and provide strong evidence that targeting the Dll4/Notch pathway may lead to exciting new therapies for clinical investigation.

COMPETING INTERESTS STATEMENT
The author declares that he has no competing financial interests.

1. Hicklin, D.J. & Ellis, L.M. *J. Clin. Oncol.* **23**, 1011–1027 (2005).
2. Noguera-Troise, I. *et al. Nature* **444**, 1032–1037 (2006).
3. Ridgway, J. *et al. Nature* **444**, 1083–1087 (2006).
4. Artavanis-Tsakonas, S., Rand, M.D. & Lake, R.J. *Science* **284**, 770–776 (1999).
5. Shutter, J.R. *et al. Genes Dev.* **14**, 1313–1318 (2000).
6. Gale, N.W. *et al. Proc. Natl. Acad. Sci. USA* **101**, 15949–15954 (2004).
7. Krebs, L.T. *et al. Genes Dev.* **18**, 2469–2473 (2004).
8. Duarte, A. *et al. Genes Dev.* **18**, 2474–2478 (2004).
9. Lawson, N.D., Vogel, A.M. & Weinstein, B.M. *Dev. Cell* **3**, 127–136 (2002).
10. Patel, N.S. *et al. Cancer Res.* **65**, 8690–8697 (2005).
11. Hainaud, P. *et al. Cancer Res.* **66**, 8501–8510 (2006).
12. Jain, R.K. *Science* **307**, 58–62 (2005).

RNA interference has second helpings

Eric A Miska & Julie Ahringer

Efficient RNA interference in *Caenorhabditis elegans* requires a distinct class of secondary short interfering RNA.

Textbook descriptions of RNA interference (RNAi) may have to be rewritten in the wake of recent work by Pak and Fire[1] and Sijen *et al.*[2]. The papers, published in *Science*, indicate that RNAi is in fact a two-step process involving primary short-interfering RNAs (siRNAs), which function as triggers, and secondary siRNAs, which act as executors. Secondary siRNAs arise by unprimed synthesis and are distinct in structure at the 5′ end from primary siRNAs. These reports shed new light on the biology of RNAi and may eventually have implications for future RNAi tools in biotechnology.

In animals and plants, siRNAs are produced by the processing of long double-stranded RNA (dsRNA) by the RNase type III enzyme Dicer into ~21-nucleotide RNAs that have 5′-monophosphate and 3′-hydroxyl groups. These siRNAs are loaded into an RNA-induced silencing complex (RISC), which contains an Argonaute (AGO) protein with a RNase H–like endonuclease activity that directs sequence-specific target RNA cleavage (**Fig. 1**). The Argonaute superfamily is large, and not all members are predicted to have catalytic activity. RDE-1, one of 27 Argonaute proteins in *C. elegans*, is found in a complex with primary siRNAs and is essential for exogenously induced RNAi silencing. The residues essential for catalytic activity in the Argonaute superfamily are conserved in RDE-1, strongly suggesting that RDE-1 is the enzyme responsible for target cleavage.

Although many proteins involved in RNAi were initially studied in the context of experimentally induced RNAi, they are part of a rich biology in many organisms that is generally much less understood and involves other small RNA species (e.g., microRNAs, endogenous siRNAs and Piwi-interacting RNAs) and diverse mechanisms, including transcriptional and post-transcriptional gene silencing, chromatin modification, chromosome segregation and genome editing (see ref. 3 for a review).

Previous work in *C. elegans* and plants suggested that RNAi can be amplified and the targeted region spread to neighboring sequences. This phenomenon was termed 'transitive' RNAi[4]. Whereas in plants spreading is bidirectional, in *C. elegans* it is strongly biased toward the region upstream of the site of interaction with the 'trigger' or primary siRNA on the target RNA[5].

Both Pak & Fire and Sijen *et al.* carry out a detailed analysis of the siRNAs generated by experimentally induced RNAi in *C. elegans*. Pak & Fire chose the standard route of inducing RNAi in *C. elegans* by introducing longer dsRNA, whereas Sijen *et al.* induced RNAi by the more unusual route of a microRNA-like transgene that is expected to give rise to a single siRNA species. Both studies revealed a new class of siRNA, termed secondary siRNA, with unique features.

First, secondary siRNA has a 5′ triphosphate, precluding cloning methods that rely on a single 5′ phosphate. This explains the apparent underrepresentation of this species observed in another large-scale sequencing study carried out by Ruby *et al.*[6]. Second, both studies confirmed previous work that secondary siRNAs are primarily antisense of the target message and that spreading is directionally biased upstream of the trigger sequence. Third, secondary siRNAs appear to be phased with the first siRNA close to the initial siRNA trigger.

