Heliothis zea nudivirus-1 (HzNV-1) is a baculiform insect virus with a circular double-stranded DNA genome. This viral genome has been sequenced and was found to encode approximately 154 open reading frames. This virus was originally regarded as a member of the Baculoviridae family, but due to its lack of an occlusion body and low sequence homology to baculoviruses, it is now temporarily re-classified with other non-occluded viruses as a new Nudivirus genus. The HzNV-1 virus has a relatively broad host range and has been reported to infect many insect cell lines. HzNV-1 virus can establish distinct latent and productive infection cycles in insect cell cultures. During productive infection, the virus generates more than 100 transcripts, and high titers of virus progeny are produced. In this infection stage, the majority of Tn-368 and SF-21 insect host cells are killed leaving a small percentage of the cells become latently infected. During viral latency, the persistency-associated gene 1 (pag1), is the only viral gene transcript detected. In this infection stage, virus genomes exist either as episomes or are inserted into the host genome, and persist through many cell passages without the releasing of viral particles. Occasionally, viruses can be released from very small proportions of latently-infected cells (usually less than 0.2%), resulting in the death of these cells. This small quantity of continuously-released virions results in the presence of low viral titers (around 10^3 pfu/ml) in the culture medium of the latently-infected cells.

Although pag1 is the only transcript detectable during latent viral infection, it is also expressed during productive viral infection. Uniquely, the transcript of pag1 is a non-coding RNA, previously also referred to as the persistency-associated transcript (PAT1), and was found to be involved in the establishment of latent HzNV-1 virus infection. The pag1 has several interesting features. Sequence analysis of pag1 revealed abundant direct and inverted repeats. The transcript of pag1 is not translated, as it does not associate with polysome, and was later shown to be a nuclear RNA. Although we can not rule out the possibility that pag1 transcript is an intron of a larger transcript, the fact that the direct upstream sequence of pag1 coding region is a promoter suggesting that this is more likely to be an independent transcript.

During productive viral infection, an abundant 6.2 kb transcript is expressed from a gene located in the fragment of HzNV-1 Hind III-1 (hhi1) of the viral genome. hhi1 is a viral gene expressed very early during viral productive infection (0.5 h post viral infection, hpi), and it was shown to involve in viral re-activation from latency. It was also shown that the suppression of hhi1 expression can switch viral infection from productive to latent.
Results

hhi1 expression is negatively regulated by pag1. In order to study the interactions of pag1 and hhi1, two expression plasmids, pKSpP1 and pKShH1, were constructed. The former used pag1 and the latter used hsp 70 promoters to express pag1 and hhi1 transcripts, respectively. Northern analysis showed that pag1 was not only expressed in the productive and latent viral infected cells, it was also properly expressed in pKSpP1 transfected cells (Fig. 1A). Both plasmids were then co-transfected into SF-21 cells to identify any possible interactions. The hhi1-expressing vector pKShH1 was also transfected into SF-21 cells with an empty vector (pBluescript II KS-, Stratagene) as a control. Northern blot analysis showed that the level of hhi1 expression decreased dramatically in the presence of pag1 (Fig. 1B). To confirm that pag1 can negatively down-regulate hhi1 expression during H2N-1 infection, cells were first transfected with pag1-expression vector pKSpP1 and the transfected cells were subsequently infected with H2N-1. Similarly, hhi1 expression from viral infection was suppressed by the transfection of pKSpP1 (Fig. 1C). In this experiment, the level of hhi1 transcript was detected at 2 hours post H2N-1 infection (hpi), because, according to our previous study, this is the timing at which hhi1 expression reaches the highest level46.

Prediction of possible pag1 miRNA precursors. Previously, sequence analysis of pag1 revealed the presence of abundant direct and inverted repeats with no significant open reading frames (ORFs) in its coding region47,48. In this experiment, our analysis showed that pag1 transcript contains abundant stem-loop structures. Therefore, it is possible that pag1 transcript may produce miRNAs to suppress the expression of hhi1. In order to explore this possibility, 23 possible miRNAs were first predicted from pag1 coding region and five of them were predicted to target to hhi1 coding region (Fig. 2).

