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Modulation of Flavivirus Population Diversity by RNA Interference

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To test the hypothesis that RNA interference (RNAi) imposes diversifying selection on RNA virus genomes, we quantified West Nile virus (WNV) quasispecies diversity after passage in *Drosophila* cells in which RNAi was left intact, depleted, or stimulated against WNV. As predicted, WNV diversity was significantly lower in RNAi-depleted cells and significantly greater in RNAi-stimulated cells relative to that in controls. These findings reveal that an innate immune defense can shape viral population structure.

Within hosts, RNA viruses exist as a dynamic population of closely related viral variants termed a quasispecies. Because quasispecies structure affects both pathogenesis and therapeutic responsiveness, considerable attention has been devoted to identifying host responses that shape quasispecies diversity (1, 2). To date, the majority of this work has focused on the impact of vertebrate adaptive immune responses on virus diversification, particularly with regard to single host viruses that establish chronic infections (e.g., HIV and hepatitis C virus) (3–7). Arthropod-borne viruses, such as West Nile virus (WNV [genus *Flavivirus*]), transiently infect vertebrate hosts but establish chronic infections in invertebrate vectors that lack adaptive immunity. Yet it is known that arthropod vectors can still contribute to flavivirus quasispecies complexity (8, 9). Arthropods defend against viral infections through several innate immune mechanisms, the most important of which is the exogenous small interfering RNA (exo-siRNA) pathway (10). This evolutionarily conserved pathway is highly sequence specific, and single nucleotide mismatches between the siRNA and target sequence, as well as mutations that alter secondary structure in the targeted sequence, can reduce or abolish silencing efficacy (11, 12). Both *Drosophila melanogaster* and mosquitoes lacking a functional exo-siRNA pathway are significantly more susceptible and often succumb to viral infection (13–16). Direct exo-siRNA targeting of the viral RNA genome has been demonstrated for WNV in infected mosquitoes (17). Furthermore, it has been observed that increased mutational diversity is associated with intense exo-siRNA-mediated targeting of the WNV genome in mosquitoes and that highly genetically diverse WNV populations are more fit in mosquitoes (17, 18). Together, these findings suggest that due to its potency and sequence specificity, the RNAi pathway may shape the rate and mode of viral evolution. Therefore, we directly assessed the impact of the exo-siRNA pathway on shaping WNV populations passaged on cells with either an intact, depleted, or stimulated exo-siRNA pathway.

To accomplish this, *Drosophila* S2 cells were treated with a control double-stranded RNA (dsRNA), dsLuc, a dsRNA specific for one of two major components of the exo-siRNA pathway, Dicer-2 (dsDcr2) and Argonaute-2 (dsAgo2), or with anti-WNV dsRNA (dsRNA directed to the same region of the genome as the sequencing amplicons), using the soaking method (Tables 1 and 2) (19–22). Using this approach, we were able to achieve ~80% suppression of each gene. One-step growth curve analysis of WNV (derived from infectious clone NY99 [23]) following a triple treatment of dsRNA (16 h prior to infection, 1 h postinfection, and 3 days postinfection [dpi]) revealed no significant difference in WNV titers between the dsDcr2 and dsAgo2 and the dsLuc control group, consistent with previously published findings (20). Conversely, when WNV-specific dsRNA was administered to the cells, WNV titers were significantly reduced at early time points but eventually rebounded to control-treated levels by 6 dpi (data not

TABLE 1 List of primers used in this study

Primer[a]	Sequence (5′→3′)
T7-Ago2 F	TAATAC GACTCA CTATAG GGG ATT ATG AA CTTG CTG CAATAC
T7-Ago2 R	TAATAC GACTCA CTATAG GGC ACATCG GCT CCA ATG TAC ATG
T7-Dcr2 F	TAATAC GACTCA CTATAG GGG ATT TTG AAG ATA AGG AAT AC
T7-Dcr2R	TAATAC GACTCA CTATAG GGCTCC ACG AAG CGGTTG TAGTTG
Ago2 QPCR F	ATT GCGTCC TACTTC CAC AG
Ago2 QPCR R	GCT GCGTACTTT ATC ATATTG GC
Dcr2 QPCR F	GCC CAA AAC ATT AAA GGA GCG
Dcr2 QPCR R	AAC AGATTT CAC CTA CCC GC
Rrp49 QPCR F	TAC AGG CCC AAG ATC GTG AA
Rrp49 QPCR R	ACC GTT GGG GTT GGT GAG

[a] Primer orientation: F, forward; R, reverse.

