

The following resources related to this article are available online at www.sciencemag.org (this information is current as of December 2, 2009):

Updated information and services, including high-resolution figures, can be found in the online version of this article at:

<http://www.sciencemag.org/cgi/content/full/312/5772/452>

Supporting Online Material can be found at:

<http://www.sciencemag.org/cgi/content/full/1125694/DC1>

A list of selected additional articles on the Science Web sites **related to this article** can be found at:

<http://www.sciencemag.org/cgi/content/full/312/5772/452#related-content>

This article **cites 18 articles**, 11 of which can be accessed for free:

<http://www.sciencemag.org/cgi/content/full/312/5772/452#otherarticles>

This article has been **cited by** 108 article(s) on the ISI Web of Science.

This article has been **cited by** 32 articles hosted by HighWire Press; see:

<http://www.sciencemag.org/cgi/content/full/312/5772/452#otherarticles>

This article appears in the following **subject collections**:

Immunology

<http://www.sciencemag.org/cgi/collection/immunology>

Information about obtaining **reprints** of this article or about obtaining **permission to reproduce this article** in whole or in part can be found at:

<http://www.sciencemag.org/about/permissions.dtl>

RNA Interference Directs Innate Immunity Against Viruses in Adult *Drosophila*

Xiao-Hong Wang,^{1*} Roghiyh Aliyari,^{2*} Wan-Xiang Li,² Hong-Wei Li,² Kevin Kim,⁴ Richard Carthew,⁴ Peter Atkinson,³ Shou-Wei Ding^{1,2†}

Innate immunity against bacterial and fungal pathogens is mediated by Toll and immune deficiency (*Imd*) pathways, but little is known about the antiviral response in *Drosophila*. Here, we demonstrate that an RNA interference pathway protects adult flies from infection by two evolutionarily diverse viruses. Our work also describes a molecular framework for the viral immunity, in which viral double-stranded RNA produced during infection acts as the pathogen trigger whereas *Drosophila* Dicer-2 and Argonaute-2 act as host sensor and effector, respectively. These findings establish a *Drosophila* model for studying the innate immunity against viruses in animals.

RNA interference (RNAi) silences gene expression through small interfering RNAs (siRNAs) and microRNAs (miRNAs). In *Drosophila melanogaster* (1), Dicer-2 (*Dcr-2*) produces siRNAs, whereas Dicer-1 (*Dcr-1*) recognizes precursors of miRNAs. The small RNAs are assembled with an Argonaute (*Ago*) protein into related effector complexes, such as RNA-induced silencing complex (RISC), to guide specific RNA silencing (1).

RNA silencing provides an antiviral mechanism in plants and animals (2–6). Plant viruses have evolved diverse strategies for evading the RNA silencing immunity, and expression of viral suppressors of RNAi (VSRs) is essential for infection and virulence (6). However, it is unknown whether antiviral silencing in plants is controlled by a specific small RNA pathway targeted by plant VSRs. Bacterial and fungal infections of *D. melanogaster* induce Toll and immune deficiency (*Imd*) pathways, leading to transcriptional induction of antimicrobial peptide effectors via nuclear factor κ B (NF- κ B)-like signaling processes (7). However, it has been unclear whether either pathway plays a role in *Drosophila* innate immunity against viruses (8, 9). Our previous work in cell culture has indicated that RNAi might mediate viral immunity in *D. melanogaster* (3). Here, we investigated whether RNAi indeed provides protection against virus infection in *Drosophila* embryos and adults.

Flock house virus (FHV) contains an RNA genome (10) divided among two plus-strand molecules, RNAs 1 and 2. RNA2 (R2) encodes