Proper expression of miRNAs from pag1 is confirmed in productively and latently infected cells, and also in cells transfected with pag1 plasmid. To confirm whether the above predicted miRNAs truly come from pag1 and are expressed in infected SF-21 cells, a stem-loop PCR49-51 was performed to analyze the miRNA expression in pag1-transfected cells. The pag1-transfected cells were harvested at 12 hpi and total RNA was extracted. The cDNAs of miRNAs were obtained using miRNA stem-loop RT primers (Table 1). Stem-loop PCR analysis was performed using these cDNAs as the templates with specific miRNA primers designed based on our predictions. In order to investigate whether if any of the predicted miRNAs are expressed in viral infected cells, total RNAs were extracted from productive and latent viral infected cells and subsequently analyzed by stem-loop PCR. Real-time PCR products were also separated by gel electrophoresis and visualized by EtBr staining as a further control to show proper expression of different PCR products (Fig. 3 and Supplemental Fig.1). The result showed that two miRNAs, hv-miR-246 5p and hv-miR-2959 5p, were expressed in the H2N-1 productively infected cells (Fig. 3A), H2N-1 latently infected cells (Fig. 3B), and pag1-transfected cells (Fig. 3C). These two miRNAs were cloned and the predicted secondary structures of hv-miR-246 5p and hv-miR-2959 5p precursors are shown in Fig. 3D, and Fig. 3E, respectively. Mature miRNA is shadowed in red and the numbers on nucleotides indicating their corresponding positions to pag1 transcript.

The melting curve for hv-miR-246 5p had two peaks in the H2N-1 productively infected cells (Fig. 3A). We cloned these two fragments into cloning vector and analyzed their sequences. The sequence of the upper band did not match to the predicted miRNA and therefore is a non-specific product. The molecule size of the upper band is also larger than the predicted miRNA. In these experiments, proper expression of let-7a miRNA gene was performed as a positive control52. To further confirm the results from stem-loop PCR, northern blot analysis showed that hv-miR-246 and hv-miR-2959 became detectable in productively infected cells at 4 hpi and 8 hpi, respectively (Fig. 3F). However, they were not detected in mock infected cells (data not shown). These results indicated that the pag1 transcript was indeed processed into miRNAs during the infection of H2N-1 (Fig. 3F).

hhi1 expression can be down-regulated by pag1 miRNAs. To further test whether these two miRNAs target hhi1 and suppress its transcripts, these miRNAs were separately transfected into SF-21 cells along with a hhi1-expressing vector and the levels of hhi1 transcript were analyzed by northern blot analysis at 12 hpi. Both miRNAs were able to reduce the amount of hhi1 transcript within the cells (Fig. 4). The sequences of hv-miR-246 and hv-miR-2959 are mapped to nucleotides 226325 to 226366 and 226527 to 226555 of the hhi1 coding region, respectively (Fig. 2). To confirm if the matching of miRNA sequences to hhi1 was important, i.e., acting as targeting sites, we constructed mutant miRNAs with three base-pair mutations in the matching regions for these two miRNAs (Fig. 4A, 4C). The mutational substituted bases are indicated by arrows. Northern analysis (Fig. 4B, 4D; left panels) and RT-PCR (Fig. 4B, 4D; right panels) showed that these two mutant miRNAs did not affect the levels of hhi1 transcript (Fig 4B, 4D), suggesting that our predicted target positions are crucial for functioning. In these experiments, the levels of actin transcript were used as a loading control.

