

# West Nile virus encodes a microRNA-like small RNA in the 3' untranslated region which up-regulates GATA4 mRNA and facilitates virus replication in mosquito cells

Mazhar Hussain<sup>1</sup>, Shessy Torres<sup>2</sup>, Esther Schnettler<sup>3</sup>, Anneke Funk<sup>2</sup>, Adam Grundhoff<sup>4</sup>, Gorben P. Pijlman<sup>3</sup>, Alexander A. Khromykh<sup>2</sup> and Sassan Asgari<sup>1,\*</sup>

<sup>1</sup>School of Biological Sciences, <sup>2</sup>Australian Infectious Disease Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD 4072, Australia, <sup>3</sup>Laboratory of Virology, Wageningen University, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands and <sup>4</sup>Heinrich-Pette-Institute for Experimental Virology and Immunology at the University of Hamburg, 20251 Hamburg, Germany

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## ABSTRACT

West Nile virus (WNV) belongs to a group of medically important single-stranded, positive-sense RNA viruses causing deadly disease outbreaks around the world. The 3' untranslated region (3'-UTR) of the flavivirus genome, in particular the terminal 3' stem-loop (3'SL) fulfils multiple functions in virus replication and virus-host interactions. Using the Kunjin strain of WNV (WNV<sub>KUN</sub>), we detected a virally encoded small RNA, named KUN-miR-1, derived from 3'SL. Transcription of WNV<sub>KUN</sub> pre-miRNA (3'SL) in mosquito cells either from plasmid or Semliki Forest virus (SFV) RNA replicon resulted in the production of mature KUN-miR-1. Silencing of *Dicer-1* but not *Dicer-2* led to a reduction in the miRNA levels. Further, when a synthetic inhibitor of KUN-miR-1 was transfected into mosquito cells, replication of viral RNA was significantly reduced. Using cloning and bioinformatics approaches, we identified the cellular GATA4 mRNA as a target for KUN-miR-1. KUN-miR-1 produced in mosquito cells during virus infection or from plasmid DNA, SFV RNA replicon or mature miRNA duplex increased accumulation of GATA4 mRNA. Depletion of GATA4 mRNA by RNA silencing led to a significant

reduction in virus RNA replication while a KUN-miR-1 RNA mimic enhanced replication of a mutant WNV<sub>KUN</sub> virus producing reduced amounts of KUN-miR-1, suggesting that GATA4-induction via KUN-miR-1 plays an important role in virus replication.

## INTRODUCTION

West Nile virus (WNV), an enveloped RNA virus with a single-stranded RNA genome of positive polarity, belongs to the *Flavivirus* genus (Family: *Flaviviridae*), an important class of arthropod-borne viruses that causes major outbreaks of potentially fatal diseases affecting humans and animals across the globe (1). Indigenous to Africa, Asia, Europe and Australia, WNV made a dramatic entry to the Americas through New York City in 1999, where an epidemic of meningo-encephalitis resulting in several human fatalities was attributed to the virus (2,3). The highly pathogenic strain isolated in New York (WNV<sub>NY99</sub>) is closely related to the Australian subtype of WNV, Kunjin (WNV<sub>KUN</sub>). Unlike WNV<sub>NY99</sub>, WNV<sub>KUN</sub> is highly attenuated and does not cause overt disease in vertebrates (4), which makes it an ideal model for the WNV life cycle and virus-host interactions. The 11 022 nt WNV<sub>KUN</sub> genome consists of one long open reading frame (ORF), which encodes

\*To whom correspondence should be addressed. Tel: +617 3365 2043; Fax: +617 3365 1655; Email: s.asgari@uq.edu.au  
Correspondence may also be addressed to Alexander A. Khromykh. Tel: +617 334 67219; Fax: +617 3365 4620; Email: a.khromykh@uq.edu.au

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three structural and seven non-structural proteins essential for the viral life cycle and is flanked by 5' and 3' untranslated regions (UTRs) (5). The 3'-UTR of WNV<sub>KUN</sub> contains a number of stem-loops (SLs) and tertiary structures conserved among members of the *Flavivirus* genus (6–8), which makes it resistant to RNase degradation and results in the accumulation of 525 nt subgenomic flavivirus RNA (sfRNA) (7). This sfRNA has been shown to be important in facilitating virus-induced cytopathicity and neuroinvasiveness (7). In addition, one of these SLs, the 3'SL located at the very end of the 3'-UTR, has been shown to be crucial for viral replication and interacts with a variety of cellular proteins (8–11).

MicroRNAs (miRNAs) are an important class of small (20–25 nt), non-coding, single-stranded RNAs that play crucial roles in cell development, proliferation, differentiation, apoptosis and host defence (12,13). The production of miRNAs involves a number of maturation steps in which a primary miRNA (pri-miRNA) is cleaved by Drosha to produce ~70 nt long precursor miRNA (pre-miRNA) consisting of an imperfectly complementary hairpin, which is in turn cleaved by the cytoplasmic RNase III enzyme Dicer to produce the single-stranded mature miRNA (14,15). Mammals and nematodes possess one Dicer enzyme, while insects have two distinct enzymes, Dicer-1, which is specific for the generation of miRNAs, and Dicer-2, which is specific for the generation of small interfering RNAs (siRNA) (14–16). Usually one arm of the pre-miRNA hairpin, the mature miRNA, is stable after Dicer-1 cleavage and loaded into the RNA-induced silencing complex (RISC) while the other arm of the pre-miRNA, miRNA\*, is rapidly degraded (14,15,17). The miRNA:RISC machinery regulates gene expression by binding to complementary sequences in the target mRNA for which usually nucleotide positions 2–8 (the seed region) in the miRNA are critical (15,18,19). Depending on the degree of complementarity, binding can result in translational repression, degradation or up-regulation of the targeted mRNA (14,19,20). In addition to cellular miRNAs, many virally encoded miRNAs have been described since the first discovery of Epstein–Barr virus encoded-miRNAs (21). Viral miRNAs can regulate both cellular and viral gene expression through modulation of cellular factors involved in the host's innate or adaptive anti-viral immune responses, mimicking cellular miRNAs or targeting viral mRNAs to regulate the viral life cycle (15,22–24). Interestingly, the majority of identified viral miRNAs are derived from DNA viruses, mainly from the herpesvirus family, and only one RNA virus, HIV-1, has been found to encode a viral miRNA so far (23–25). In addition, it has been shown recently that functional cellular/viral miRNAs can be produced by cytoplasmic RNA viruses when pre-miRNA sequence is incorporated into the virus genome. For example, replicating in the cytoplasm, flavivirus tick-borne encephalitis virus (TBEV) was shown to express an Epstein–Barr virus miRNA by incorporating its miR-BART2 precursor in the 3'-UTR of the TBEV genome (26). In another example, miR-124, a cellular miRNA, was expressed from another cytoplasmic positive strand RNA virus,

alphavirus Sindbis, without miRNA-mediated targeting of viral RNAs, thereby permitting the virus to produce an miRNA without a negative impact on viral replication (27). These examples from cytoplasmic RNA viruses demonstrate that pri-miRNA SLs can be processed in the cytoplasm by dicer-dependent non-canonical mechanisms without nuclear involvement of Drosha. Further, a murine  $\gamma$ -herpesvirus (a DNA virus) pri-miRNA (MHV68) was shown to be processed by cellular tRNase Z rather than Drosha (28).

Here we show that the sfRNA and in particular the highly conserved 3'SL of the WNV<sub>KUN</sub> genome serves as a likely source for the generation of a mature miRNA, which we termed KUN-miR-1, in infected mosquito cells. This miRNA was detected in abundance in RNAi pathway competent *Ae. aegypti* Aag2 cells as well as in RNAi pathway defective *Ae. albopictus* C6/36 mosquito cells (29,30). In addition, silencing of *Dicer-1* in Aag2 cells abolished processing of pre-KUN-miR-1 to mature miRNA. Using a novel miRNA target identification method (31) combined with miRNA target analysis software and northern blot detection, we show that KUN-miR-1 targets mosquito GATA4 mRNA that leads to its up-regulation in cells. Inhibition of KUN-miR-1 or depletion of GATA4 mRNA both led to reduced WNV<sub>KUN</sub> RNA replication, while induction of GATA4 mRNA by KUN-miR-1 RNA mimic enhanced replication of a mutant virus producing decreased amounts of KUN-miR-1.

## MATERIALS AND METHODS

### Prediction of KUN-miR-1 and its cellular targets

Vmir (32–34) was used to analyse the WNV<sub>KUN</sub> 3'-UTR region (GenBank accession number: AY274504) for possible pre-miRNA hairpin structures, using stringent filtering parameters as described before (32). The potential target sequences of KUN-miR-1 were analysed using RNAHybrid (35) and RNA22 software (IBM).