These unique characteristics point toward distinct biogenesis pathways for secondary siRNAs and primary exogenous siRNAs. Secondary siRNAs, but not primary siRNAs, require RNA-dependent RNA polymerases (RdRPs), which are present in *C. elegans* and plants but appear to be lacking in *Drosophila melanogaster* and mammals. An RdRP that probably synthesizes secondary siRNA in response to an exogenous RNAi trigger in *C. elegans* is RRF-1, which is required for an efficient RNAi response[2]. The 5′-triphosphate makes it unlikely that RdRPs synthesize secondary siRNAs by priming off of primary siRNAs. Strong evidence for unprimed synthesis comes from the fact that secondary siRNAs overlapping the primary trigger siRNA are perfectly complementary to the target RNA when given mismatched primary siRNA as a trigger[2].

It remains to be determined how secondary siRNAs are made and why they are phased. One explanation for phasing is that only one secondary siRNA is made per round. Possible routes for such synthesis are self-termination of RdRP activity, or more processive RdRP activity coupled with a specific endonuclease or exonuclease that cuts or trims the secondary siRNA to 22 or 23 nt (**Fig. 1**). Dicer is unlikely to be such an endonuclease because it generates 5′-monophosphate rather than 5′-triphosphate groups. The ERI-1 exonuclease, which limits the RNAi response, is an attractive candidate for a trimming activity (**Fig. 1**). Any synthesis route is likely to require additional co-factors, probably including Argonaute proteins.

Primary siRNA is in a complex with the Argonaute RDE-1. However, secondary siRNAs induced by an exogenous RNAi trigger are not found in association with RDE-1 (ref. 1). A recent study in *C. elegans* by Yigit *et al.*[7] suggests that the SAGO-1 and SAGO-2 Argonautes might associate with secondary siRNAs. These Argonautes are rate limiting for RNAi efficiency and lack residues essential for endonuclease activity[7]. As a consequence, secondary RNAi may not lead to direct target cleavage as does primary RNAi, but may instead induce indirect mRNA destabilization by targeting to cellular RNA-degrading enzymes or, alternatively, may induce transcriptional gene silencing (**Fig. 1**).

It is intriguing that secondary siRNAs only spread a few hundred nucleotides from the input trigger. Length-restricted secondary siRNA synthesis could prevent out-of-control

Eric A. Miska and Julie Ahringer are at the Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QN, UK
e-mail: eam29@cam.ac.uk or jaa@mole.bio.cam.ac.uk