the single virion structural protein, whereas RNA1 (R1) encodes protein A, the viral RNA-dependent RNA polymerase (RdRP), and B2, a VSR (3, 4, 11) expressed after RNA1 replication from its own mRNA, RNA3 (fig. S1). In the absence of R2, R1 replicated autonomously, accumulated to high levels, and produced abundant RNA3 in wild-type (WT) *D. melanogaster* embryos 30 hours after injection with R1 transcripts synthesized in vitro (Fig. 1, lane 2). No FHV RNAs accumulated in WT embryos injected with R1fs transcripts that contain a frameshift mutation in the RdRP open reading frame (ORF) (Fig. 1, lane 1). FHV RNAs were also not readily detected in WT embryos injected with a second mutant of R1, R1 Δ B2, which does not express the VSR (Fig. 1, lane 3). However, abundant accumulation of R1 Δ B2 (Fig. 1, lane 9) but not FR1fs (Fig. 1, lane 7) occurred in mutant *Drosophila* embryos that carried a homozygous null mutation in *ago-2* (*ago-2*¹⁴), which is essential for RNAi in *Drosophila* (1, 12, 13). These data indicated that viral RNA replication in *Drosophila* embryos triggers an RNAi-mediated virus clearance in an *Ago-2*-dependent manner and that effective RNAi suppression by B2 is necessary to achieve normal accumulation of FHV RNAs.

In *Drosophila*, *Ago-2* acts downstream of Dicer-2 (*Dcr-2*) to recruit siRNAs, the products of *Dcr-2* activity, into the siRNA-dependent RISC (siRISC) (1, 14). Thus, a genetic requirement for *ago-2* in FHV RNA clearance implicates *Dcr-2* in the RNAi antiviral effector mechanism. To test this hypothesis, we injected R1, R1fs, and R1 Δ B2 transcripts into embryos carrying a homozygous *dcr-2* null mutation, *dcr-2*^{L811fsX}. Northern blot hybridizations showed that, although FHV RNAs remained undetectable in *dcr-2*^{L811fsX} embryos injected with R1fs (Fig. 1, lane 4), viral RNA accumulation was rescued in the *dcr-2*^{L811fsX} embryos injected with R1 Δ B2 transcripts (Fig. 1, lane 6). This result shows that *Dcr-2* is required to initiate RNAi-mediated clearance of FHV RNAs in *Drosophila* embryos.

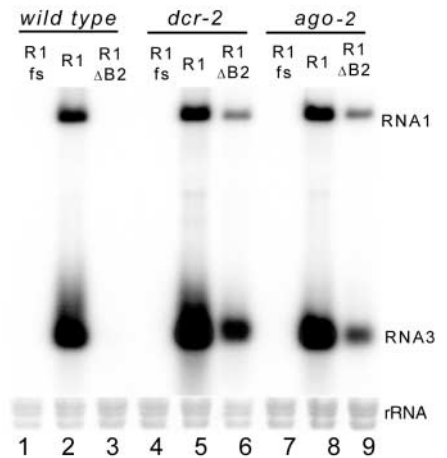


Fig. 1. Antiviral silencing in *Drosophila* embryos requires *Dcr-2* and *Ago-2*. Northern blot detection of FHV RNA accumulation in *wt*, *dcr-2*^{L811fsX}, and *ago-2*¹⁴ embryos microinjected with in vitro synthesized transcripts R1, R1fs, and R1 Δ B2, as shown in fig. S1.

To investigate whether the RNAi pathway protects *Drosophila* from virus infection, we injected adult flies of either WT or *dcr-2*^{L811fsX} genotype with purified FHV virions. The FHV isolate was of low virulence in WT flies, because about 50% of infected flies survived 15 days postinoculation (dpi) (Fig. 2A) despite a detectable virus load (Fig. 2B, lanes 1 to 6). Inoculation with the same dose of FHV resulted in 60% mortality by 6 dpi and 95% by 15 dpi in *dcr-2*^{L811fsX} flies (Fig. 2A). Mock inoculation with buffer had little effect on either WT or *dcr-2*^{L811fsX} flies for as long as observations were made. Both Northern and Western blot analyses revealed that the virus accumulated more rapidly and to much greater levels in *dcr-2*^{L811fsX} than WT flies (Fig. 2, B and C). Thus, *dcr-2* mutants exhibit enhanced disease susceptibility to FHV in comparison with WT flies, demonstrating that *Dcr-2* is also required to mount an immune response that protects adult *Drosophila* against FHV infection.

