RNase III nucleases from diverse kingdoms serve as antiviral effectors

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In contrast to the DNA-based viruses in prokaryotes, the emergence of eukaryotes provided the necessary compartmentalization and membranous environment for RNA viruses to flourish, creating the need for an RNA-targeting antiviral system1,2. Present day eukaryotes employ at least two main defence strategies that emerged as a result of this viral shift, namely antiviral RNA interference and the interferon system2. Here we demonstrate that Drosha and related RNase III ribonucleases from all three domains of life also elicit a unique RNA-targeting antiviral activity. Systemic evolution of ligands by exponential enrichment of this class of proteins illustrates the recognition of unbranched RNA stem loops. Biochemical analyses reveal that, in this context, Drosha functions as an antiviral clamp, confling steric hindrance on the RNA-dependent RNA polymerases of diverse positive-stranded RNA viruses. We present evidence for cytoplasmic translocation of RNase III nucleases in response to virus in diverse eukaryotes including plants, arthropods, fish, and mammals. These data implicate RNase III recognition of viral RNA as an antiviral defence that is independent of, and possibly predates, other known eukaryotic antiviral systems.

Life, as far back as self-replicating genetic material, demanded the capacity to generate and maintain sufficient diversity to allow for adaptation and evolution. In the approximately 4 billion years that followed, self-replicating genetic entities, such as viruses, co-evolved with early cellular life, creating an unremitting host–pathogen arms race6–8. From prokaryotic systems such as clustered regularly interspaced short palindromic repeats (CRISPR) to the RNA interference (RNAi) systems of plants and invertebrates, many diverse antiviral strategies have proved successful in combating viral pathogens. As vertebrates transitioned from RNAi to interferon (IFN), remnants of this past system remained, including the two catalytically active RNase III members, Drosha and Dicer, both of which are critical for the biogenesis of microRNAs (miRNAs), a regulatory system designed on the same principles and machinery as antiviral RNAi3.

As miRNA biology stemmed from the development of RNAi4, but was not subjected to the selective pressures imposed by the biological arms race between host and pathogen, these components probably reflect the early eukaryotic RNAi machinery. This idea is supported computationally in model systems such as Drosophila where the antiviral Dicer (Dicer-2) underwent significant evolutionary changes as opposed to its miRNA counterpart (Dicer-1)5. Interestingly, of the two human RNase III nucleases, Drosha has greater homology than Dicer to the ancestral founder of this ancient domain6. The close relationship of Drosha to its antiviral counterparts in invertebrates is particularly noteworthy given that this nuclease has recently been found to translocate to the cytoplasm after infection6–8.

To better understand Drosha biology as it relates to the cellular response to virus infection, we disrupted the gene encoding Drosha in a previously characterized human Dicer-deficient cell line (NoDice cells) that did not alter RNA virus replication9 (Extended Data Fig. 1a). We characterized these cells (herein referred to as RNaseIII−/− cells)

Figure 1 | Drosha mediates miRNA-independent antiviral activity. a, Northern blot of RNA from NoDice and RNaseIII−/− cells reconstituted with indicated plasmids. Blots probed for miR-93 and U6. b, Western blot of whole-cell extract from NoDice and RNaseIII−/− cells infected with SINV (multiplicity of infection (MOI) = 0.01) at 4, 8, and 12 hours post-infection (h.p.i.). Blot probed for SINV Capsid (SIN-C) and pan-Actin (Actin). c–f, Western blots of Ross River virus (RRV) (MOI = 0.1) (c), Langat virus (LGTV) (MOI = 0.1) (d), influenza virus (IAV) (MOI = 1) (e), and GFP-encoding Sendai virus (SeV) (MOI = 1) (f). Protein levels were assessed at 24 h.p.i. using virus-specific or GFP antibodies as indicated. g, RNA sequencing correlation analyses of NoDice and RNaseIII−/− cells at baseline. h, As described in g, except cells were treated with dsRNA for 8 h. Graphs in g and h depict data from biological replicates.

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**Figure 2** | The RNA binding domain of Drosha is essential for virus inhibition. a, Core domains and variants of Drosha including proline-rich (P-rich), arginine-serine-rich (RS-rich), conserved central domain (CED), RNaseIII domain (RIIID), and double-stranded RNA binding domain (dsRBD). Black boxes indicate point mutations (mut). b, Northern (NB) and western (WB) blots of RNaseIII−/− cells transfected with the indicated Drosha variants and cytoplasmic miR-124. Western blot depicts Drosha, Dicer, and pan-Actin. c, d, Western blot (c) and titres (d) from RNaseIII−/− cells expressing the indicated transcripts and SINV RNA 24 hours post-transfection (h.p.t.). Graph denotes average titres obtained; p.f.u., plaque-forming units; error bars, s.d. from three independent experiments. NS, not significant; all other conditions had P < 0.05 by Student’s t-test. e, Western blot of input and Flag-immunoprecipitated (IP) fractions derived from HEK293T cells expressing indicated transcripts and Flag–DGCR8.

