Chapter II

A baculovirus-encoded microRNA (miRNA) suppresses its host miRNA biogenesis by regulating the Exportin-5 cofactor Ran
2.1 Abstract

miRNAs have emerged as key players in the regulation of various biological processes in eukaryotes, including host-pathogen interactions. Recent studies suggest that viruses encode miRNAs to manipulate their host gene expression to ensure their effective proliferation, whereas host limits virus infection by differentially expressing miRNAs that target essential viral genes. Here, I demonstrate that an insect virus, BmNPV modulates the small RNA-mediated defense of its host, B. mori by encoding a miRNA (bmnpv-miR-1), which downregulates the expression of its host GTP-binding nuclear protein, Ran, an essential component of Exportin-5-mediated nucleocytoplasmic transport machinery mainly involved in small RNA transport from nucleus to cytoplasm. I have demonstrated the sequence dependent interaction of bmnpv-miR-1 with Ran mRNA using cell culture and in vivo assays, including RNAi of Ran. Our results clearly show that bmnpv-miR-1 represses Ran leading to a reduction in the host small RNA population, and consequently the BmNPV load increases in the infected larvae. Blocking of bmnpv-miR-1 resulted in higher expression of Ran and a decrease in BmNPV proliferation. In contrast, blockage of host miRNA, bmo-miR-8, which targets immediate early gene of the virus and whose production was repressed upon bmnpv-miR-1 and Ran dsRNA administration, resulted in significant increase in the virus load in the infected B. mori larvae. The present study provides insights into one of the evasion strategies used by the virus to counter the host defense for its effective proliferation, and has relevance in the development of insect virus control strategies.
2.2 Introduction

In the course of evolution of host-virus interactions viruses have developed very dynamic and sophisticated mechanisms for interacting with host cells. Such interactions are mostly for enhancing production of viruses by abrogating the molecular arsenals mounted by the host cells in the form of induced immune response. Since the discovery of miRNAs (Lee et al. 1993; Reinhart et al. 2000), a number of studies have been carried out to understand the role of miRNAs in almost all the biological processes in eukaryotes (He and Hannon 2004). Apart from eukaryotes, viruses have also been shown to encode miRNAs (Pfeffer et al. 2004; Grundhoff and Sullivan 2011). Recently, miRNAs have been shown to play a critical role in intricate host-viral interactions (Gottwein and Cullen 2008; Ghosh et al. 2009). Generally, structural and regulatory viral proteins do the job for virus to manipulate host system for its own advantage, but now looking at the recent advancements in small RNA field and their functional characterization in virus, it is very likely that some viral miRNAs can perform similar role (Boss and Renne 2010). Conceptually, miRNAs can combat immune evasion directly by suppressing the components of the host defense system, and indirectly by limiting the expression levels of the viral proteins (Cullen 2013). The small size of miRNAs, and their ability to sequence specifically regulate the expression of various cognate targets, together make them an ideal tool for viruses to convert the cellular environment favorable for their own replication (Cullen 2006). Given the often subtle nature of miRNA-mediated regulation, it is most likely that virus-derived miRNAs have a
hidden role during lytic stage of virus infection, in which robust changes occur in
the host and viral gene expression (Cullen 2011). Plenty of recent reports suggest
that viral miRNAs are the key players in controlling the viral infection in the host
cell via several diverse mechanisms (Lu and Cullen 2004; Choy et al. 2008; Shapiro
et al. 2010; Grundhoff and Sullivan 2011). Some of the well known strategies
adapted by viruses to achieve their establishment and persistent infection in the
host cells include; modulation of host immune response and escaping recognition by host immune
system (Stern-Ginossar et al. 2007; Xia et al. 2008), anti-apoptosis (Choy et al. 2008; Xu et
al. 2011), cell cycle arrest, and mimicking of host miRNAs (Gottwein et al. 2007;
Skalsky et al. 2007; Zhao et al. 2009). Mostly, viruses with large DNA genomes are
found to encode miRNA (examples are listed in the Table 1.1), due to their
requirement during latency phase, where, generally virus gene expression remains
quiescent (Cullen 2009). Initially, after discovery of virus-derived miRNAs it was a
difficult task to find out the correct target for a viral miRNA by only relying on the
computational methods, because viral miRNAs are not conserved as their host
counterparts and same is pertinent for their targets, but later High-throughput
sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) and
other experimental approaches made it easy to fetch out the more cognate targets

The Herpesvirus, Polyomavirus, Ascovirus, Baculovirus, Iridovirus, and
Adenovirus are the virus families, which are known to produce miRNAs, and among
all, Herpesvirus family accounts for more than 50% of the reported viral miRNAs
The functional analysis of virus-encoded miRNA was first reported in Simian vacuolating virus 40 (SV40), a Polyomavirus in which SV-40-derived miRNAs referred as SVmiRNAs, was found to negatively regulate its own gene, T antigen, which expresses in the late stage of infection, and triggers cytotoxic T cells mediated lysis of host cell (Sullivan et al. 2005). Research on viral miRNA-governed gene regulation of host genes is restricted to only mammalian systems, and barely studied in insects due to several reasons, first, best suited insect genetic model organism i.e. Drosophila, does not have any known DNA virus as pathogen, which are mostly found to encode miRNAs, second, except a few, most of the other insects or their respective virus genomes are not yet sequenced, and third, insect viruses are strictly host-specific (Asgari 2011).

In insect viruses, Heliothis virescens ascovirus is the only virus, which is explored for miRNAs and their functional implication in the virus infection (Hussain et al. 2008). This virus encodes a miRNA, which regulates its own DNA polymerase to control viral replication (Hussain et al. 2008). Here, I have shown that BmNPV encodes a miRNA that modulates the host defense machinery by regulating the production of host GTP-binding nuclear protein Ran (Ras-related nuclear protein). Ran, a 25kDa protein, is a member of the Ras family of small GTP-binding proteins, which plays a vital role in nucleocytoplasmic transport of various non-coding RNAs and proteins (Bischoff and Ponstingl 1991). During nuclear export of pre-miRNAs, Exportin-5 binds to the pre-miRNAs in a RanGTP-dependent manner and depletion of Ran results in a severe reduction of pre-miRNA export (Yi
et al. 2003; Bohnsack et al. 2004; Kim 2004; Lund et al. 2004; Wang et al. 2011). Ran exists in two forms inside the cell, RanGTP and RanGDP, which are the active and inactive states of Ran, respectively. These two forms are firmly maintained by chromatin bound guanine nucleotide exchange factor RCC1 (conversion of RanGDP to RanGTP) and cytoplasmic GTPase-activating protein RanGAP (conversion of RanGTP to RanGDP) (Bischoff and Ponstingl 1991; Izaurralde and Adam 1998). In humans, Ran also interacts with other transporters like Exportin-1 and Exportin-t, which are involved in the transport of small nuclear RNAs (snRNAs) and tRNAs, respectively (Shibata et al. 2006). In Drosophila, Exportin-5 is known to transport both pre-miRNAs and tRNAs (Shibata et al. 2006), whereas in B. mori, small RNAs export pathways and important components are yet to be investigated.