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TABLE 2 Sequences of A and B adaptors, Roche Barcode identifiers, and WNV primers

Adaptor sequence[a]	Extended multiplex identifier set sequences[b]	WNV primer sequence[c]
A: CGTATCGCCTCCCTCGCGCCATCAG	ACGAGTGCGT	WNV F: GAGCTGACAAACTTAGTAGTGTTTG
B: CTATGCGCCTTGCCAGCCCGCTCAG	ACGCTCGACA	WNV R: CCGTCATCATCACCTTCCCTTGGAAG
	CATAGTAGTG	
	AGCACTGTAG	
	ATCAGACACG	
	ATATCGCGAG	
	CGTGTCTCTA	
	CTCGCGTGTC	
	TAGTATCAGC	
	CGAGAGATAC	
	ATACGACGTA	
	TCACGTACTA	
	CGTCTAGTAC	
	TCTACGTAGC	
	TGTACTACTC	
	ACGACTACAG	
	CGTAGACTAG	
	TACGAGTATG	

[a] Barcodes A and B were added to the 5′ end of every forward (F) and reverse (R) amplicon primer, respectively.
[b] Roche Barcode identifiers were added 3′ of the adaptors so that samples could be pooled.
[c] Viral priming sequences found at the 3′ end of the amplicon primers.

shown). Because we had previously demonstrated the time course of effective dsRNA-mediated silencing of Dcr2 and Ago2 protein levels in S2 cells, suppression was confirmed by quantitative reverse transcription-PCR (qRT-PCR) in these studies (24).

Subsequently, we examined the effect of knockdown on WNV population diversity. A 438-bp amplicon spanning the 5′ untranslated region (UTR)-capsid-premembrane of WNV from populations produced after a single round of infection or five passages in