The specificity of this activity was assessed by infecting cells with positive- versus negative-stranded RNA viruses including Ross River virus (Fig. 1c) and Langat virus (Fig. 1d), as well as influenza A virus (Fig. 1e) and Sendai virus (Fig. 1f), respectively. These data demonstrated that only viruses with positive polarity had enhanced protein production in the absence of Drosha. Transcriptome profiling of NoDice versus RNaseIII−/− cells at baseline revealed the induction of primary miRNAs and DGCR8, a known target for Drosha-mediated cleavage[10,11] (Fig. 1g, Supplementary Table 1 and Extended Data Fig. 1c). Reconstitution of Drosha restored the transcriptome to that of NoDice cells (Extended Data Fig. 1d). In response to double-stranded RNA (dsRNA), NoDice versus RNaseIII−/− also showed little differential expression (Fig. 1h). Furthermore, we observed no defects relating to the IFN-1 system (Extended Data Fig. 2a, b). This phenotype was corroborated in primary Drosha (Rnasen−/−) conditional mouse fibroblasts (Extended Data Fig. 2c–e).

We next sought to identify the molecular basis of the impact of Drosha on positive-stranded RNA viruses. To ensure that Drosha’s antiviral activity was not due to nuclear sequestration of a host RNA, we first generated a cell line expressing endogenous Drosha and a recombinant green fluorescent protein (GFP)–Drosha S300A/S302A (herein referred to as Drosha–2A), which localized exclusively to the cytoplasm[12] (Extended Data Fig. 3a). Drosha–2A maintained enzymatic activity (Extended Data Fig. 3b) and, as predicted by our knockout studies, demonstrated an approximate 1-log decrease in SINV titres (Extended Data Fig. 3c). These data implicate cytoplasmic Drosha directly in the observed antiviral activity. Next, to map the domains required for this activity, we generated six deletion- and/or site-specific Drosha mutants (Fig. 2a). Among these mutants, only the catalytic mutant E1045Q/E1222Q10 (herein referred to as RIIIDmut) (Fig. 2b) failed to associate with its canonical RNA binding partner, DGCR8. Western blot as described in c with Drosha–RBmut.

Corollary graphs and figures are shown in Extended Data Figs. 1–3. © 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
RNase III nucleases are known to recognize RNA stem-loop structures in all three domains of life\(^1\). To identify RNA that associated with the RNase III domain of Drosha, we performed systemic evolution of ligands by exponential enrichment (SELEX) with RBmut. Five rounds of enrichment identified a subset of unique RNAs that lacked conserved sequences but folded into miRNA-like hairpin structures (Fig. 3a). GFP pull-downs yielded two dominant RNA species with complex predicted secondary structures that were evident in all samples, which were subsequently used as controls for specificity (Fig. 3b). Using a recombinant Drosha-RB fragment, we corroborated specific association between Drosha’s RNase III domain and an RNA hairpin identified by SELEX via electrophoretic mobility shift assay (EMSA), suggesting that no other host factors were required for recognition of the RNA \textit{in vitro} (Fig. 3c). These data were further corroborated using immunoprecipitated proteins from whole-cell extract (Fig. 3d).

Given that the genomes of positive-stranded RNA viruses frequently utilize stem loops similar to those identified by SELEX\(^{14,15}\), we next investigated whether this RNase III domain could engage SINV RNA. Compared with Flag-tagged SeV-NP, immunoprecipitated RBmut protein–RNA complexes in SINV-infected cells showed a 1-log enrichment of viral RNA (Extended Data Fig. 4a, b). Moreover, RNA-EMSA confirmed that RBmut interacted with a specific hairpin at the 5’ end of the genome (Extended Data Fig. 4c, d).

To better understand how Drosha engagement of these structures impedes replication, we used a \textit{Gausia} luciferase-encoding Sindbis replicon system (Extended Data Fig. 5a). RNaseIII\(^{-/-}\) cells produced significantly more genomic RNA, sub-gRNA, luciferase activity, and antigenome than the NoDice parental cells (Fig. 3e–g). These results suggest that Drosha is affecting RNA stability or translation, and/or directly blocking RNA-dependent RNA polymerase (RdRp) processivity. To assess the RNA stability hypothesis, we used a temperature-sensitive RdRp (RdRp\(^{TS}\)) SINV mutant that was inactive at 40°C (ref. 16) (Extended Data Fig. 5b). At this non-permissive temperature, no significant differences in genomic RNA decay in infected NoDice and RNaseIII\(^{-/-}\) were observed (Extended Data Fig. 5c). To determine whether Drosha impacted translation, we measured the activity of a firefly luciferase-encoding Sindbis construct \textit{in vitro} (SIN-\textit{nsP3L}) and found Drosha presence to be inconsequential (Extended Data Fig. 5d, e). Lastly, we assessed whether RdRp itself was directly impeded. To this end, we used a vaccinia virus-based system to generate functional minus-strand-specific replicase complexes, as previously described\(^{17}\). Cellular fractions containing both SINV replicon and Drosha were isolated and assayed \textit{in vitro}, demonstrating that Drosha could reduce RdRp output by almost 50% (Fig. 3h, i).

