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Reduced expression of *Autographa californica* nucleopolyhedrovirus ORF34, an essential gene, enhances heterologous gene expression

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ABSTRACT

Autographa californica multiple nucleopolyhedrovirus ORF34 is part of a transcriptional unit that includes ORF32, encoding a viral fibroblast growth factor (FGF) and ORF33. We identified ORF34 as a candidate for deletion to improve protein expression in the baculovirus expression system based on enhanced reporter gene expression in an RNAi screen of virus genes. However, ORF34 was shown to be an essential gene. To explore ORF34 function, deletion (KO34) and rescue bacmids were constructed and characterized. Infection did not spread from primary KO34 transfected cells and supernatants from KO34 transfected cells could not infect fresh Sf21 cells whereas the supernatant from the rescue bacmids transfection could recover the infection. In addition, budded viruses were not observed in KO34 transfected cells by electron microscopy, nor were viral proteins detected from the transfection supernatants by western blots. These demonstrate that ORF34 is an essential gene with a possible role in infectious virus production.

Introduction

Baculoviruses are large rod shaped DNA viruses that infect insects. They have practical applications as biological insecticides and protein expression vectors. *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) is the type species of the *Alphabaculovirus* genus of the family *Baculoviridae* (International Committee on Taxonomy of Viruses, 2009). Two clades, group I and group II, have been identified in *Alphabaculovirus* (Zanotto et al., 1993), which differ in the envelope fusion protein of the budded virus (BV) (Ijkel et al., 2000; Pearson et al., 2000). AcMNPV is member of group I and has a 134 Kb genome encoding approximately 150 proteins (Rohrmann, 2011). The functions of approximately 50% of these are known (Cohen et al., 2009). AcMNPV is a lytic virus and its life cycle involves the production of two morphological forms of the virus and a regulated cascade of gene expression (for reviews see (Miller, 1997; Rohrmann, 2011). The first form of the virus produced during infection is BV, which is formed when nucleocapsids are transported from their site of replication and assembly in the nucleus to the plasma membrane and acquire an envelope as they bud from the infected cells. The second form, occlusion derived virus (ODV), is produced late in infection when nucleocapsids retained in the nucleus acquire an envelope, most likely derived from the inner nuclear membrane (Braunagel and Summers, 2007), and are occluded in large protein crystals called occlusion bodies. The primary component of the occlusion bodies is a single virus protein called polyhedrin (polh). AcMNPV gene expression comprises four gene classes. Immediate early genes, which do not require viral gene products, and delayed early genes are transcribed by the host RNA pol II and encode proteins required for virus replication. Late and very late genes are transcribed by a virus RNA polymerase from virus-specific promoters and encode primarily structural proteins. The very late or occlusion-specific genes, which encode polh and another small protein, p10, are expressed to very high levels at the end of the replication cycle. At this stage in the infection cycle polh makes up approximately 50% of the newly synthesized protein in the infected cells. The high-level of expression from the *polh* promoter is achieved through the use of the strong virus RNA polymerase. In addition, the production of large amounts of virus DNA that accumulates in the nucleus effectively amplifies

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Table 1
AcMNPV ORFs silenced by RNAi.

ORF(s) targeted by dsRNA	Annotation	dsRNA size bp	dsRNA template positions in AcMNPV genome (bp)
ORF1	ptpase	451	524–974
ORF3	Ctx	150	2245–2119
ORF21	arif-1	485	16938–16454
ORF46	Odv-e66	497	37898–38394
ORF61	Fp25k	374	49086–48713
ORF94	(Odv-e25) p25	370	79974–80343
ORF105	He65	496	93011–92516
ORF148	Odv-e56	405	130128–129724
ORF15	egt	681	11852–12513
ORF22	pif-2	665	17702–18366
ORF23		692	19020–19711
ORF27	iap1	684	22609–23292
ORF32	fgf	534	27581–27048
ORF34+ORF35 (v-ubi)		535	28655–29189
ORF49+ORF48+ORF47 (PCNA)		540	39713–39174
ORF63+ORF64 (gp37)		554	51969–51416
ORF71	iap2	633	61118–61750
ORF109		631	95518–94888
ORF115	pif3	503	99703–99202
ORF119	pif1	616	101140–101755
ORF131	pe/pp34	604	110989–111592
ORF136 (p26)+ORF137 (p10)+ORF138 (p74)		1172	118536–119707
ORF134	94K	989	115972–114984
ORF106+ORF107		601	93750–94334
ORF112+ORF113		669	96584–97253
ORF12		480	9108–9480
ORF2	Bro	664	1783–1120
ORF11		640	8853–8214
ORF12		416	9065–9480
ORF13		491	10379–9889
ORF18		563	15119–14557
ORF26		319	22214–22532
ORF29		200	24259–24060
ORF30		507	25531–25025
ORF31	Sod	425	25835–26259
ORF33		474	28235–27762
ORF42	gta	504	34829–35332
ORF43		201	35569–35769
ORF44		354	35768–36121
ORF45		501	36155–36655
ORF51		565	43335–43899
ORF52		340	44708–44369
ORF55		191	46418–46608
ORF56		251	46634–46884
ORF57		461	47073–47533
ORF58		150	47733–47584
ORF60		259	48363–48105
ORF63		403	50820–51222
ORF68		517	58740–59256
ORF69		596	59315–59910
ORF73		251	62302–62052
ORF74		516	63044–62529
ORF78		299	65276–64978
ORF79		303	65602–65300
ORF82	tlp	472	67918–67447
ORF83	p95	605	68012–68616
ORF84		401	71247–71647
ORF86	pnk/pnl	602	73269–72668
ORF87	p15	301	74397–74697
ORF88	cg30	604	75505–74902
ORF91		502	78490–77989
ORF93		423	79523–79945
ORF96		455	84406–84860
ORF108		220	94696–94477
ORF110+ORF111		313	96321–95961
ORF114		750	98868–98119
ORF116		150	99958–99809
ORF117		200	99939–100138
ORF118		411	100661–100251
ORF120		201	102321–102521
ORF122		151	102897–102747
ORF123	pk2	531	103561–103031
ORF124		634	103854–104487
ORF129	p24	413	109900–110312
ORF130	gp16	306	110524–110829

Table 1 (*continued*)

ORF(s) targeted by dsRNA	Annotation	dsRNA size bp	dsRNA template positions in AcMNPV genome (bp)
ORF132		643	111874–112516
ORF145		205	126310–126514
ORF146		587	127116–126530
ORF149		200	130388–130189
ORF150		207	130189–130751
ORF152		250	132360–132111
ORF153	pe38	621	132695–133315
ORF154		203	133620–133822

the template available for transcription (Rohrmann, 2011). Viral DNA is likely accessible for transcription through the entire infection cycle because a large proportion (approx. 70%) of viral DNA synthesized in cells is not packaged (Vanarsdall et al., 2006).

AcMNPV open reading frame 34 (ORF34) is a previously uncharacterized late gene that is located from 28,294 to 28,939 bp on the genome and transcribed in a counterclockwise direction relative to the circular map (Ayres et al., 1994). ORF34 homologs are found in all other group I and most group II *Alphabaculovirus* genomes (Cohen et al., 2009), and gene knockouts could not be obtained for *Bombyx mori* nucleopolyhedrovirus (BmNPV), suggesting that it may be an essential gene (Rohrmann, 2011). ORF34 is located immediately upstream of a transcriptional unit that includes ORF33 and fibroblast growth factor (*fgf*) (ORF32) (Detvisitsakun et al., 2005), and is transcribed in the same direction. A viral ubiquitin gene (*ubi*) (ORF35) is located upstream of ORF34 and transcribed in the opposite direction (Guarino, 1990). While this manuscript was in revision, a paper describing characterization of an AcMNPV ORF34 deletion mutant was published (Cai et al., 2012). This study identified a domain with similarity to S-phase kinase-associated protein 1 (SKP1), characterized ORF34 expression as both early and late, and showed the protein localized to both nucleus and cytoplasm, but not virions. They also report obtaining viruses with ORF34 deletions that were characterized by reduced BV production, smaller plaques, and delayed late gene expression.

In this study we identified ORF34 as an essential virus gene that is required for the production of infectious BV. The study was initiated to identify virus genes that could be deleted from the AcMNPV genome to improve protein expression in the baculovirus expression vector system (BEVS). Because secretory proteins, especially those that are highly glycosylated, and integral membrane proteins (IMPs) are more difficult to express than cytoplasmic proteins (Grisshammer and Tate, 1995; Jarvis, 1997), we focused on a model IMP as a reporter for improved protein expression. We selected 92 AcMNPV open reading frames (ORFs) that were not previously shown to be essential for virus replication, and screened for their impact on the expression of the model protein gene fused to an enhanced green fluorescent protein (EGFP) using BEVS by RNAi. We report that dsRNA targeting ORF33 increased expression of the model IMP in this screen, as well as that of other model proteins expressed using BEVS. However, deleting ORF33 from the AcMNPV genome failed to enhance expression of the model IMP. Further investigation using gene knockouts, RNAi, and qRT-PCR revealed that reduced expression of ORF34, part of a transcriptional unit that includes ORF33 and *fgf*, was responsible for the increased expression of the model IMP. We were unable to generate recombinant viruses with ORF34 deletions, using bacmid technology. Cells transfected with ORF34 knock-out bacmids resulted in single infected cells. This phenotype could be rescued by expressing ORF34 inserted at the *polh* locus. BV was not observed in transmission electron microscopy of ORF34 knockout bacmids. Together these data demonstrate that ORF34 is an essential gene that is required for virus spread.