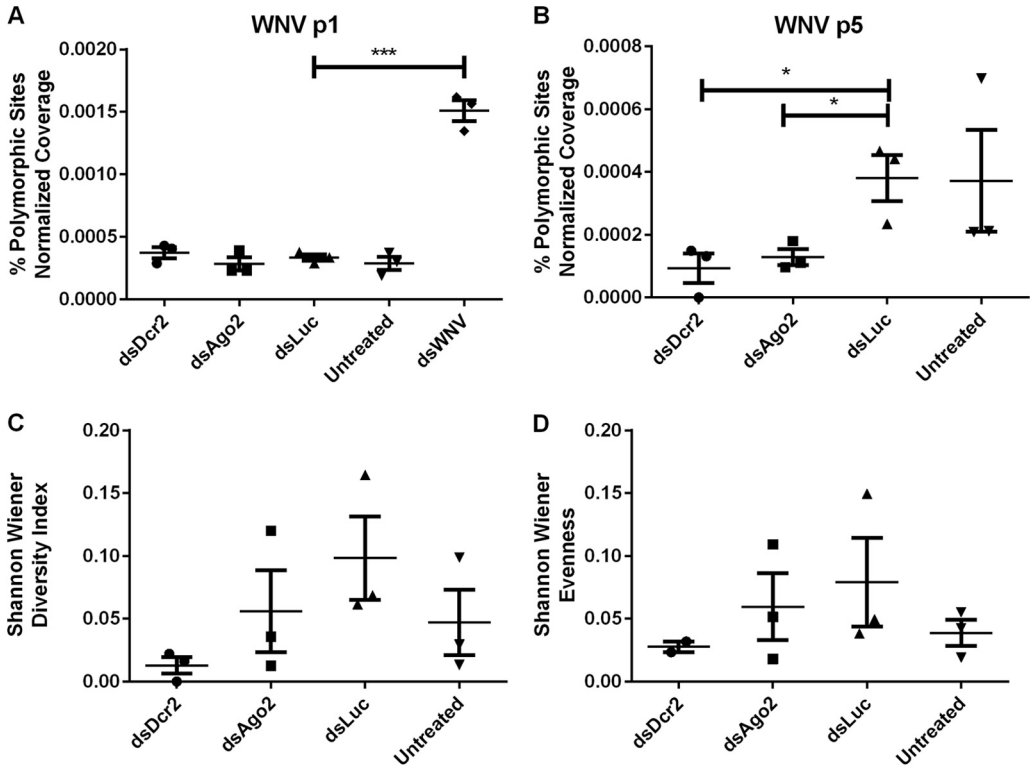

FIG 1 WNV quasispecies diversity is reduced in RNAi-depleted cells and enhanced in cells pretreated with WNV dsRNA. Shown are the percentages of polymorphic sites normalized to read coverage during WNV passage 1 (p1) (A) and WNV passage 5 (p5) (B) in designated treatments. (C and D) Shannon-Wiener diversity (C) and evenness (D) of WNV p5 samples after passage in RNAi-depleted cells. Values represent the mean ± standard error of the mean (SEM) from three independent replicates, with the exception of the dsDcr2 evenness group (D), in which one of the samples contained only one haplotype, and therefore the evenness was undefined. *, $P < 0.05$, and ***, $P < 0.001$, by two-tailed unpaired *t* test individually comparing experimental versus control treatments.

TABLE 3 Sample and sequencing characteristics and V-phaser output

Sample	No. of passages	Harvest time (dpi)[a]	Titer (log_{10} PFU/ml)	Total no. of reads	No. of polymorphic sites[b]	Haplotype	% nucleotide diversity[c]
WNV plasmid	NA[d]	NA	NA	2,331	0	NA	0
WNV transcript	NA	NA	NA	1,138	0	NA	0
Input	NA	NA	6.30	682	0	NA	0
dsAgo2 r1	5	6	5.00	2,067	1	2	0.0013
dsAgo2 r2	5	6	5.20	2,538	2	3	0.0055
dsAgo2 r3	5	6	4.65	2,376	1	2	0.0004
dsDcr2 r1	5	6	2.90	1,536	1	2	0.0007
dsDcr2 r2	5	6	4.11	1,732	1	2	0.0005
dsDcr2 r3	5	6	4.61	1,096	0	1	0
dsLuc r1	5	6	3.20	1,552	3	4	0.0024
dsLuc r2	5	6	4.47	1,948	2	3	0.0082
dsLuc r3	5	6	2.60	3,427	7	5	0.0019
Untreated	5	6	4.32	2,167	2	2	0.0008
	5	6	4.48	1,638	5	6	0.0033
	5	6	3.90	1,097	1	2	0.0010
WNV plasmid	NA	NA	NA	10,159	4	ND[e]	0.0009
WNV transcript	NA	NA	NA	12,698	19	ND	0.0019
Input	NA	NA	4.22	9,525	17	ND	0.0039
dsWNV r1	1	3	2.95	2,712	16	ND	0.0067
dsWNV r2	1	3	2.47	12,535	86	ND	0.0218
dsWNV r3	1	3	2.47	15,943	113	ND	0.0292
dsIrr r1	1	3	5.04	8,447	14	ND	0.0059
dIrr r2	1	3	4.95	14,922	22	ND	0.0075
dsIrr r3	1	3	5.11	2,365	3	ND	0.0019
dsDcr2 r1	1	3	5.07	11,452	18	ND	0.0048
dsDcr2 r2	1	3	4.84	9,007	16	ND	0.0041
dsDcr2 r3	1	3	5.07	11,165	21	ND	0.0062
dsAgo2 r1	1	3	5.20	9,913	17	ND	0.0036
dsAgo2 r2	1	3	4.95	15,808	16	ND	0.0050
dsAgo2 r3	1	3	5.11	17,666	18	ND	0.0054
Untreated	1	3	5.27	15,562	13	ND	0.0043
	1	3	5.34	4,295	7	ND	0.0023
	1	3	4.95	14,419	19	ND	0.0038

[a] Samples were harvested on the days postinfection (dpi) shown.
[b] Number of polymorphic sites that were not present in the input.
[c] Percentage of nucleotide diversity present at SNPs not found in the input.
[d] NA, not applicable.
[e] ND, not determined.