BmNPV is a natural pathogen of the domesticated silkmoth, B. mori, belongs to the baculoviruses family, Baculoviridae. Viral pathogens of this family infects several insects of the economically important order, Lepidoptera (GF. 2008). In B. mori, BmNPV inflicts heavy larval mortality and thus causes severe economic damage to silk production.

In silico prediction of BmNPV-encoded miRNAs targets had shown Ran as one of the potential target of bmnpv-miR-1. Here, I have carried out a functional analysis of bmnpv-miR-1 to show that it acts as a suppressor of Ran in both cell culture and in vivo in B. mori. I have found that bmnpv-miR-1 significantly represses the expression of Ran, both at the transcript and protein levels, resulting in the impairment of RanGTP-mediated small RNA transport in the host.
Further, I analyzed the effect of \textit{bmnpv-miR-1}-mediated downregulation of Ran on viral proliferation, and found a significant increase in the viral load upon Ran knockdown. Consistent with this result, blocking of \textit{bmnpv-miR-1} by specific LNA (Locked Nucleic Acid) resulted in higher Ran transcript level and decrease in virus titre in BmNPV infected larvae. Further, I assessed the virus load by blocking one of the host miRNAs, \textit{bmo-miR-8}, which was suppressed by administration of \textit{bmnpv-miR-1} and Ran dsRNA, by specific LNA. The virus load was dramatically increased upon inhibition of this host miRNA. These results provide convincing evidence that BmNPV suppresses the small RNA-mediated host defense to successfully proliferate in the host cells by employing \textit{bmnpv-miR-1}. The present study thus provides an insight into yet another layer of complex host-viral interactions mediated by a virus-encoded miRNA.
2.3 Materials and Methods

2.3.1 *In silico* prediction of *bmnpv-miR-1* binding site on Ran transcript

Various computational tools are available for searching appropriate targets of miRNA based on various criteria. miRanda target prediction program was used (Enright et al. 2003), which is based on dynamic-programming alignments and many other statistical parameters, to identify the targets of BmNPV-encoded miRNAs (Singh et al. 2010). Hits obtained from miRanda were further subjected to more stringent filters to reduce false positive miRNA targets (for details of the host targets of BmNPV-encoded miRNAs please refer appendix-I). Prediction results of miRanda program showed that *bmnpv-miR-1* has binding site on 3’UTR of Ran mRNA, which was reconfirmed by employing RNAhybrid program, which calculates minimum free energy of miRNA::mRNA duplex (Rehmsmeier et al. 2004).

2.3.2 RNA isolation and RT-qPCR analysis

Infection of BmNPV to *B. mori* larvae was done as described in the materials and method section of Chapter1 (1.3.1). Total RNA was extracted from fat body tissues of uninfected and BmNPV infected *B. mori* larvae using TRIzol reagent (Invitrogen®) and subsequently treated with DNase I (Invitrogen®). 2 µg total RNA was reverse transcribed using SuperScript® III First-Strand Synthesis System (Invitrogen®) in 20 µl reaction mixture. 1 µl of the reverse transcription product was used to perform the qPCR-amplification on RT-7500 system (Applied
Biosystems®) using primers specific to Ran. 25 µl RT-qPCR reaction mixture included 1 µl of cDNA or DNA, 12.5 µl of 2X SYBR Green Master Mix (Applied Biosystems®), 5 µM of primers and nuclease free water to make up the total volume. The relative quantification was performed on RT-7500 system (Applied Biosystems®) with the initial denaturation for 2 min at 50°C and 10 min at 95°C followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Each of the reactions was performed three times independently, in triplicates, and the results were normalized with constitutively expressing 18S ribosomal RNA gene of *B. mori*. All the primer sequences are provided in the Table 2. RT-qPCR data was analyzed by using RT-7500 system (Applied Biosystems®) software and was further verified using standard delta-delta-Ct (ddCt) method.

### 2.3.3 Counting of OBs

To determine the BmNPV load in *B. mori* larvae, OBs were isolated from hemolymph of the infected larvae by centrifugation (1000rpm for 1 min.) followed by resuspension of the viral pellet in 1X PBS. The OBs were then counted using an automated cell counter, Countess (Invitrogen®), and these results were also verified by scoring OBs in hemocytometer.

### 2.3.4 HeLa cell culture and luciferase assays

HeLa cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Thermo scientific®) containing 2 mM L-glutamine and 10% fetal bovine serum (FBS) (Invitrogen®) at 37°C in 5% CO₂. Luciferase construct was prepared using pmirGLO, a Dual luciferase miRNA Target Expression vector (Promega®) fused
with \textit{bmnpv-miR-1} predicted target site on the Ran. 3'UTR sequence of Ran mRNA (Ran-3'UTR) containing the 23bp long \textit{bmnpv-miR-1} binding site along with some flanking sequence was amplified (primer sequences are provided in Table 2.2) and cloned into pmirGLO vector according to the manufacturer’s instructions using double restriction digestion with \textit{SacI} and \textit{XhoI}. Insert orientation was determined by amplifying plasmid with insert and vector specific primers in combination and the concentration of selected plasmids (pmirGLO-Ran-3’UTR) was quantified using Nanodrop 2000c spectrophotometer (Thermo Scientific®) as well as by comparing it with Lambda DNA/HindIII Marker, 2 (Fermentas®) on an agarose gel. An unrelated target sequence of Pro-phenoloxidase 3'UTR (pmirGLO-PPO-3'UTR) was also cloned in the same vector as described above (sequence details are given in Table 2.2). Around $10^5$ HeLa cells/well were seeded in to 24-well plate a day prior to transfection. Cells were first transfected with 300 ng pmirGLO-Ran-3’UTR construct and after 24 hrs, 200 nM of perfectly complementary \textit{bmnpv-miR-1} duplexes was added. For miRNA inhibitor assays, the cells were co-transfected with \textit{bmnpv-miR-1} duplexes along with its 200 nM anatogomir LNA-1 i.e. miRCURY® LNA knockdown probe (Exiqon), whereas random-LNA was used as a negative control (sequences of miRNA duplexes and LNAs are listed in Table 2.1). All the transfections were done using Lipofectamine (Invitrogen®) according to the manufacturer's protocol. Luciferase assay was performed 48 hrs post transfection using the Dual luciferase Reporter Assay System (Promega®) according to the manufacturer’s instructions. Luciferase activity was normalized against the control
plasmid harboring the unrelated insert (Pro-phenoloxidase) without the bmnpv-
miR-1 binding site. Assays were done independently three times in triplicates for each sample.