As RNA hairpins, often essential for virus replication, are structurally conserved and evolutionarily constrained, they represent optimal targets for an antiviral system\(^{16}\). To determine whether Drosha from other phyla can recognize this putative pathogen-associated molecular pattern as demonstrated both in mammals and in arthropods\(^{19}\), we used miRNA-containing viruses to determine whether cytoplasmic processing, as an indicator of virus-induced translocation, could be observed. In zebrafish (\textit{Danio rerio}), we demonstrated Drosha-dependent processing of an SINV-derived artificial miRNA in inoculated embryos (Fig. 4a and Extended Data Fig. 6a, b). We next examined cytoplasmic processing in plants. To accomplish this, we cloned pre-miR-124 into turnip crinkle virus (TCV) and infected \textit{Arabidopsis} protoplast cultures, which, like zebrafish, supported cytoplasmic miRNA processing (Fig. 4b and Extended Data Fig. 6c, d).

Given the retention of this ancient activity, we turned to RNase III members from all domains of life (Extended Data Fig. 7a). Expression of each of these diverse nucleases, from bacteria, to archaea, to yeast, demonstrated robust antiviral activity against positive- but not negative- stranded viruses (Fig. 4c, d and Extended Data Fig. 7b). We next sought to demonstrate the antiviral activity of endogenous Drosha in an invertebrate. SINV-infected \textit{Drosophila melanogaster} cells were pre-treated with dsRNA to knockdown Drosha as previously described\(^{18}\). Consistent with the results from mammalian cultures, loss of Drosha enhanced capsid expression (Extended Data Fig. 7c).

Small RNA sequencing demonstrated that while more viral-derived RNAs were present in Drosha-depleted cells, the contours of mapped alignments were indistinguishable, ruling out a role for RNAi (Fig. 4e). This same phenotype was observed with \textit{Drosophila} C virus in \textit{D. melanogaster}, and SINV in human cells (Fig. 4f and Extended Data Fig. 7d).

These results suggest that the RNase III family of nucleases, used in all domains of life for the maturation of ribosomal and other structural RNAs, may represent an ancient RNA recognition platform that helped eukaryotes combat viruses. Interestingly, of the two RNase III proteins encoded in the human genome, Drosha is more related to bacterial RNase III than is Dicer\(^{4}\). In contrast, Dicer has been suggested to be the ancestor that gave rise to MDA5, which later duplicated and generated RIG-I\(^{21}\). Given these evolutionary connections, it is tempting to speculate that the RNase III domain of prokaryotes may have provided an early defence system for eukaryotes. In its most simple form, a Drosha-like homologue would recognize and engage RNA as an antiviral clamp but perhaps later evolve to utilize its enzymatic activity to give rise to RNAi. As the arms race continued, duplication, domain swapping, and time provided chordates with the opportunity to use RNase III domains to create new RNA sensors and the eventual development of the IFN-1 system, which is believed to be fundamentally incompatible with RNA\(^{21,23}\). As RNAi was functionally replaced with IFN-1 during vertebrate development, the roles of Dicer and...
Drosha became largely dedicated to post-transcriptional regulation. Despite all the changes that have accrued over this expansive time, it would seem that fossils of this evolutionary arms race may remain in the basic biology of our miRNA machinery.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 13 September 2016; accepted 11 May 2017.

Published online 28 June 2017.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank J. K. Lim and A. G. Pletnev for Langat virus reagents, B. Lee for Sendai virus reagents, R. W. Hardy for SINV reagents, B. R. Cullen for NoDice cells, K. K. Conzelmann for BSR-T7 cells, and B. Ramratnam for pEGFP-Drosha. Recombinant IFN-β was provided by the National Institute of Health’s Biodefense and Emerging Infections Research Resources Repository (HuFlN-β, NR-3080). This material is based upon work supported in part by the Burroughs Wellcome Fund, which provides support for both S.C. and B.R.T. S.C. is also supported by the National Institute of Allergy and Infectious Diseases (NIAID) (R01AI1074951). J.P.L. is supported by the D.M. Albert, Council Regional d’Ille-de-France. L.C.A. is partly supported by the American Heart Association (15PRE2430012). A.E.S. is supported by National Science Foundation (MCB-1411836) and NIAID (R21AI117882). J.M. is supported by National Institute of General Medicine (F32 GM119235). B.R.T. is also supported by NIAID (R01AI10575).