Results

Transfecting Sf21 cells with ds33 enhanced the protein production of integral membrane proteins as well as cytosolic proteins driven by the polh promoter

To identify AcMNPV genes that affect protein expression in the baculovirus expression system, we conducted an RNAi screen of AcMNPV genes. We used a model integral membrane protein, human transient receptor potential cation channel subfamily V member 4 (TRPV4), fused to EGFP expressed in a recombinant AcMNPV from the *polh* promoter (vTRPV4) for readout. TRPV4 is a receptor cation channel comprised of four subunits with six predicted transmembrane domains that interact to form a tetrameric channel similar to voltage dependent K^+ channels (Everaerts et al., 2010). To conduct the screen we knocked down each of 92 AcMNPV ORFs using dsRNAs. Fluorescent intensity was measured using a plate reader. Comparisons of the fluorescence measurements were made between infected cells treated with dsRNAs directed against AcMNPV ORFs and that of non-specific control dsRNA, the drosophila nautilus gene (dsC). The 92 ORFs were selected to exclude all genes known to affect virus replication or budded virus production. A total of 83 dsRNAs were used in the screen (Table 1), however because some predicted ORFs are very small and others overlapped with adjacent ORFs, 92 ORFs were effectively targeted for silencing. To monitor the efficiency of gene silencing during the RNAi screen, dsRNA targeting EGFP (the reporter gene) was used. The RNAi screen was conducted by transfecting the Sf21 cells with dsRNAs 18 h prior to infection with vTRPV4. From the data collected at 48 h post-infection (p.i.), we observed enhanced fluorescent intensity of the EGFP comparing to the control when transcripts of several different ORFs were depleted. Knocking down transcripts in ORF33, using complementary double-stranded RNA (ds33), showed the highest median fluorescence intensity (MFI) compared to two controls, dsC and dsRNA targeting the chloramphenicol acetyl transferase (CAT) gene (dsCAT), an overall enhancement of 2.3 fold (Fig. 1A). Using in-gel fluorescence to compare TRPV4-EGFP expression in cells transfected with ds33 with dsC, we found that the fusion protein product was the correct size (128 kDa) and confirmed that expression was enhanced 1.5 fold (Fig. 1B). To correlate increased MFI to the increases in protein production, SDS-PAGE and western blots were conducted (Fig. 1C). The band intensity of the proteins isolated from the Sf21 cells transfected with ds33 prior to vTRPV4 infection was 1.8 fold greater than the control. Enhancement was also detected for the human cannabinoid receptor 2 (CNR2), a G-protein coupled receptor (GPCR), and cytosolic EGFP expressed as a non-fusion protein both expressed from the *polh* promoter, 1.3 and 1.9 fold respectively, when cells were transfected with ds33 compared to controls prior to infection with the viruses expressing CNR2 (vCNR2) or EGFP (Fig. 1D and E). This indicated that enhanced expression from the polh promoter resulting from ORF33 knockdown was not limited to IMPs.

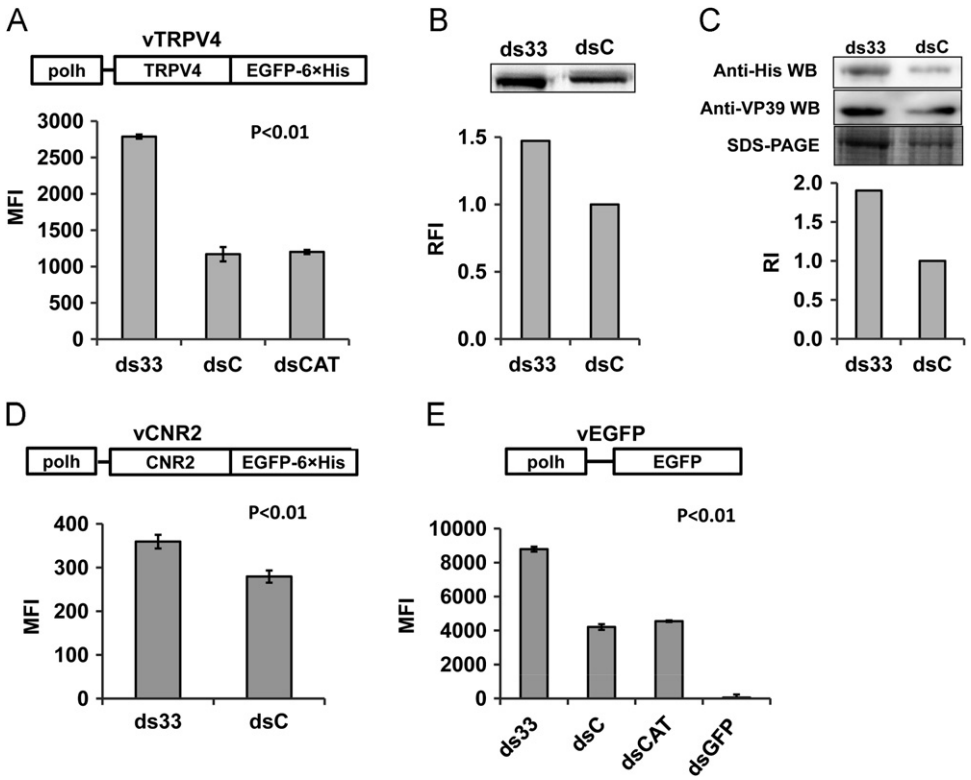

Fig. 1. Silencing ORF 33 with dsRNA enhanced expression levels of proteins driven by the *polh* promoter. (A) Median fluorescence intensity (MFI) of Sf21 cells transfected with ds33 18 h prior to vTRPV4 infection and measured by FACS at 48 h p.i. (B) In-gel fluorescent of TRPV4-EGFP protein from representative experiment shown in (A). Total proteins extracted from the same number of cells transfected with either ds33 or dsC prior to vTRPV4 infection were run on SDS-PAGE and fluorescence was detected using a Versadoc imaging system (Bio-Rad). (C) SDS-PAGE and western blot (WB) showing TRPV4-EGFP expression at 48 h p.i. after ORF 33 silencing. Anti-His and anti-VP39 antibody was used for WB. The SDS-PAGE gel was stained with Coomassie Blue. Relative intensity (RI) of the TRPV4 band was normalized to VP39 and analyzed by Quantity One software. (D) MFI of human cannibinoid receptor (vCNR2) at 48 h p.i., measured by microplate reader (SpectraMax® M5). (E) The results of FACS analyzing the MFI of cells infected with vEGFP at 48 h p.i. after treatment with ds33, dsC or dsGFP. dsGFP was used as an additional control for gene silencing efficiency. Standard errors from three independent experiments are shown on each graph. The significance differences were determined by *t*-test.

Targeting ORF33 with ds33 reduced the levels of ORF33 transcripts and those of neighboring ORFs on polycistronic transcripts

ORF33 is encoded on the complementary strand of the AcMNPV genome (nucleotides; 27733–28281) as part of a transcriptional unit that includes *fgf* (Fig. 2A). Upstream is ORF35, *ubi,* transcribed in the opposite direction (Guarino, 1990). In a previous study three abundant transcripts were detected on northern blots using a complement probe for *fgf* (Detvisitsakun et al., 2005). These transcripts are an early 0.6 kb *fgf* transcript, a late 1.4 kb ORF33/*fgf* transcript, and a late 3.1 kb transcript (Fig. 2A). Only the two late transcripts (1.4 and 3.1 kb) were detected with a complementary probe for ORF33. The 3.1 kb transcript hybridized with ORF33 and FGF probes but its start and end sites were not determined (long dotted line in Fig. 2A) (Detvisitsakun et al., 2005). It is not known if ORF34 is transcribed as part of the 3.1 kb transcript or if ORF34 monocistronic transcripts are produced (short dotted line in Fig. 2A). Of note are two canonical late transcription start sites upstream of ORF34 within the *ubi* coding region. To examine the transcripts spanning this region and determine their expression levels following treatment with ds33, we used reverse transcriptase and real time PCR.

One-step reverse transcriptase PCR (one-step RT-PCR), using the primers shown in Fig. 2A, was used on total RNA extracted from Sf21 cells transfected with ds33 prior to vTRPV4 infection, harvested at 48 h p.i. and compared to the control. At 22 cycles, the ORF33 transcripts were reduced in the RNA sample isolated from cells transfected with ds33 relative to dsCAT (Fig. 2B). Reductions in ORF34 and FGF transcripts were not apparent from this experiment, but were clearly observed in qRT-PCR experiments (Fig. 3, see below). A similar reduction in transcripts was seen when multiple ORFs were targeted (34/33, 33/FGF, and 34/33/FGF) (Fig. 2C). This experiment indicated that polycistronic transcripts spanned the ORF34/ORF33/FGF region (solid line with asterisk in Fig. 2A; black box with asterisk in Fig. 2C), however, it was not determined if the 3.1 kb transcript observed by Detvisitsakun et al. (2005) is a polycistronic transcript of the three ORFs. A non-specific band (arrow, Fig. 2B and C) was observed when the F1 primer was used. We used this non-specific band as an internal control to confirm the change in the level of transcripts detected by F1/R3 (black box, Fig. 2C) in cells transfected with ds33 compared to the control. Primers specific to the 28S ribosomal RNA were used as a second control and showed no difference in band intensity between the experimental and control samples.

To verify the effects of ds33 on transcripts spanning the adjacent ORFs in this region, qRT-PCR was performed. We compared the relative levels of ORF33, ORF34, FGF, and TRPV4-EGFP transcripts in Sf21 cells transfected with ds33 or dsC prior to vTRPV4 infection (Fig. 3). The level of transcripts detected in the control was considered one and the relative level of the transcripts in the cells transfected with ds33 was measured accordingly. At 24 h p.i., the transcripts in the Sf21 cells transfected with ds33 were reduced by approximately 6, 2, and 15 fold for ORF33, ORF34, and FGF, respectively, when compared to the control (Fig. 3B). In contrast, the TRPV4-EGFP transcript increased 2.4 fold. At 48 h p.i., a similar pattern was observed with reductions of approximately 3, 2, and 7 fold for ORF33, ORF34, and FGF, respectively, and a 1.7 fold increase for TRPV4-EGFP (Fig. 3C).