### Northern blot analysis of virus-infected mosquito cells

*Aedes albopictus* C6/36 cells were infected with MOI = 1 of the wild-type WNV<sub>KUN</sub> and IRA $\Delta$ CS3 mutant WNV<sub>KUN</sub> defective in the generation of sfRNA (7). Mock-infected cells were used as control. Total RNA was isolated with TRIzol (Invitrogen) at different time points after infection, enriched for small RNAs using PureLink™ miRNA isolation kit (Invitrogen). Small RNA samples (20  $\mu$ g) were separated on 15% denaturing polyacrylamide gels, electroblotted to nylon membrane and ultraviolet cross-linked. The blots were subjected consecutively to three different probes: a '5' probe' complementary to a sequence derived from the 5' stem of the pre-miRNA (5'-GTGTGGCTGGTTGTGCAGAGCAG AAGATCT), a '3' probe' to detect the 3' stem of the pre-miRNA (5'-AGATCCTGTGTTCTCGCACCA) and a 'terminal loop (TL) probe' complementary to the sequence of the terminal loop, (5'-CCACCATTGTCCG CGCACTGTGCCGTGTGG) (Figure 1A). The probes were generated by labelling of DNA oligonucleotides

with [ $\alpha^{32}$ P]dCTP using a terminal nucleotide transferase. All the probe hybridizations and washings were done at 50°C.

The transcripts of target and non-target genes were detected by northern analyses of total RNA (10  $\mu$ g) run on 1.2% agarose formaldehyde gels as described previously (36).

### Cloning KUN-miR-1

To determine the sequence of the detected viral miRNA, we used two independent miRNA-specific cloning methods (37,38). Briefly, RNA from WNV<sub>KUN</sub>-infected C6/36 cells was isolated at 7 days post-infection (dpi), fractionated on a 15% urea polyacrylamide gel and bands of 18–25 nt in size were isolated. Isolated RNAs were polyadenylated using the Ncode miRNA first-strand cDNA synthesis kit (Invitrogen) as per the manufacturer's instructions followed by first-strand cDNA synthesis using a poly-dT primer (Invitrogen). The first-strand cDNA was then used as a template for PCR with three different forward primers and oligo dT as reverse primer. Forward primers were 15-mer oligonucleotides located progressively closer to the 5'-end of the predicted mature miRNA strand (KUN-miR1-1-GGTGGCTGGTGGTGC, KUN-miR1-2-GGCTGGTGGTGGCAG, KUN-miR-1-3-TGGTGCGAGAACACA).

For the second approach, 5' and 3' adaptor oligonucleotides were used as previously described (38). PCR products were cloned using the pGEM-T Easy vector system (Promega) and sequenced.

### Expression of KUN-miR-1 in mosquito cells

The pre-KUN-miR-1 (coordinates 10944–11022) was cloned into the pIZ/V5-His vector containing an insect-specific promoter (Invitrogen). The resulted pIZ/pre-KmiR-1 plasmid was transfected into C6/36 cells using Cellfectin (Invitrogen) as a transfection reagent. Control cells were transfected with the empty pIZ/V5-His vector. RNA was analysed from the cells 48 h after transfection by northern blot with the 3' probe (see above). In addition, pre-KUN-miR-1 (3'SL, last 80 nt of the viral genome) and the complete sRNA (last 525 nt of the viral genome) were PCR amplified from pBS-3'XX plasmid (7) and cloned into the 3'-UTR region of Semliki Forest virus replicon pSFV-1 (Invitrogen) expressing GFP (pSFV1-GFP, Khromykh, A., unpublished data) immediately downstream of the GFP gene. Replicon RNAs were packaged into secreted virus-like particles (VRPs) by co-electroporating replicon RNAs with pSFV-Helper2 RNA (Invitrogen). VRPs were activated by incubation with 20  $\mu$ g/ml of  $\alpha$ -chemotrypsin (Sigma) followed by inactivation of  $\alpha$ -chemotrypsin with 5  $\mu$ g/ml of aprotinin (Sigma). VRP titres were determined by counting GFP-positive cells in a flow cytometer. C6/36 cells were infected with VRPs at MOI of ~1 to 5 and incubated for 3 days before isolating total cellular RNA. KUN-miR-1 RNA was detected by northern blot with the 3' probe (see above).

### RNAi-mediated gene silencing

For RNAi, we used dsRNA synthesized *in vitro* by using MEGAscript T7 kit (Ambion) according to the manufacturer's instructions. T7 promoter sequences (TAATACGACTCACTATAGGG) were incorporated in both forward and reverse primers designed to amplify 450 bp of *Ae. aegypti Dicer-1* (Forward-CCCGGACCAAGTCCTAGTA; reverse-CAACTCTTTCGGCACGTAA), *Dicer-2* (Forward-GCATTGACGACGAAATCATCGTC; reverse-ACCATGGCATCCGCCGGTGTCT), *GATA4* (Forward-AGAAGTGAAGTTCAGCGCGACG; reverse-AGAACAGCTGGTTGTGATGTG) and *GFP* genes. For dsRNA synthesis, 1  $\mu$ g PCR product was used in 16 h incubation at 37°C, DNase treated and precipitated by Lithium chloride method. We used 0.5  $\mu$ g dsRNA for transfection of Aag2 cells, which were transfected again 72 h after the first transfection. Twenty four hours after the second transfection, cells were inoculated with WNV<sub>KUN</sub>. Cells and media containing viruses were collected for analysis at various times after infection.

### Cloning of sRNA plasmids, miRNA sensor constructs and pSuper plasmids

The insect expression vector of the maltose binding protein (MBP) pIB-MBP and the inducible expression vectors of Firefly luciferase (pMT-Fluc) and Renilla luciferase (pMT-Rluc) have previously been described (39,40). The insect expression vectors encoding sRNA were constructed via Gateway cloning using pDonor207 and destination vector pIB-GW (Invitrogen), respectively.

The miRNA sensor constructs contain repeats of (complementary) 5' and 3' regions (harbouring KUN-miR-1) of the WNV 3'SL, designated A1A2 and C1C2 (Figure 4A). The WNV miRNA sensor constructs were made by tandem repetition PCR using 40 nt long primers WNV-miRNA-A1A2, -B1B2, -C1C2 and -D1D2 (Table 1). In this PCR, the last 20 nt of primers WNV-miRNA-A1A2 or -C1C2 are complementary to the last 20 nt of the WNV-miRNA-B1B2 or -D1D2 primers, respectively. A PCR yields a ladder of PCR products consisting of A1A2 (or C1C2) repeat elements. PCR products of ~500 nt (~12 repeats) were isolated, cloned into pJET1.2 plasmid (Fermentas) and sequenced (Eurofins Operon, Germany). A short phosphorylated XbaI linker (NotI-XbaI linker-Phos, Table 1) was inserted into the NotI site of these plasmids and the A1A2 and C1C2 repeat regions were cloned in both orientations as XbaI fragments downstream of Fluc genes in the insect expression vector pMT-Fluc (39). The first set of sensor constructs contains 12 repeats of the A1A2 sequence, and the other set contains 13 repeats of the C1C2 sequence (Figure 4B).

### miRNA sensor experiments

Schneider (S)-2 cells were grown in Schneider's medium (Invitrogen) supplemented with 10% heat inactivated fetal calf serum (FCS) (Gibco) at 28°C. To reach a confluence of 60–70% at the time of transfection, cells were seeded 24 h pre-transfection in a 96-well plate (Greiner) at a

concentration of  $5 \times 10^4$  cells per well. Cells were co-transfected, using Cellfectin II (Invitrogen) according to the manufacturers' instructions, with luciferase-expressing plasmids (3.5 ng pMT-Fluc constructs and 2 ng pMT-Rluc) and 120 ng of either pIB-sfRNA or the negative control pIB-MBP (encoding the maltose binding protein). Expression of the luciferase constructs was induced 19 h post-transfection by  $5 \mu\text{M}$   $\text{CuSO}_4$  and the luciferase expression was determined 24 h post-induction using self-made buffers for the Dual luciferase reporter assay (41).

### Identification of KUN-miR-1 cellular target using a cloning approach

We used a recently described novel method (31) to identify potential KUN-miR-1 targets in the cellular genome of *Ae. aegypti*. Reverse transcription-polymerase chain reaction (RT-PCR) using KUN-miR-1 as reverse complementary primer (5'-TGGTGCAGAACACAGGATCT-3') and a 5' adaptor (5'-TGACCACGCGTATCGGGCACCACGTATGCTATCGATCGTGAGATGGG-3') was performed on total RNA from uninfected and infected Aag2 cells to amplify a sequence which KUN-miR-1 binds to. The PCR product was cloned into the pGEM-T Easy vector (Promega) and sequenced.

### KUN-miR-1 RNA mimics and inhibitors

A KUN-miR-1 mimic dsRNA (5'-UGGUGCGAGAACACAGGAUCU-3', 5'-AUCCUGUGUUCUCGCACC AUU-3'), and two versions of the mimic with mutations in the seed region were chemically synthesized (Genepharma). The mutated mimics were mut1 (5'-UGCCGCGAGAACACAGGAUCU-3', 5'-AUCCUGUGUUCUCGCAGCAUU-3') and mut2 (5'-UGGUGCCGAACACAGGAUCU-3', 5'-AUCCUGUGUUCG GGCACCAUU-3'). Mutated residues are underlined. An unrelated mimic (5'-UUCUCCGAACGUGUCACG UTT-3', 5'-ACGUGACACGUUCGGAGAATT-3') was also used as a negative control. One hundred nanogram of the mimics was transfected into  $10^6$  Aag2 cells using Cellfectin transfection reagent (Invitrogen). Cells were analysed 72 h after transfection with reverse transcription-quantitative PCR (RT-qPCR).