pag1 miRNA functions in establishing latent viral infection. Previously, we showed that pag1 gene can promote the establishment of latent viral infection7. To test whether these miRNAs alone are enough to promote latent viral infection, SF-21 cells were first transfected with miRNA followed by H2N-1 infection. The number of colonies was then recorded at 12 dpi. Most of the SF-21 cells died when they were infected with H2N-1 virus alone and only a small percentage of cells became latently infected (Fig. 5A). However, the number of latently-infected cell colonies increased dramatically upon transfection of individual hv-miR-246 or hv-miR-2959 (Fig. 5B). We also analyzed the gene expression of pag1 and hhi1 in H2N-1 infected cells with or without miRNA treatments at 12 dpi (Fig. 5C). hhi1 expression could only be

latent infection4,16. In contrast with the expression of most immediate-early genes of baculovirus, the proper expression of hhi1 requires viral factors49.

Recently the herpes simplex virus (HSV-1) was shown to use the miRNAs derived from a latent transcript to block the function of early genes, without the proper function of early transcripts, it is speculated that virus may enter latency48, however, a proof is still lacking. In this study, we found that H2N-1 can serve as a convenient system to investigate such possibility. We provided evidences showing that the pag1 is capable of down-regulating hhi1 transcript in this virus. Subsequent experiments showed that pag1 functions through the expression of two microRNAs (miRNAs), which target to and degrade hhi1 transcript. These miRNAs could be detected in viral infected cells and our data indicated that the miRNA derived from pag1 can establish latent viral infection in the cells.

The establishment of latent viral infection through miRNA-producing non-coding viral RNA represents a simple yet highly effective way for H2N-1 to achieve latent viral infection in the host cells. Although different from H2N-1, a further prove for the miRNA to establish viral latency in HSV-1 may be still needed, the similarity between HSV and H2N-1 in using miRNA derived from latent-specific transcripts to control early transcript, and subsequently result viral latency represents an interesting viral convergent evolution. Furthermore, miRNA as a switch for the transition of productive to latent viral infections may be a widely-spread phenomenon of virus/host interactions in nature.
detected in HzNV-1 productively infected cells (Fig. 5C, lane 1) whereas pag1 expression could be detected in productive and latent cells SFP4 (Fig. 5C, lanes 1 and 3). Cells transfected with these two miRNAs, hv-miR-246 and hv-miR-2959, produced only pag1 transcript but not hhi1 transcript, this is an evidence of viral latency (Fig. 5C, lanes 4 and 5). These results indicate that miRNAs alone can function to establish or at least to strongly promote latent viral infection. Furthermore, pag1 transcript, but not that of hhi1,
was expressed in the latent cells induced by the transfection of miRNA. Besides, these latent cells can be cultured for long-term passages. All these evidences indicate strongly that the establishment of latent viral infection by miRNA is not a transient effect; it is a long term latent viral infection in the cells.

In these and previous experiments, we have observed that the number of latently-infected cell colonies increased upon miRNA or pag1 transfection. However, whether these miRNA or pag1 can only promote viral latency or are essential for the establishment of latent viral infection remain unknown. In order to confirm the function of the miRNA and pag1, we further constructed a pag1-null HzNV-1 virus for infection experiment. Sf-21 cells were infected with pag1-null virus or transfected with a siRNA targeting to pag1 transcript followed by HzNV-1 infection. RT-PCR confirmed that the suppression of pag1 gene expression by siRNA was successful (Fig. 6A) and also, no pag1 expression was detected from pag1-null HzNV-1 virus (Fig. 6B). Further experiments showed that upon knocking down (Fig. 6C, lane 5) or deletion of pag1 transcripts (Fig. 6C, lane 1), the number of latent colony dropped significantly, and none of the cell colony can survive two weeks after viral infection. However, the number of latently-infected cell colonies increased dramatically upon transfection of individual hv-miR-246 or hv-miR-2959 (Fig. 6C, line 3, 4, 7 and 8). These cells keep on viable for long time (at least 2 months up to now). These experiments demonstrated that pag1 and its derived miRNAs play a crucial role in the establishment of HzNV-1 latent viral infection.
Figure 3 | Cloning and analysis of the predicted miRNA by stem-loop PCR and northern blot. Stem-loop PCR was performed to clone and analyze the proper expression of the predicted miRNAs in (A) HzNV-1 productively infected cells, (B) pag1-transfected cells, and (C) HzNV-1 latently infected cells. (D, E) Predicted secondary structures of hv-miR-246 5′p (D), and hv-miR-2959 5′p (E), precursors. (F) Small RNAs harvested from HzNV-1 productively infected cells at various time points were analysis by northern blots with probes against predicted HzNV-1 miRNAs (top panels) or let-7a miRNA as a positive control (bottom panels).
Discussion