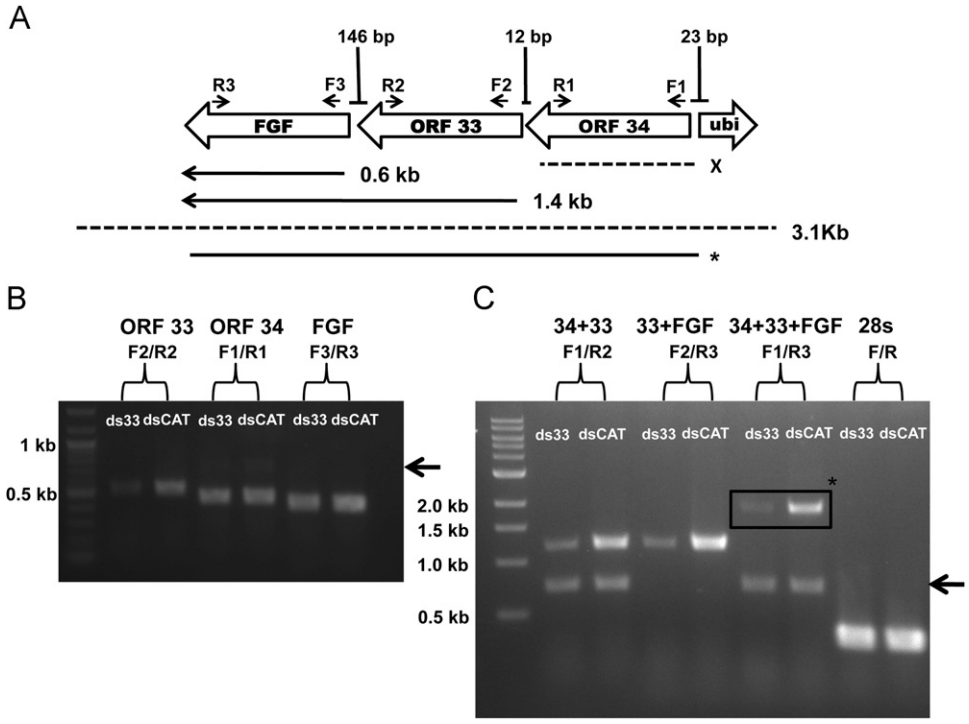

Fig. 2. One-step RT-PCR analysis of transcripts at the ORF 33 locus. (A) Diagram of ORFs and transcripts at the ORF 33 locus (after Detvisitsakun et al., 2005). The viral ubiquitin gene (*ubi*), encoded on the opposite strand with transcripts originating within ORF34, is also included in the diagram. Solid arrows are previously mapped transcripts and dashed lines indicate an observed transcript whose 5′ and 3′ ends have not been mapped (3.1 Kb) and a putative ORF 34 transcript (X). The solid line (*) represents a polycistronic message confirmed by one-step RT-PCR. The locations of primers used for analysis are shown. (B) Transcripts through each ORF at 48 h p.i. with vTRPV4 following transfection with ds33 or dsCAT. ORFs, primer pairs and dsRNAs are indicated. (C) Transcripts across adjacent ORFs as indicated at the top of the panel. Primer pairs and dsRNAs are indicated. 28S RNA was used as a control. Black box in (C) and * in (A) and (C) indicate a transcript that includes FGF, ORF33 and ORF34. A non-specific band (~750 bp) was detected when F1 primer was used (arrow, in B and C).

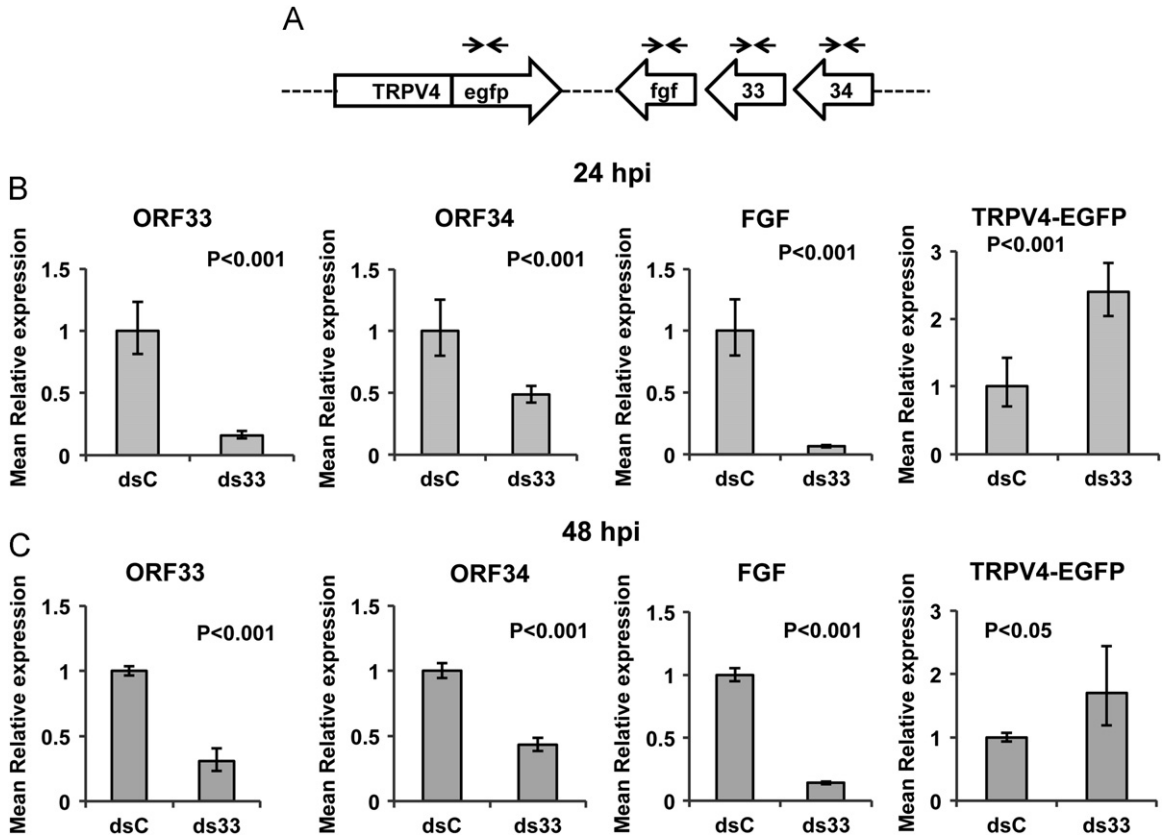

Fig. 3. Analysis by qRT-PCR showed that targeting ORF33 with dsRNA reduces the level of ORF33, ORF34 and FGF transcripts and increased levels of TRPV4-EGFP transcripts. (A) Diagram showing the locations of primers used for analysis. Relative levels of transcripts in Sf21 cells infected with vTRPV4 following transfection with ds33 or dsC at 24 (B) and 48 h p.i. (C) dsRNAs are indicated on the X-axis and amplified transcripts at the top of each graph. Bars indicate standard deviation. Mean relative expression was calculated based on $2^{-\Delta\Delta}Ct$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Significant differences between transcript levels in ds33 and dsC treated cells are shown ($p < 0.001$, $p < 0.05$).

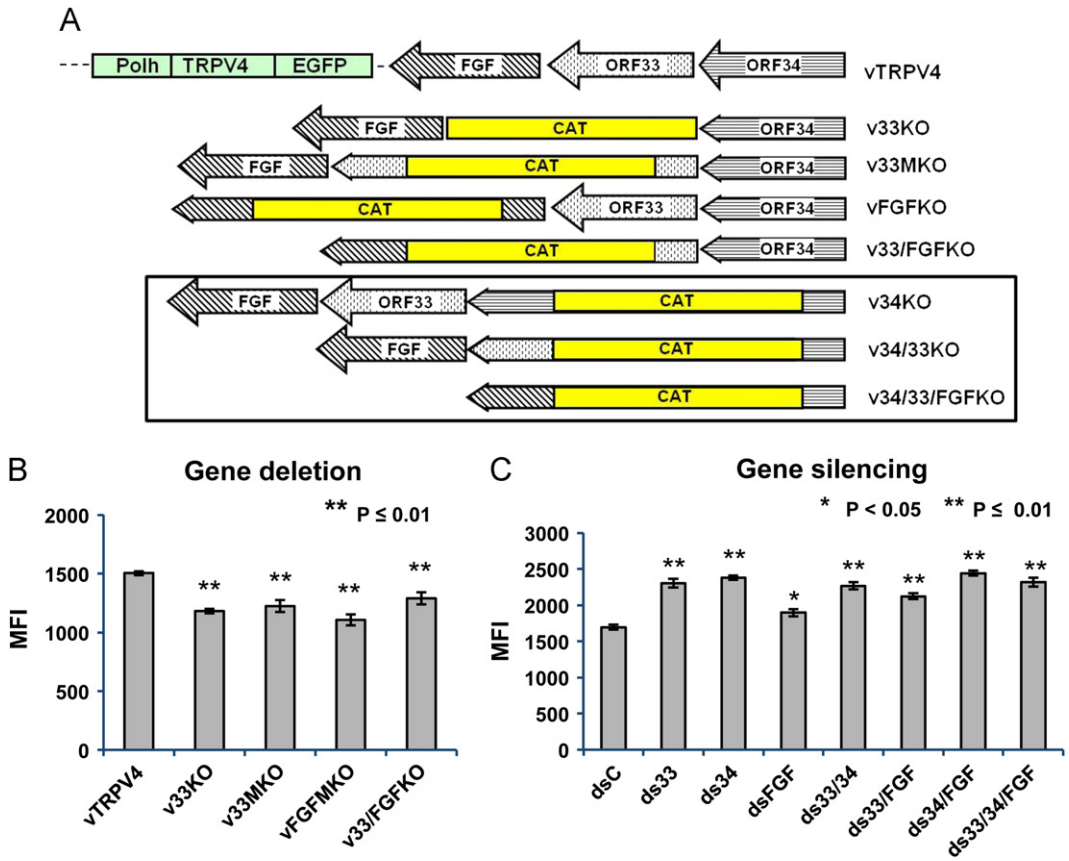

Fig. 4. Knockout of ORF33 did not improve expression of TRPV4-EGFP. (A) Diagram showing gene knockouts at the ORF33 locus. Deletions were made in the TRPV4-EGFP bacmid by replacement with the CAT gene using λ Red recombination. ORFs are drawn to scale. Viruses that included an ORF34 deletion (black box) could not be recovered. (B) MFI of Sf21 cells 48 h p.i. with knockout viruses relative to parental vTRPV4. Knockout viruses are indicated on the x-axis. (C) MFI of Sf21 cells transfected with dsRNA as indicated on the x-axis 48 h p.i. with vTRPV4. Data were obtained by FACS. Standard errors from three independent experiments are shown on each graph. The significance differences in fluorescent intensity between controls and experimental treatments were determined by *t*-test.

ORF33 deletion did not have the same effect on TRPV4-EGFP expression as dsRNA knockdown

To confirm that knockdown of ORF33 was responsible for the enhanced expression of TRPV4-EGFP, recombinant viruses with gene disruptions of each of the genes, both singly and in combination, in this transcriptional unit were generated. Genes were replaced with or disrupted by the insertion of the CAT gene into the TRPV4-EGFP bacmid using λ-Red recombination (Datsenko and Wanner, 2000). For all of the double and triple deletions, a portion (approximately half of the ORF) at the 5′end of the first ORF and a portion (approximately half of the ORF) at the 3′end of the last ORFs were maintained. We were successful in obtaining recombinant viruses with the following gene disruptions: replacement of ORF33 with CAT (v33KO), replacement of approximately 100 bp in the middle of ORF33 (v33MKO), replacement of approximately100 bp in the middle of FGF (FGFKO), and replacement of ORF33 and FGF (v33/FGFKO) (Fig. 4A). However, we failed to obtain the following recombinant viruses: replacement of 100 bp in the middle of ORF34 (v34KO), replacement of ORF34 and ORF33 (v34/33KO), and replacement of ORF34, ORF33, and FGF (v34/33/FGFKO) (boxed in Fig. 4A). When cells were transfected with any bacmid in which ORF34 was disrupted we observed fluorescence in many single cells and occasionally a small fluorescent plaque comprising fewer than 10 infected cells. However, when supernatants from these transfections were passaged no fluorescent cells were observed, suggesting that ORF34 is required for production of infectious BV. FACS was used to measure the MFI of the Sf21 cells infected with the obtained viruses (v33KO, v33MKO, FGFKO, and v33/FGFKO) and data were compared with the MFI of cells infected with vTRPV4 (Fig. 4B). In contrast to the enhanced expression we observed for the dsRNA knock-down of ORF33, all recombinant viruses with ORF33 gene disruptions showed significant reductions ($p \leq 0.01$) in the level of TRPV4-EGFP expression when compared with vTRPV4. The reductions were approximately 21%, 18%, 26% and 14% for v33KO, v33MKO, FGFKO, and v33/FGFKO, respectively.