An RNA inhibitor for KUN-miR-1 (5'-AGAUCUG UGUUCUCGCACCA-3') and a control RNA inhibitor (a random sequence; 5'-UCUACUCUUUCUAGGAGG UUGUGA-3') were synthesized by Genepharma. One hundred nanogram of the KUN-miR-1 inhibitor or the control inhibitor was transfected into Aag2 cells using

Cellfectin transfection reagent. Forty eight hours following transfection, cells were infected with WNV<sub>KUN</sub> and collected after 72 h to analyse virus replication with RT-qPCR as below using specific primers to genomic RNA (gRNA) in the capsid gene region (Forward, 5'-GCGAGC TGTTTCTTAGCACGA-3'; Reverse 5'-CCGTGAACCT AAAAAACGCC-3').

### RT-qPCR

GATA4 transcript levels were analysed by RT-qPCR using specific primers to *GATA4* (forward: 5'-GGGACC GATTCTACGTATG-3'; reverse: 5'-CGTAGAATGTTC AATCTGC-3'), while specific primers to the mosquito gene *RPS17* (forward: 5'-CACTCCCAGGTCCGTGGT AT-3'; reverse 5'-GGACACTTCCGGCAGTAGT-3') were used as reference. For GATA4 RNAi experiment, two biological replicates, respectively, with three technical replicates were analysed and for the rest three technical replicates were analysed (as they were also confirmed with northern hybridization) in a Rotor-Gene thermal cycler (QIAGEN) under the following conditions: 50°C for 2 min; 95°C for 2 min; and 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 15 s, followed by the melting curve (68°C to 95°C). For an inhibitor experiment, two biological replicates with three technical replicates were analysed. Melting curves for each sample were analysed after each run to check the specificity of amplification. The relative ratio of GATA4 mRNA or WNV<sub>KUN</sub> gRNA to cellular RNA (*RPS17* used for normalizing data) was determined using the specific primers given above and *RPS17* specific primers. The *t*-test was used to compare differences in means between different treatments. Fold changes in gRNA and GATA4 were calculated first by normalizing data against *RPS17* cellular gene followed by normalizing data against mock or control treatment. For SFV VRP infections, GATA4 and GFP values were normalized to *RPS17* values first, then fold inductions were calculated for infected versus non-infected samples.

## RESULTS

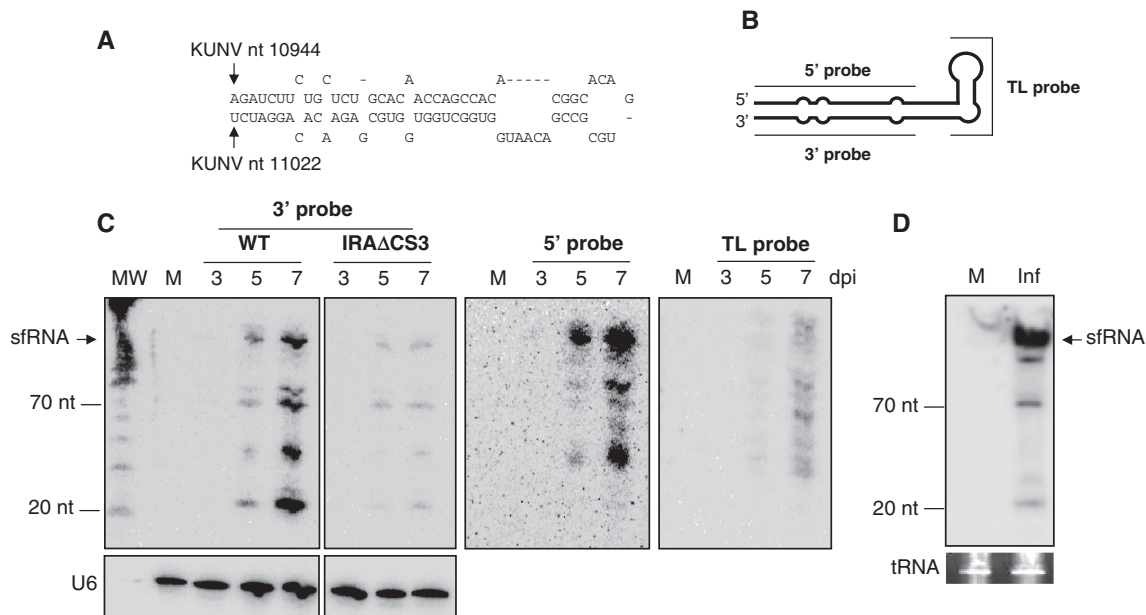
### Bioinformatic prediction of a pre-miRNA in the 3'-UTR of the West Nile virus RNA genome

Considering extensive secondary structures in the 3'-UTR of WNV RNA genome, and its important role in the viral life cycle, we analysed this region for the presence of potential pre-miRNAs. Vmir was used to analyse the

**Table 1.** Oligonucleotides

Primer name	Sequence 5'-3'
NotI-XbaI linker-Phos	<b>GGCCGCTCTAGAC</b>
WNV-miRNA-A1A2	GAGATCTTCTGCTCTGCACAACCAGCCACACGGCACAGTG
WNV-miRNA-B1B2	TGTGCAGAGCAGAAGATCTCCACTGTGCCGTGTGGCTGGT
WNV-miRNA-C1C2	CGCCGACAATGGTGGCTGGTGGTGGCAGAACACAGGATCT
WNV-miRNA-D1D2	ACCAGCCACCATTGTCCGGCAGATCCTGTGTTCTCGCACC

(Partial) restriction sites are in bold italics.



**Figure 1.** Prediction and experimental detection of  $WNV_{KUN}$  miRNA. (A) Sequence and secondary structure of the predicted  $WNV_{KUN}$  pre-miRNA hairpin (pre-KUN-miR-1). Nucleotide positions in the  $WNV_{KUN}$  genome are shown. (B) Location of probes used for KUN-miR-1 detection in the 3'SL. 5' probe is complementary to the 5' stem of pre-miRNA, 3' probe is complementary to the 3' stem of pre-miRNA, TL probe is complementary to the terminal loop. (C) Northern blot analysis using 3'SL-specific probes of small RNAs from C6/36 cells infected with the wild-type  $WNV_{KUN}$  and  $IRA\Delta CS3$  mutant  $WNV_{KUN}$  virus defective in the generation of sfRNA (7). dpi, days post-infection; MW, molecular weight marker; M, mock-infection; WT, wild-type. U6 RNA serves as loading control. The same blot was used for all 3 probes. (D) Northern blot analysis of RNA from Aag2 cells infected (5 days) with  $WNV_{KUN}$  using the 3' probe.

$WNV_{KUN}$  RNA genome for possible pre-miRNA hairpin structures, using stringent filtering parameters as described before (32). An imperfect SL was predicted to be located between nt 10944 and 11022 co-linear with the viral genome and thus corresponds to the highly conserved 3'SL (Figure 1A).

#### Detection of WNV-derived miRNA in RNAi-incompetent C6/36 and RNAi-competent Aag2 mosquito cells

To investigate whether the miRNA predicted to be derived from the 3'SL is indeed produced in infected cells, *Aedes albopictus* C6/36 cells were infected with  $WNV_{KUN}$ . Enriched small RNAs were subjected to northern blot consecutively with three different probes: a '5' probe' complementary to a sequence derived from the 5' stem of the pre-miRNA, a '3' probe' to detect the 3' stem of the pre-miRNA and a 'terminal loop (TL) probe' complementary to the sequence of the terminal loop (see 'Materials and Methods' section for sequences) (Figure 1B). KUN-miR-1 of 21 nt as well as a larger RNA of ~70 nt presumably representing the pre-miRNA, were clearly detected using the 3' probe, while only larger RNAs and no 21 nt RNA were detected using either the 5' probe or the TL probe (Figure 1C). In addition, the pre- and mature miRNAs were also detected in *Ae. aegypti* Aag2 cells 5 days after infection with  $WNV_{KUN}$  (Figure 1D). Our attempts to detect KUN-miR-1 in infected mammalian BHK cells using the same experimental conditions failed (data not shown). This correlates with the failure to detect WNV-derived miRNAs in several cell lines published previously (42). However, no mosquito cell lines

were tested in the study. Tissue-specific expression of cellular and viral miRNAs is very well established; therefore, the lack of detection of WNV-encoded miRNA(s) in mammalian cell lines does not exclude the possibility of their expression in mosquito vectors.