RNA interference (RNAi)-depleted and RNAi-intact S2 cells were sequenced by pyrosequencing. Using the highly sensitive viral variant caller V-phaser (25), we quantified two diversity estimates: the percentage of polymorphic sites normalized to sequencing coverage and the Shannon-Wiener diversity (*H*) and evenness (*E*) estimates. No differences in percentages of polymorphic sites were observed for populations of WNV derived from a single passage (p1) in Dcr2- or Ago2-depleted cells (WNV p1) (Fig. 1A). However, when passaged five times (p5), WNV populations from cells treated with dsDcr2 and dsAgo2 showed significant reductions in the percentage of polymorphic sites compared to populations passaged in cells treated with dsLuc (Dcr2, $P = 0.03$; Ago2, $P = 0.03$) (Fig. 1B). Additionally, WNV populations passaged in dsDcr2- and dsAgo2-treated cells were lower in both diversity and evenness than the dsLuc controls, but these differences were not significant (Fig. 1C and D, respectively). Baseline error rates for the

TABLE 4 WNV p1 Shannon Wiener diversity and evenness and percentage of unique clone estimates determined by manual alignment and analysis

Sample	No. of reads analyzed[a]	% of unique haplotypes[b]	H[c]	E[d]
WNV plasmid	1,011	2.0	0.16	0.05
WNV transcript	1,008	3.5	0.30	0.08
Input	1,008	3.9	0.34	0.09
dsAgo2 r1	1,018	2.7	0.25	0.08
dsAgo2 r2	1,007	2.1	0.26	0.09
dsAgo2 r3	1,006	3.3	0.30	0.09
dsDcr2 r1	1,004	1.6	0.14	0.05
dsDcr2 r2	1,011	3.3	0.31	0.09
dsDcr2 r3	1,002	2.7	0.25	0.08
dsLuc r1	1,001	4.9	0.51	0.13
dsLuc r2	1,007	3.7	0.38	0.10
dsLuc r3	1,008	3.0	0.29	0.09
Untreated	1,005	2.9	0.23	0.07
	1,001	2.6	0.26	0.08
	1,008	2.2	0.19	0.06
dsWNV r1	1,004	8.0	0.99	0.23
dsWNV r2	1,006	5.9	0.63	0.16
dsWNV r3	1,003	8.0	0.79	0.18

[a] Number of reads per subset used in determining the diversity estimates.
[b] Percentage of mutant haplotypes calculated as (no. of haplotypes carrying at least 1 mutation relative to the most common haplotype/no. of reads) × 100.
[c] Shannon Weiner diversity (H) calculated as the absolute value of $\sum p_i (\ln p_i)$, where i signifies a unique haplotype.
[d] Shannon Weiner evenness (E) calculated as H/ln(no. of haplotypes).

amplicon preparation and sequencing controls, which were substantially lower than those of the WNV populations themselves, are presented in Tables 3 and 4.

The differences between WNV p1 and WNV p5 are consistent with our previous findings that the selective pressures of RNAi have a cumulative effect that is detectable only after viruses have experienced relatively long (14-day) exposure to RNAi (17). As with any study employing RNAi-mediated knockdown, the potential for off-target effects must be considered (26, 27); however, because we used long dsRNA, we judge it unlikely that the observed results are attributable to off-target effects (28). Another possible confounder is that suppression of RNAi may have affected other host factors that in turn influenced quasispecies diversity. For instance, it has recently been shown that the expression of Vago, a protein that suppresses WNV replication in mosquito cells, is dependent on Dcr2 binding to dsRNA (29, 30). Interestingly, the same studies found that suppression of Ago2 did not affect the expression levels of Vago (29). The fact that we observed significantly reduced quasispecies diversity in WNV passaged in both Dcr2- and Ago2-depleted cells supports the conclusion that the exo-siRNA pathway directly modulates the complexity of the viral mutant swarm.