Previously, we found that hhil can activate the expression of many early HzNV-1 genes and reactivate HzNV-1 virus from latency. We had also discovered that the transcript of pagl, a non-coding RNA, plays a critical role to block hhil-induced apoptosis and to the establishment of latent viral infection. In this study we found that pagl transcript could suppress hhil expression remarkably through the targeting of hhil transcript by miRNAs. Interestingly, these two miRNAs, hv-miR-246 or hv-miR-2959, were individually sufficient for strong promotion and/or establishment of latent viral infection. The observation that pagl-null HzNV-1 virus was unable to establish latent infection further suggested that pagl plays a key role in the establishment of latent infection.

Most miRNA target sites have perfect pairing to the seed region located near the miRNA 5’ end. Nevertheless, recent reports have also demonstrated that perfect pairing between target sequence and the miRNA can also take place near the 3’ end of miRNA. Our results also showed that the 3’ ends of miRNAs derived from pagl have perfect base pairings to the target sequences, and mutagenesis experiments showed that these pairing are critical for functioning. To further confirm that inhibition of hhil expression was mediated through miRNA pathway, the effects of silencing dicer1 and ago1 expressions (Supplemental Fig. 2A) on the production of pagl-derived miRNA and control let-7a were assessed in Drosophila S2 cells (Supplemental Fig. 2B). It is known that dicer1 is an important component of the miRNA progenesis; whereas, ago1 is not involved.

Figure 4 | Down-regulation of hhil expression by pagl, hv-miR-246, and hv-miR-2959. (A, C) sequences of hv-miR-2959 and hv-miR-246 were shown, and miRNAs with mutations were denoted as hv-miR-246m and hv-miR-2959m, separately. (B, D) levels of hhil transcript in various treatments were analyzed by northern hybridization (left panel) and RT-PCR (right panel).
in the generation of mature RNAs, rather, is involved in the interaction between miRNA and target transcript. Thus, it is reasonable that levels of miRNAs hv-miR-246, hv-miR-2959 and let-7a were not affected by ago1 knockdown using stem-loop RT-PCR analysis (Supplemental Fig. 2B). We also found that hhi1 expression was not down-regulated by pag1 in either dicer1 or ago1 knocked down cells (Supplemental Fig. 2C), suggesting that miRNA pathway is essential for the production of pag1-derived miRNAs.

HzNV1 virus is an insect-specific virus. In our studies, we found it shares striking similarity in the gene regulation networks during viral latency with the vertebrate virus, HSV-134. Despite the high numbers of viral transcripts produced during productive viral infections, both viruses silence the expression of all genes except the expression of latency-associated transcript (LAT) in HSV-134, and pag1 transcript in HzNV-134-4, during latent viral infection. It is interesting to note that HzNV-1 is a baculiform insect virus and HSV, on the other hand, is an icosahedra virus. They are quite distinct in shape and genomic sequences and are obviously not evolved from the same origin. Nevertheless, LAT is a latency-associated transcript, which contains a non-coding intron35 and pag1 transcript is similarly a non-coding transcript predominately expressed during viral latency3. Such striking similarity between these transcripts strongly suggests that the strategy for producing a non-coding RNA during latency is not an isolated viral infection phenomenon restricted to the HSV of vertebrates.