When C6/36 cells were infected with a mutant  $WNV_{KUN}$  virus incapable of generating 3'-UTR-derived sfRNA normally produced in abundance in wild-type virus-infected cells (7), significantly less pre- and mature KUN-miR-1 were detected (Figure 1C,  $IRA\Delta CS3$ ), which also coincided with previously shown less efficient viral replication in mosquito cells (7). Highly diminished but still detectable amount of KUN-miR-1 produced by the mutant virus is likely to be explained by the smaller RNA fragment (sfRNA3) containing 3'SL (pre-miRNA) that is produced by the mutant virus albeit in greatly reduced amounts (7,43). The diminished amount of KUN-miR-1 RNA in cells infected with sfRNA-deficient mutant virus and its resulting effect on virus replication indicate that KUN-miR-1 is likely to be the product of  $WNV_{KUN}$  sfRNA and is essential for efficient virus replication in mosquito cells.

#### Depletion of Dicer-1, but not Dicer-2, in Aag2 cells diminishes expression of KUN-miR-1

One of the key enzymes in the biogenesis of miRNAs is Dicer-1 that cleaves the pre-miRNA SL to generate mature miRNA/miRNA\* duplex (14). To further confirm that KUN-miR-1 is a product of miRNA biogenesis, *Dicer-1* was silenced in Aag2 cells using RNAi approach followed

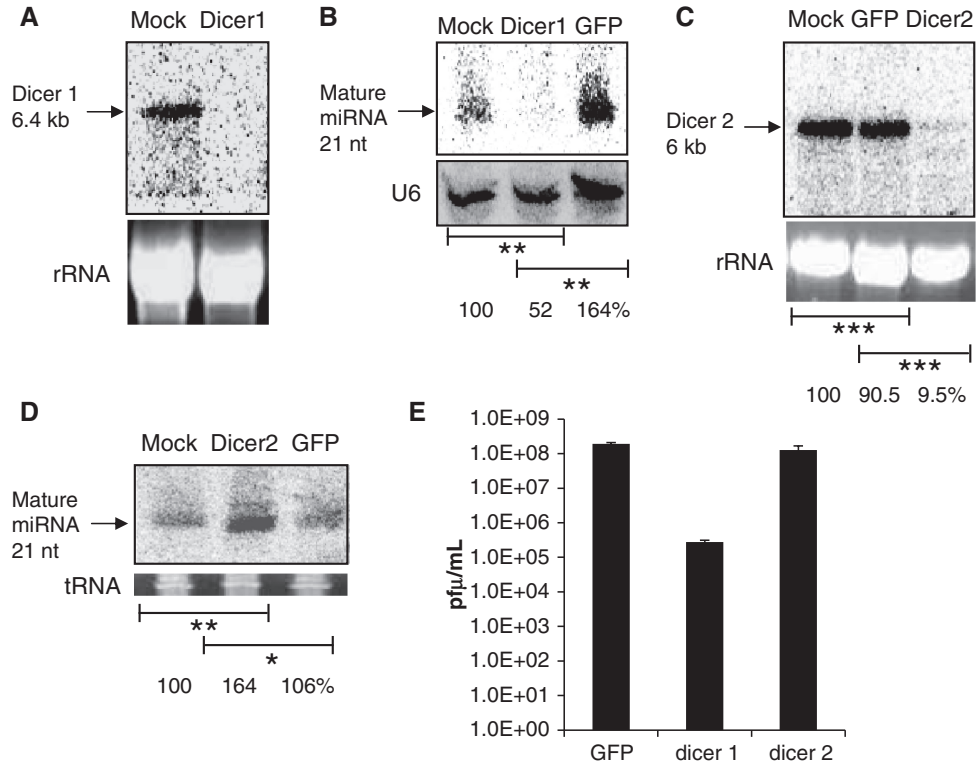
by the infection of cells with WNV<sub>KUN</sub>. Silencing of *Dicer-1* mRNA was confirmed by northern hybridization (Figure 2A). As expected, there was a significant decline in KUN-miR-1 production in *Dicer-1*-silenced cells compared with mock- or GFP-dsRNA-transfected cells despite the detected presence of pre-KUN-miR-1 in all the samples (Figure 2B). However, when *Dicer-2* which is normally involved in the degradation of dsRNA was silenced (Figure 2C), production of KUN-miR-1 did not decline but even increased compared with mock or GFP dsRNA transfections (Figure 2D). The increase is likely due to more efficient virus replication in the absence of cellular RNAi response because of the *Dicer-2* knockdown [e.g. (44)]. This further confirmed that KUN-miR-1 is not a viral dsRNA degradation product processed by *Dicer-2*. In addition, we investigated the effect of *Dicer-1* and *Dicer-2* depletions on WNV<sub>KUN</sub> replication and showed that only *Dicer-1* knockdown and not *Dicer-2* reduces virus replication (Figure 2E).

**Cloning and ectopic expression of KUN-miR-1**

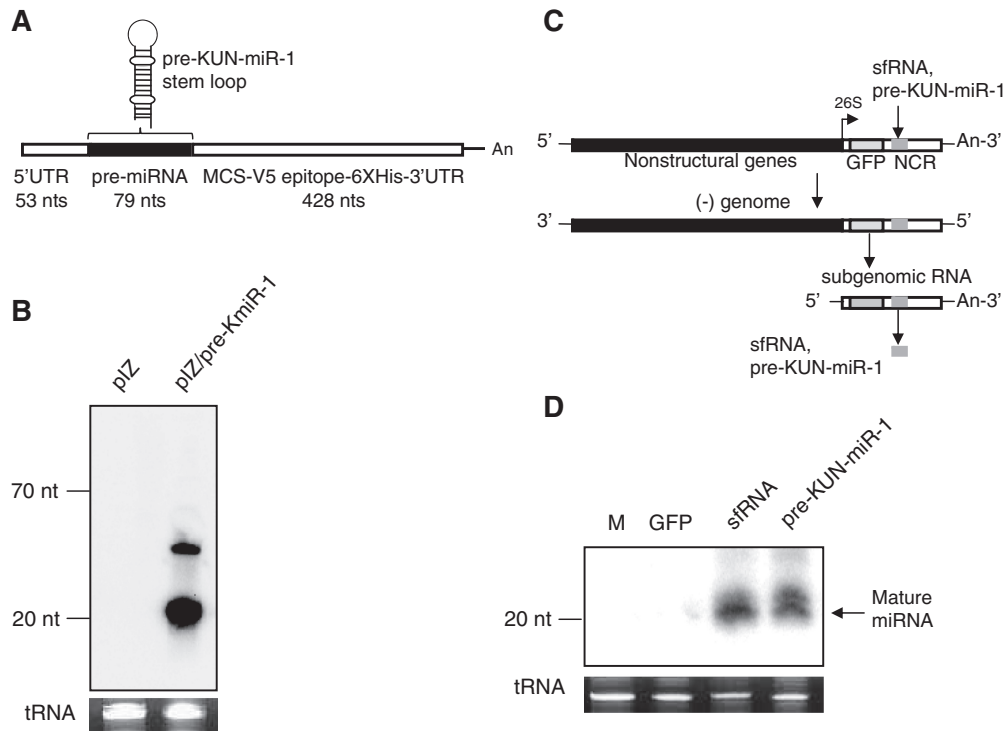
To determine the sequence of the detected viral miRNA, we used two independent miRNA-specific cloning methods (37,38). Using both methods, after sequencing a

total of 60 clones, 12 clones were found to all contain the KUN-miR-1 with the exact sequence of 5'-UGGUGCGA GAACACAGGAUCU-3' (corresponding to nucleotides 11002-11022 in WNV<sub>KUN</sub> genomic RNA). All the other 48 clones contained random cellular sequences (mostly ribosomal) of various lengths that could not be located on the viral genome. Thus, the sequence of KUN-miR-1 was found to represent the very last 21 nt of the viral genomic RNA. To the best of our knowledge, this is the first report demonstrating generation of virus-encoded miRNA by any RNA virus with the exception of HIV.

To determine whether KUN-miR-1 can be generated from the pre-miRNA in isolation from other viral or virus-induced host factors and is not a degradation product from the end of the viral genome, we cloned the pre-KUN-miR-1 sequence in the insect expression plasmid vector pIZ/V5-His, transfected the resulting pIZ/pre-KmiR-1 plasmid into C6/36 cells and analysed RNA from transfected cells by northern blot with the 3' probe. It is worth mentioning that the transcript produced from this plasmid contained a 5'-UTR from the vector (53 nt) followed by 79 nt pre-KUN-miR-1 sequence, and followed by another 428 nt of vector sequences comprising parts of the multi-cloning site, V5 epitope, 6×His tag and 3'-UTR



**Figure 2.** *Dicer-1* silencing led to reductions in KUN-miR-1 levels. (A) Northern hybridization showing silencing of *Dicer-1* in Aag2 cells after mock and *Dicer-1* dsRNA transfections. rRNA is shown as loading control. (B) Northern blot analysis of Aag2 cells after mock-transfection and infection with WNV<sub>KUN</sub> (7 days; Mock), *Dicer-1* dsRNA transfection and infection with WNV<sub>KUN</sub> (Dicer1) and GFP dsRNA transfection and infection with WNV<sub>KUN</sub> (GFP). U6 is shown as loading control. (C) Northern hybridization showing silencing of *Dicer-2* in Aag2 cells after mock, GFP and *Dicer-2* dsRNA transfections. rRNA is shown as loading control. (D) Northern blot analysis of Aag2 cells after mock-transfection and infection with WNV<sub>KUN</sub> (7 days; Mock), *Dicer-2* dsRNA transfection and infection with WNV<sub>KUN</sub> (Dicer2) and GFP dsRNA transfection and infection with WNV<sub>KUN</sub> (GFP). tRNA is shown as loading control. Densitometry of northern blot signals were carried out based on three independent determinations. Significant differences in expression levels detected using *t*-test (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001). (E) Plaque assay results showing WNV<sub>KUN</sub> titer 72 h after infection of Aag2 cells previously transfected with GFP dsRNA, *Dicer-1* dsRNA or *Dicer-2* dsRNA.