Because RNAi does not eliminate all transcripts, we were able to use ds34 in RNAi experiments to explore the possibility that reduced expression of ORF34 rather than ORF33 might be responsible for enhanced expression of TRPV4-EGFP . For these experiments, each of the ORFs in the transcription unit was targeted both singly and in combination with dsRNAs complementary to the central regions of each ORF, to avoid potential regulatory regions of adjacent ORFs. Changes in fluorescent intensity relative to the control were measured by flow cytometry (Fig. 4C). There was a significant increase in TRPV4-EGFP expression ($p \leq 0.01$ for all except dsFGF $p < 0.05$); approximately 35%, 40%, 12%, 34%, 25%, 44% or 37% increases in the MFI when Sf21 cells were transfected with ds33, ds34, dsFGF, ds33/34, ds33/FGF, ds34/FGF, or ds33/34/FGF, respectively, compared to the control.

Production of infectious BV is reduced and levels of virus DNA increases in vTRPV4-infected Sf21 cells transfected with ds33 or ds34

To investigate the effects of knocking down ORFs 33 and 34 on the virus lifecycle and gain some insight into how this improved expression of TRPV4-EGFP, we examined the effects of ds33 and

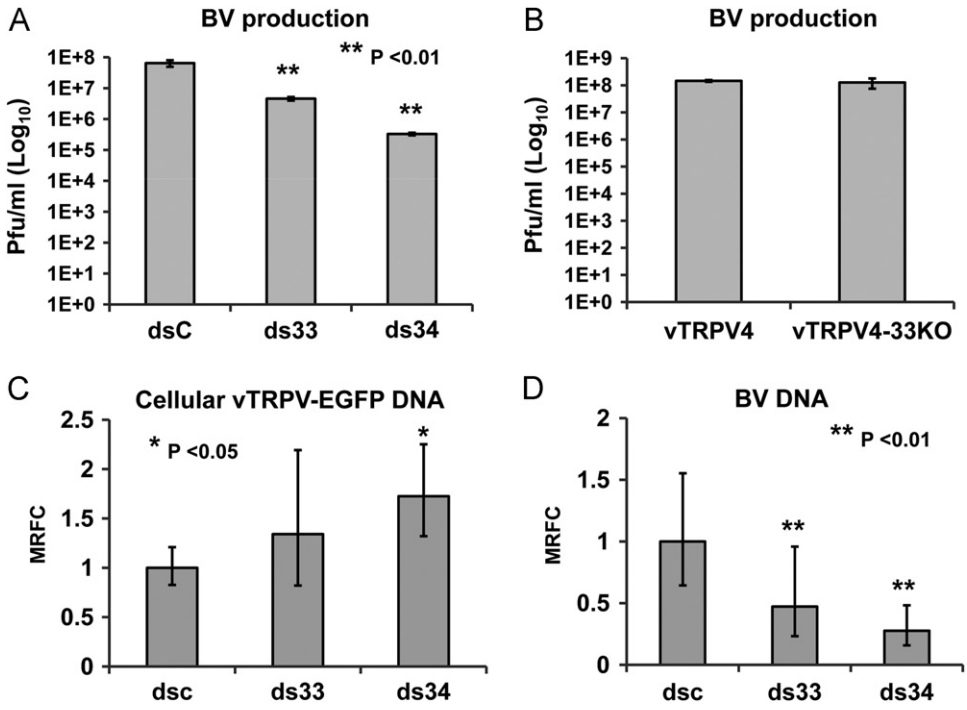

Fig. 5. Transfecting Sf21 cells with ds33 and/or ds34 reduces infectious BVs and increases viral genome retention in the cells. (A) BV production by Sf21 cells that were transfected with 5 μg dsRNAs as indicated on the x-axis followed by infection with vTRPV4 (MOI 5) at 48 h p.i. (B) BV production by Sf21 cells infected with vTRPV4 or v33KO (MOI 5) at 48 h p.i. (C) qPCR of intracellular AcMNPV genome and BV DNA (D) for cells transfected with ds33 and ds34, as indicated in A, respectively. Primers were designed for the vp39 gene and the mean relative fold change (MRFC) was measured. Mean relative expression was calculated based on $2^{-\Delta\Delta}Ct$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The significant differences between the tested samples (ds33 and ds34) and the control (dsC) are shown as *p*-values.

ds34 on BV production and virus DNA accumulation in vTRPV4-infected cells. Sf21 cells were transfected with ds33, ds34, or dsC prior to vTRPV4 infection. At 48 h p.i. the cells and medium were collected. Plaque assays were performed on the medium to quantify the infectious BVs (Fig. 5A). The average titers of the BV in the medium of Sf21 cells transfected with ds33, ds34, or dsC were 4.6×10^6, 3.3×10^5, or 6.5×10^7 pfu/ml, respectively. Thus the infectious BVs released in the media from cells transfected with ds33 or ds34 were 14 or 197 fold less than the BVs released from the cells transfected with dsC, respectively. DNA was extracted from the cells and a quantitative PCR (qPCR) was performed. We found that there was a subtle increase in the mean levels of virus DNA from cells transfected with ds33 (1.3 fold increase) or ds34 (1.7 fold increase) when compared with dsC (Fig. 5C). The increase in cellular virus DNA was statistically significant for ds34-treated cells ($p<0.05$), but not for ds33-treated cells. A second qPCR performed on BV DNA, extracted from fractions of medium collected and used for plaque assays, showed 2 or 3.6 fold reduction in virus DNA when ds33 or ds34 were used, respectively (Fig. 5D), providing further evidence that BV production is reduced when ORFs 33 or 34 are knocked-down . The data were normalized to the 28S gene to eliminate any background contamination from cellular DNA obtained during the DNA extraction from the BVs. When we compared the BV titers from vTRPV4- and v33KO-infected Sf21 cells there were no significant differences (Fig. 5B). These data suggested that the reduced titers observed when cells were transfected with ds33 or ds34 were most likely the result of knocking down ORF34 expression, rather than a consequence of knocking down ORF33 expression.

Reducing the levels of an essential gene reduced BV titers and appeared to increase the intracellular levels of virus DNA. This suggested that increased viral DNA in the nucleus providing additional template for gene transcription could be a possible mechanism for enhanced reporter gene expression. To test this, expression of the major viral capsid protein, vp39, was knocked down with dsRNA prior to infections with vTRPV4, to reduce BV production. Expression of TRPV4 was not enhanced (Supplementary Fig. 1A). When virus DNA was measured by qPCR, there was a significant reduction in BV DNA relative to controls and a small, although not significant, increase in intracellular virus DNA (Supplementary Fig. 1B). These results indicate that reducing virus BV production may result in a small increase in intracellular virus DNA, but does not enhance heterologous gene expression. Although this experiment does not rule out the possibility that increased template DNA may contribute to enhanced gene expression from the *polh* promoter in ORF34 knockdown experiments, it suggests that it is not a key mechanism.

Knockdown of ORF34 enhances TRPV4-EGFP expression in cells infected with v33KO

To verify the role of reduced expression of ORF34 in enhancing expression of the TRPV4-EGFP reporter, we conducted RNAi experiments on v33KO-infected Sf21 cells. Cells were transfected with ds33 or ds34 prior to infection, as previously described, and fluorescent intensity was measured at 48 h p.i. by flow cytometry. We found that ds34 but not ds33 significantly enhanced TRPV4-EGFP expression relative to controls (Fig. 6A). We verified the reduction in ORF34 transcripts by RT-PCR (Fig. 6B). Because the two transcriptional start-sites of *ubi* map to immediately upstream and within ORF34 (Guarino, 1990), we also examined *ubi* transcription by RT-PCR . We found no difference in the levels of *ubi* transcripts relative to 28S controls (Fig. 6B). These data support the idea that enhanced TRPV4-EGFP expression is the result of reducing the levels of ORF34 transcripts.

ORF34 is an essential gene required for budded virus production

To confirm that ORF34 is an essential gene, as suggested by our inability to obtain recombinant viruses when cells were

transfected with any bacmid with an ORF34 deletion (Fig. 4A), we constructed ORF34 deletion bacmids in the same manner as the TRPV4-EGFF-expressing v34KO (Fig. 7A). Successful deletion of ORF34 was confirmed by PCR (Fig. 7B). An EGFP reporter driven by the *polh* promoter was then inserted at the *polh* locus either alone or together with ORF34 driven by its own promoter (Fig. 7C). Two different rescue constructs were made, orienting ORF34 in either the same direction as GFP (KO34-polhGFP-Res34F) or in the opposite orientation (KO34-polhGFP-Res34R) (Fig. 7B). Successful deletion of ORF34 and re-insertion were confirmed by PCR (Fig. 7D).

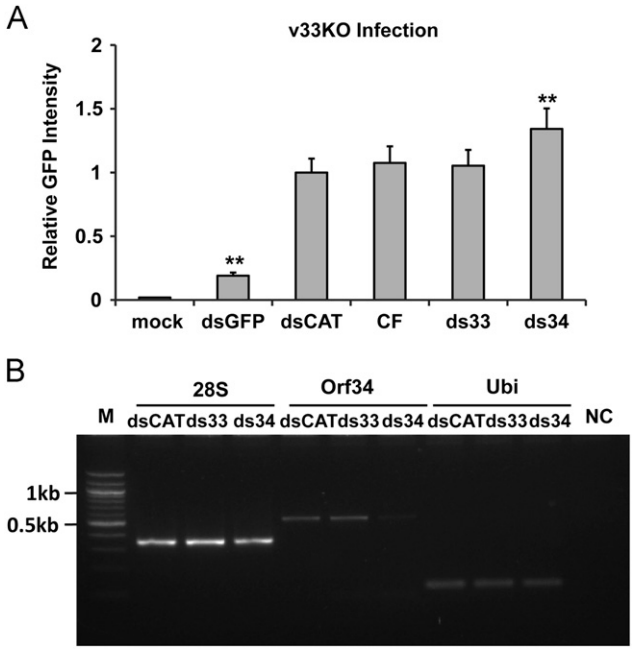

Fig. 6. Silencing ORF34 with dsRNA enhanced expression levels of TRPV4 protein in Sf21 cells infected with an ORF33 knockout virus. (A) Sf21 cells were transfected with 2 μg dsRNA and 24 h later cells were infected with v33KO. Flow cytometry was used to analyze the relative GFP intensity at 48 h p.i. CF, cellfectin transfection reagent. (B) The expression levels of ORF34 and *ubi* were determined by RT-PCR. After transfection with dsRNA, cells were infected with v33KO and RNA was isolated at 48 h p.i. M, 100 bp ladder; NC, negative control. ** indicate $P < 0.01$ (*t*-test).