**Table 2.1 Sequences of miRNA duplexes and LNAs used in this study**

<table>
<thead>
<tr>
<th>Name of miRNA/LNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>bmnpv-miR-1</td>
<td>Sense: 5’ AAAUGGGCGGCGUACAGCUGG 3’</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’ AGCUGUACGCGGCCCUAUUUGG 3’</td>
</tr>
<tr>
<td>bmnpv-miR-3</td>
<td>Sense: 5’ GAAAGCCAAACGAGGGCAGGCG 3’</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’ CCUGCCCUCGUUUGGCUUUCCG 3’</td>
</tr>
<tr>
<td>LNA-1</td>
<td>5’ CCAGCTGTACGCCGCCCATTT 3’</td>
</tr>
<tr>
<td>Random-LNA</td>
<td>5’ CATGAGCTGACCGGAACAGCT 3’</td>
</tr>
</tbody>
</table>

**2.3.5 Ran 3’UTR mutational analysis**

To perform the mutational analysis of bmnpv-miR-1 binding site on Ran mRNA, I designed oligonucleotides either with intact (WT) or mutated target (Mut) site of bmnpv-miR-1 (Figure 2.1C). The restriction sites for SacI and XhoI were added to these oligonucleotides at their 5’ and 3’ ends respectively, and were digested with the same enzymes and ligated to SacI and XhoI digested pmirGLO vector. The ligation reaction was carried out by taking vector and insert in 3:1 ratio, using T4 DNA Ligase in to 10 µl volume as per instructions of manufacturer (New England Biolabs). The mutations in the target site were confirmed by sequencing. Transfections and luciferase assays were done in HeLa cells as described above.
2.3.6 BmN cell culture and transfections

BmN cells were cultured in TC-100 insect medium (PAN Biotech GmbH®) supplemented with 10% FBS at 27°C. Around 10^6 cells were plated in six well plates, overnight prior to transfection. 1pmole of bmnpv-miR-1 perfect duplexes and siRNA duplexes against GFP (Sense 5’ GCUACCUGUUCCAUGGCCATT 3’ and antisense 5’ UGGCCAUGGAACAGGUAGCTT 3’) were transfected using Cellfectin (Invitrogen®). After 72 hrs of transfection, the cells were harvested and total RNA was isolated by TRIzol method (Invitrogen®).

2.3.7 In vivo assay

bmnpv-miR-1 was administered to B. mori larvae (5th instar, 2nd day) by injecting 100 pmoles of bmnpv-miR-1 perfect duplexes with 100 pmoles of specific or nonspecific LNA as antagonir. Larvae injected with 10 µl DEPC-treated water were taken as negative control. After 60 hrs, the fat body tissues were extracted from the injected larvae, and total RNA was isolated. For blocking the endogenous expression of bmnpv-miR-1, 100 pmoles each of specific LNA of bmnpv-miR-1 (LNA-1) and random-LNA was injected 24 hrs post BmNPV infection to larvae on 2nd day of 5th instar. Cellular miRNA, bmo-miR-8, inhibition was also done by injecting 100pmoles LNA specific to bmo-miR-8 (5’- GACATCTTTACCTGACAGTATTA -3’) in BmNPV infected larvae on 2nd day of 5th instar. The random-LNA (5’ GTGTAACACGTCTATACGCCTA 3’) was used as a negative control. Three
independent experiments were carried out in three replicates, each with a set of 3 larvae.

2.3.8 RNAi analysis

Double-stranded RNA (dsRNA) for Ran, Dicer-1, Dicer-2 and GFP were synthesized by using in vitro transcription kit Megascript (Ambion®) according to manufacturer’s instructions. Ran, Dicer-1 and Dicer-2 cDNA (primer sequence details are given in Table 2.2) was cloned into pCRII-TOPO vector (Invitrogen®) and amplified using M13 primers. The generated templates with flanking T7 and SP6 promoters were utilized for in vitro transcription. The sense and antisense RNA strands were transcribed using T7 and SP6 RNA polymerases (Ambion®), respectively. Residual DNA template was removed by treatment with DNase I and RNA was purified using lithium chloride precipitation. Equimolar amounts of both the RNA strands were annealed by heating at 95°C for 5 min followed by gradual cooling at room temperature for 12 hrs. To monitor annealing, RNA samples were electrophoresed on a 2% agarose gel compared with its dsDNA template and then finally quantified by Nanodrop 2000c spectrophotometer. Similarly, dsRNA for GFP (Green Fluorescent Protein) was also synthesized and used as an experimental negative control. 10 µg dsRNA was injected into 5th instar two days old larvae at their fourth thoracic abdominal leg. GFP dsRNA injected larvae of the same developmental stage were maintained as negative control. After 3 days of injection, fat body tissues were extracted and total RNA was isolated from both injected as well as control larvae.
2.3.9 Northern blot analysis

For host miRNAs detection by Northern blotting, 20 µg of total RNA (for bmnpv-
miR-1 50 µg of total RNA was used) was resolved on a 15% denaturing
polyacrylamide gel and transferred to nylon-Hybond+ membrane (Amersham) using
Semi-dry transfer cell (Trans-blot® SD Bio-Rad). UV cross-linked membranes were
hybridized overnight at 37°C in ULTRAhyb-Oligo hybridization buffer (Ambion)
with $\gamma^{32}$-ATP (specific activity $>1\times10^8$ cpm/pmol) labelled probes (sequences of DNA
probes are given in Table 2.2) and scanned using FLA-9000 Starion phosphorimager
(Fujifilm Global).
### Table 2.2 Sequences of primers, probes and oligonucleotides used in this study