R2D2 contains tandem double-stranded RNA (dsRNA)-binding domains and forms a heterodimer with *Dcr-2* in vivo that is required for siRNA loading into RISC (1, 15). We found that flies homozygous for a loss-of-function mutation in *r2d2* exhibited a phenotype of enhanced disease susceptibility to FHV infection similar to that of *dcr-2*^{L811fsX} (Fig. 2). Thus, R2D2 also participates in the innate immunity pathway that protects adult flies from FHV infection. Notably, although FHV accumulated to extremely high levels in both *dcr-2* and *r2d2* mutant flies, abundant viral siRNAs were detected only in *r2d2* mutant flies, and viral siRNAs were below the level of detection in *dcr-2*^{L811fsX} flies (Fig. 2D). Thus, FHV infection is detected by *Dcr-2*, leading to production of FHV siRNAs. However, R2D2 is not required for the production but is essential

¹Graduate Program for Microbiology, ²Department of Plant Pathology and Center for Plant Cell Biology, Institute for Integrative Genome Biology, ³Department of Entomology, University of California, Riverside, CA 92521, USA. ⁴Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208, USA.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: dingsw@ucr.edu

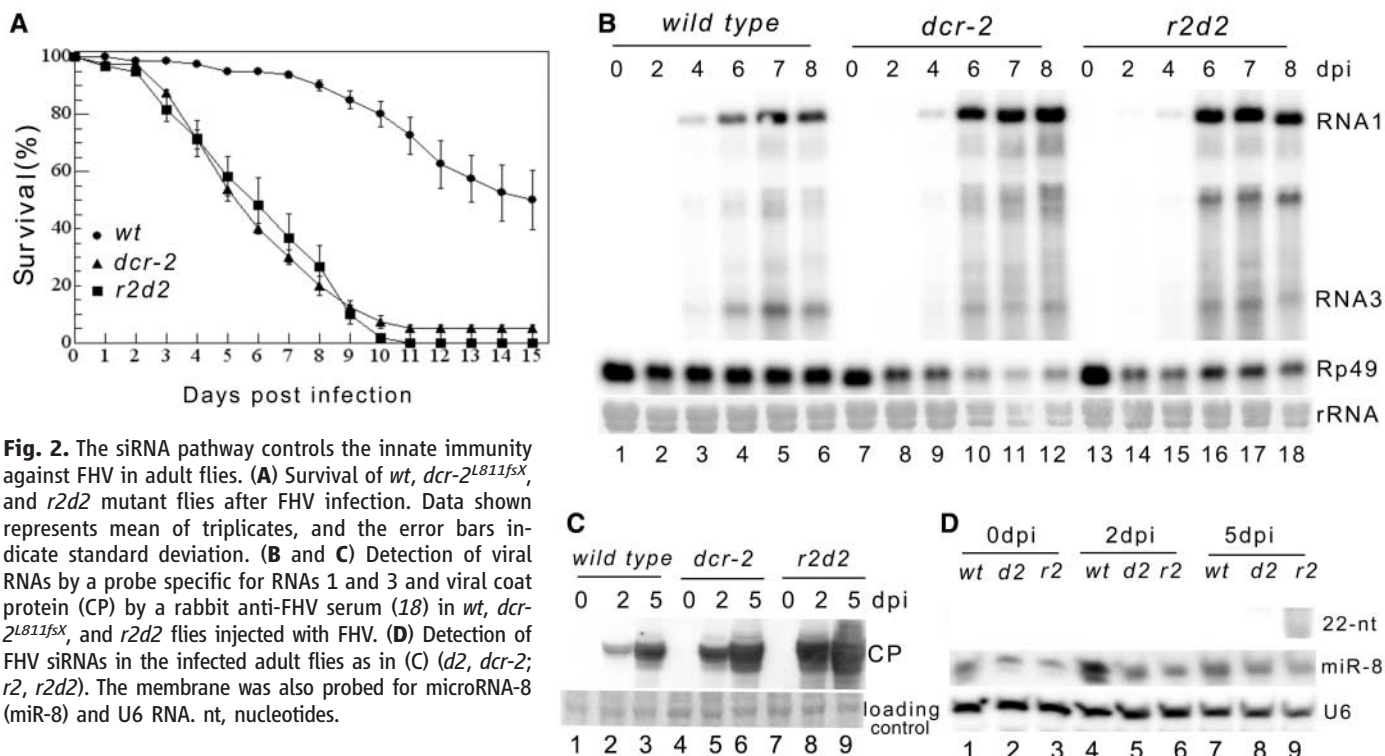


Fig. 2. The siRNA pathway controls the innate immunity against FHV in adult flies. **(A)** Survival of *wt*, *dcr-2^{LS11fsX}*, and *r2d2* mutant flies after FHV infection. Data shown represents mean of triplicates, and the error bars indicate standard deviation. **(B and C)** Detection of viral RNAs by a probe specific for RNAs 1 and 3 and viral coat protein (CP) by a rabbit anti-FHV serum (18) in *wt*, *dcr-2^{LS11fsX}*, and *r2d2* flies injected with FHV. **(D)** Detection of FHV siRNAs in the infected adult flies as in **(C)** (*d2*, *dcr-2*; *r2*, *r2d2*). The membrane was also probed for microRNA-8 (miR-8) and U6 RNA. nt, nucleotides.

for the function of viral siRNAs, which is consistent with the genetic requirements for processing the artificially introduced dsRNA (1, 15).