**Figure 3.** Ectopic expression of KUN-miR-1. (A) Diagram representing the RNA transcript of the ectopically expressed pre-KUN-miR-1 in pIZ vector. MCS, multi-cloning site. (B) Northern blot of RNA derived from C6/36 cells transfected with plasmid DNA pIZ/pre-KmiR-1 (48 h after transfection) encoding WNV<sub>KUN</sub> pre-miRNA using 3' probe specific for KUN-miR-1. pIZ, empty vector. tRNA is shown as loading control. (C) Schematic diagram of Semliki Forest virus (SFV) replicon constructs expressing WNV<sub>KUN</sub> sfRNA and pre-KUN-miR-1. sfRNA and pre-KUN-miR-1 were inserted in the 3'NCR of the SFV replicon downstream of the GFP gene. SFV replicon RNA is first transcribed into the complementary (–) strand from which subgenomic RNA encoding GFP gene and 3'NCR is produced in large excess to the (+) strand genomic RNA. Both WNV<sub>KUN</sub> RNAs are produced primarily from this subgenomic RNA. (D) Northern blot hybridization with KUN-miR-1-specific probe of C6/36 cells infected with VRPs containing encapsidated GFP-expressing SFV replicon RNAs encoding pre-KUN-miR-1 RNA or sfRNA. RNA was isolated at 4 days post-infection. The faint upper band is the intermediate band also observed in mosquito virus-infected (Figure 1B) or plasmid-transfected cells (Figure 3B). M, mock-infected cells; GFP, SFV1-GFP VRP-infected cells; sfRNA, SFV1-GFP-sfRNA VRP-infected cells; pre-KUN-miR-1, SFV1-GFP-pre-KUN-miR1 VRP-infected cells.

containing a polyA signal (Figure 3A); therefore, the pre-miRNA SL was not located at the very end of the transcript but flanked by vector sequences. A 21-nt RNA representing KUN-miR-1 was clearly detected (Figure 3B), thus confirming that KUN-miR-1 is indeed produced from the pre-miRNA sequence and is not an artefact of degradation from the end of the viral genome. An RNA band of 40 nt between mature miRNA and pre-miRNA, which was also detected in WNV<sub>KUN</sub>-infected mosquito cells (Figure 1C), was visible in pIZ/pre-KmiR-1-transfected cells. Intermediate fragments are frequently observed for other miRNAs [e.g. (25,45)].

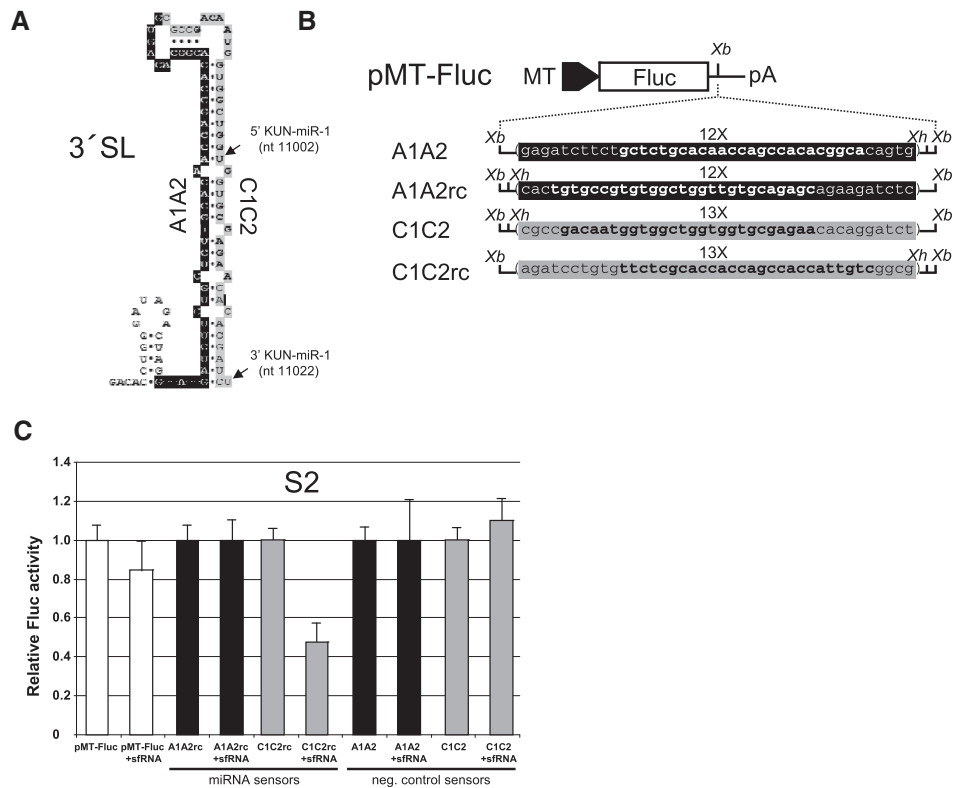
To further confirm that the WNV miRNA can be produced by cytoplasmic pathway and that 3'SL as well as sfRNA serves as a source of it, we cloned pre-KUN-miR-1 (3'SL) as well as the entire sfRNA into the Semliki Forest virus replicon pSFV1 expressing GFP (Figure 3C), generated secreted VRPs (see 'Materials and Methods' section) and used these VRPs to infect C6/36 cells. Northern blot of total RNA from VRP-infected cells showed production of mature miRNA by both replicons (Figure 3D), demonstrating that (i) KUN-miR-1 RNA can be produced from an unrelated cytoplasmic RNA

virus, and (ii) that both 3'SL and sfRNA can serve as a source of KUN-miR-1.

### Functionality of KUN-miR-1 in insect cells

To act as miRNA, it is essential that the KUN-miR-1 is loaded into the RISC complex, which in turn can interact with the mRNA sharing complementarity to KUN-miR-1. To validate the functionality of KUN-miR-1, a sensor construct was designed by fusing several repeats of the complementary sequence of the 3'part of 3'SL (Figure 4A) and insertion in the 3'-UTR of Firefly luciferase (pMT-Fluc-C1C2rc). Another sensor construct, harbouring the complementary sequence of the 5'part of the 3'SL (pMT-Fluc-A1A2rc) was used to ensure the incorporation of KUN-miR-1 into RISC and not the putative corresponding KUN-miR-1\* strand. As controls, vectors harbouring the sequences of either 3' or 5'part fused to the Firefly luciferase (pMT-Fluc-C1C2 and -A1A2) (Figure 4B) or a vector encoding Firefly luciferase alone were constructed.

*Drosophila melanogaster* (S2) cells were co-transfected with the expression vector encoding sfRNA, Renilla



**Figure 4.** Validation of KUN-miR-1 functionality by Firefly luciferase-based sensor constructs. (A) Schematic representation of the WNV 3'SL. A1A2 and C1C2 sequences for tandem repeat cloning into miRNA sensor constructs are indicated in black and grey highlights, respectively. Sequences used for shRNA cloning into pSuper plasmids are indicated in bold. The arrows indicate KUN-miR-1. (B) Schematic representation of miRNA sensor constructs for expression in insect (pMT-Fluc) cells. A1A2 and C1C2 tandem repeats and their reverse complements (rc) are indicated in black and grey highlights, respectively. Sequences used for shRNA cloning into pSuper plasmids are indicated in bold. Fluc, Firefly luciferase; MT, metallothionein promoter; pA, polyadenylation signal; Xb, XbaI; Xh, XhoI restriction sites. (C) Silencing of miRNA sensor constructs by sfRNA expression in *Drosophila* S2 cells. Cells were co-transfected with pMT-Renilla, pMT-Fluc(sensor constructs) and either piB-sfRNA or piB-MBP (negative control). After induction, the relative luciferase expression (Firefly/Renilla) was determined 24 h post-induction (hpi) and the mean of two independent experiments in duplicate is shown with standard error.

luciferase and the Firefly luciferase sensor constructs. Only transfection with the sensor construct harbouring the reverse complement of the 3' part of 3'SL (C1C2rc), but not the other sensor constructs, led to a significant reduction in luciferase expression of ~45% (Figure 4C), compared with the negative control (MBP). Similar results were observed in mosquito cells (U4.4), although the silencing effect was less intense (data not shown); probably due to the low transfection efficiency observed in these cells (<1%). The results demonstrate that KUN-miR-1 produced from sfRNA is functionally active in insect cells. Similar experiments using mammalian expression reporter constructs failed to detect an effect of sfRNA-derived miRNA on luciferase expression (data not shown).