<table>
<thead>
<tr>
<th></th>
<th><strong>Real time PCR primers</strong></th>
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</thead>
<tbody>
<tr>
<td>Ran</td>
<td><strong>Forward</strong>: 5’ GCCTGCCCTTCTGCCACCAG 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong>: 5’ TGGGCACTGGGTACATCCGT 3’</td>
</tr>
<tr>
<td>18S rRNA</td>
<td><strong>Forward</strong>: 5’ CGATCCGCGCAGTTACTACA 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong>: 5’ GTCCGGGCTTGGTGAGATTT 3’</td>
</tr>
<tr>
<td>BmNPV <em>ie-1</em></td>
<td><strong>Forward</strong>: 5’ GTCCGTGTTGCGTGTGCGCT 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong>: 5’ CGGCCGCTTGAGATTGTG 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Primers used for cloning</strong></td>
</tr>
<tr>
<td>PmirGLO vector</td>
<td><strong>Forward</strong>: 5’ TGACCGGCAAGTTGGACGCC 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong>: 5’ GGCCGCCCAAGGGGTTATG 3’</td>
</tr>
<tr>
<td>Ran</td>
<td><strong>Forward</strong>: 5’ TGATGAGCTCGCTGGCCCTTCTGTCCACCAG 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong>: 5’ ACTGCTCGAGACGCTACACTGAACACATTGTGATGA 3’</td>
</tr>
<tr>
<td>Pro-phenoloxidase (PPO)</td>
<td><strong>Forward</strong>: 5’ CGACTATTGAGCTCTCTACCGGATCTCGTCTTT 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong>: 5’ ACATTTTGCTCGAGCGCAAGTTCATGACCAACAG 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Primers for RNAi</strong></td>
</tr>
<tr>
<td>Ran</td>
<td><strong>Forward</strong>: 5’ CCATACGAACCGCGGCGCCCAA 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong>: 5’ TGGGCACCTGGGTACATCCGT 3’</td>
</tr>
<tr>
<td>Dicer-1</td>
<td><strong>Forward</strong>: 5’ TGAAGCCGGGGGTAGGTGTTC 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong>: 5’ GGGAGTGAGGAGGTTGAGTGC</td>
</tr>
<tr>
<td>Dicer-2</td>
<td><strong>Forward</strong>: 5’ ACCGAAGAGGAAGTAATGACCGGT 3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong>: 5’ ACGACGAGTGAGACGACGCGGT 3’</td>
</tr>
<tr>
<td></td>
<td><strong>DNA probes for Northern blot analysis</strong></td>
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<tr>
<td><em>bmo-miR-1</em></td>
<td>5’ CTCCATACCTTCTATCCATCCA 3’</td>
</tr>
<tr>
<td><em>bmo-miR-276a</em></td>
<td>5’ AGAGCAGGTATGAGTGCTCTTA 3’</td>
</tr>
<tr>
<td><em>bmo-miR-8</em></td>
<td>5’ GACATCTTTACGTGACGATTTA 3’</td>
</tr>
<tr>
<td><em>bmo-let-7</em></td>
<td>5’ ACTATAAACGTTACACTCCTCA 3’</td>
</tr>
<tr>
<td><em>B. mori</em> lysine tRNA</td>
<td>5’ CGCCCAACGTGCTCCAGACCCCAACCGACCGAGGATTAAGAGTTCATGCTCTAC 3’</td>
</tr>
<tr>
<td><em>B. mori</em> 5S rRNA</td>
<td>5’ GTTGCTTCTCTGTGACGAGAGAACCCGGGTGATTCAACA</td>
</tr>
</tbody>
</table>
### Oligonucleotides used for Ran 3’UTR mutational analysis

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PmirGLO-Ran-WT</td>
<td>5’TTCCAGAGCTCATATGATCAACGGATGTACCCAAGTGCATGCTTTTGTGATTGGAGGATCA</td>
</tr>
<tr>
<td></td>
<td>3’TGATTGGAGGATCATGCAAATGTGTCTCGAGTCGA</td>
</tr>
<tr>
<td>PmirGLO-Ran-Mut</td>
<td>5’TTCCAGAGCTCATATGATCAACGGATGTACCCAAGTGCATGCTTTTG</td>
</tr>
<tr>
<td></td>
<td>3’TGATTGGAGGATCATGCAAATGTGTCTCGAGTCGA</td>
</tr>
</tbody>
</table>

### 2.3.10 Western blot assay

50 μg protein from Ran knockdown and *bmnpv-miR-1* administered samples was resolved on a 12% SDS-polyacrylamide gel and transferred on to PVDF (Polyvinylidene fluoride) membrane by Semi-dry transfer cell (Trans-blot® SD Bio-Rad) followed by blocking in NAP-BLOCKER (G-Biosciences®) for overnight at 4°C. The membrane was then incubated for 2 hrs at room temperature with polyclonal antibody (1:1000 dilution) generated in rabbit against Ran (SCL® group). Detection was done with donkey anti-rabbit IgG conjugated to HRP (1/2000 dilution) and ECL detection reagent (Amersham).

### 2.3.11 DNA and protein isolation

DNA was isolated from different *B. mori* fat body samples using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturers’ instructions and protein extraction was done by grinding tissues in 1X PBS in presence of protease inhibitor cocktail followed by centrifugation.
**2.3.12 Densitometry analysis**

For Northern and Western blots, band intensity was evaluated by Densitometry using ImageJ software (available at [http://rsb.info.nih.gov/ij](http://rsb.info.nih.gov/ij)). In each case, band intensity was normalized against denominator gene (endogenous control, for Northern blot-5S ribosomal RNA and for Western blot-α-tubulin).

**2.3.13 Statistical analysis**

I carried out three independent experiments, each with three replicates for every assay. The results obtained were analyzed using a one-way ANOVA. This was followed by a post-hoc test i.e. Tukey’s test for pair-wise comparison between each pair of treatments. All the tests were performed at p-value<0.05. In all the histograms representing qPCR, the results were normalized against control samples.
2.4 Results and Discussion

2.4.1 *In silico* prediction of Ran as a target of *bmnpv-miR-1*

Targets for BmNPV-encoded miRNAs were predicted in our lab using different bioinformatics tools and Ran was identified as one of the putative host targets of *bmnpv-miR-1* (Singh et al. 2010). The viral miRNA, *bmnpv-miR-1* was found to have binding site in the 3’UTR of Ran transcript with the complete complementarity in the *seed region* (Krek et al. 2005; Lewis et al. 2005) i.e., consecutive Watson-Crick matches from the position 2 to 8 at the 5’ end of the miRNA (Figure 2.1A). The thermodynamic stability of *bmnpv-miR1::Ran* mRNA duplex was further confirmed using another target prediction program, RNAhybrid (Rehmsmeier et al. 2004) that gave a value of -26.7kcal/mol as minimum free energy of hybridization.
Figure 2.1 Schematic representation of the *bmnpv-miR-1* binding site on Ran mRNA and the luciferase constructs. (A) Ran mRNA showing the putative binding site of *bmnpv-miR-1*. (B) Luciferase reporter constructs containing the Ran 3′UTR (as shown in panel A) and the 3′UTR of the unrelated target PPO (prophenoloxidase) fused at the 3′end of the firefly luciferase (luc) gene. The firefly and Renilla luciferase genes are driven by human phosphoglycerate kinase (PGK) and simian virus 40 (SV40) promoters, respectively. (C) The WT or the Mut 3′UTR of Ran encompassing the binding site of *bmnpv-miR-1* was cloned into the pmirGLO vector downstream of the firefly luciferase gene (pmirGLO-Ran-WT and pmirGLO-Ran-Mut, respectively). The 4 mutated nucleotides (pmirGLO-Ran-Mut) in the seed region of the *bmnpv-miR-1* binding site are highlighted in red.
2.4.2 *bmnpv-miR-1* negatively regulates expression of Ran by binding to the 3'UTR of Ran mRNA