Author Contributions L.C.A. and S.S. designed and conducted experiments. M.P. performed SELEX. J.M., L.C.A., and A.E.S. were responsible for plant data. J.V.S. generated the RNase III inhibition of virus replication is highly conserved. a, Northern blot of RNA from zebrafish embryos treated with indicated morpholinos, and inoculated 2 days later with wild-type SINV (WT) or a strain encoding an artificial miRNA (amiRNA). Blot depicts amiRNA and U6 at 40 h.p.i. b, Northern blot of RNA from Arabidopsis thaliana protoplasts treated with a TCV containing a scrambled sequence (Scb) or miR-124 (124). c, d, Western blot from RNaseIII+ cells, co-transfected with the indicated plasmids and either in vitro transcribed SINV gRNA or Langat virus rescue plasmid. e, f, Small RNA sequencing of DL1 cells treated with indicated dsRNA for 3 days and subsequently infected with SINV (MOI = 1) (e) or Drosophila C virus (MOI = 7) (f). Graphs depict the number of reads mapping to indicated positions along the viral genomes.
METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Viruses. Recombinant SIN, SINV124, and SINVamiRNA were previously described27,31. To generate SINV-GFP, the open reading frame of enhanced GFP was cloned under control of the additional subgenomic promoter of the SIN’ TE1I2Q construct2−3, replacing mir-I24. Virus infections in cell culture were performed in DMEM supplemented with 10% FBS and 1× penicillin/streptomycin. HEK293T cells were purchased from ATCC (CRL-3126). NoDicer HEK293T cells were a gift from B. R. Cullen9. BSR-T7 cells were provided by K.-K. Conzelmann. RNaseI/II primary lung fibroblasts were a gift from T. Littman and are described elsewhere27. HEK293T cells stably expressing GFP-tagged Drosha-WT or Drosha-2A were generated using a lentiviral expression vector and selected with puromycin. HEK293T cells lacking a functional Drosha protein, we PCR amplified and sequence verified the construct. A control sequence was subsequently inserted by replacing miR-124 in SINV minigenome RNA. For each experimental setting the average normalized read count was calculated (reads per million) between two experimental settings. A Pearson correlation coefficient (r) was calculated using the corr function in R with default parameters.

Cell culture and reagents. Drosophila cells (DL1) were grown, maintained, and treated with dsRNA as previously described20,28. All mammalian cells were cultured in DMEM supplemented with 10% FBS and 1× penicillin/streptomycin. Cells were purchased from ATCC (VR-373) and amplified on BHK21 cells. Langat (strain TP1) and Sendai-GFP viruses were gifts from J. Lin and B. Lee. Vaccinia viruses encoding T7 DNA-dependent RNA polymerase, ubiquitin-tagged SINV nsP4, and the SINV polyprotein 123 C-frame of Gaussia luciferase fused to 76 nucleotides of the SINV capsid (noLuc2) were gifts from B. Lee. The pMini(SeV-L) SINV minigenome was purchased from DNA2.0. NoDice cells were transfected with this plasmid and selected with puromycin.

Primers, oligonucleotides, and other reagents were purchased from DNA2.0. NoDice cells were transfected with this plasmid and selected with puromycin.

Antiviral assays. SINV RNA was amplified using a lentiviral expression vector and selected with puromycin. For each experimental setting the average normalized read count was calculated first using two biological replicates. Small RNA sequencing of infected DL1 cells was performed as previously described20. Briefly, cells were treated with indicated dsRNA for 3 days and subsequently infected with SINV (MOI = 1) or Drosophila C virus (MOI = 7) for 96 h before RNA extraction. Total RNA (40 μg) was separated on a 15% TBE-urea gel. The 15 to 29 nucleotide fraction was extracted, eluted, and ethanol precipitated. Libraries were prepared using a Small RNA Sample Prep Kit, version 1.5 (Illumina, San Diego, California, USA) according to the manufacturer’s instructions and sequenced on an Illumina Genome Analyzer II. Libraries for small RNA sequencing of HEK293T and RNaseIII−/− cells were prepared using 1 μg of total RNA and a Small RNA Sample Prep Kit, version 1.5 (Illumina) according to the manufacturer’s instructions. The entire small RNA fractions (~15 to 200 nucleotides) were gel extracted and barcoded samples were PCR amplified for an additional 25 cycles before sequencing on an Illumina MiSeq platform with a MiSeq Reagent Kit v3 or on a HiSeq 2500. Sequence reads were analysed with the RNA Express application (Illumina, BaseSpace) or custom pipelines. Alignment of reads to viral genomes was performed using Bowtie and subsequently visualized using the Integrative Genomics Viewer25. The correlation analyses were performed by comparing the natural logarithm of the normalized read counts (reads per million) between two experimental settings. A Pearson correlation coefficient (r) was calculated using the corr function in R with default parameters.