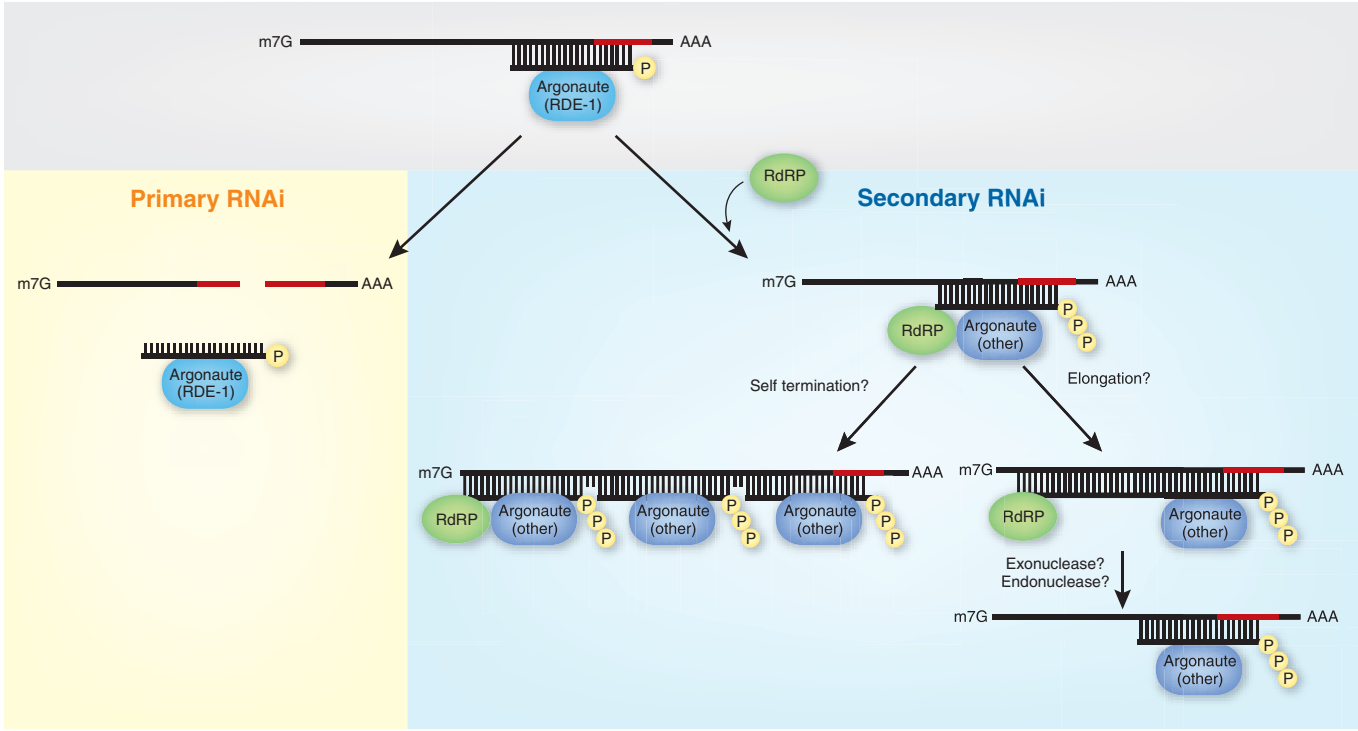

Figure 1 In *C. elegans*, RNAi has two pathways: primary RNAi and secondary RNAi. Primary siRNAs, which are 21–23 nucleotides long and are 5′-monophosphorylated, bind in complex with the Argonaute protein RDE-1 to a target mRNA, resulting in cleavage of the target mRNA cleavage. Secondary siRNAs are synthesized by an RNA-dependent RNA polymerase (RdRP) and are 22 or 23 nucleotides in length. Their length may be determined by self-termination of the RdRP, endonuclease cleavage or exonuclease cleavage. Secondary siRNAs are 5′-triphosphorylated and associate with non-RDE-1 Argonautes. It is unclear whether secondary siRNAs lead to direct target cleavage, indirect destabilization of the target or transcriptional gene silencing.

RNAi amplification. This might be beneficial, as extensive spreading would be more likely to lead to production of secondary siRNAs that could target messages related in sequence. This may be particularly pertinent for *C. elegans*, where exogenous dsRNA induces systemic RNAi[8]. Does the absence of RdRPs in *D. melanogaster* and mammals suggest that these species do not have a secondary RNAi pathway or an alternative one? Perhaps the interferon response in mammals is an alternative mechanism for preventing an excessive RNAi response[9].

Pak & Fire and Sijen *et al.* have introduced some order into the emerging siRNA zoo. Of key importance now is to find out how secondary siRNAs contribute to exogenous RNAi-induced silencing and their roles in endogenous gene regulation. Although it is too early to assess whether secondary RNAi pathways could be exploited as new tools for manipulating gene expression for biotechnology, the elucidation of these pathways should offer insight into this question.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

1. Pak, J. & Fire, A. *Science* **315**, 241–244 (2007).
2. Sijen, T., Steiner, F.A., Thijssen, K.L. & Plasterk, R.H. *Science* **315**, 244–247 (2007).
3. Zamore, P.D. & Haley, B. *Science* **309**, 1519–1524 (2005).
4. Cerutti, H. *Trends Genet.* **19**, 39–46 (2003).
5. Sijen, T. *et al. Cell* **107**, 465–476 (2001).
6. Ruby, J.G. *et al. Cell* **127**, 1193–1207 (2006).
7. Yigit, E. *et al. Cell* **127**, 747–757 (2006).
8. Feinberg, E.H. & Hunter, C.P. *Science* **301**, 1545–1547 (2003).
9. Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H. & Schreiber, R.D. *Annu. Rev. Biochem.* **67**, 227–264 (1998).

Toll-free vaccines?

Arthur M Krieg

Strong immune responses can be activated in the absence of a major immune pathway.

In a recent report in *Nature*, Nemazee and colleagues[1] put forward a proposal that verges on heresy to some immunologists involved in vaccine development. Working with mice, they show that it is possible to stimulate strong antibody responses using an experimental antigen and common vaccine adjuvants without any contribution from Toll-like receptor (TLR) pathways. Although TLR ligands have been an important focus of recent vaccine research, the authors propose that their exclusion from vaccines may avoid unwanted side effects.

Vaccination has been the single greatest success of biomedical science, enabling eradication or control of smallpox, polio and many other diseases. Nevertheless, there is still much room for further development as we have no effective vaccines against malaria, AIDS and many other infectious diseases. Recent research on vaccine development has focused on improving adjuvants (from the Latin *adjuvare,* to

Arthur M. Krieg is at Coley Pharmaceutical Group, Inc., 93 Worcester Street, Suite 101, Wellesley, Massachusetts 02481, USA
e-mail: akrieg@coleypharma.com