Sf21 cells were transfected with the bacmids and observed over time post-transfection . Images taken at 72 h post transfection are shown in Fig. 8A. Similar to our observations of cells transfected with ORF 34 knock-out bacmids expressing TRPV4-EGFP (v34KO, v34/33KO, and v34/33/FGFKO) (Fig. 4A), only single fluorescent cells were observed when cells were transfected with KO34-polhGFP (KO34). In contrast, fluorescent plaques were observed in cells transfected with both rescue bacmids. When supernatants from these transfections were used to infect fresh monolayers of Sf21 cells, no fluorescent cells were observed for KO34-polhGFP, whereas robust infections were initiated with supernatants from both rescue bacmid transfections (Fig. 8B). DNA was prepared from the supernatants of infected cells harvested at 72 h p.i. and analyzed for the presence of BV by PCR (Fig. 8C) and qPCR (Fig. 8D) using primers targeting vp39. No amplification was observed from KO34-polhGFP samples, whereas all of the samples of rescue viruses had levels of virus DNA orders of magnitude higher than KO34-polhGFP .

Because our results conflicted with those reported by (Cai et al., 2012), we re-examined our constructs and our data. The region of ORF34 deleted for each of our constructs, v34KO, v34/33KO, v34/33/FGFKO, and KO34 (Fig. 4A and Fig. 7A) was nearly identical to their deletion, which was in the same region but was more extensive comprising an additional 48 nucleotides, 47 towards the 3′end, yet in contrast we were unable to isolate deletion viruses. We came up with two possible explanations for these differences. Perhaps a portion of the remaining ORF34 gene was expressed in their constructs and provided some essential function allowing viable virus to be recovered. Another possibility was that intact ORF34 was present at low levels. We hypothesized that the differences in levels of infection, the presence of plaques, and the ability to recover infectious virus was due to low levels of bacmid DNA with intact ORF34 in the transfections. To determine

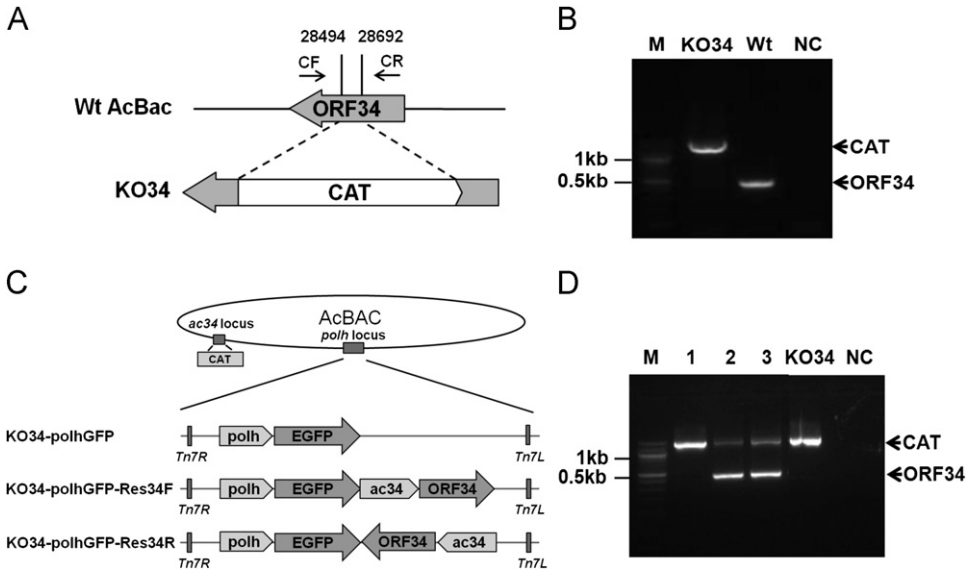

Fig. 7. Construction of an ORF34-knockout bacmid (KO34) and rescue bacmids. (A) A diagram showing the knockout of the ORF34 gene by insertion of the CAT gene. CF and CR indicate the primers located outside the ORF34 deletion region (from 28494 to 28692, Gene accession no. NC_001623). (B) PCR was used to verify the deletion of ORF34 with primers CF and CR. (C) Diagram showing ORF34 rescue constructs. GFP under *polh* promoter was put into the *polh* locus of the KO34 as a control (KO34-polhGFP). ORF34 under its own promoter was cloned downstream of EGFP in either the forward (KO34-polhGFP-Res34F) or reverse direction (KO34-polhGFP-Res34R). (D) PCR analysis of the rescue bacmids with the CF and CR primers. M, 100 bp ladder; 1, KO34-polhGFP; 2, KO34-polhGFP-Res34F; 3, KO34-polhGFP-Res34R; the KO34 bacmid, used as a positive control; NC, negative control using water as template.

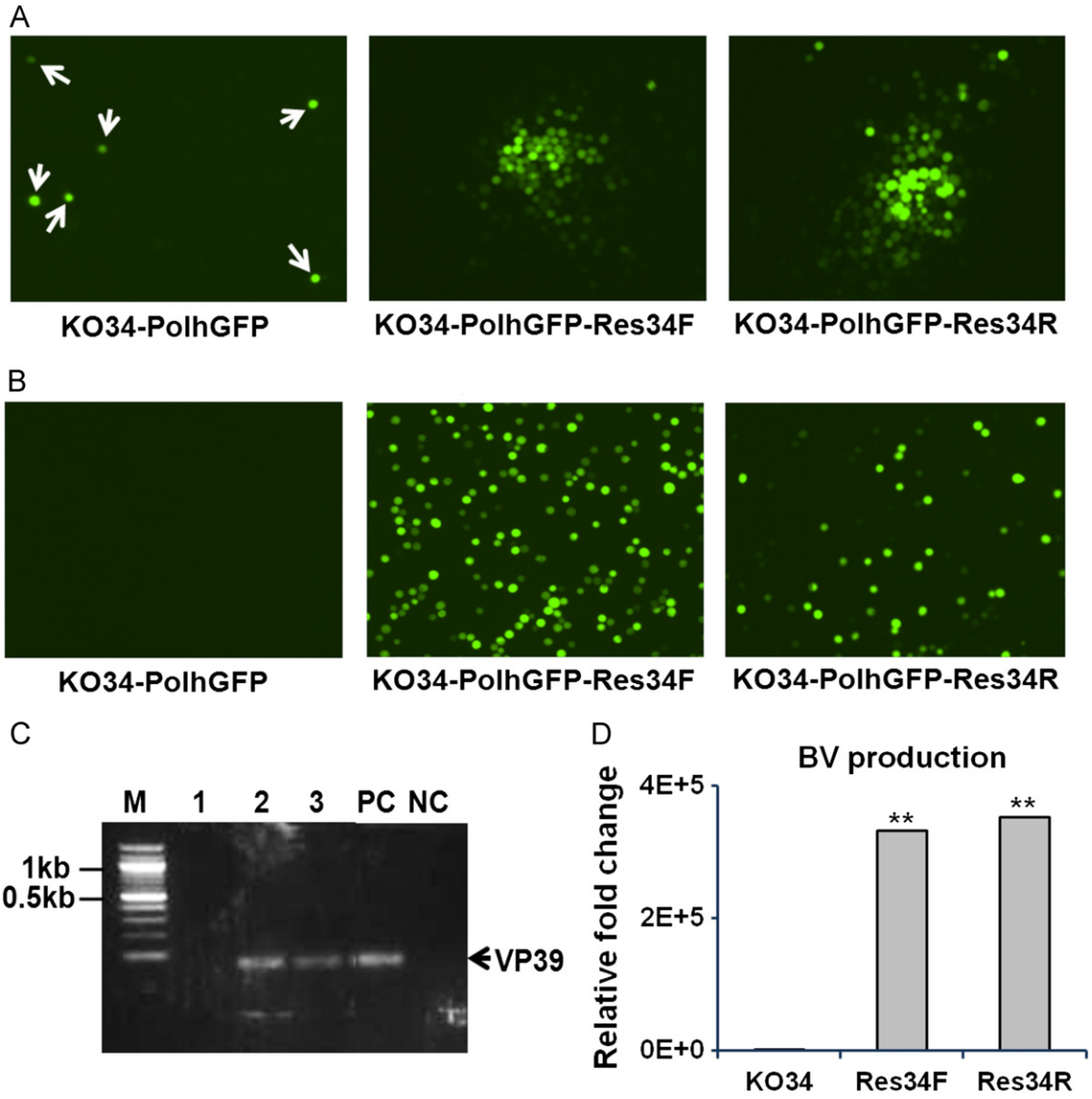

Fig. 8. Analysis of virus propagation and BV production in Sf21 cells. (A) Sf21 cells were transfected with the knockout or rescue bacmids and cells were visualized for GFP fluorescence under microscope at 72 h post transfection. (B) The supernatant of transfected cells were collected at 96 h post transfection. Cells were removed and half the volume of supernatant was used to infect Sf21 cells. Images were taken at 48 h p.i. (C) DNA was isolated from BV harvested from the rescue virus infected Sf21 cells at 72 h p.i. and PCR was used to detect the virus vp39 gene. M, 100 bp ladder; 1, KO34-polhGFP; 2, KO34-polhGFP-Res34F; 3, KO34-polhGFP-Res34R; PC, Wild type Acbacmid was used as a positive control; NC, negative control using water as template. (D) The result of real-time PCR analyzing the relative fold change of the BV production. DNA was extracted from the BV collected at 72 h p.i. and primers qvp39-F and qvp-39-R were used for real-time PCR. KO34, KO34-polhGFP; Res34F, KO34-polhGFP-Res34F; Res34R, KO34-polhGFP-Res34R. ** indicate $P < 0.01$ (t-test).

how the course of infection would appear, we co-transfected cells with KO34 bacmid and AcMNPV bacmid (AcBac) (10:1) and compared these cells with those transfected with KO34 or Res34F over time. We observed virus spread and plaque formation, but at a slower rate than in cells transfected with Res34F (Supplementary Fig. 2). Based on these observations we repeated transfection experiments with KO34, Res34F, and AcBac, and titrated the supernatants by plaque assay. In this experiment we observed 15 small plaques on the titration plates from undiluted transfection supernatants from KO34 transfected cells harvested at 72 h post transfection (Supplementary Fig. 3A). We harvested the transfection supernatants from each time point and infected fresh cells with equal volumes (200 μl) of the supernatants. The supernatants from the infected cells were harvested at 48 h pi and titrated. We did not observe any plaques in cells infected with supernatants from KO34 transfections (Supplementary Fig. 3B).

