing are not clear. Finally, Ghidiyla *et al.*[6] used yet a different strategy; they reasoned that any endogenous siRNAs were likely to be modified at their 3′ ends, as this is where siRNAs produced from exogenous dsRNA are methylated. Accordingly, they prepared libraries of small RNAs enriched by virtue of their 3′ end modifications. They were thereby able to identify a population of RNAs that did not correspond to miRNAs or piRNAs. Similarly to the results reported by Czech *et al.*[2] and Kawamura *et al.*[3], most of the RNAs were derived from transposable elements; others clearly were derived from protein-coding sequences. One unique aspect of this approach was the discovery of what may be a fourth class of small RNA, piRNA-like RNAs. Further experiments will be necessary to elucidate the role(s) of these mysterious RNAs.

Collectively, these five papers provide compelling evidence of the existence of endogenous siRNAs in *D. melanogaster*. Two additional studies document similar findings in mouse oocytes. Both Tam *et al.*[4] and Watanabe *et al.*[5] used deep sequencing to examine the small RNA population in oocytes, and both uncovered, in addition to piRNAs and miRNAs, a family of what seemed to be esiRNAs. These RNAs were clearly distinct in size from piRNAs and in many cases were derived from distinct genomic loci. In this regard, mapping of the esiRNAs revealed that, much like in *D. melanogaster*, the small RNAs were derived from a variety of sources: long hairpin structures, overlapping transcription units and transposable elements. Most intriguingly, a fraction of esiRNAs were apparently processed from overlapping regions of functional genes and cognate pseudogenes. The presence of esiRNAs spanning exon-exon boundaries indicated that spliced mRNAs are capable of annealing *in trans* with transcribed pseudogenes. This exciting finding suggested that pseudogenes, previously thought to be nonfunctional, may actually regulate the expression of their founder gene.

Although the evidence for the existence of esiRNAs in *D. melanogaster* and mice (and presumably every other animal) is overwhelming, do these RNAs have biological function? All seven papers address this question, and the answer is unambiguously yes. Most strikingly, esiRNAs have a role (analogous to that of piRNAs) in suppressing the expression of mobile genetic elements. Mice deficient for Dicer (mammals, unlike *D. melanogaster*, have only one Dicer gene) show elevated expression

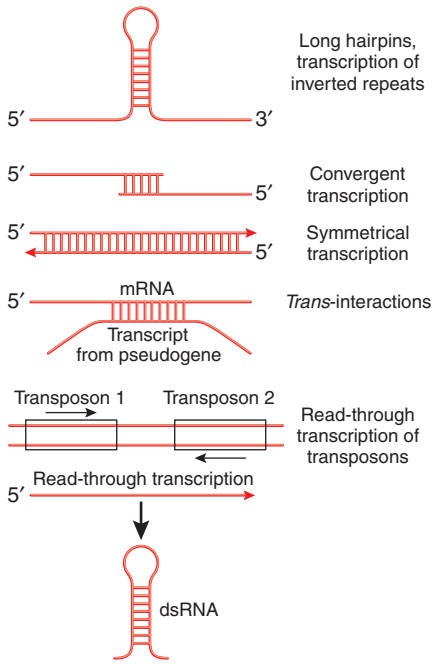


Figure 1 Sources of endogenous double-stranded RNAs. See text for details.

of transposable elements. Interestingly, there is a level of specificity in this phenomenon; that is, only certain transposons seem to be affected by the esiRNA pathway[4,6].

In *D. melanogaster*, flies deficient in AGO2 also show increased expression of transposons, and in this organism esiRNAs may be the primary mechanism for silencing mobile elements in somatic cells, which lack the piRNA pathway (reviewed in ref. 8). In addition to their function in controlling the expression of transposons and other selfish genetic elements, it seems clear that esiRNAs also regulate specific protein-coding transcripts. Nevertheless, the extent and biological relevance of this type of regulation awaits further investigation. In this regard, it seems possible that the esiRNA pathway may, at least in part, serve a 'passive' function in mammals; that is, the destruction of long cellular dsRNAs would prevent the inappropriate activation of cellular defense mechanisms such as the dsRNA-activated protein kinase (PKR).

Whereas the discovery of esiRNAs is in itself highly significant, the mechanism by which these RNAs are produced is also extremely interesting. As noted above, *D. melanogaster* has two distinct Dicers, each of which has a 'partner' dsRNA binding protein: Dicer-2 and r2d2; and Dicer-1 and Loquacious. Not surprisingly, Dicer-2 is responsible for production of esiRNAs. Remarkably, however, flies deficient in its partner, r2d2, show no defect in the amount of esiRNAs; rather, mutants in Loquacious are highly depleted in esiRNAs. This striking

observation raises several intriguing questions. First, how does Dicer-2 'know' which partner to choose when confronting exogenous and endogenous dsRNA. One possibility is that there is a subpopulation of Dicer-2, perhaps post-translationally modified, that specifically recruits Loquacious and is devoted to the esiRNA pathway. Further biochemical analysis may reveal whether this conjecture is correct.