#### RNA inhibitor of KUN-miR-1 suppresses viral RNA replication

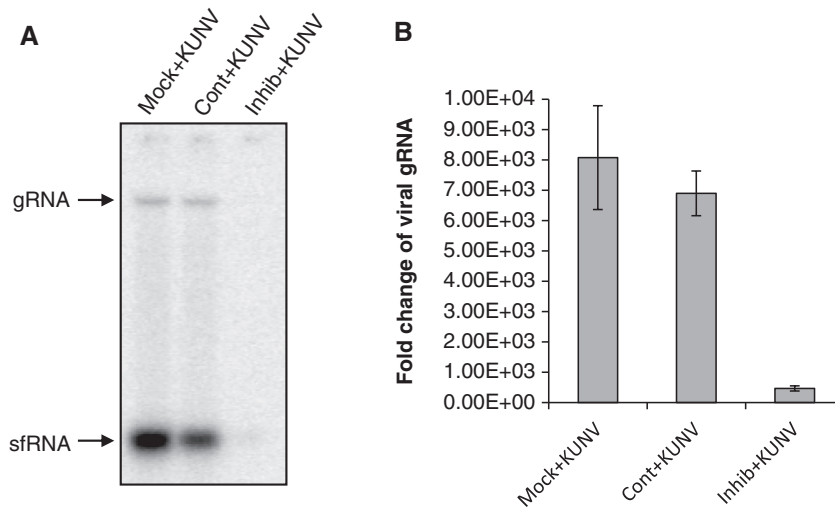
To explore the effect of KUN-miR-1 on virus replication, we used a synthetic RNA inhibitor complementary to KUN-miR-1 sequence to inhibit the miRNA function. A miRNA sequence-specific inhibitor binds to miRNA thus preventing its binding to its target; consequently, the effect of the miRNA on the target is inhibited. Aag2 cells were

transfected with KUN-miR-1 RNA inhibitor, a control RNA inhibitor with random sequences and a mock. Forty eight hours after transfection, cells were infected with WNV<sub>KUN</sub> and analysed for viral RNA replication. Northern blot of total cellular RNA with 3'-UTR-specific radiolabelled cDNA probe showed dramatic decrease in accumulated WNV<sub>KUN</sub> genomic and sfRNA in cells treated with a specific inhibitor, whereas only slight decrease was observed in the control inhibitor sample (Figure 5A). Quantitative analysis of viral RNA using RT-qPCR with primers specific for the capsid coding region of gRNA normalized against mosquito *RPS17* gene and mock-infection confirmed significantly reduced replication of viral RNA in cells treated with KUN-miR-1-specific inhibitor (Figure 5B; ~17 fold reduction,  $P < 0.0001$ ), but not in cells treated with control non-specific inhibitor (Figure 5B; <0.5 fold reduction,  $P > 0.05$ ).

#### KUN-miR-1 up-regulates GATA4 mRNA and facilitates virus replication

Viral miRNAs have been shown to fulfil important functions in the regulation of host cell gene expression (15). Consequently, we investigated whether KUN-miR-1





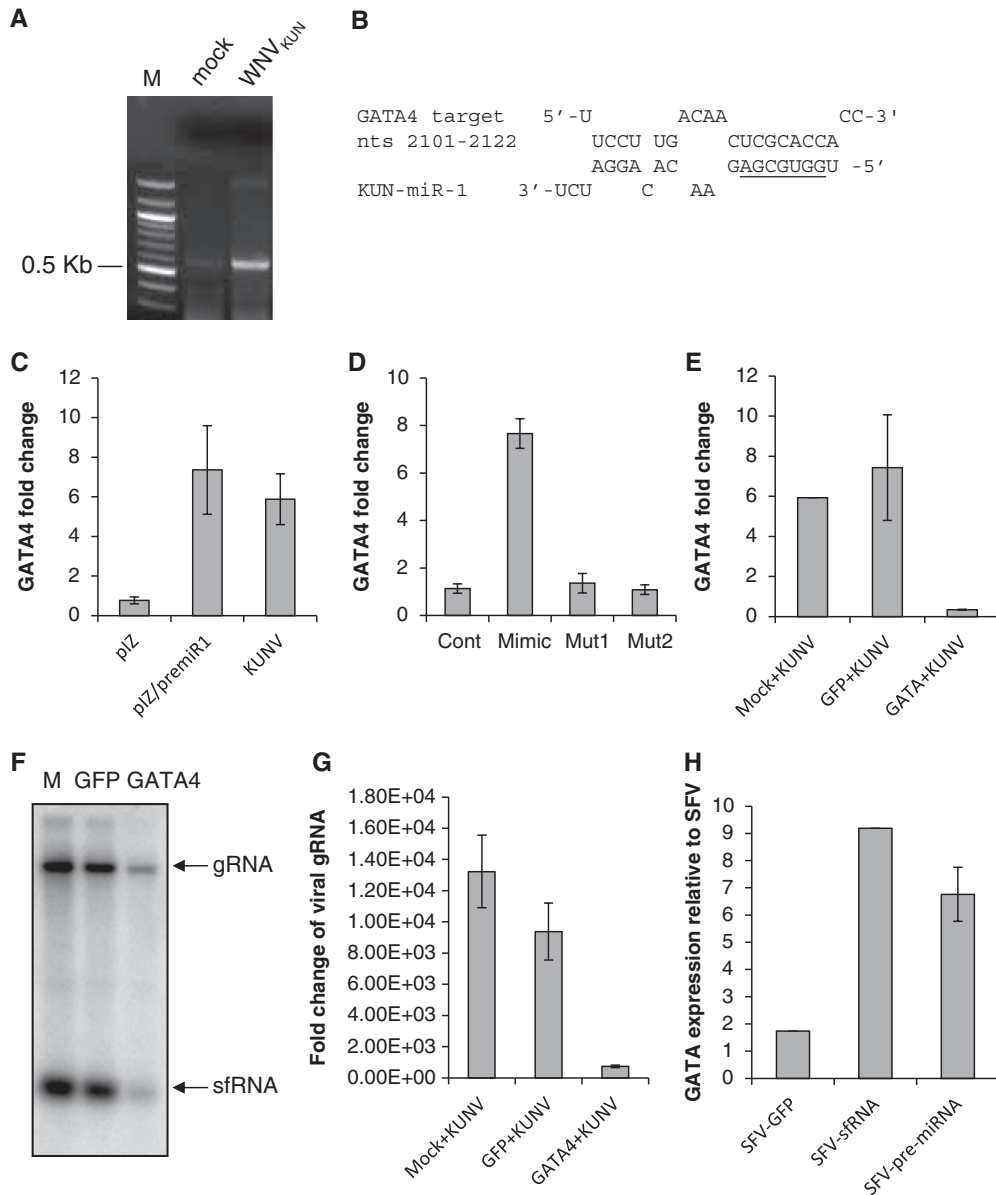
**Figure 5.** RNA inhibitor of KUN-miR-1 reduces replication of WNV<sub>KUN</sub> viral RNA. (A) Northern blot hybridization with WNV<sub>KUN</sub> 3'-UTR specific of total RNA from Aag2 cells transfected with a control inhibitor (Cont) and KUN-miR-1 specific inhibitor (Inhib) for 48 h and then infected with WNV<sub>KUN</sub> (KUNV) for 72 hours. (B) RT-qPCR analysis of RNA samples from (A). Specific primers to the capsid coding region of WNV<sub>KUN</sub> gRNA were used and data were first normalized against RPS17 gene transcript levels and then a fold change in genomic RNA was calculated against mock-infected values. Error bars indicate standard deviations of averages from two biological and three technical replicates.

regulates expression of cellular genes. To identify potential KUN-miR-1 targets in the cellular genome of *Ae. aegypti*, we used a recently described novel method (31). Accordingly, RT-PCR using KUN-miR-1 as reverse complementary primer and a 5' adaptor was performed on total RNA from uninfected and infected *Ae. aegypti* Aag2 cells to amplify an RNA which KUN-miR-1 binds to. A PCR product of ~0.6 kb in size was amplified (Figure 6A) and sequenced following cloning. Note that the amount of this amplified product was increased in infected cells compared with non-infected cells (Figure 6A). The PCR product from two different PCRs was sequenced and the obtained sequence was analysed using BLAST (NCBI) against the *Ae. aegypti* genome, which revealed that the amplified product was corresponding to the GATA4 sequence (GenBank accession number XM\_001654324). This suggested that GATA4 mRNA may be a target for KUN-miR-1.