To determine whether *bmnpv-miR-1* binds to its predicted target site on 3'UTR of Ran mRNA sequence specifically and regulates its expression (Figure 2.1A), I constructed a dual luciferase reporter vector (Firefly and Renilla) by inserting 186 bases (631-816) of Ran cDNA sequence, comprising the *bmnpv-miR-1* binding site at the downstream of firefly luciferase gene (Figure 2.1B). Renilla luciferase served as an endogenous control. The vector was transfected into the HeLa cells and *bmnpv-miR-1* duplexes were added after 24 hrs. The luciferase activity was found to be reduced by > 36% in these cells, which was significantly rescued when *bmnpv-miR-1* was blocked by its specific antagomir LNA (LNA-1). The addition of random-LNA sequences, on the other hand, did not alter the luciferase activity (Figure 2.2A). To confirm that the inhibitory effect on the luciferase activity is due to sequence-specific binding of *bmnpv-miR-1* to its target site on Ran, I mutated 4 nucleotides on the binding site of Ran mRNA corresponding to the *seed region* of *bmnpv-miR-1* (Figure 2.1C), and analyzed the interaction using luciferase assay. For luciferase assay, the HeLa cells were transfected either with pmirGLO-Ran-WT or with pmirGLO-Ran-Mut constructs followed by *bmnpv-miR-1* transfection. I did not observe any significant reduction in the luciferase activity in the cells transfected with pmirGLO-Ran-Mut, whereas >50% repression was observed in the cells harbouring pmirGLO-Ran-WT (Figure 2.2B). I also analysed the effect of an unrelated miRNA (*bmnpv-miR-3*) on Ran and *bmnpv-miR-1* effect on unrelated target (Pro-phenoloxidase 3'UTR) (Figure 2.1B) by luciferase assay. As shown in
Figure 2.2C, unrelated miRNA did not alter the luciferase activity from reporter vector carrying bmnpv-miR-1 binding site. Similarly, upon introduction of bmnpv-miR-1 duplexes, no effect was observed in the luciferase level of the reporter vector lacking bmnpv-miR-1 binding site (carrying Pro-phenoloxidase 3’UTR). From these results I conclude that bmnpv-miR-1 indeed binds sequence specifically to its target site located in the 3’UTR of the Ran mRNA and represses its translation.

Figure 2.2 bmnpv-miR-1 negatively regulates expression of Ran by binding to its 3’UTR in HeLa cells. (A) Luciferase assay showed that bmnpv-miR-1 represses the luciferase activity when it binds to Ran 3’UTR and this repression was rescued when the cells were treated with bmnpv-miR-1 specific antagonist, LNA-1, but the expression remains repressed upon random-LNA treatment. (B) Mutation in the seed region of bmnpv-miR-1 binding site on 3’UTR of Ran mRNA leads to derepression of luciferase activity. (C) To determine the specificity of bmnpv-miR-1::Ran 3’UTR interaction, pmirGLO-Ran construct was transfected with an unrelated BmNPV miRNA (bmnpv-miR-3). Also, bmnpv-miR-1 was subjected to an unrelated target site containing the reporter vector (pmirGLO-PPO). In both the cases, no significant change was observed in luciferase activity. Assays were done independently three times in triplicates for each sample. Significant difference (p-
value<0.05) is denoted by different letters on top of the error bars. Data are presented as Mean ± SD (N=3).
2.4.3 Ran and *bmnpv-miR-1* levels are inversely correlated in BmNPV infected larvae

In order to determine the expression level of Ran upon BmNPV infection, I first analyzed the transcript level of Ran upon BmNPV infection. RT-qPCR was carried out using RNA isolated from the fat body tissues from uninfected and infected (4 days post BmNPV infection) larvae. Ran transcript level was found to decrease >3 fold upon BmNPV infection (Figure 2.3A). Then I analyzed the expression of Ran in BmNPV infected samples at different time points post infection by RT-qPCR and I observed a gradual decrease in expression of Ran with the increase of BmNPV infection (Figure 2.3B). To establish that downregulation of Ran upon BmNPV infection is due to *bmnpv-miR-1*, I determined *bmnpv-miR-1* expression by Northern blotting and noted that its expression was enhanced in the late stages of infection (Figure 2.3C). This contradiction may be attributed to the saturation of Ran-mediated transport by pre-*bmnpv-miR-1*. These results indicate that Ran and *bmnpv-miR-1* expression levels are inversely correlated.
Figure 2.3 Expression of Ran decreases as *bmnpv-miR-1* level increases post BmNPV infection in *B. mori* larvae. (A) RT-qPCR analysis of Ran transcript from control fat body (CFB) and BmNPV infected fat body (IFB), post 4 days BmNPV infection. (B) Ran transcript levels upon BmNPV infection at different time points examined by RT-qPCR in larvae. Each of the reactions was performed in triplicate three times independently, and the results were normalized against endogenous 18S rRNA. Data are presented as Mean±SD (N=3). (C) *bmnpv-miR-1* expression analyzed by Northern blotting in BmNPV infected RNA samples used in experiment 3B. Blots were probed with *bmnpv-miR-1* (upper panel) and 5S rRNA (lower panel), an endogenous control.
2.4.4 *bmnpv-miR-1* downregulates Ran in *B. mori* larvae and cells

To test the effect of *bmnpv-miR-1* on expression of Ran in *B. mori* larvae, double stranded *bmnpv-miR-1* perfect duplexes with or without its LNA, were administered into the larvae, and the Ran transcript and protein levels in the fat body tissues were determined by RT-qPCR and Western blot, respectively. The results showed >4 fold decrease in the Ran transcript level in the *bmnpv-miR-1* administered larvae as compared to that in the control larvae (administered with DEPC-H₂O) (Figure 2.4A). Whereas no significant change was observed in the expression level of Ran in the larvae treated with both *bmnpv-miR-1* and its specific LNA (LNA-1) (Figure 2.4A). However, no inhibition of *bmnpv-miR-1* was observed in larvae administered with random-LNA. Inhibitory effect of *bmnpv-miR-1* on expression of Ran was also confirmed at protein level by Western blotting (Figure 2.4B). *bmnpv-miR-1*-mediated negative regulation of Ran was also observed in the *B. mori* cells (BmN cell line) by analyzing the transcript level of Ran upon transfection of *bmnpv-miR-1* (Figure 2.4C). Considering these results I conclude that *bmnpv-miR-1* effectively represses the expression of Ran in *B. mori* larvae as well as in the cells.
Figure 2.A *bmnpv-miR-1* downregulates expression of Ran in *B. mori* larvae and in BmN cells. (A) RT-qPCR analysis of Ran transcripts in the fat body tissues of *bmnpv-miR-1* and with or without its specific LNA administered *B. mori* larvae. DEPC-H2O administered larvae were used as negative control. (B) Western blots showing decrease in the protein level of Ran in *bmnpv-miR-1* administered larvae as compared to the control larvae (DEPC-H2O administration). Band ratio was determined by densitometry and normalized against internal control α-tubulin. (C) Ran transcript analysis by RT-qPCR in BmN cells upon *bmnpv-miR-1* transfection. For transfection, siGFP was used as negative control. For RT-qPCR analysis, three independent experiments were carried out in three replicates each with a set of 3 larvae and the results were normalized against endogenous 18S rRNA. Data are presented as Mean±SD (N=3).
2.4.5 Abolition of Ran function by RNAi results in suppression of host small RNA population