We picked the five largest plaques from the titration of KO34 transfection supernatants from 72 h post transfection and used them to infect fresh cells. By using sparsely seeded Sf21 cells, to reduce overgrowth during a long incubation, we were able to amplify two of these plaques. After one week, infection from plaque 1 had spread to nearly 50% of the cells in a 6-well plate, whereas infection from plaque 2 was closer to 20% of the cells. We then examined total cellular DNA and BV DNA from these amplified plaques for the presence of ORF34 by PCR and qPCR, using primers ORF34F(CF) and ORF34R(CR) or qORF34F(q34F) and qORF34R(q34R), respectively (Supplementary Fig. 4A). We observed a faint band indicative of intact ORF34 in PCR amplified cellular DNA from plaque 1, but not from plaque 2 (Supplementary Fig. 4B, arrowhead). When we used qPCR targeting ORF34 we observed DNA amplification from both plaque 1 (Ct 26.9) and plaque 2 (Ct 26.9) at appoximately10^6-fold lower levels than for Res34F (Ct 10.0) (Supplementary Fig. 4C). We were

also able to detect ORF34 DNA in BV from plaque 1 amplification (Ct 29.9), but not from plaque 2, at levels around 3×10^5-fold lower than Res34F (Ct 15.0) (Supplementary Fig. 4D). These data indicated that low levels of ORF34 DNA were present in the small plaques we were able to amplify, and these low levels were sufficient to support virus spread and plaque formation.

To determine if ORF34 affected viral DNA replication and accumulation, qPCR was conducted to detect vp39 on DNA isolated from cells transfected with KO34 and ORF34 rescue (Res34F) bacmids at 24 and 48 h post transfection. Both the intracellular and supernatant qPCR data were normalized to the 28S gene to eliminate any background. The levels of intracellular virus DNA in cells transfected with KO34 at 24 and 48 h post transfection were similar to Res34 at 24 h post transcription, with a small increase between 24 and 48, reflecting DNA replication in individually transfected cells. In contrast, there was an over 100 fold increase in intracellular virus DNA in cells transfected with the rescue bacmid, as the result of virus spread and DNA replication in the newly infected cells (Supplementary Fig. 5A and B). Supernatants from these KO34 and Res34F transfections were also analyzed by qPCR for the presence of viral DNA as evidence for the presence of BVs. An increase in viral DNA in the supernatants of the rescue bacmid, but not KO34 was observed (Supplementary Fig. 5C). The differences in supernatant DNA in KO34 samples from 24 to 48 h post transfection is most likely the result of difficulties in DNA isolation owing to lack of or low levels of BV in these samples. Western blots of pelleted transfection supernatants were also performed to see if BV proteins could be detected. In these experiments we detected vp39 and gp64 protein expression from Res34F but not KO34 (Supplementary Fig. 6). Together with the ORF34 knockdown experiments where BV was reduced over 100 fold (Fig. 5A) and lack of evidence for infectious virus production in cells transfected with KO34 bacmids (Fig. 8D) these data suggest that ORF34 plays a role in BV production.

Transmission electron microscopy studies provide evidence for DNA replication and suggest lack of virus budding

To better understand the defect in BV production that occurs when ORF34 expression is reduced or eliminated, transfected cells were examined by transmission electron microscopy. Two sets of experiments were done. For knockdown experiments ORF34 dsRNA was used to knockdown ORF34 expression prior to infection with wt AcMNPV and compared with cells transfected with dsCAT RNA prior to infection. Cells were processed 48 h p. i. For

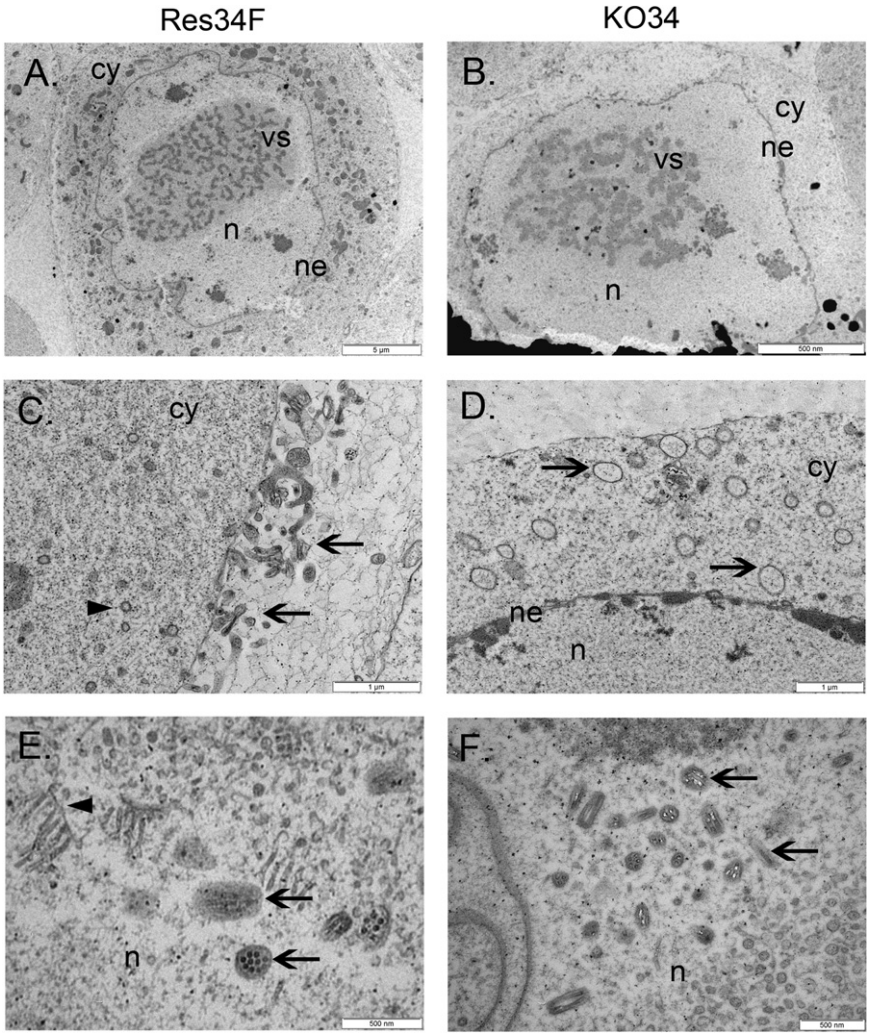

Fig. 9. Transmission electron microscopy of KO34 and Res34F transfected cells 48 h post transfection. Virogenic stroma (vs) in rescue (A) and (B) KO34 bacmid transfected cells. (C) BV in rescue bacmid transfected cells, arrowhead indicates small vesicles and arrows indicate BV. (D) Vesicles observed in KO34 transfected cells (arrows). Enveloped nucleocapsids (arrows) in the nucleus of rescue (E) and KO34 transfected cells (F), arrowhead indicates nucleocapsid array in rescue bacmid transfected cells (E). *n*=nucleus; cy=cytoplasm; ne=nuclear envelope. White bars indicate scale.

ORF34 knockout experiments cells were transfected with KO34 and Res34F bacmids and processed at 48 h post transfection. In ORF34 knockdown experiments polyhedra formed but enveloped virions were not occluded (data not shown). In comparing cells transfected with KO34 and ORF34 repair Res34F we observed that cells transfected with both KO and Res34F contained virogenic stroma indicating that virus DNA was replicated (Fig. 9A and B). BV were clearly observed in cells transfected with the rescue bacmid (Fig. 9C, arrows), but we were unable to find clear evidence for BV budding from cells transfected with KO34. However we observed what appeared to be membrane vesicles in the cytoplasm of KO34 transfected cells (Fig. 9D, arrows). These were larger than similar structures observed in rescue bacmid transfected cells (Fig. 9C, arrowhead). In the nucleus of cells transfected with the rescue bacmid, capsid structures were observed in arrays associated with membranes (Fig. 9 E, arrowhead), but similar arrays were not observed in KO34 transfected cells. However, individual capsids were observed in KO34 transfected cells near the virogenic stroma (data not shown). Enveloped nucleocapsids were observed in both KO34 and rescue bacmid transfected cells (Fig. 9E and F, arrows). However, it was unclear if the apparent nucleocapsids in the KO34 transfected cells contained DNA.

Discussion

As a result of an RNAi screen of AcMNPV ORFs, we found that reducing ORF34 expression via dsRNA knock-down significantly increased the expression of a TRPV4-EGFP reporter driven by the *polh* promoter in a recombinant AcMNPV. ORF34 is part of a transcriptional unit that includes two other ORFs, ORF32 (*fgf*), and ORF33. In a previous study, three abundant transcripts were identified on northern blots when probes complementary to ORF33 and FGF were used (Detvisitsakun et al., 2005) (Fig. 2A). Two of them, 1.4 and 3.1 kb transcripts, were overlapping and spanned both ORF33 and FGF. Based on one-step RT-PCR data, we found that the 3.1 kb transcript also spans ORF34 (Fig. 2C). When cells were transfected with ds33 expression of all three ORFs was reduced (Figs. 2 and 3). However, ORF34 transcripts were reduced less relative to either ORF33 or *fgf* transcripts (Fig. 3), indicating that a subset of transcripts in this region include only ORF34 (Fig. 2A, transcript X). Reducing transcription of these genes by transfecting ds33 also improved the expression of another IMP, CNR2 a G-protein coupled receptor, as well as that of cytoplasmic EGFP (Fig. 1), indicating that enhanced expression was not specific for IMPs.