Quantitative PCR. dsRNA for 3 days and subsequently infected with SINV (MOI = 1) or Drosophila C virus (MOI = 7) for 96 h before RNA extraction. Total RNA (40 μg) was separated on a 15% TBE-urea gel. The 15 to 29 nucleotide fraction was extracted, eluted, and ethanol precipitated. Libraries were prepared using a Small RNA Sample Prep Kit, version 1.5 (Illumina, San Diego, California, USA) according to the manufacturer’s instructions and sequenced on an Illumina Genome Analyzer II. Libraries for small RNA sequencing of HEK293T and RNaseIII−/− cells were prepared using 1 μg of total RNA and a Small RNA Sample Prep Kit, version 1.5 (Illumina) according to the manufacturer’s instructions. The entire small RNA fractions (~15 to 200 nucleotides) were gel extracted and barcoded samples were PCR amplified for an additional 25 cycles before sequencing on an Illumina MiSeq platform with a MiSeq Reagent Kit v3. Adapters were trimmed and analysis was performed using the Small RNA Sequencing App from Illumina. Raw and processed data have been deposited in the Gene Expression Omnibus under accession numbers GSE86610, GSE89790, GSE98889, GSE43031, and GSE98135.

Immunoprecipitations. For immunoprecipitations of Flag−GFP and –DCCGR8, 2 × 106 HEK293T cells were transfected with 2 μg each of the indicated plasmids. For each, cells were lysed in 50 mM Tris pH 7.5, 10 mM glycerol, 1 mM EDTA, 100 μM DTT, 0.5% NP-40, and 1% protease inhibitor cocktail and sonicated once for 15 s. Lysates were rotated overnight at 4 °C with 5 μl Anti-Flag M2 antibody before addition of 25 μl protein G agarose beads. Samples were rotated for 1.5 h, washed in the same buffer used for lysis and then analysed by western blot.

RNA immunoprecipitation was performed as previously described25. Briefly, 36 h.p.t. of RNaseIII−/− cells with the indicated plasmids, cells were infected with SINV at an MOI of 3. At 7 h.p.t., cell lysates were prepared in lysis buffer (15 mM Tris/HCl pH 7.5, 150 mM NaCl, 15 mM MgCl2, and 1% (v/v) Triton X-100 supplemented with 10 mM of Ribonucleoside Vanadyl complex (NEB S1402S) and 0.1% (v/v) formaldehyde). RNA was isolated using TRIzol (Thermo Fisher) and reversed transcribed using SuperScript II (Thermo Fisher) according to the manufacturer’s instructions. Complementary DNA was amplified with gene-specific primers using a KAPA SYBR Green Master Mix (KAPA Biosystems) and analysed on a LightCycler 480 (Roche). Sequences of gene-specific primers are listed in Supplementary Table 2.

RNA sequencing. For messenger RNA (mRNA) sequencing, libraries from two biological replicates per condition were prepared with a TruSeq RNA Library Preparation Kit v2 (Illumina) according to the manufacturer’s instructions. In brief, 1 μg of total RNA was enriched for mRNA using oligo-dT beads. Purified RNA was fragmented and reverse transcribed, followed by second-strand synthesis, end repair, A-tailing, adaptor ligation, and PCR amplification. Prepared libraries were then quantified using a universal complete KAPA Library Quantification Kit (KAPA Biosystems). Libraries were sequenced on an Illumina MiSeq platform with a MiSeq Reagent Kit v3 or on a HiSeq 2500. Sequence reads were analysed with the RNA Express application (Illumina, BaseSpace) or custom pipelines. Alignment of reads to viral genomes was performed using Bowtie and subsequently visualized using the Integrative Genomics Viewer25. The correlation analyses were performed by comparing the natural logarithm of the normalized read counts (reads per million) between two experimental settings. A Pearson correlation coefficient (r) was calculated using the corr function in R with default parameters.