Ran is known to be involved in pre-miRNA transport from nucleus to the cytoplasm by binding and inducing a conformational change in the key export protein, Exportin-5 (Lund et al. 2004). In B. mori, the function of Ran has not yet been demonstrated. Hence, to establish its role in pre-miRNA transport, I carried out dsRNA-mediated knockdown of Ran and analyzed its effects on the expression of host miRNAs. As a result of RNAi, Ran transcript level was markedly reduced (Figure 2.5A). Western blot analysis also confirmed the knockdown of Ran (Figure 2.5B). Next, I assessed the effect of Ran repression on host miRNAs expression. Northern blot analysis was carried out for 4 randomly selected highly expressed known host miRNAs i.e. bmo-let-7, bmo-miR-1, bmo-miR-276a and bmo-miR-8 (Jagadeeswaran et al. 2010). As expected, expression levels of all the 4 miRNAs were found to be reduced as compared with their respective GFP dsRNA injected control larvae (Figure 2.5C). As positive control for miRNAs processing I performed knockdown of Dicer-1 (Swevers et al. 2011) in B. mori larvae and checked the expression level of two of these miRNAs (bmo-miR-1 and bmo-miR-8) by Northern blotting. Unexpectedly I could not see any change in the levels of these miRNAs although Dicer-1 knockdown was efficient (Figure 2.6). Then I analyzed the host miRNAs expression upon Dicer-2 (NM_001193614) knockdown, and I found reduced levels of host miRNA expression (Figure 2.7) similar to that observed in Ran knockdown larvae, clearly implicating the role of Ran in the miRNA biogenesis.
In addition to pre-miRNAs, Ran is also known to transport other small RNAs like tRNAs (Arts et al. 1998; Kutay et al. 1998). Hence I checked the effect of Ran knockdown on the lysine tRNA expression, and did not find any significant change; this might be due to the fact that maturation of tRNAs occurs in the nucleus and if their transport is abrogated, the tRNAs may accumulate in the nucleus, which in turn may inhibit tRNAs production. Consequently tRNA amount may decrease to a negligible extent (Figure 2.5D). Similarly, Dicer-2 knockdown did not alter the expression of lysine tRNA (Figure 2.7), as it is known to specifically process stem-loop pre-miRNAs and dsRNAs into miRNAs and siRNAs, respectively and not tRNAs (Macrae et al. 2006).
Figure 2.5 Functional analysis of Ran by RNAi in *B. mori* larvae. (A) dsRNA-mediated knockdown of Ran in larvae analyzed by RT-qPCR. GFP dsRNA injected larvae were used as a negative control for all the knockdown experiments. Three independent experiments were carried out in three replicates each with a set of 3 larvae and the results were normalized against endogenous 18S rRNA. Data are presented as Mean±SD (N=3). (B) Western blot showing marked decrease in Ran protein level upon dsRNA injection in larvae. α-tubulin was used as an internal control. (C) Downregulation of host miRNAs upon Ran knockdown as determined by Northern blot analysis. (D) Northern blot showing expression of lysine tRNA in Ran knockdown larvae. The probes used are mentioned beneath each of the respective blots. Band ratio was obtained by densitometry and normalized against endogenous control 5S rRNA.
Figure 2.6 *B. mori* miRNAs expression remained unaltered upon knockdown of Dicer-1 in larvae. (A) Dicer-1 knockdown confirmed by RT-PCR in *B. mori* larvae. (B) Cellular miRNAs, *bmo-miR-8* and *bmo-let-7* expression in Dicer-1 knockdown larvae examined by Northern blot. Band ratio was determined by densitometry and normalized against endogenous controls 18S and 5S rRNA for Dicer-1 and cellular miRNAs, respectively.

Figure 2.7 *B. mori* miRNAs and lysine tRNA expression analysis upon Dicer-2 knockdown. (A) RT-PCR analysis of dsRNA-mediated Dicer-2 knockdown in fat body tissues. dsRNA against GFP and Dicer-2 are represented as dsGFP and dsDicer-2 respectively. 18S rRNA was used as loading control. (B) Northern blot analysis of *bmo-miR-1*, *bmo-miR-8* and lysine tRNA expression upon Dicer-2 knockdown in fat body tissues. Band ratio was determined by densitometry and normalized against endogenous controls 18S and 5S rRNA for Dicer-2 and cellular miRNAs, respectively.
2.4.6 *bmnpu-miR-1* inhibits Ran function

To demonstrate that *bmnpu-miR-1*-mediated downregulation of Ran mRNA indeed results in impairment of its function, I checked the expression levels of 4 host miRNAs that I had selected earlier as well as lysine tRNA in the *bmnpu-miR-1* administered larvae by Northern blot analysis. Again, I noticed reduction in the expression levels of all the 4 host miRNAs but lysine tRNA expression level remained unchanged (Figure 2.8A). The experiments carried out in the BmN cell line also yielded results consistent with the *in vivo* experiments (Figure 2.8B). Expression of these 4 host miRNAs and lysine tRNA were also analyzed 4 days after *bmnpu-miR-1* administration, and I found similar results for all the 4 miRNAs as I observed after 60 hrs of treatment (Figure 2.9), whereas no noticeable change was observed in the expression level of lysine tRNA (Figure 2.10). Together, these results suggest that BmNPV utilizes its miRNA to repress the host miRNAome.
Figure 2.8 *bmnpv-miR-1*-mediated repression of Ran hampers small RNA transport in *B. mori* larvae and in BmN cells. (A) Northern blot analysis of *B. mori* miRNAs and lysine tRNA expression in *bmnpv-miR-1*-administered larvae. DEPC-H2O-administered larvae were used as negative control. (B) *bmnpv-miR-1* effects on host miRNAs, *bmo-miR-1*, *bmo-let-7* and lysine tRNA were analyzed by Northern blotting. SiGFP and *bmnpv-miR-1* transfected BmN cells are represented as SiGFP and *bmnpv-miR-1* respectively. The probes used are mentioned beneath each of the respective blots. Band ratio was determined by densitometry and normalized against endogenous control 5S rRNA.
Figure 2.9 Host miRNAs expression after 4 days of *bmnpv-miR-1* duplex administration in the *B. mori* larvae determined by Northern blot. Band ratio was determined by densitometry and normalized against endogenous control 5S rRNA.

Figure 2.10 Northern blots showing, *B. mori* lysine tRNA expression after 4 days of *bmnpv-miR-1* and Ran dsRNA administration in BmNPV infected larvae. Band ratio was determined by densitometry and normalized against endogenous control 5S rRNA.
2.4.7 Blocking of a cellular miRNA, \textit{bmo-miR-8}, results in higher BmNPV load

After observing \textit{bmnpv-miR-1}-mediated inhibition of Ran, question arises here what advantage BmNPV derives by suppressing the host miRNA population. To answer this and further understand the functional importance of \textit{bmnpv-miR-1}-mediated Ran inhibition in the host-pathogen interaction, I looked for the targets of the 4 selected host miRNAs, which were found repressed upon inhibition of Ran by \textit{bmnpv-miR-1} and Ran dsRNA (Figure 2.5C and 2.8A), both in the virus as well as in the host using miRanda program. The putative functions of some of these targets are listed in Table 2.3. The known biological functions of these targets clearly indicate that the role of host miRNAs is not only restricted to regulating its own immune genes, but also includes various viral genes required for viral entry and its establishment inside the host, as demonstrated in various mammalian systems (Lecellier et al. 2005; Pedersen et al. 2007; Hussain and Asgari 2010; Nuovo et al. 2010; Zhang et al. 2010).
Table 2.3 The putative targets of the selected four host miRNAs