LAT was previously identified as an 8.3 kb transcript expressed from the genome of HSV32. This RNA is spliced and yields a stable intronic non-coding region of 2 kb and an unstable exonic RNA. This unstable exonic RNA was further processed to produce several miRNAs, which are found to suppress protein expression of early HSV genes, ICP0 and ICP417,26. More recently, two sRNAs of 62 nt and 32 nt, but not miRNA, were predicted to be located on the 2 kb stable intronic region. Shen et al. (2009) found that these sRNAs play a role through an unidentified mechanism to inhibit apoptosis and productive infection27. Although these authors showed that these miRNAs or sRNAs were likely responsible for latent HSV infection due to the suppression of two important early genes, direct proof of latent establishment due to these miRNAs or sRNAs is still lacking.

In our results, due to HzNV-1 can establish latent viral infection in insect cells, we were able to show that HzNV-1 virus establishes latent viral infection through the suppression of the hhi1 transcript using non-coding transcript pag1. We also showed that even miRNAs from pag1 transcript alone are sufficient to establish latent viral infection. Since the transiently transfected miRNA is sufficient for the triggering of latent viral infection using pag1-null HzNV-1, suggesting that once entering viral latency, viral gene expression is somehow restricted without the need of a continuous supply of pag1 transcript for hhi1 suppression.

Our experiments showed that, although HzNV-1 virus is a herpes virus and pag1 transcript has no sequence homology with LAT, shutting-off of all genes except the expression of one non-coding transcript during latency of these two viruses is a striking convergent evolution of viral-host interactions. In addition, the use of miRNAs derived from the non-coding RNA for similar functions by two unrelated viruses suggests that miRNA can be an important and effective mechanism for switching between productive and latent infections in a wide-range of host cells. Furthermore, pag1 transcript is a stable non-coding RNA consistently observed during productive and latent viral infections3–4, which resembles the intron stable non-coding RNA of LAT. Although in the study of herpes virus, miRNA was not found from the stable non-coding RNA of LAT, we showed that pag1 transcript can generate functional miRNAs.

In Fig. 7, we summarize the interactions between hhi1 and pag1 to better illustrate how they function as molecular switches to determine should the viruses enter productive or latent infections in the

Figure 5 | Establishment of latent viral infection by miRNAs. (A) Sf21 cells were transfected with or without miRNA followed by HzNV-1 infection. (B) Column representation of the results of panel (A). (C) Confirmation of hhi1 and pag1 expressions in various cells by RT-PCR.

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Figure 6 | Suppression of HzNV-1 viral latency by knocking down pag1 expression. (A) RT-PCR showed that artificial siRNA can efficiently suppress pag1 expression in HzNV-1 infected cells. (B) pag1 expression is not detectable by RT-PCR in the pag1-null HzNV-1-infected cells. (C) Formation of latent colony is not observed by the infection of pag1-null HzNV-1.

Figure 7 | A comprehensive model for the establishment of productive and latent HzNV-1 viral infections through hhi1 and pag1 interactions.
host cells. The initial infection of HzNV-1 results in high levels of hhi1 expression (Fig. 5C, lane 1)\(^\text{16}\), and a moderate level of pag1 expressions (Fig. 5C, lane 1)\(^\text{3–4}\). The newly produced abundant hhi1 transcript can tolerate some RNA degradation caused by the miRNAs derived from pag1. Then, the hhi1 transcript, before degradation or survived from initial degradation, can quickly transactivate stimulation or survived from initial degradation, resulting in virus production (Fig. 7C)\(^\text{16}\). If pag1 gene or its derived miRNAs (Fig. 6C lanes 3–4) were transfectioned into the cells prior to the initial viral infection, the chance of cells entering latent viral infection becomes strongly boosted (Fig. 7D). Different from the function of miRNAs produced from LAT of the herpes virus, which blocks protein expression, miRNAs derived from pag1 transcript degrade the transcript of hhi1. More significantly, our evidences proved that pag1 transcript and its derived miRNAs can establish latent viral infection in the cells.