Northern blot analysis. Total RNA was isolated using TRIzol (Thermo Fisher) according to the manufacturer’s instructions. Small RNA northern blotting was performed as previously described27,31. For large RNA northern analysis, 5 μg of total RNA in loading buffer (30% (v/v) formamide, 3% (v/v) formaldehyde, 5% (v/v) glycerol, 1 × MOPS, and 0.01% (w/v) bromophenol blue) was separated in a 1% agarose gel in 1 × MOPS buffer. RNA was transferred by capillary action in 20 × SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0) onto Hybond-NX nylon membrane (GE Healthcare). RNA was then ultraviolet cross-linked to the membrane (0.2 mJ cm−2) and incubated in blocking buffer (6 × SSC and 7% SDS) for 1 h at 65 °C. Next, the membrane was incubated with a radio-labelled DNA oligonucleotide overnight at 42 °C in 6 × SSC and 7% SDS, washed three times for 10 min in 3 × SSC and 0.1% SDS, and exposed to film. Sequences of probes are listed in Supplementary Table 2.

To generate SIN-RdRpts, total RNA was isolated with TRIzol (Thermo Fisher) and reversed transcribed using SuperScript II (Thermo Fisher) according to the manufacturer’s instructions. Complementary DNA was amplified with gene-specific primers using a KAPA SYBR Green Master Mix (KAPA Biosystems) and analysed on a LightCycler 480 (Roche). Sequences of gene-specific primers are listed in Supplementary Table 2.

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CustomArray. The DNA oligonucleotide library was PCR amplified, followed by *in vitro* transcription using a MEGAScript T7 Transcription Kit (Thermo Fisher) according to the manufacturer’s instructions. RNA was heated at 65 °C for 5 min and immediately put on ice before the selection process. Flag-tagged proteins were purified from RNaseIII−/− cells at 24 h.p.i. in lysis buffer (15 mM Tris/HCl pH 7.5, 150 mM NaCl, 15 mM MgCl₂, and 1% (v/v) Triton X-100) supplemented with 1× protease inhibitor cocktail (Roche). Cell lysates were incubated with anti-Flag affinity resin (Sigma A2220) at 4 °C overnight, followed by four washes of 5 min each with lysis buffer. Purified microgram of virus RNA in lysis buffer was added to the beads and incubated at 4 °C for 2 h, followed by four washes of 5 min each with lysis buffer. Protein–RNA complexes were eluted with 300 μg ml⁻¹ Flag-peptide (Sigma F3290) and purified RNA was extracted using TRizol. Purified RNA was reverse transcribed, PCR amplified, and *in vitro* transcribed to repeat the selection process. A total of five rounds of selection were performed.

**EMSA.** EMSAs were performed as previously described²⁷. Flag-tagged proteins were purified from RNaseIII−/− cells at 24 h.p.i. in lysis buffer (15 mM Tris/HCl pH 7.5, 150 mM NaCl, 15 mM MgCl₂, and 1% (v/v) Triton X-100) supplemented with 1× protease inhibitor cocktail (Roche). Cell lysates were incubated with anti-Flag affinity resin (Sigma A2220) at 4 °C overnight, followed by four washes of 5 min each with lysis buffer. Bound protein was eluted with 300 μg ml⁻¹ Flag-peptide (Sigma F3290) in 20 mM Tris/HCl pH 8.0, 100 mM KCl, and 0.2 mM EDTA. Alternatively, a fragment of recombinant human Drosophila protein was purchased from Abcam (ab94010) and 0.25–1 μg of this purified protein was used for EMSAs. Radioactively labelled RNA was *in vitro* transcribed from a PCR-amplified template using a MAXIScript Kit (Ambion). For complex formation protein and RNA, protein eluate or recombinant protein was incubated with radiolabelled RNA (~100,000 counts per minute per reaction) for 30 min at room temperature in 20 mM Tris/HCl pH 8.0, 100 mM KCl, and 0.2 mM EDTA, supplemented with 0.1 μg ml⁻¹ BSA and 1 mM DTT. Reactions were run on a 6% polyacrylamide gel.

**In vitro cleavage assay.** *In vitro* cleavage assays with Drosophila were performed as previously described³⁴. Briefly, 15 μg of T7-transcribed TCV genomic RNA was transcribed into 7 × 10⁵ cells in a 60 mm Petri dish and incubated in the dark at 22 °C for 40 h. Samples were collected at 13,000 r.p.m. Total RNA was isolated using RNA extraction buffer (50 mM Tris/HCl pH 7.5, 5 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS), followed by phenol–chloroform extraction and ethanol precipitation.

**Fluorescence in situ hybridization.** Fluorescence in situ hybridization (FISH) of *A. thaliana* protoplasts was performed following an established protocol³⁵. Briefly, mock and TCV-infected protoplasts were fixed with methanol and hybridized with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Cells were imaged using a laser scanning LSM 710 AxioObserver confocal microscope (Carl Zeiss). Images were acquired using a 63×/1.40 oil differential interference contrast (DIC) objective with 405 nm and 561 nm filters for DAPI and Cy3 respectively.