Mutations of ORF33, FGF and ORF34 singly or in combination using vTRPV4 bacmids resulted in reduced TRPV-GFP expression when ORF33 or FGF were mutated (Fig. 4B). Although the previous study showed no negative impact on the virus replication or budded virus production when FGF was deleted (Detvisitsakun et al., 2006), our study is the first to demonstrate that deleting FGF may have a slight negative effect on the expression of an IMP using BEVS. The reasons for this are unknown. In contrast to FGF and ORF33, we were unable to obtain any recombinant viruses in which ORF34 had been disrupted. Although individual green fluorescent cells were observed in Sf21 cells transfected with each of the bacmids with ORF34 disruptions (Fig. 4A, boxed), viruses lacking ORF34 could not be propagated. This indicated that ORF34 was essential.

Our data supports the idea that ORF34 is essential, but it may not be required at high levels. We hypothesized that ds33 knocked down a transcript(s) comprising both ORF33 and ORF34, but low level of these transcripts remaining were sufficient to insure virus viability. Because ORF34 deletions could not be obtained we explored this possibility using RNAi targeting ORF34. Targeting ORF34 alone or in combination with ORF33 and/or FGF enhanced TRPV4-EGFP expression to the same levels as ds33 (Fig. 4C). These data support the idea that enhanced expression of TRPV4-EGFP was due to the reduced expression of ORF34. Knockdown of ORF34 expression in v33KO-infected cells enhanced TRPV4-EGFP expression confirming the role of reduced ORF34 transcripts in enhancing protein expression (Fig. 6A).

When we examined the kinetics of BV production, we found that transfecting cells with either ds33 or ds34 resulted in significantly reduced BV yields (Fig. 5A). However a similar reduction in BV was not observed in cells infected with the ORF33 knockout virus v33KO (Fig. 5B), indicating that the reduction in BV production resulted from reduced ORF34 expression and not from changes in ORF33 expression. Because a previous report showed that a frame-shift mutation in *ubi* (ORF35) resulted in viable virus but with reduced BV titers (Reilly and Guarino, 1996), we considered the possibility that reduced BV production was due to knock-down of *ubi*. UBI is encoded on the strand complementary to FGF, ORF33 and ORF34 on the virus genome, immediately upstream of ORF34 with no overlap (Ayres et al., 1994; Guarino, 1990). It is transcribed from two late promoter elements that map to a site immediately upstream of ORF34 and to a site within the ORF34 coding sequence, respectively (Guarino, 1990). The double-stranded RNA targeting ORF34 was designed to exclude the *ubi* transcriptional start sites, and knocking down ORF34 expression with ds34 did not reduce *ubi* transcription (Fig. 6B). However, our results provide an explanation for why an AcMNPV *ubi* deletion could not be constructed (Reilly and Guarino, 1996). Deleting *ubi* would effectively eliminate both late transcriptional start sites for *orf34*, and an early start site (Cai et al., 2012), which lie within the *ubi* ORF. In addition, because the frame-shift eliminated the proximal *orf34* transcriptional start site it also explains the reduced BV titers in cells infected with *ubi* frame-shift mutants (Reilly and Guarino, 1996). In contrast, when *ubi* was disrupted in BmNPV it did not affect BV production (Katsuma et al., 2011). Examination of the region of the BmNPV sequence encompassing the initiator codon of Bm25 (the Ac34 homolog), *ubi*, and *ubi* upstream sequence (Accession no. NC_0011962; nucleotides 25012–25500) revealed that the single late transcriptional start site upstream of *Bm25* was not altered by the disruption strategy used in this study.

Another report on the role of AcMNPV ORF34 was recently published (Cai et al., 2012). In agreement with our study they saw no effects of ORF34 deletion on virus DNA replication. In contrast to our study, they observed BV in Ac34KO bacmid transfections, although BV production was delayed and titers reduced relative to rescue bacmids. They were also able to recover recombinant viruses. This was very puzzling considering that the ORF34 mutations were quite similar to ours, but we could not recover virus and only rarely observed plaques, which were always very small. Based on our dsRNA knockdown studies only low levels of ORF34 were required for BV production and virus spread. Thus, one possible explanation for virus recovery might be the presence of low levels of ORF34 or truncated ORF34 remaining in the deletion being expressed. Spiking KO34 transfection with bacmids having intact ORF34 gave a phenotype consistent with this hypothesis (Supplementary Fig. 2). In addition, we succeeded in amplifying two of the small plaques we occasionally observed in transfections with our ORF34KO bacmids. We detected low levels of ORF34 in cells infected with each of these (Supplementary Fig. 4). These results support the plausibility of this hypothesis.

Failed attempts to generate ORF34-knockout viruses indicated that ORF34 was an essential gene. Reduced virus titers in ds34 transfected cells (Fig. 5A) suggested a role for ORF34 in BV infectivity, virion packaging, trafficking from nucleus to the plasma membrane, or in budding. Transfecting cells with ORF34

knockout and rescue bacmids confirmed that ORF34 was essential for the spread of virus infection from initially transfected cells (Fig. 8A). The supernatants from ORF34-knockout bacmid transfected cells were unable to infect fresh cells, indicating that infectious BV was not produced (Fig. 8B–D). Electron microscopy showed virus DNA replication and formation of capsid structures, although we were unable to unequivocally determine if the capsids contained DNA. We were unable to find clear evidence for BV by electron microscopy, or by western blot analysis suggesting a role for ORF34 in budding. It is possible that ORF34 may also play a role in DNA packaging. Further study is needed to determine the specific role of ORF34 in the baculovirus lifecycle and how reducing its expression enhances the expression of heterologous proteins.

Materials and methods

Cells and viruses

Spodoptera frugiperda IPLB-Sf21 cells (Vaughn et al., 1977) were maintained at 27 °C in TC100 medium (United States Biologicals) supplemented with 10% fetal bovine serum (Atlanta Biologicals). vEGFP a recombinant AcMNPV expressing EGFP from the *polh* promoter was a gift from Xiao-Wen Cheng (Miami U., Oxford, OH). vTRPV4 and vCNR2 were constructed using the Bac-to-Bac and Gateway systems (Invitrogen). cDNA clones of TRPV4 (Accession no. NM_021625.4) and CNR2 (Accession no. BC069722) were obtained from the American Type Culture Collection and amplified by PCR to incorporate attB sites and inserted into pDONR201. The PCR primers were: **TRPV4FP** 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCatggcggattccagcgaagg-3′;

TRPV4RP 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCgagcggggcgtcctcagtc-3′; **CNR2FP** 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCatggaggaatgctgggtgacagagat-3′; and

CNR2RP 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTAgcaatcagagaggtctagatctctgga-3′.

The TRPV4 and CNR2 coding sequences were transferred from the donor plasmids to a modified pDEST8 vector, to generate an in-frame fusion to an EGFP-His-tag with a TEV protease site between the cloned genes and EGFP. These constructs, pDEST-TRPV4 and pDEST-CNR2 were used to generate the recombinant viruses according to the Bac-to-Bac manual (Invitrogen). Recombinant viruses were plaque purified, amplified, and verified by sequencing. Infections and plaque assays followed standard procedures (O'Reilly et al., 1992). Plaque assays were performed in duplicate on three independent samples.

Virus gene deletions

To produce vTRPV4 with gene deletions, λ Red recombination was used to replace the targeted ORFs with a CAT cassette in the TRPV4 bacmid as previously described (Datsenko and Wanner, 2000; Vanarsdall et al., 2004). The TRPV4 bacmid DNA was electroporated into, and subsequently maintained in, the BW25113/pKD46 strain. Primers were designed to amplify CAT gene with flanking sequences (50 bp) homologous to the ORFs targeted for deletion. A total of 100 ng of the PCR products were electroporated into 40 μl of competent BW25113/pKD46/TRPV4 cells. The primers used to generate the knockout viruses were as the following: **1–CatF33 ,** ATGTGGACGTTACAGCAGCCCGATTTGTATGCGTACG CGGTTCATGACGGCGGTGTAGGCTGGAGCTGCTTC; **CatR33,** GTTGTATAAACTGCTC.

TGGCGTGTAAAACTGCAGACTTAAGTTTTTTGCAAAATGGGAATTAGCCATGGTCC (v33KO); **2–CatF33M ,** CTTCCGTGCGCACACAAG CTAAAGCGTTTGTACGAATTAGGC.

TACGATTTGTGTAGGCTGGAGCTGCTTC; **CatR33M,** AATATGCGTAAACTGTTTGGCC.

ATCTCGCGCCACATTCCCGTGTCGGGCTATGGGAATTAGCCATGGTCC (v33MKO); **3–CatF34 ,** GAGGCTGACGACGATTTTAAGG;TGAATCAAACGCGGAATCTAAGCTGCAA.

GTGTAGGCTGGAGCTGCTTC; **CatR34,** CTCGACGTAGTTGTTGCATGTTATGTCGCG.

TGTGCCGCGATACGCGTGATATGGGAATTAGCCATGGTCC (v34MKO); **4–CatFFGF ,** GAAATCGCATCGTCATTCAAAACGCCATCACGTGTGTGTACCTGTGCATGGTGTAGGCTGGAGCTGCTTC; **CatRFGF,** GGAGTACCGTCGTTCTTCAGTGCCACATACGTCAAC.

TTGCGATCGTACACATGGGAATTAGCCATGGTCC (vFGFMKO); **5–CatF34 and CatR33M (**v34/33KO); and **6–CatF34 and CatRFGF (**v34/33/FGFKO). Gene deletions in positive colonies were verified using the following primers: **1–Orf33F ,** GTTACAGCAGCCCGATTTGT; **Orf33R,** TGCTCTGGCGTGTAAAACTG (bac33MKO); **2–ORF34F ,** GACAACGGTTGCTGTGAATG; **ORF34R,** GTGCTCGACGTAGTTGTTGC (bac34MKO); **3–FGFF ,** GACGGAGCTGTTTACGGAAC; **FGFR,** GACATTTACGATGGCG.

AACA (bacFGFMKO); 4–**Orf33F** and **FGFR (**v34/33KO and v34/33/FGFKO).

ORF34 Knockout virus (KO34) was constructed the same way using the λ Red recombination system. **CatF34 and CatR34** (v34MKO) primers were used to replace the region from 28494 to 28692 of ORF34 with a CAT cassette in the Acbacmid. To make the ORF34 rescue viruses, EGFP under the *polh* promoter and ORF34 under its own promoter were inserted to the *polh* locus using the Bac-to-Bac system. The primers used for cloning ORF34 into the pDEST 8 vector were ResORF34F-FP (5′-GGGAAGCTTGGTTAGCGATAATACACAAG-3′) and ResORF34F-RP (5′-GGGACTAGTTTACTCAAAGTCCATCAATTC-3′); ResORF34R-FP 5′-GGGAAGCTTTTACTCAAAGTCCATCAATTC-3′ and ResORF34R-RP GGGACTAGTGGTTAGCGATAATACACAAG-3′). The deletion and rescue of ORF34 were verified by PCR using primers ORF34F and ORF34R.