To investigate whether the RNAi pathway in *Drosophila* is specific against nodaviruses and not other classes of RNA viruses, we assessed the susceptibility of WT, *dcr-2^{LS11fsX}*, and *r2d2* mutant flies to cricket paralysis virus (CrPV). CrPV contains a nonsegmented plus-strand RNA genome but belongs to a group of picorna-like viruses (16). CrPV is substantially more virulent than FHV in *Drosophila*, because injection of CrPV at much lower titers resulted in mortality of 70% of WT flies by 15 dpi (Fig. 3A). We found that CrPV also induced enhanced disease susceptibility in both *dcr-2* and *r2d2* mutant flies (Fig. 3A). About 60% of the infected mutant flies were dead by 6 dpi, and more than 95% were dead by 15 dpi (Fig. 3A). In addition, Northern blots indicated that the virus accumulated more rapidly and to greater levels in the mutant flies (Fig. 3B). Thus, both *dcr-2* and *r2d2* are required for protection of *Drosophila* against CrPV.

CrPV infection of cultured S2 cells induced antiviral silencing, illustrated by detection of CrPV-specific siRNAs (Fig. 4A). Antiviral silencing against FHV in S2 cells induced by FR1gfp as described previously (11) was suppressed by CrPV superinfection, leading to depression of green fluorescent protein (GFP) (Fig. 4B, left). Two ORFs are encoded by the CrPV RNA genome (16) (fig. S2). We did not

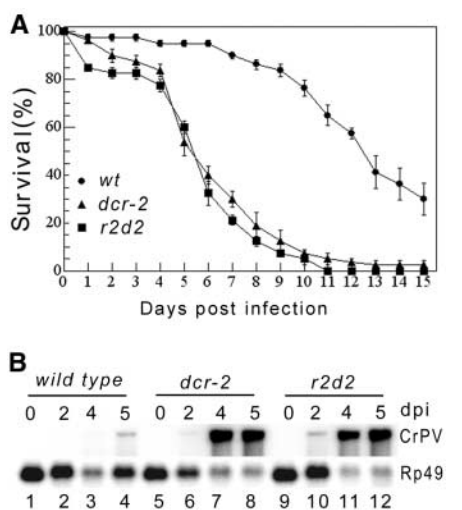


Fig. 3. Mutant *dcr-2* and *r2d2* flies exhibit enhanced disease susceptibility to CrPV. **(A)** Survival of *wt*, *dcr-2^{LS11fsX}*, and *r2d2* mutant flies after CrPV infection. Data shown represents mean of triplicates, and the error bars indicate standard deviation. **(B)** Accumulation of CrPV genomic RNA in WT, *dcr-2^{LS11fsX}*, and *r2d2* mutant adults injected with CrPV.

observe suppression of antiviral silencing in S2 cells cotransfected with a plasmid expressing either the entire downstream ORF of CrPV or the individual mature virion proteins processed from the polyprotein (Fig. 4C, lanes 4 to 10). In contrast, RNAi suppression was detected after cotransfection with a plasmid expressing either

the entire upstream ORF of CrPV or its N-terminal 140 codons (Fig. 4C, pA in lane 1). However, the suppressor activity was not detected after a frameshift mutation was introduced into pA (Fig. 4C, lane 2), thus identifying the N-terminal fragment of 140 amino acids of the CrPV nonstructural polyprotein as a VSR.