Methods

Cells and virus. Spodoptera frugiperda IPL-B SF-21 was incubated in TC-100 insect cell culture medium, which contained 10% of FBS at 26°C (Gibco BRL)\(^\text{1–2}\). Standard HzNV-1 virus was derived by a serial dilution of the stock viral solution and isolated by plaque purification. Latently infected colonies were calculated as described in Wu et al.\(^\text{1–2}\). The titers of the virus clones were estimated by both Q-PCR\(^\text{30}\) and TCID\(_\text{50}\)\(^\text{26}\) assays from total RNAs using miRNA specific stem-loop primers (Table 1) according to criteria described previously\(^\text{18}\). All miRNAs duplex were synthesized by MDbio Inc. The siRNAs used in this study were designed for the PCR amplification of Hz-pag1 and actin as follows: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’; actin-F: 5’-CGTGAATGGGCTGGATGTCAG-3’; actin-R5’-CTAATGTCAGACGAGTCCTT-3’. The number of latently infected colonies was calculated at 12 dpi\(^\text{4}\). Primers designed for the detection of hhi1 and pag1 by RT-PCR are listed as following: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’, and hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’. The number of latently infected colonies was calculated at 12 dpi\(^\text{4}\). Primers designed for the detection of hhi1 and pag1 by RT-PCR are listed as following: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’. The number of latently infected colonies was calculated at 12 dpi\(^\text{4}\). Primers designed for the detection of hhi1 and pag1 by RT-PCR are listed as following: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’. The number of latently infected colonies was calculated at 12 dpi\(^\text{4}\). Primers designed for the detection of hhi1 and pag1 by RT-PCR are listed as following: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’. The number of latently infected colonies was calculated at 12 dpi\(^\text{4}\). Primers designed for the detection of hhi1 and pag1 by RT-PCR are listed as following: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’. The number of latently infected colonies was calculated at 12 dpi\(^\text{4}\). Primers designed for the detection of hhi1 and pag1 by RT-PCR are listed as following: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’. The number of latently infected colonies was calculated at 12 dpi\(^\text{4}\). Primers designed for the detection of hhi1 and pag1 by RT-PCR are listed as following: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’. The number of latently infected colonies was calculated at 12 dpi\(^\text{4}\). Primers designed for the detection of hhi1 and pag1 by RT-PCR are listed as following: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’. The number of latently infected colonies was calculated at 12 dpi\(^\text{4}\). Primers designed for the detection of hhi1 and pag1 by RT-PCR are listed as following: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’.

RNA interference. pag1 knockdown experiments, all siRNAs were predicted and synthesized by MDbio Inc. The siRNAs used in this study were designed to silence Hz-pag1 and Hz-pag1

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// Construction of pag1-null HzNV-1. A fragment containing egfp gene flanked by EcoRV sites was inserted down-stream of the hsp 70 promoter (p-hsp) of plasmid pBShsp70, generating plasmid pBShE. Cells were transfected with designed siRNAs and, at 4 hpt, the cells were infected with HzNV-1 virus (MOI = 1). The number of transfected experiments, all miRNAs were synthesized by MDbio Inc. Sf-21 cells (4 × 10^5) in 96-well plates were transfected with 50 nM of the siRNA using Silencer siRNA transfection kit (Applied Biosystems). Cells were transfected with designed siRNAs and, at 4 hpt, the cells were infected with HzNV-1 virus (MOI = 1). The number of latently infected colonies was calculated at 12 dpi. Primers designed for the detection of hhi1 and pag1 by RT-PCR are listed as following: hhi1-1F: 5’-CGATATGAATCAAAGTGATGACGTCC-3’; hhi1-1R: 5’-AACAGGGAATCAAAGTGATGACGTCC-3’; pag1-F: 5’-ACGGGAATCCTGGGAGGACTT-3’; pag1-R: 5’-CATGTCCTAGAACCTCTCCTTT-3’.
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Additional information
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