**Western blot analysis.** Whole-cell extracts were separated on Mini Protein TGX gels and transferred onto nitrocellulose membrane (BioRad). SINV-specific ascsfs (ATCC-VR-1248AF) and antibodies specific to actin (Thermo Fisher, MS-1295-1), and HEK293T Drosha-2A cells and 2 μg of SINV-nps³⁶ RNA were added to the reaction components and incubated for 2 h at 30 °C before analysis for firefly luciferase and Renilla luciferase activity.

**In vitro translation assay.** *In vitro* translation assays were performed using a Rabbieticulocyte Lysate System (Promega) according to the manufacturer’s instructions. Membrane fractions (10 μg of protein) of T7-transcribed TCV genomic RNA were added and the incubation continued for another 20 min. The reactions were terminated by the addition of SDS to 2.5% and proteinase K at a final concentration of 100 μg ml⁻¹. RNA was isolated with TRizol, equal volumes were run on a 1.25% agarose gel, transferred onto Hybond-NX nylon membrane (GE Healthcare), and exposed to film.

**SuperSignal West Femto Maximum Sensitivity** (Thermo Fisher) substrates were used according to the manufacturer’s instructions.

**Luciferase assays.** To assess translational inhibition by Drosophila viral RNA binding, SINV replicon constructs (described above) were *in vitro* transcribed using an SP6 mMessage Machine Kit (Ambion) and co-transfected with a control *Gaussia* luciferase construct used as a hairpin controls. Luciferase expression was analysed at the indicated time points after transfection using a Promega Dual-Luciferase Reporter Assay System. In all experiments, *Gaussia* luciferase expression was normalized to full-length luciferase.

**In vivo infections.** Zebrafish embryos (AB strain) were randomly pooled in groups and injected with 8 ng of control (GAAAGCTGGCAGCTGATCATGA) or of Drosophila translation blocking (AGTGAAGTTTGAACACCTGTCGAA) morpholinos at the one-cell stage (sex was not determined). At 2 days after fertilization, embryos were inoculated intraperitoneally and intracranially, receiving a total of ~1,000 plaque-forming units of SINWv or SINvAmiRNA. RNA was extracted from surviving larvae at 40 h.p.i. Each sample was a pool of ~11 larvae. Zebrafish experiments were performed in accordance with the Institut Pasteur Animal Care and Use Committee (reference CEEA 89).

Protoplasts were prepared from callus cultures of *A. thaliana* (Col-0 strain). Transfections were performed using polyethylene glycol as previously described³⁸. Briefly, 15 μg of T7-transcribed TCV genomic RNA was transfected into 7 × 10⁵ cells in a 60 mm Petri dish and incubated in the dark at 22 °C for 40 h. Samples were collected at 13,000 r.p.m. Total RNA was isolated using RNA extraction buffer (50 mM Tris/HCl pH 7.5, 5 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS), followed by phenol–chloroform extraction and ethanol precipitation.

**Data availability.** Deep sequencing data have been deposited in the Gene Expression Omnibus under accession numbers GSE86610, GSE89790, GSE96889, GSE43031, and GSE98135. All other data are available from the corresponding author upon reasonable request.

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Extended Data Figure 1 | Characterization of RNaseIII−/− cells.
a, Sequence alignment of genetic alterations in the two alleles encoding Drosha in RNaseIII−/− cells. The deletion and insertion result in a frameshift and early stop codon. b, The ten most abundant miRNAs in each condition, parental HEK293T (WT) or RNaseIII−/− either mock-treated or SINV-infected for 24 h as determined by Illumina small RNA deep sequencing. c, Quantitative PCR (qPCR) analysis of DGCR8 mRNA levels in mock-treated and SINV-infected (MOI = 0.1, 8 h.p.i.) NoDice and RNaseIII−/− cells; error bars, s.d. from three independent experiments; all conditions had P < 0.05 by Student's t-test. d, Transcriptome profiling and correlation analyses of NoDice cells at baseline and RNaseIII−/− cells transfected with GFP-tagged human Drosha for 72 h. Graph depicts data from two biological replicates per condition.
Extended Data Figure 2 | Drosha depletion does not alter the response to IFN-I. a, qPCR analysis of IFIT1 mRNA levels in NoDice and RNaseIII−/− fibroblasts treated with IFN-β (100 U ml−1, 8 h); error bars, s.d. from three independent experiments; all conditions had P < 0.05 by Student's t-test. b, Western blot of NoDice and RNaseIII−/− fibroblasts infected with SINV for 1 h before administration of indicated amounts of IFN-β for 24 h. c, d, Northern blot (c) and western blot (d) of primary Rnasenfl/− ear-derived fibroblasts treated with indicated AdVs for 2–3 days and then treated with either 100 U IFN-β for 6 h (c) or infected with SINV for 24 h (d). e, mRNA sequencing of total RNA from samples in c. Heatmap depicts known mouse ISGs and IFN downregulated genes with a log2(fold change) greater than 1, as defined by the Interferome database (www.interferome.org).
Extended Data Figure 3 | Characterization of Drosha-2A cells.