In *D. melanogaster*, Imd signaling is stimulated by Gram negative (Gram⁻) bacterial infection, whereas Toll signaling is triggered by Gram positive (Gram⁺) bacterial infection (7, 17). To determine whether loss of the RNAi pathway initiated by Dcr-2 had an impact on the Toll and Imd signaling processes, we subjected WT, *dcr-2^{LS11fsX}*, and *r2d2* mutant flies to immune challenge by inoculation with *Escherichia coli* (Gram⁻) or *Micrococcus luteus* (Gram⁺). Northern blot hybridizations detected substantial transcriptional induction of the antimicrobial peptide gene *Diptericin A* 6 hours postimmune challenge (hpi) with either *E. coli* or *M. luteus*, which declined at 24 hpi (fig. S3) as described (17). Similar induction patterns for *Diptericin A* were observed in *dcr-2^{LS11fsX}* and *r2d2* mutant flies inoculated with Gram⁺ and Gram⁻ bacteria (fig. S3). Furthermore, we found that induction of either *Attacin A* or *Drosomycin* by Gram⁺ and Gram⁻ bacteria was also not altered in *dcr-2^{LS11fsX}* and *r2d2* mutant flies as compared to WT flies (fig. S3). These results indicate that induction of antimicrobial peptide genes via Toll and Imd signaling pathways is not compromised in *dcr-2^{LS11fsX}* and *r2d2* mutant flies.

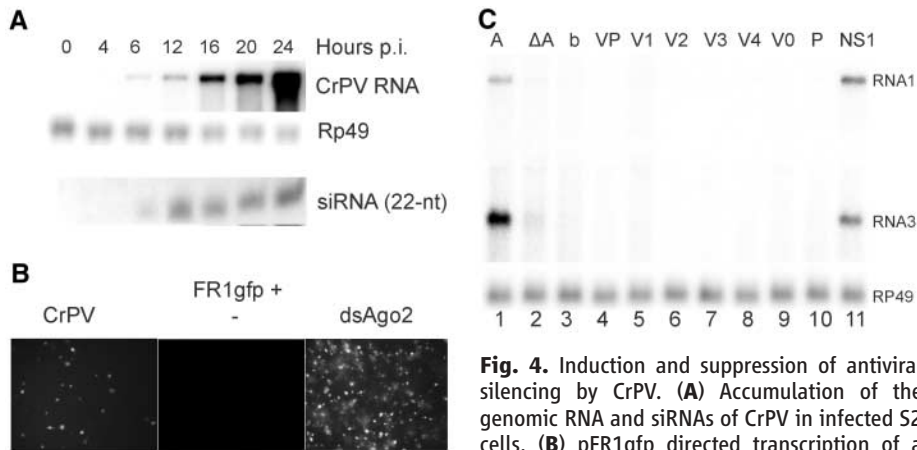


Fig. 4. Induction and suppression of antiviral silencing by CrPV. **(A)** Accumulation of the genomic RNA and siRNAs of CrPV in infected S2 cells. **(B)** pFR1gfp directed transcription of a recombinant FHV RNA1 in which the coding sequence for B2 was replaced by that of GFP. S2 cells were transfected with pFR1gfp alone (middle) or pFR1gfp plus either dsRNA-targeting Ago-2 (right) or CrPV superinfection (left). **(C)** Identification of CrPV RNAi suppressor. Cells were cotransfected with pFR1gfp and a plasmid as indicated on top of each lane (fig. S2), and total RNA was analyzed for the accumulation of pFR1gfp-encoded RNA1 and RNA3. P, the empty plasmid; VP, the virion protein precursor; V1, VP1; V2, VP2; V3, VP3; V4, VP4; V0, VPO (precursor for VP3 and VP4); A, the first 140 codons of the upstream ORF; ΔA, a frameshift mutant of A; and b, the first 107 codons of the upstream ORF. NS1 is an RNAi suppressor of influenza A virus as described previously (11).