We also predicted that stimulation of RNAi should increase quasispecies diversity. As predicted, we observed a significant increase in polymorphic sites in WNV populations from the cells treated with dsWNV (3 dpi, p1) compared to the control ($P = 0.0002$) (Fig. 1A). The Shannon-Wiener diversity and evenness of WNV from dsWNV-treated cells were also significantly greater than those of the control (Table 2). These data support previous findings that targeting viral genomes with siRNAs can lead to the rapid emergence of escape mutants containing mutations within the complementary sequence (31, 32).

Thus, the results of this study reveal for the first time, a mechanism by which the innate immune system directly influences quasispecies diversity of an RNA virus. In light of the controversy surrounding two recent papers that reported an antiviral effect of RNAi in undifferentiated mouse and hamster cells and suckling mice, it would be particularly interesting to monitor WNV quasispecies development in HEK-293 and Dicer-deficient HEK-293 cells to determine whether patterns in quasispecies diversification differed from those observed in the RNAi-intact and RNAi-depleted insect cells utilized in this study (33–36).

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REFERENCES

1. **Miura M, Maekawa S, Takano S, Komatsu N, Tatsumi A, Asakawa Y, Shindo K, Amemiya F, Nakayama Y, Inoue T, Sakamoto M, Yamashita A, Moriishi K, Enomoto N.** 2013. Deep-sequencing analysis of the association between the quasispecies nature of the hepatitis C virus core region and disease progression. J Virol **87:**12541–12551. http://dx.doi.org/10.1128/JVI.00826-13.
2. **Perales C, Iranzo J, Manrubia SC, Domingo E.** 2012. The impact of quasispecies dynamics on the use of therapeutics. Trends Microbiol **20:**595–603. http://dx.doi.org/10.1016/j.tim.2012.08.010.
3. **Plauzolles A, Lucas M, Gaudieri S.** 2013. Hepatitis C virus adaptation to T-cell immune pressure. ScientificWorldJournal **2013:**673240. http://dx.doi.org/10.1155/2013/673240.
4. **Woo HJ, Reifman J.** 2012. A quantitative quasispecies theory-based model of virus escape mutation under immune selection. Proc Natl Acad Sci U S A **109:**12980–12985. http://dx.doi.org/10.1073/pnas.1117201109.
5. **Cale EM, Hraber P, Giorgi EE, Fischer W, Bhattacharya T, Leitner T, Yeh WW, Gleasner C, Green LD, Han CS, Korber B, Letvin NL.** 2011. Epitope-specific $CD8^+$ T lymphocytes cross-recognize mutant simian immunodeficiency virus (SIV) sequences but fail to contain very early evolution and eventual fixation of epitope escape mutations during SIV infection. J Virol **85:**3746–3757. http://dx.doi.org/10.1128/JVI.02420-10.
6. **Erickson AL, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, McKinney D, Sette A, Hughes AL, Walker CM.** 2001. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. Immunity **15:**883–895. http://dx.doi.org/10.1016/S1074-7613(01)00245-X.
7. **Valkenburg SA, Quiñones-Parra S, Gras S, Komadina N, McVernon J, Wang Z, Halim H, Iannello P, Cole C, Laurie K, Kelso A, Rossjohn J, Doherty PC, Turner SJ, Kedzierska K.** 2013. Acute emergence and reversion of influenza A virus quasispecies within $CD8^+$ T cell antigenic peptides. Nat Commun **4:**2663. http://dx.doi.org/10.1038/ncomms3663.
8. **Jerzak G, Bernard KA, Kramer LD, Ebel GD.** 2005. Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests

quasispecies structure and strong purifying selection. J Gen Virol **86:** 2175–2183. http://dx.doi.org/10.1099/vir.0.81015-0.
9. **Jerzak GVS, Bernard K, Kramer LD, Shi PY, Ebel GD.** 2007. The West Nile virus-mutant spectrum is host-dependent and a determinant of mortality in mice. Virology **360:**469–476. http://dx.doi.org/10.1016/j.virol.2006.10.029.
10. **Prasad A, Brackney D, Ebel G.** 2013. The role of innate immunity in conditioning mosquito susceptibility to West Nile virus. Viruses **5:**3142–3170. http://dx.doi.org/10.3390/v5123142.
11. **Pusch O, Boden D, Silbermann R, Lee F, Tucker L, Ramratnam B.** 2003. Nucleotide sequence homology requirements of HIV-1-specific short hairpin RNA. Nucleic Acids Res **31:**6444–6449. http://dx.doi.org/10.1093/nar/gkg876.
12. **Westerhout EM, Ooms M, Vink M, Das AT, Berkhout B.** 2005. HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. Nucleic Acids Res **33:**796–804. http://dx.doi.org/10.1093/nar/gki220.
13. **Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, Imler J-L.** 2006. Essential function *in vivo* for Dicer-2 in host defense against RNA viruses in *Drosophila*. Nat Immunol **7:**590–597. http://dx.doi.org/10.1038/ni1335.
14. **Keene KM, Foy BD, Sanchez-Vargas I, Beaty BJ, Blair CD, Olson KE.** 2004. RNA interference acts as a natural antiviral response to O'nyong-nyong virus (*Alphavirus*; *Togaviridae*) infection of *Anopheles gambiae*. Proc Natl Acad Sci U S A **101:**17240–17245. http://dx.doi.org/10.1073/pnas.0406983101.
15. **Myles KM, Wiley MR, Morazzani EM, Adelman ZN.** 2008. Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. Proc Natl Acad Sci U S A **105:**19938–19943. http://dx.doi.org/10.1073/pnas.0803408105.
16. **Wang XH, Aliyari R, Li WX, Li HW, Kim K, Carthew R, Atkinson P, Ding SW.** 2006. RNA interference directs innate immunity against viruses in adult *Drosophila*. Science **312:**452–454. http://dx.doi.org/10.1126/science.1125694.
17. **Brackney DE, Beane JE, Ebel GD.** 2009. RNAi targeting of West Nile virus in mosquito midguts promotes virus diversification. PLoS Pathog **5:**e1000502. http://dx.doi.org/10.1371/journal.ppat.1000502.
18. **Fitzpatrick KA, Deardorff ER, Pesko K, Brackney DE, Zhang B, Bedrick E, Shi PY, Ebel GD.** 2010. Population variation of West Nile virus confers a host-specific fitness benefit in mosquitoes. Virology **404:**89–95. http://dx.doi.org/10.1016/j.virol.2010.04.029.
19. **Feinberg EH, Hunter CP.** 2003. Transport of dsRNA into cells by the transmembrane protein SID-1. Science **301:**1545–1547. http://dx.doi.org/10.1126/science.1087117.
20. **Chotkowski HL, Ciota AT, Jia Y, Puig-Basagoiti F, Kramer LD, Shi PY, Glaser RL.** 2008. West Nile virus infection of *Drosophila melanogaster* induces a protective RNAi response. Virology **377:**197–206. http://dx.doi.org/10.1016/j.virol.2008.04.021.
21. **Rogers SL, Rogers GC.** 2008. Culture of *Drosophila* S2 cells and their use for RNAi-mediated loss-of-function studies and immunofluorescence microscopy. Nat Protoc **3:**606–611. http://dx.doi.org/10.1038/nprot.2008.18.
22. **Xu J, Cherry S.** 2014. Viruses and antiviral immunity in *Drosophila*. Dev Comp Immunol **42:**67–84. http://dx.doi.org/10.1016/j.dci.2013.05.002.