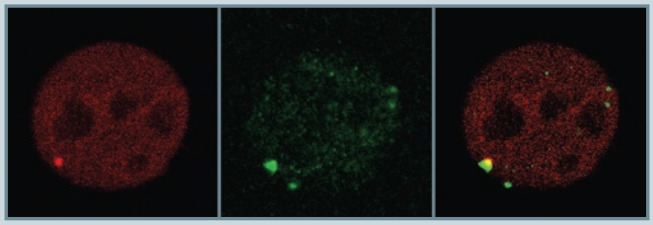
A second mysterious question raised by the esiRNA pathway is, where in the cell does Dicer processing take place? In this regard, Dicers have been thought to be cytoplasmic enzymes. However, it seems highly unlikely that the substrates for esiRNA production would be exported to the cytoplasm for processing. Surprisingly, none of the seven papers comments on this issue. In the absence of evidence to the contrary, it seems possible and perhaps likely that there may be a dedicated nuclear RNAi apparatus in animal cells. If this proves to be true, it raises the prospect that small RNAs could be involved in every level of gene expression in animals; perhaps we are not so different from plants after all.

1. Okamura, K. *et al. Nature* advance online publication, doi:10.1038/nature07015 (7 May 2008).
2. Czech, B. *et al. Nature* advance online publication, doi:10.1038/nature07007 (7 May 2008).
3. Kawamura, Y. *et al. Nature* advance online publication, doi:10.1038/nature06938 (7 May 2008).
4. Tam, O.H. *et al. Nature* advance online publication, doi:10.1038/nature06904 (10 April 2008).
5. Watanabe, T. *et al. Nature* advance online publication, doi:10.1038/nature06908 (10 April 2008).
6. Ghildiyal, M. *et al. Science* published online, doi:10.1126/science.1157396 (10 April 2008).
7. Okamura, K., Balla, S., Martin, R., Liu, N. & Lai, E.C. *Nat. Struct. Mol. Biol.* **15**, 581–590 (2008).
8. Aravin, A.A., Hannon, G.J. & Brennecke, J. *Science* **318**, 761–764 (2007).
9. Kim, V.N. *Nat. Rev. Mol. Cell Biol.* **6**, 376–385 (2005).
10. Zamore, P.D. & Haley, B. *Science* **309**, 1519–15124 (2005).
11. Eulalio, A., Huntzinger, E. & Izaurralde, E. *Cell* **132**, 9–11 (2008).

Damage control

Efficient repair of DNA damage is essential to all organisms. Cells must sense the damage and activate the appropriate signals to allow DNA to be repaired. If DNA damage is extensive, the cell might transiently delay the cell cycle to ensure the damage is repaired before the cell divides. In more extreme cases, cell cycle arrest may be permanent, resulting in senescence, or the cell may undergo cell death.

The DNA damage response (DDR) in mammalian cells comprises early damage sensors, such as the MRN complex (MRE11/Rad50/NBS1), that detects double-stranded breaks; mediator proteins, MDC1 and 53BP1, which are recruited by MRN; and activated kinases such as ATM that phosphorylate the histone variant H2AX. H2AX phosphorylation marks the chromatin at the site of DNA damage and serves as a signal to help recruit the DNA repair machinery in a complex cascade of events whose sequence and interdependencies have been difficult to define. Recently, two groups independently investigated the consequences of accumulating specific DDR proteins at a defined DNA site, and found that the DDR can be activated without DNA damage.



DDR proteins accumulate in distinct DNA repair foci at the sites of DNA lesions. To reconstruct DDR foci in the absence of DNA damage, both groups fused genes for DDR proteins to those encoding a fluorescent protein and the bacterial LacR repressor protein. The fusion proteins were then expressed in cells containing an array of repeats of the LacR target sequence, *lacO*, where they became immobilized to this DNA site, forming a focus that can be visualized by fluorescence microscopy. Adding IPTG causes the focus to disassemble, so one can tell the effects of overproduction of the protein from those due to its concentration on a chromatin region.

Soutoglou and Misteli (*Science*, published online 15 May 2008; doi 10.1126/ science.1159051) found that tethering of NBS1 led to recruitment of MRE11 as well as MDC1 and 53BP1. Likewise, tethering of MRE11 led to accumulation of NBS1, MDC1 and 53BP1. Moreover, targeting of NBS1, MRE11, MDC1 or ATM was sufficient to activate the DDR in mouse cells, as indicated by several phosphorylation marker events and cell cycle delay (left hand panel immobolization of a simple repair factor on chromatin (red), middle panel DDR activation is indicated by phosphorylation of H2AX (green), right hand panel colocalization). The fact that the DDR can be triggered without a DNA lesion indicates that although the lesion itself is important for triggering the response, it is not instrumental for subsequent steps of the DDR. The authors also provided some insight into who recruits whom—as expected, they found that tethered upstream factors recruited downstream components, but they also noticed that downstream factors such as MDC1 could recruit upstream factors, pointing to a feedback loop that might be important for amplification of the initial DDR signal. H2AX was required for the checkpoint response, indicating that chromatin modifications are important for full DDR activation.

Toczyski and colleagues (*Mol. Cell* **30**, 267–276) tethered a different set of DDR proteins to yeast chromatin, those recruited to single-stranded DNA overhangs formed during processing of double-stranded breaks: the damage-specific DNA clamp, called the 9-1-1 complex, and the complex Mec1–Ddc2. Mec1 is the yeast ortholog of the mammalian ATR. It phosphorylates the adaptor molecule Rad9 (an ortholog of 53BP1) at the site of DNA damage, leading to recruitment and activation of the checkpoint kinase Rad53 (a Chk2 ortholog). Tethering of both the 9-1-1 and the Mec1-Ddc2 complexes to a chromatin site could activate the checkpoint responses, with Rad53 phosphorylation and downstream events including cell cycle arrest occurring in the absence of DNA damage. Specific chromatin modifications were required for efficient checkpoint signaling.

Both these papers used an elegant and powerful technique to dissect the events involved in DNA damage signaling and checkpoint responses, and this approach may be useful in studying other protein complexes involved in DNA-dependent processes.

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