Nodaviruses and the polio-like CrPV belong to two different superfamilies of animal RNA viruses. We demonstrate that the same set of RNAi pathway genes is required for *Drosophila* defense against FHV and CrPV and that both viruses encode a potent VSR. These results collectively show that RNAi pathway functions as a common viral immunity mechanism in *Drosophila* and that RNAi suppression represents a general counterdefensive strategy used by insect viruses. Furthermore, a genetic requirement for Dcr-2, R2D2, and Ago-2 in antiviral silencing establishes a molecular framework for the innate immunity against viruses in *D. melanogaster*. None of Dcr-2, R2D2, and Ago-2 plays a detectable role in either the production or function of miRNAs in *D. melanogaster* (1). Thus, our work identifies the dsRNA-siRNA pathway of RNAi as providing the innate immunity against virus infection in *Drosophila* and establishes

that dsRNA produced during virus replication acts as the pathogen trigger whereas Dcr-2 and Ago-2 act as host sensor and effector of the immunity, respectively. These results support and extend the previous findings on antiviral silencing in *C. elegans* (4, 5).

Although NF- κ B-like signaling in the Toll and Imd pathways do not appear to play a role in the RNAi-directed viral immunity mechanism in *D. melanogaster*, the fly mutant defective in the Janus kinase (JAK) Hopscotch exhibited a modest increase in susceptibility to infection with *Drosophila C* virus, suggesting an antiviral role for JAK-signal transducer and activator of transcription (STAT) signaling (8). Nonetheless, we believe that RNAi-based immunity is independent of JAK-STAT signaling, because virus infection is not known to induce the RNAi pathway in *Drosophila* (8) and FHV induction of the JAK-STAT respon-

sive gene *vir-1* was unaltered in the *dcr-2* and *r2d2* mutants, as shown by our recent work. Because the Toll and Imd pathways are highly conserved in vertebrates (7), the *Drosophila* model established for RNAi may also be useful for the analyses of the innate antiviral immunity in vertebrates.

References and Notes

1. Y. Tomari, P. D. Zamore, *Genes Dev.* **19**, 517 (2005).
2. A. J. Hamilton, D. C. Baulcombe, *Science* **286**, 950 (1999).
3. H. W. Li, W. X. Li, S. W. Ding, *Science* **296**, 1319 (2002).
4. R. Lu *et al.*, *Nature* **436**, 1040 (2005).
5. C. Wilkins *et al.*, *Nature* **436**, 1044 (2005).
6. O. Voinnet, *Nat. Rev. Genet.* **6**, 206 (2005).
7. J. A. Hoffmann, *Nature* **426**, 33 (2003).
8. C. Dostert *et al.*, *Nat. Immunol.* **6**, 946 (2005).
9. R. A. Zambon, M. Nandakumar, V. N. Vakharia, L. P. Wu, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 7257 (2005).
10. L. A. Ball, K. L. Johnson, *Adv. Virus Res.* **53**, 229 (1999).
11. W. X. Li *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 1350 (2004).
12. S. M. Hammond, S. Boettcher, A. A. Caudy, R. Kobayashi, G. J. Hannon, *Science* **293**, 1146 (2001).
13. K. Okamura, A. Ishizuka, H. Siomi, M. C. Siomi, *Genes Dev.* **18**, 1655 (2004).
14. Y. S. Lee *et al.*, *Cell* **117**, 69 (2004).
15. Q. Liu *et al.*, *Science* **301**, 1921 (2003).
16. J. E. Wilson, M. J. Powell, S. E. Hoover, P. Sarnow, *Mol. Cell. Biol.* **20**, 4990 (2000).
17. P. Irving *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 15119 (2001).
18. X. F. Dong, P. Natarajan, M. Tihova, J. E. Johnson, A. Schneemann, *J. Virol.* **72**, 6024 (1998).
19. We thank J. L. Imler for a stimulating discussion, A. Schneemann for providing CrPV and the anti-FHV serum, A. Raikhel for the bacterial strains, and members of the Ding and the Atkinson laboratories for comments and technical assistance. This project was supported by NIH grant AI052447 and U.S. Department of Agriculture grant 2005-35319-15331 (to S.W.D.). Partial support for this work was provided by NIH grants (to P.A. and R.W.C.).

Supporting Online Material

www.sciencemag.org/cgi/content/full/1125694/DC1
 Materials and Methods
 Figs. S1 to S3
 References

1 February 2006; accepted 14 March 2006
 Published online 23 March 2006;
 10.1126/science.1125694
 Include this information when citing this paper.