The potential target sequence of KUN-miR-1 in GATA4 mRNA was further analysed using RNAHybrid and RNA22 software (IBM). This confirmed the target position at nucleotide 2101 to 2122 of the GATA4 mRNA with the KUN-miR-1 seed region fully complementary to nucleotides 2113–2121 (Figure 6B). RT-qPCR analysis revealed that the amount of GATA4 mRNA was increased ~6-fold in Aag2 cells infected with WNV<sub>KUN</sub> 96 h after infection (Figure 6C;  $P < 0.0001$ ). Similar ~7-fold increase was also observed in cells transfected with pIZ/pre-KmiR-1 (Figure 6C;  $P < 0.0001$ ), but not in cells transfected with empty pIZ vector (Figure 6C;  $P > 0.05$ ). These were also confirmed by northern hybridization (data not shown). However, transcript levels of another cellular mRNA encoding the inhibitor of apoptosis 2 (IAP2) were not affected under the same experimental conditions (data not shown). To further confirm the specific up-regulation of its target by KUN-miR-1, we

transfected Aag2 cells with the mature KUN-miR-1 RNA duplex (mimic). Subsequently, the status of GATA4 transcript levels were analysed by RT-qPCR. Interestingly, we observed ~8-fold increase in GATA4 mRNA in cells transfected with specific mimic RNA, but not in cells transfected with a non-specific (control) mimic RNA (Figure 6D;  $P < 0.0001$ ). However, when cells were transfected with KUN-miR-1 mimics containing mutations in their seed regions (mut1 and mut2), GATA4 mRNA induction was not observed (Figure 6D;  $P > 0.05$ ). These results were also confirmed with northern hybridizations (data not shown). This confirmed that the increase in the amount of GATA4 mRNA was caused specifically by KUN-miR-1 RNA and not by any other viral or virus-induced cellular factors.

To find out if GATA4 is essential for virus replication, Aag2 cells were transfected twice with *in vitro* synthesized dsRNAs specific to *GATA4* and *GFP* (control) genes and subsequently infected with WNV<sub>KUN</sub>. In cells transfected with dsRNA specific to GATA4, substantially less GATA4-specific transcripts were detected relative to cells transfected with GFP dsRNA or mock (Figure 5E). This also confirmed successful knockdown of GATA4 (Figure 5E). To determine the effect of GATA4 knockdown on viral RNA replication, we performed northern blot of total cellular RNA with 3'-UTR-specific radiolabelled cDNA probe and showed clear decrease in accumulated WNV<sub>KUN</sub> genomic and sfRNA in cells treated with GATA4-specific dsRNA (Figure 6F). No decrease in viral RNA accumulation was observed in cells treated with a control (GFP-specific) dsRNA (Figure 6F). Quantitative analysis of viral RNA using RT-qPCR with primers specific for the capsid coding region of gRNA normalized against mosquito *RPS17* gene and mock-infection confirmed reduced replication of viral RNA in cells treated with GATA4-specific dsRNA



**Figure 6.** Identification of GATA4 mRNA as a target for KUN-miR-1 miRNA. (A) RT-PCR of RNA from mock- and WNV<sub>KUN</sub>-infected Aag2 cells. (B) Predicted target of KUN-miR-1 miRNA in the GATA4 mRNA coding region. Seed region is underlined. (C) RT-qPCR analysis of RNA from Aag2 cells with GATA4-specific primers transfected with pIZ empty vector, plasmid pIZ/pre-KmiR-1 encoding pre-miRNA or mock-transfected and infected with WNV<sub>KUN</sub>. Mock-transfected cells (uninfected) were also used as control. (D) RT-qPCR analysis of RNA from Aag2 cells with GATA4-specific primers 72 h after transfection with control mimic (Cont), KUN-miR-1 mimic (Mimic), mutant 1 mimic (Mut1) and mutant 2 mimic (Mut2) (see ‘Materials and Methods’ section for sequences and calculation of fold induction). (E) RT-qPCR analysis of RNA from Aag2 cells with GATA4-specific primers 72 h after infection with WNV<sub>KUN</sub>, which were previously transfected twice with dsRNA specific to GFP or GATA4 genes and mock-transfected. (F) Northern blot hybridization showing reduced viral RNA accumulation at 72 h after infection of Aag2 cells previously mock-transfected (M), or transfected with GFP dsRNA or GATA4 dsRNA. (G) RT-qPCR results of RNA samples from (F) for the WNV<sub>KUN</sub> genomic RNA with primers specific for the capsid region. Data were first normalized against RPS17 gene transcript levels and then a fold change in genomic RNA was calculated against mock-infected values. (H) RT-qPCR with GATA4-specific primers of RNA from C6/36 cells infected with VRPs containing encapsidated GFP-expressing SFV replicon RNAs encoding pre-KUN-miR-1 RNA (SFV-pre-KUN-miR-1) or sfRNA (SFV-sfRNA). Control cells were infected with SFV-GFP VRPs. RNA was isolated at 4 days post-infection and used for RT-qPCR. Shown fold inductions for GATA4 normalized to the levels of GFP-expressing subgenomic SFV RNA were calculated as in ‘Methods and Materials’ section. Error bars in all the graphs in this figure indicate standard deviations of averages from replicates indicated in the ‘Materials and Methods’ section.

(Figure 6G; ~17 fold reduction,  $P < 0.0001$ ), but not in cells treated with control GFP dsRNA (Figure 6G; <0.2 fold reduction;  $P > 0.05$ ).

To further confirm that the cytoplasmically produced KUN-miR-1 is functional, we infected C6/36 cells with

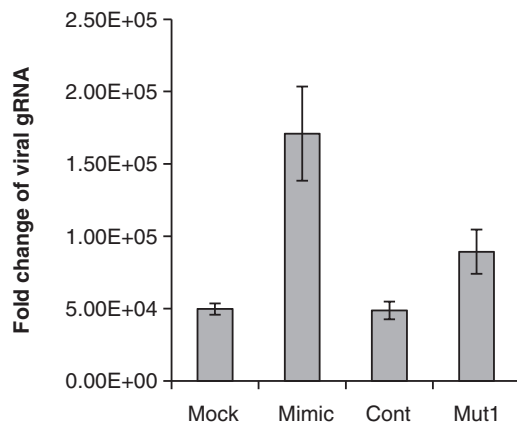
SFV VRPs that produce KUN-miR-1 either from pre-miRNA or from sfRNA (Figure 3C and D) and showed that GATA4 was induced only in cells infected with SFV1-GFP-sfRNA and SFV-GFP-pre-KUN-miR-1 constructs and not in SFV-GFP-infected cells (Figure 6H;

$P < 0.0001$ ). A slight difference in the levels of SFV-GFP subgenomic RNA between the samples detected by GFP-specific primers was taken into account when calculating fold induction values by normalizing data to GFP expression. The expression levels of two host genes tested alongside, *RPS17* and *actin* did not change in any of the treatments (data not shown).

To further confirm the role for KUN-miR-1 and induction of GATA4 in viral replication, we transfected Aag2 cells with KUN-miR-1 mimic, which was shown to induce expression of GATA4 (Figure 6D), and then infected the cells with inefficiently replicating IRA $\Delta$ CS3 mutant WNV<sub>KUN</sub> virus largely defective in KUN-miR-1 production (Figure 1C). RT-qPCR results demonstrated that virus genomic RNA replication was increased by ~3.5 fold compared with cells either mock-transfected or transfected with a control mimic (Figure 7;  $P < 0.001$ ). There was also a slight increase in viral genomic RNA replication in mut1 mimic RNA-transfected cells (~1.7-fold), but the difference was not statistically significant ( $P > 0.05$ ). The results show that KUN-miR-1 mimic, which induces GATA4, is able to enhance replication of the mutant virus that is largely deficient in miRNA production.

## DISCUSSION

In this study, we used bioinformatics analysis to predict a SL structure with imperfect complementarity in the highly conserved terminal 3'SL sequence of the WNV<sub>KUN</sub> RNA genome as having potential characteristic of pre-miRNA. We demonstrated that this SL structure serves as a precursor for the production of a 21-nt small viral regulatory RNA (svrRNA), which has typical characteristics of an miRNA. Therefore, we coined this small RNA, KUN-miR-1. In addition to being produced in the



**Figure 7.** KUN-miR-1 mimic increases virus RNA replication of IRA $\Delta$ CS3 mutant WNV<sub>KUN</sub> virus defective in generation of svrRNA. RT-qPCR results of RNA samples from Aag2 cells infected for 72 h with IRA $\Delta$ CS3 mutant WNV<sub>KUN</sub> virus, which were previously mock-transfected or transfected with KUN-miR-1 mimic, control mimic or mut1 mimic for 48 h. To quantify viral gRNA, specific primers to the virus capsid region were used. Data were first normalized against RPS17 gene transcript levels and then a fold change in gRNA was calculated against mock-infected values. Error bars indicate standard deviations of averages from two biological and three technical replicates.

WNV<sub>KUN</sub>-infected mosquito cells, KUN-miR-1 was also shown to be generated independently from the precursor SL structure (pre-KUN-miR-1) either cloned into a plasmid expression vector or in a cytoplasmically replicating SFV RNA replicon. However, the small RNA was not detected in WNV<sub>KUN</sub>-infected mammalian cells under the same experimental conditions, consistent with previous studies (42). At this stage, it is not clear why KUN-miR-1 is not produced in mammalian cells infected with the virus. Considering that differential regulation of miRNA biogenesis in different tissues has been well established in the field, we can only speculate at this stage that additional regulatory factors in mammalian cells interacting with the 3'SL may inhibit processing of the SL to mature miRNA. Further investigations are required to identify these factors. Alternatively, KUN-miR-1 may be produced in much smaller quantities in mammalian cells and therefore not detectable under the experimental conditions used.