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<th>Known Function</th>
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<td>HQ179970</td>
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<td>B. mori cuticle protein</td>
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<td></td>
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<td>AB009987</td>
<td>BmNPV DNA-directed RNA polymerase component lef8</td>
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<td>BmNPV tyrosine phosphatase NPV-PTP gene</td>
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To assess the direct role of cellular miRNAs in viral proliferation, I analyzed the effect of one of the host miRNAs, *bmo-miR-8*, which was found to be repressed in *bmnpv-miR-1* and Ran knockdown samples, on virus proliferation. *bmo-miR-8* has multiple putative binding sites on BmNPV immediate early gene (*ie-1*) mRNA as well as on other important genes of BmNPV. The *ie-1* gene encodes a trans-activator protein required for initial establishment of the virus in the host cells. On blocking of *bmo-miR-8* by specific LNA I observed ~ 3 fold increase in the *ie-1* transcript level and ~8 fold increase in BmNPV accumulation in fat body tissues of infected larvae as quantified by RT-qPCR and qPCR, respectively, whereas no such effect was seen in the random-LNA administered sample (Figure 2.11A and 2.11B). The marked effect of *bmo-miR-8* on increase of virus load might be because of its multiple targets in the viral genome. As expected, *bmo-miR-8* expression was found to be repressed upon BmNPV infection (Figure 2.11C). Taken together, these results suggest that in order to counterattack the miRNA-mediated host defense, the virus uses its miRNA resources to block the host miRNAome by modulating some of the key components of miRNA biogenesis pathway in its own favour. Further, the functional basis of Ran inhibition by *bmnpv-miR-1* was determined by analyzing the virus load upon repression of Ran by *bmnpv-miR-1* and Ran dsRNA administration in BmNPV infected *B. mori* larvae.
Figure 2.11 Inhibition of cellular miRNA, bmo-miR-8, resulted in higher BmNPV proliferation in *B. mori* larvae. (A) Transcript analysis of BmNPV *ie-1* by RT-qPCR in LNA-8 and random-LNA administered BmNPV infected larvae. (B) BmNPV load in LNA-8 and random-LNA administered BmNPV infected larvae were determined by qPCR of viral DNA using BmNPV *ie-1* specific primers. For qPCR analysis, three independent experiments were carried out in three replicates each with a set of 3 larvae, and the results were normalized against endogenous 18S rRNA. Data are presented as Mean±SD (N=3). (C) Northern blots showing reduction in expression of *bmo-miR-8* upon BmNPV infection in *B. mori* larvae (CFB: control fat body, IFB: infected fat body). Band ratio was determined by densitometry and normalized against endogenous 5S rRNA.
2.4.8 Inhibition of bmnpv-miR-1 effectively suppresses BmNPV proliferation

To confirm the role of bmnpv-miR-1 in BmNPV proliferation, I first blocked the endogenous expression of bmnpv-miR-1 by injecting its antagonir LNA in the BmNPV infected larvae, and then measured the expression of Ran and viral titre in the fat body tissues by RT-qPCR and qPCR respectively, and the results were normalized against infected fat body (IFB) samples without LNA treatment. The viral titre was determined by quantifying the viral DNA levels using the ie-1 gene of BmNPV and also by scoring OBs in the specific (LNA-1) and random-LNA injected BmNPV infected larvae. I found a significant increase in the expression of Ran in the LNA-1 administrated larvae (Figure 2.12A), and consequent decrease in the viral load (Figure 2.12B and 2.12C). No such change was observed either in expression of Ran levels or on viral accumulation in the infected larvae treated with random-LNA (Figure 2.12B and 2.12C).
Figure 2.12 Blocking of *bmnpu-miR-1* by LNA leads to decrease in BmNPV accumulation in *B. mori* larvae. (A) RT-qPCR analysis showing increase in expression of Ran upon inhibition of *bmnpu-miR-1* by specific LNA (LNA-1), while random-LNA has no effect compared to respective control samples (IFB: Infected fat body). (B) BmNPV titre, determined by qPCR using *ie-1* of BmNPV, showing drastic reduction in virus load when *bmnpu-miR-1* was blocked by specific LNA (LNA-1), whereas no significant decrease in virus load was observed in larvae administered with random-LNA compared to control samples. Three independent experiments were carried out in three replicates each with a set of 3 larvae and the results were normalized against constitutively expressing 18S rRNA gene of *B. mori*. (C) Virus load was determined by scoring the OBs from hemolymph of LNA administered BmNPV infected larvae. Three independent experiments were performed with a set of 3 larvae. Data are presented as Mean±SD (N=3).
2.4.9 BmNPV proliferation enhances upon Ran knockdown

To check the viral proliferation upon Ran knockdown, dsRNA against GFP and Ran were injected in the BmNPV infected larvae and expression of \textit{ie-1} was quantified by qPCR. As shown in Figure 2.13A, a very high viral load was observed in the Ran dsRNA injected larvae as compared to that seen in the control larvae and similar result was also observed when OBs were scored in the same samples (Figure 2.13B). To further confirm that the higher virus load upon Ran knockdown is due to inhibition of miRNAome of the host and not because of some other function of Ran, I also checked the virus titre in the Dicer-2 knockdown larvae. I noticed a significant increase in the virus load in the Dicer-2 knockdown larvae as was observed upon Ran knockdown (Figure 2.13B), suggesting that the higher viral accumulation is due to suppression of the host miRNAs. No observable difference was noticed in the phenotypes of Ran and Dicer-2 knockdown larvae (Figure 2.14). Interestingly, blocking of \textit{bmnpv-miR-1} resulted in marked decrease (~8 fold) in BmNPV load (Figure 2.12B), whereas knockdown of Ran and Dicer-2 larvae showed only ~1.5 and 2.5 fold increase in the virus accumulation (Figure 2.13A), respectively. This may possibly be due to \textit{bmnpv-miR-1} targeting other host genes that are involved in host defence. Based on these results I conclude that BmNPV-encoded miRNA, \textit{bmnpv-miR-1} combats the host small RNA-mediated antiviral attack by suppressing the expression of Ran, an important component of the small RNA transport machinery of the cell, as proposed in the model shown in Figure 2.15.
Viruses are known to evade host defense by different ways. Recently, viral derived miRNAs have emerged as potential modulators of the host immune system. Many mammalian viral miRNAs are shown to regulate both the host as well as their own genes which otherwise are obstacle to virus in gaining entry or proliferation inside the host (Gottwein and Cullen 2008). Similarly, there are many reports of cellular miRNAs of host modulating the expression of various viral genes (Lecellier et al. 2005; Pedersen et al. 2007; Hussain and Asgari 2010; Nuovo et al. 2010; Zhang et al. 2010), hence it is likely that B. mori miRNAs directly or

Figure 2.13 B. mori miRNAs suppression increases BmNPV proliferation in larvae. (A) Quantitative PCR analysis of ie-1 of BmNPV to determine the viral load in Ran and Dicer-2 knockdown larvae. GFP dsRNA injected larvae were used as a negative control. Three independent experiments were carried out in three replicates each with a set of 3 larvae and the results were normalized against endogenous 18S rRNA. (B) Virus load was determined by scoring the OBs from hemolymph of Ran knockdown BmNPV infected larvae. Three independent experiments were performed with a set of 3 larvae. Data are presented as Mean±SD (N=3).