RNAi experiments

Unless specified otherwise, RNAi experiments were performed in 24 well microtiter plates. A total of 2×10^5 Sf21 cells were seeded per well and 5 μg of each of the dsRNAs were transfected into the Sf21 cells using 5 μl cellfectin II according to the instructions of the cellfectin II manual (Invitrogen). The transfection was conducted overnight (18 h) at 27 °C on a rocker platform at 2.5 rpm. The transfection mixture was removed and the cells were infected with virus at MOI of 5. For transfections with multiple dsRNAs, 5 μg of each dsRNA was transfected and the amount of cellfectin II was increased proportionally. The primers used for synthesis of dsRNA are listed below. dsGFP-FP 5′-TAATACGACTCACTATAGGGACGTAAACGGCCACAAGTT-3′, dsGFP-RP 5′-TAATACGACTCACTATAGGGTGTTCTGCTGGTAGTGGTCG-3′, ds33FP 5′-TAATACGACTCACTATAGGGACGGCGCTAAAAGAACCAAA-3′, ds33RP 5-TAATACGACTCACTATAGGGGCTCTGGCGTGTAAAACTGC-3′, ds34FP 5′-TAATACGACTCACTATAGGGCCTTCAACGGCAATTGATTT-3′, ds34RP 5′-TAATACGACTCACTATAGGGGCCATTAGATCGGATAGCGA-3′, dsCAT-FP 5′TAATACGACTCACTATAGGGATCCCAATGGCATCGTAAAG-3′, dsCAT-RP 5′-TAATACGACTCACTATAGGGATCACAAACGGCATGATGAA-3′, dsFGF-FP 5-TAATACGACTCACTATAGGGTCGCTTGCTGGCACTTGTAG-3′, dsFGF-RP 5′-TAATACGACTCACTATAGGGTGACATTTACGATGGCGAACA-3′, ds39-FP 5′-TAATACGACTCACTATAGGGTTTTAATTCCAAGCGCAACC-3′, ds39-RP 5′-TAATACGACTCACTATAGGGAATTCCTCCGTGTCGATTTG-3′, ds39*-FP 5′-TAATACGACTCACTATAGGGATGCAAGCCGAACAGCTAAT-3′, ds39*-RP 5′-TAATACGACTCACTATAGGGGCAAACGATTGGGTTGACTT-3′.

RNAi ORF library screening

AcMNPV bacmid DNA was used as a template to produce PCR templates to synthesize dsRNA targeting AcMNPV ORFs. The primers used to generate the PCR templates are listed in Supplementary Table 1. Megascript kit (Ambion) was used to synthesize the dsRNAs according to the instruction manual. The RNAi screen was performed in triplicate as described above and the fluorescence data were collected at 48 h p.i. using a plate reader (see below).

Plate reader

SpectraMax® M5 Microplate Reader (Molecular Devices) was used to measure the mean fluorescence intensity in Sf21 cells. Fluorescence was measured from the bottom side of the well at 530 nm. For each well, 100 reads were taken and averages were calculated. The data were collected from three independent samples. The significant difference between controls (dsC or dsCAT) and the experiments was calculated by *t*-test.

Fluorescent-Activated Cell Sorter (FACS)

The median fluorescence intensity (MFI) representing the average fluorescence intensity that used to compare the cellular EGFP expression was computed using the Fluorescent-Activated Cell Sorter (FACS). Flow cytometry was performed using a BD FACS Vantage SE instrument (BD-Biosciences, San Jose, CA). Light scatter was produced and fluorescence was excited using an argon-ion laser at 100 mW and fluorescence detection was done using a 530/30 nm band pass filter. Data were collected and analyzed using "Cell Quest Version 3.3" (BD-Biosciences). For each sample, 15,000 cells were analyzed.

In-gel fluorescence

Cells from ds33 and dsC treatments were collected at 48 h p.i. and washed with 1 × PBS buffer (pH 7.4) twice and resuspended in 50 μl of 1 × PBS. Cells were lysed with equal volume (50 μl) of solubilization buffer 200 mM Tris–HCl (pH8.8), 20% Glycerol, 5 mM EDTA (pH 8.0), 0.02% bromophenol blue, and stored at −20 °C; 200 μl 20% SDS, and 100 μl 0.5 M DDT were added before loading the gels (Drew et al., 2006). A total of 20 μl of each sample was loaded on 10% SDS-PAGE . Following electrophoresis, images were acquired by VersaDoc™ imaging system (BioRad). Relative fluorescent intensity (RFI) was measured by Quantity One software.

Western blot analysis

Total proteins were extracted from ds33 and dsC treated cells at 48 h p.i. The cell pellets were resuspended in 50 μl 1 × PBS (pH 7.4) and lysed using 50 μl of solubilization buffer (see In-gel Fluorescence). All samples were incubated on ice for 30–45 min. Samples were boiled for 5 min and spun down for loading. A total of 20 μl of each sample was resolved on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Amersham). The TRPV4-EGFP-His fusion protein was detected with mouse monoclonal anti-His antibody (Qiagen) diluted 1:2000 followed by anti-mouse IgG (1:1000 dilution) as the secondary antibody (Thermo Scientific). Anti-VP39 and anti-GP64 antibody were diluted by 1:2000 or 1:500 followed by anti-rabbit or anti-mouse IgG respectively. Visualization was performed by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) according to instructions. VersaDoc MP4000 system (Bio-Rad) was used to image the western-blot . Relative intensity (RI) was measured by Quantity One software.

Total RNA extraction

A total of 0.8×10^6 Sf21 cells in each well of the 6-well plate were transfected overnight (18 h) with 20 μg dsRNAs and 20 μl cellfectin II followed by 1 h infection with vTRPV4. Total RNA was extracted from the cells at 48 h p.i. following the instruction manual of RNeasy Mini kit (Qiagen). Samples were digested twice with the RNase-Free DNase set (Qiagen).

One-step reverse transcriptase polymerase chain reaction (RT-PCR)

The reaction was conducted according to Access RT-PCR kit (Promega) with modifications. A total of 100 ng of DNA-free total RNAs were used and the reaction was as the following: 10.6 μl Nuclease-Free Water, 4 μl AMV/*Tfl* 5 × Reaction Buffer, 0.4 μl dNTP Mix (10 mM each dNTP), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 1.2 μl 25 mM MgSO4, 0.4 μl AMV Reverse Transcriptase (5 u/μl), and 0.4 μl *Tfl* DNA Polymerase (5 u/μl). The thermocycler (PerkinElmer) program was as the following: 45 °C for 45 min; 94 °C for 2 min; 22 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 5 min; and 72 °C for 7 min. The primers were the same as those used for generating PCR products for gene deletions, see above FGFR(R3), FGFF (F3) Orf33R (R2), Orf33F (F2), ORF34F(F1) and ORF34R (R1). The primers for determining virus *ubi* expression were Ubi-FP 5′-GCCGAAACG-GAACCCGCAGA-3′ and Ubi-RP 5′-GAATCTTCCAGTTGTTTGCCCGC-3′. 28S RNA amplified with primer F (AGCTGGCTTGATCCAGATGT) and primer R (AACCAAATGTCCGAAACTGC) was used as a control.

Quantitative real-time polymerase chain reaction

For qRT-PCR a total of 1 μg of the DNase-treated total RNA was used for cDNA synthesis following the manual of the iScript cDNA synthesis kit (Bio-Rad). The qPCR was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems). Three biological samples for each treatment and two technical replicates were used in the qRT-PCR . For each sample, 10 ng RNA-equivalent cDNA were used as a template using 300 nM of each primer (forward and reverse) in addition to the 2 × Power SYBR Green PCR Master Mix in a 15 μl reaction. The relative expression of each gene was calculated according to the $2^{-\Delta\Delta}Ct$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). 28S RNA was used as a reference. The relative expression of transcripts in the cells transfected with dsC was considered to be 1 and the relative expression of transcripts in the cells transfected with ds33 was calculated accordingly. qRT-PCR was done on the 24 h p.i. and 48 h p.i. samples. A Student's *t*-test was used to determine the significant differences between samples. Primers used for qRT-PCR were the following: qORF33F, CACGGTCCAAATTTCCAAAAA; qORF33R, TCGTACAAACGCTTTAGCTTGTG; qORF34F, GACTGG-TTGTGGTGTGATTTTCA; qORF34R, GCTCGACGTAGTTGTTGCATGT; qFGF-F, GAACCATTGAATCGGACAACGT; qFGF-R, AATGACGATGC-GATTTCTGTCA; qEGFP-F, CTGCTGCCCGACAACCA; qEGFP-R, TGT-GATCGCGCTTCTCGTT; q28s_163962980_F_15, CTGGCTTGATCCA-GATGTTCAG; q28s_163962980_R_72, GGATCGATAGGCCGTGCTT.

Viral DNA analysis

A total of 3.5×10^5 Sf21 cells were seeded in each well of 12-well plate. In three independent experiments, cells were transfected overnight with 10 μg of ds33, ds34, or dsC and then infected with vTRPV4 at MOI of 5. At 48 h p.i., cells were collected and washed with 1 × PBS (pH 7.4) and 100 μl of the media were

saved for plaque assay. BVs were recovered from equal volumes of supernatant by centrifugation (O'Reilly et al., 1992). DNA was extracted from both the cells and BV according to the manual of the DNeasy Blood & Tissue kit (Qiagen). The mean relative fold change of the vTRPV4 genome in cells was measured by qPCR (as described above). Reactions used 50 ng of DNA extracted from infected Sf21 cells as a template and primers targeting the major capsid gene, vp39 (qvp39-F, TGTCGCGAGACGAATTGC; qvp39-R, CCAGCACCGCCTCGAA). The 28S RNA gene was used as a reference to normalize for any background from cellular DNA contamination obtained during BV DNA isolation.

Transmission electron microscopy

Transected cells were harvested at 48 h post transfection, scraped from plates, washed in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 2 mM KH_2PO_4, pH 7.4), and fixed in 2.5% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Hatfield, PA). The fixed cells were pelleted in 2% agarose, postfixed in 1% osmium tetroxide (in 0.1 M cacodylate buffer), dehydrated in an acetone series, and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were prepared on a Power Tome XL ultramicrotome (RMC, Boeckeler Instruments, Tucson, AZ) and mounted on copper grids, stained for 30 min with saturated aqueous uranyl acetate and 15 min with lead citrate. Sectioned cell pellets were visualized using a JEOL100 CXII (Japan Electron Optics Laboratories) transmission electron microscope. Images were captured using a Soft Imaging Systems, MegaView III digital camera.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.10.022.

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