In insects, Dicer-1's function is mainly implicated in miRNA biogenesis, whereas Dicer-2 is primarily involved in RNAi response generating short interfering RNAs (siRNAs) (16). Our results revealed that silencing of *Dicer-1*, and not *Dicer-2*, in Aag2 cells led to significant reductions in KUN-miR-1 levels as well as virus replication. Further, KUN-miR-1 and its corresponding pre-miRNA were detected in RNAi-incompetent C6/36 cells after infection with WNV<sub>KUN</sub>. Chotkowski *et al.* (44) showed that WNV<sub>NY</sub>-infected C6/36 cells did not produce WNV-homologous siRNAs, suggesting that the virus does not induce an RNAi response. This was further confirmed in two independent recent reports demonstrating that C6/36 cells are RNAi incompetent, whereas Aag2 cells are RNAi competent (29,30). This was shown to be due to the defective expression and/or function of Dicer-2 in C6/36 cells. Taken into account the diminishing effect of Dicer-1, and not Dicer-2, knockdown on KUN-miR-1 production in Aag2 cells and considering that C6/36 cells are not able to generate siRNAs upon viral infection, the 21-nt band detected in WNV-infected mosquito cells is likely to represent a WNV<sub>KUN</sub>-encoded miRNA.

Host- or virus-encoded miRNAs have been shown to play significant roles in host-virus interactions [reviewed in (46)]. So far, over 200 virus-encoded miRNAs have been reported; mostly from DNA viruses (46). From RNA viruses, miRNA has only been reported from HIV (25). The initial scepticisms regarding the potential HIV-1-encoded miRNAs originated partly from the lack of information with regard to the existence of non-canonical pathways of miRNA biogenesis and partly due to the fact that they have not been cloned from HIV-infected cells (perhaps due to low abundance) and their detection have not been consistent. In our study, KUN-miR-1 was cloned from WNV-infected cells using two independent cloning strategies and consistently detected in virus-infected or plasmid-transfected mosquito cells. It is still unknown how RNA viruses that replicate in the cytoplasm can produce miRNAs by bypassing the Drosha processing step of the pri-miRNA. However, recent evidence strongly suggests that non-canonical Dicer-dependent mechanisms exist that are able to

process pri-miRNA SLs generated by RNA viruses in the cell cytoplasm [e.g. (27,28)]. For example, successful expression of miRNAs has been recently reported from a cellular miRNA precursor inserted into the viral genome of the alphavirus Sindbis (27) and a related flavivirus tick-borne encephalitis virus (26) implying that miRNA biogenesis can indeed be used by RNA viruses that replicate in the cytoplasm to generate virus-encoded mature miRNAs. Notably, tick-borne encephalitis virus belongs to the same virus genus as WNV and has the same replication strategy. We also showed that SFV replicons, which replicate in the cytoplasm, were able to produce KUN-miR-1 from the pre-KUN-miR-1 or sRNA in C6/36 RNAi-incompetent cells. The mechanism of miRNA biogenesis by cytoplasmically replicating RNA viruses remains largely unknown with different studies suggesting either nuclear-initiated or purely cytoplasmic processes. Studies with tick-borne encephalitis virus-encoded cellular miRNA showed that down-regulation of Droscha by shRNAs resulted in the alteration of a plasmid-based miRNA-dependent reporter gene activity in infected cells, which prompted authors to suggest a role for canonical nuclear-initiated mechanism for miRNA generation, either via re-distribution of Droscha to the cytoplasm or by viral RNA presence in the nucleus. The experiment in the TBEV study however, did not directly show the effect of Droscha knockdown on the generation of miRNA, and the assumption on the potential nuclear localization of viral RNA is based on highly debatable, and unconfirmed by others, single report on nuclear RNA replication of flaviviruses (47). In contrast, studies with Sindbis virus-encoded cellular miRNA used Droscha-knockout cell lines and clearly demonstrated that it did not have any effect on the generation of Sindbis-encoded cellular miRNA. Based on the above findings and our results, we believe that it is highly unlikely that nuclear-initiated mechanism is involved in the generation of miRNAs from cytoplasmically replicating RNA viruses, although further extended investigations which are beyond the scope of this study are clearly required to establish the exact mechanism.

Our target studies showed that the mosquito GATA4 mRNA is the target of KUN-miR-1. The expression levels of GATA4 mRNA in mosquito cells was significantly increased in the presence of KUN-miR-1 produced by WNV<sub>KUN</sub>, pre-miRNA cloned in plasmid or SFV RNA replicon, sRNA cloned in SFV RNA replicon or synthetic KUN-miR-1 mimic. Mutations in the seed region of KUN-miR-1 mimic abolished induction of GATA4 mRNAs. The synthetic mimic also increased virus RNA replication of a largely miRNA-deficient WNV<sub>KUN</sub> mutant (IRA $\Delta$ CS3). In addition, RNAi silencing of GATA4 or inhibition of KUN-miR-1 with a sequence-specific synthetic inhibitor led to a significant reduction in virus RNA replication. All these findings suggest that the production of KUN-miR-1 and subsequent induction of GATA4 play an important role in the regulation of WNV<sub>KUN</sub> replication. However, the exact mechanism of how KUN-miR-1 can increase GATA4 mRNA accumulation in cells is not clear and requires further

investigation. One possibility is that, since miRNA-RISC complex competitively binds to mRNA, it can inhibit the activity of proteins involved in RNA degradation and therefore enhance mRNA stability as was shown for IL-10 in TLR-triggered macrophages (48). Up-regulation rather than down-regulation of target gene transcription by miRNAs has also been shown in other instances [e.g. (19,49,50)]. Importantly, we did not detect up-regulation of other mosquito mRNAs tested, demonstrating that this up-regulation by KUN-miR-1 is specific for GATA4 mRNA.

In vertebrates and invertebrates, GATA zinc-finger transcription factors play critical roles in the development, differentiation and innate immunity (51). In *Ae. aegypti*, GATA4 is expressed after a blood meal and acts as a transcriptional activator of vitellogenin (*vg*), an important precursor gene in the target of rapamycin (TOR) signalling pathway, a nutrient sensing pathway (52,53). More importantly, *Ae. aegypti* GATA4 is involved in the regulation of *Lipophorin (Lp) Receptor fat body (LpRfb)* genes, which encode protein receptors essential for lipid trafficking in adult female mosquitoes (54). In insects, the re-usable *Lp* is the main lipid carrier protein transporting lipids to various organs through a shuttle mechanism involving a multiprotein complex called lipid transfer particle (54). Up-regulation of *Lp* has been reported upon pathogen entry, such as *Plasmodium* infection of *Ae. aegypti*, while the *LpRfb* gene contains GATA and NF- $\kappa$ B binding motifs in its promoter region that are required for immune-mediated activation (54). Although it is not clear at this stage how up-regulation of GATA4 mRNA by KUN-miR-1 miRNA may benefit WNV<sub>KUN</sub> replication in mosquito cells, one possible benefit could be a potential positive influence of GATA4 on lipid recruitment to the sites of virus replication through the up-regulation of *Lp*. This may support formation of virus-induced membranes, which represent a scaffold for flavivirus RNA replication and virus assembly (55–57). Clearly, further studies are required to identify the mechanism by which KUN-miR-1 induces the up-regulation of GATA4 mRNA and how this may facilitate virus replication as well as to identify other potential targets of KUN-miR-1 miRNA.

In conclusion, we have successfully identified and cloned a WNV-encoded small regulatory viral RNA (srvRNA), and showed that this small RNA is derived from the highly conserved 3'SL sequence of sRNA located at the end of the 3'-UTR in the viral genome. The srvRNA, coined KUN-miR-1, has most of the characteristics of an miRNA. It was detected in RNAi-defective C6/36 mosquito cells and *Dicer-2*-silenced Aag2 cells but not in *Dicer-1*-silenced cells, it was ectopically expressed from the pre-miRNA sequence independently of virus replication and it has the sequence and the structural characteristics of an miRNA. Inhibition of KUN-miR-1 using a synthetic RNA inhibitor also reduced replication of WNV<sub>KUN</sub>, suggesting that expression of the miRNA is essential for virus replication. Importantly, we have shown that this virally encoded small RNA targets and increases the accumulation of GATA4 mRNA, encoding a protein which belongs to a family of

transcription factors that have been implicated in lipid trafficking and immune recognition. RNAi silencing of GATA4 mRNA led to reduced replication of WNV<sub>KUN</sub> while up-regulation of GATA4 by KUN-miR-1 mimic enhanced replication of a miRNA-deficient virus, suggesting that KUN-miR-1 and its target GATA4 play an important role in virus replication in mosquito cells.

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*Conflict of interest statement.* None declared.

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