23. **Shi PY, Tilgner M, Lo MK, Kent KA, Bernard KA.** 2002. Infectious cDNA clone of the epidemic West Nile virus from New York City. J Virol **76:**5847–5856. http://dx.doi.org/10.1128/JVI.76.12.5847-5856.2002.
24. **Mukherjee S, Hanley K.** 2010. RNA interference modulates replication of dengue virus in *Drosophila melanogaster* cells. BMC Microbiol **10:**127. http://dx.doi.org/10.1186/1471-2180-10-127.
25. **Macalalad AR, Zody MC, Charlebois P, Lennon NJ, Newman RM, Malboeuf CM, Ryan EM, Boutwell CL, Power KA, Brackney DE, Pesko KN, Levin JZ, Ebel GD, Allen TM, Birren BW, Henn MR.** 2012. Highly sensitive and specific detection of rare variants in mixed viral populations from massively parallel sequence data. PLoS Comput Biol **8:**e1002417. http://dx.doi.org/10.1371/journal.pcbi.1002417.
26. **Aleman LM, Doench J, Sharp PA.** 2007. Comparison of siRNA-induced off-target RNA and protein effects. RNA **13:**385–395. http://dx.doi.org/10.1261/rna.352507.
27. **Jackson AL, Burchard J, Schelter J, Chau BN, Cleary M, Lim L, Linsley PS.** 2006. Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. RNA **12:**1179–1187. http://dx.doi.org/10.1261/rna.25706.
28. **Myers JW, Chi JT, Gong D, Schaner ME, Brown PO, Ferrell JE.** 2006. Minimizing off-target effects by using diced siRNAs for RNA interference. J RNAi Gene Silencing **2:**181–194.
29. **Deddouche S, Matt N, Budd A, Mueller S, Kemp C, Galiana-Arnoux D, Dostert C, Antoniewski C, Hoffmann JA, Imler J-L.** 2008. The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in *Drosophila*. Nat Immunol **9:**1425–1432. http://dx.doi.org/10.1038/ni.1664.
30. **Paradkar PN, Trinidad L, Voysey R, Duchemin J-B, Walker PJ.** 2012. Secreted Vago restricts West Nile virus infection in *Culex* mosquito cells by activating the Jak-STAT pathway. Proc Natl Acad Sci U S A **109:**18915–18920. http://dx.doi.org/10.1073/pnas.1205231109.
31. **Boden D, Pusch O, Lee F, Tucker L, Ramratnam B.** 2003. Human immunodeficiency virus type 1 escape from RNA interference. J Virol **77:**11531–11535. http://dx.doi.org/10.1128/JVI.77.21.11531-11535.2003.
32. **Gitlin L, Stone JK, Andino R.** 2005. Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches. J Virol **79:**1027–1035. http://dx.doi.org/10.1128/JVI.79.2.1027-1035.2005.
33. **Bogerd HP, Skalsky RL, Kennedy EM, Furuse Y, Whisnant AW, Flores O, Schultz KL, Putnam N, Barrows NJ, Sherry B, Scholle F, Garcia-Blanco MA, Griffin DE, Cullen BR.** 2014. Replication of many human viruses is refractory to inhibition by endogenous cellular microRNAs. J Virol **88:**8065–8076. http://dx.doi.org/10.1128/JVI.00985-14.
34. **Cullen BR, Cherry S, ten Oever BR.** 2013. Is RNA interference a physiologically relevant innate antiviral immune response in mammals? Cell Host Microbe **14:**374–378. http://dx.doi.org/10.1016/j.chom.2013.09.011.
35. **Li Y, Lu J, Han Y, Fan X, Ding SW.** 2013. RNA interference functions as an antiviral immunity mechanism in mammals. Science **342:**231–234. http://dx.doi.org/10.1126/science.1241911.
36. **Maillard PV, Ciaudo C, Marchais A, Li Y, Jay F, Ding SW, Voinnet O.** 2013. Antiviral RNA interference in mammalian cells. Science **342:**235–238. http://dx.doi.org/10.1126/science.1241930.