Viruses are known to evade host defense by different ways. Recently, viral derived miRNAs have emerged as potential modulators of the host immune system. Many mammalian viral miRNAs are shown to regulate both the host as well as their own genes which otherwise are obstacle to virus in gaining entry or proliferation inside the host (Gottwein and Cullen 2008). Similarly, there are many reports of cellular miRNAs of host modulating the expression of various viral genes (Lecellier et al. 2005; Pedersen et al. 2007; Hussain and Asgari 2010; Nuovo et al. 2010; Zhang et al. 2010), hence it is likely that B. mori miRNAs directly or
indirectly can inhibit the BmNPV proliferation. In the present study I have shown that Ran is involved in small RNA trafficking in *B. mori* and BmNPV-encoded miRNA, *bmnpv-miR-1* controls expression of Ran by destabilizing its mRNA followed by translation repression that leads to suppression of cellular miRNA production which in turn results in enhanced virus proliferation.

![Image of B. mori larvae with dsRNA injection](image)

**Figure 2.14** Comparing phenotypes of Ran and Dicer-2 knockdown *B. mori* larvae. Similar phenotypes were observed upon dsRNA-mediated knockdown of Ran and Dicer-2 in the BmNPV infected larvae.

The contradiction that I came across in this study is higher expression of *bmnpv-miR-1* in the late stage of infection despite effective repression of Ran
(Figure 2.3B & 2.3C). The pertinent question here is: when the \textit{bmnpv-miR-1} pre-miRNA is not transported out of the nucleus as a result of Ran repression, how such a higher load of processed \textit{bmnpv-miR-1} could still be seen in the cell? This suggests that BmNPV may deploy a variety of strategies to effectively replicate in the host cell. One such strategy could be that the baculovirus may not fully block RanGTP-mediated transport by its \textit{bmnpv-miR-1}, and as a result, transport of host pre-miRNAs in small quantity may still occur in the infected larvae. This strategy allows the virus to proliferate in the host cell by keeping the small RNAs-mediated host defense in check, and at the same time keeping the larvae viable to enhance its proliferation. During the late stage of infection massive quantity of virus is produced in the host cell giving rise to higher amount of viral-derived pre-\textit{bmnpv-miR-1} which saturates Ran-mediated transport machinery resulting in higher level transport of pre-\textit{bmnpv-miR-1} into the cytoplasm, and at the same time limiting the transport of host pre-miRNAs. These speculations draw credence from the recent studies, which uncover ingenious and complex strategies employed by the viruses to enhance their accumulation in the host cells. For example, Adenovirus derived small RNAs massively produced in the late stage of infection are able to deplete Dicer expression by saturating the Exportin-5 and abrogating the nuclear export of Exportin-5 dependent Dicer mRNA. As a result, maturation of small RNA is terminated by the virus using this strategy (Lu and Cullen 2004; Aparicio et al. 2010; Bennasser et al. 2011; Carnero et al. 2011; Kamel et al. 2013). Similarly, influenza virus encoded NS1 protein is shown to inhibit host mRNA export pathway
to render higher permissiveness of host cells to influenza virus replication (Lund et al. 2004). It is also possible that BmNPV may explore alternative strategies including utilization of mRNA transport pathway that is independent of Ran (Kohler and Hurt 2007). In my previous report (Singh et al. 2010), I found that \textit{bmnpv-miR-1} is derived from the coding region of Cathepsin which is expressed heavily during the late stage of infection, as it is required for liquefaction of infected larvae (Hawtin et al. 1997). Hence \textit{bmnpv-miR-1} may escape Drosha cleavage and gets transported as mRNA to be processed subsequently by canonical or non-canonical pathways in cytoplasm to give rise to mature \textit{bmnpv-miR-1} (Cheloufi et al. 2010; Shapiro et al. 2010; Skalsky and Cullen 2010; Cullen 2011; Yang and Lai 2011).
Figure 2.15 The proposed model for *bmnpv-miR-1*-mediated evasion of host defense response mounted by small RNAs, for effective proliferation of BmNPV in infected larva. The model proposed here shows that BmNPV, upon infection of silkworm larvae encodes a miRNA, *bmnpv-miR-1*, which represses Ran, an important component of small RNA export from nucleus to cytoplasm, by binding its 3’ UTR. As a result, the small RNA population is reduced in the cytoplasm leading to proliferation of the virus.
The important question which remains to be addressed further is: how does the virus control the expression of \textit{bmnpv-miR-1}? Ran has been shown to be an important component of nucleocytoplasmic transport of small RNAs as well as proteins (Bischoff and Ponstingl 1991; Yi et al. 2003; Bohnsack et al. 2004; Lund et al. 2004; Wang et al. 2011) and its complete depletion may be lethal, but I did not observe such drastic effect in either \textit{bmnpv-miR-1} or dsRNA-mediated knockdown of Ran, although the treated larvae showed stunted growth (Figure 2.14). These observations point to the role of miRNAs as fine-tuners of gene expression and may not act as phenotype switchers (Sevignani et al. 2006; Flynt and Lai 2008; Krol et al. 2010; Mukherji et al. 2011). Since viruses do not possess any miRNA biogenesis component in their genome, it is interesting to investigate how BmNPV generates its own miRNAs when it blocks its host miRNA transport.

Can we generalize this strategy to other viruses, especially latent viruses? As miRNAs are also required for regulation of many cellular genes, which are important for survival of the host, and inhibition of miRNAs processing would have serious effect on these genes and may lead to cell death. Hence, in case of persistent or latent infections, instead of blocking the miRNA biogenesis, virus may try to escape the silencing machinery. Explanation to all these questions might also reveal some of the complicated aspects of virus-governed fine-tuning of the host genes.
2.5 Conclusion

This study revealed one of the strategies employed by the virus to modulate host defence. Functional characterization of BmNPV-encoded miRNA, *bmnpv-miR-1* studied here showed how a baculovirus-derived miRNA controls host miRNA population by suppressing the Ran, an important component of miRNA biogenesis. I have demonstrated that Ran-mediated miRNAs transport is conserved in *B. mori* as reported in human and *Drosophila*. As in mammals, where host miRNAs are shown to target virus genes to prevent its infection, I also found *B. mori* miRNAs functioning as antiviral factors against BmNPV. Thus the present study provides useful insights into the multi-layered complexity of intricate host-viral interactions, and has implications for future applications in insect virus control. To the best of my knowledge this is the first report showing an insect viral-encoded miRNA regulating the host gene expression to its advantage.