a, Immunofluorescence of human fibroblasts stably expressing GFP-tagged Drosha-WT or Drosha-2A (S300A/S302A). b, The indicated Flag-tagged proteins were immunoprecipitated from whole-cell extracts (WCE) and incubated at 37 °C with in vitro transcribed genome of SIN124. Production of pre-miR-124 was determined by small RNA northern blot. c, Indicated cell types were infected with SINV at an MOI of 0.001 and viral titres were determined at 16 h.p.i. Shown is the average and s.d. of three independent experiments, with P < 0.05 as determined using a two-tailed Student's t-test.
Extended Data Figure 4 | Drosha-RB-RHIDmut recognizes stem-loop structures in SINV RNA. a, Immunoprecipitation of exogenously expressed Flag-tagged proteins. Shown is protein expression in the whole-cell extract and after immunoprecipitation (IP) with a Flag-specific antibody. b, Cells were transfected with Flag-tagged SeV-N or Drosha-RBmut and infected at 36 h.p.t. with SINV at an MOI of 3. At 8 h.p.i., Flag-tagged proteins were immunoprecipitated and bound RNA was isolated to perform qPCR. Graph shows SINV RNA levels relative to input and normalized to tubulin. The average of three independent experiments is shown. Error bars, s.d.; *P < 0.05 using a one-tailed Student’s t-test. c, Prediction of the structure of the 5' 200 nucleotides of the SINV genome using RNAfold. d, EMSA was performed with the indicated immunoprecipitated proteins and radio-labelled in vitro transcribed RNA comprising the 5' 200 nucleotides of the SINV genome. Unbound genome is indicated as 'Free RNA.'
Extended Data Figure 5 | Using virus engineering to discern Drosha’s antiviral mechanism. a, Schematic of the SINV replicon encoding Gaussia luciferase in place of the structural polyprotein used in Fig. 3e–g. b, Schematic of the SINV temperature-sensitive mutant (SIN-RdRpts). Star denotes ts point mutant. c, NoDice and RNaseIII−/− cells were infected with virus depicted in b, at an MOI of 10 and incubated at 40 °C, a temperature at which the mutant viral RdRp is completely inactive. Levels of genomic (g) SINV RNA were determined by qPCR at the indicated times after infection. Data are representative of two independent experiments where each condition was done in triplicate; error bars, s.d. d, Schematic of the SINV encoding firefly luciferase in the nsP3 region and an inactive RdRp (SIN-nsP3Luc). e, Graph depicts levels of in vitro translation of firefly luciferase produced from virus in d, in the presence of membrane fractions from control or Drosha-2A. The data shown are the average of three independent experiments; error bars, s.d.
Extended Data Figure 6 | Localization of and miRNA production from cytoplasmic viruses in diverse eukaryotes. a, Zebrafish embryos were inoculated with SINV for 24 h and then analysed by FISH using a probe complementary to the capsid region of the genome. b, qPCR analysis of zebrafish embryos treated with the indicated morpholinos for 2 days ($n = 4$); error bars, s.d. c, A. thaliana protoplasts were mock-treated or TCV-infected for 40 h and then analysed by FISH using a Cy3-labelled probe complementary to bases 1210–1259 of the TCV genome. d, Quantification of mature miR-124 production from recombinant TCV was performed using the TaqMan miRNA assay on RNA from Fig. 4b. All samples were normalized to endogenous snoR66. Quantifications of each sample were performed in triplicate; error bars, s.d. from two biological replicates.
Extended Data Figure 7 | The impact of diverse RNase III members on virus infection. a, Schematic depicting core domains of human Drosha, C-terminal region of C. intestinalis Drosha, or full-length RNase III of S. pombe, M. maripaludis, and S. pyogenes. Domains depicted include Proline-rich (P-rich), arginine-serine-rich (RS-rich), conserved central domain (CED), RNaseIII domain (RIIID), and double-stranded RNA binding domain (dsRBD). b, Western blots from BSR-T7 cells, co-transfected with the indicated RNase-III-expression plasmids and SeV rescue plasmids encoding SeV-GFP genome, SeV-N, SeV-P, and SeV-L genes. RNase III expression was determined at 48 h.p.t. and virus replication at 72 h.p.t. c, Western blot of DL1 cells treated with indicated dsRNA for 3 days and subsequently infected with SINV (MOI = 1) for 96 h. d, HEK293T (WT) or RNaseIII−/− cells were infected with SINV for 24 h. Graphs depict the number of SINV reads mapping to indicated positions along the viral genomes from the small RNA deep sequencing performed in Extended Data Fig. 1b.