**Title**: Roles of cellular NSF protein in entry and nuclear egress of budded virions of Autographa californica multiple nucleopolyhedrovirus

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**Abstract**

In eukaryotic cells, the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins comprise the minimal machinery that triggers fusion of transport vesicles with their target membranes. Comparative studies revealed that genes encoding the components of the SNARE system are highly conserved in yeast, insect, and human genomes. Upon infection of insect cells by the virus AcMNPV, the transcript levels of most SNARE genes were initially up-regulated. We found that overexpression of dominant-negative (DN) forms of NSF or knock-down of the expression of NSF, the key regulator of the SNARE system, significantly affected the infectious AcMNPV production. In cells expressing DN NSF, entering virions were trapped in the cytoplasm or transported to the nucleus with low efficiency. The presence of DN NSF also moderately reduced trafficking of the viral envelope glycoprotein GP64 to the plasma membrane but dramatically inhibited production of infectious BV. TEM analysis of infections in cells expressing DN NSF revealed that progeny nucleocapsids were retained in a perinuclear space surrounded by inner and outer nuclear membranes. Several baculovirus conserved (core) proteins (Ac76, Ac78, GP41, Ac93, and Ac103) that are important for infectious budded virion production, were found to associate with NSF, and NSF was detected within the assembled BV. Together, these data indicate that the cellular SNARE system is involved in AcMNPV infection and that NSF is required for efficient entry and nuclear egress of budded virions of AcMNPV.

**Importance**

Little is known regarding the complex interplay between cellular factors and baculoviruses during viral entry and egress. Here we examined the cellular SNARE system, which mediates the fusion of vesicles in healthy cells, and its relation to baculovirus infection. Using a dominant negative (DN) approach and an RNAi knockdown, we demonstrated that a general disruption of the SNARE machinery significantly inhibited the production of infectious budded virions (BV) of AcMNPV. The presence of a DN NSF protein resulted in low efficiency entry of BV and the retention of progeny nucleocapsids in the perinuclear space during egress. Combined with these effects, we also found that several conserved (core) baculovirus proteins closely associate with NSF, and these results suggest their involvement in the egress of BV. Our findings are the first to demonstrate that the SNARE system is required for efficient entry of BV and nuclear egress of progeny nucleocapsids of baculoviruses.

## Introduction

In eukaryotic cells, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins constitute the minimal machinery that mediates the fusion of transport vesicles with target membranes (1, 2). These evolutionarily conserved SNARE proteins constitute a large family of approximately 50 or more proteins in mammals and are classified as either v-SNAREs (found on vesicle membranes) or t-SNAREs (found on target membranes) (1). During the membrane fusion process, a highly stable four-helix bundle is formed from an interaction of the v-SNARE and t-SNARE proteins that are anchored in opposing membranes. Most v-SNARE and t-SNARE protein encodes a 50-60 amino acid SNARE motif, a motif that contains a heptad repeat that forms a coiled coil structure. Four SNARE proteins called SNAP-23 (synaptosome-associated protein of 23 kDa), SNAP-25, SNAP-29, and SNAP-47, each contain two tandem SNARE motifs separated by a linker region. The coiled coil structures from each of four v-SNAREs and four t-SNAREs interact to form the four-helix bundle during fusion. The center of the four-helix bundle contains 16 stacked layers of interacting side chains, which are largely hydrophobic, except for a central “0” layer that contains three highly conserved glutamine (Q) residues and one highly conserved arginine (R) residue, each contributed by one of the subunit proteins (see Figure 2 in reference (1)). Based on these conserved residues and the similarity of SNARE motifs, SNARE proteins are classified into four main types: Qa- (SNAREs containing a SNARE motif that is close to that of syntaxin 1, 2, 3, 4, 5, 7, 11, 13, 16, 17 or 18), Qb- (SNAREs containing a SNARE motif that is similar to the N-terminal SNARE motif of SNAP-25), Qc-

(SNAREs containing a SNARE motif that is similar to the C-terminal SNARE motif of SNAP-25) and R-SNAREs (SNAREs containing a SNARE motif that is close to that of vesicle-associated membrane protein (VAMP) proteins). Functional SNARE complexes that drive membrane fusion need one of each of the above four SNARE types (1, 3). These four main types of SNARE proteins can be further classified into 20 different conserved groups which participate in diverse intracellular trafficking processes (4). At a late step of membrane fusion, SNARE complexes are disassembled into individual SNARE proteins for recycling (1). This disassembly process is catalyzed by a protein called N-ethylmaleimide-sensitive factor (NSF) and its adaptor protein, α-soluble NSF attachment protein (α-SNAP) (5-7). NSF is an ATPase that belongs to the AAA+ ATPase family, a family of proteins that are involved in a variety of cellular activities (8). NSF functions as a homohexamer and each subunit consists of the N-terminal domain (NSF-N) and two AAA+ domains (NSF-D1 and NSF-D2). The N domain is required for interaction with the α-SNAP-SNARE complex. The D1 domain provides ATPase activity associated with SNARE disassembly, and the D2 domain is involved in nucleotide-dependent hexamerization (9-11). Two well-characterized mutations of NSF ($NSF^{E329Q}$ and $NSF^{R385A}$) result in failure of NSF to bind or hydrolyze ATP. Inactivation of NSF function by overexpressing these dominant-negative (DN) forms of NSF leads to failure in disassembly of SNARE complexes and consequent disruption of SNARE function (10, 12, 13).

A number of studies have demonstrated an important role of the cellular SNARE machinery in the replication of certain mammalian viruses. In human cytomegalovirus (HCMV) infected cells, SNARE protein SNAP-23 is found in

112 the region of viral assembly in the cytoplasm., and depletion of SNAP-23 by
113 RNA interference significantly reduced infectious HCMV production and
114 disrupted virion assembly or maturation (14). For another herpesvirus, human
115 herpes virus 6 (HHV-6), viral glycoproteins M and N (gM/gN) interact with
116 VAMP3, a SNARE involved in vesicular transport. During the late phase of
117 virus infection, the expression level of VAMP3 was significantly up-regulated
118 and VAMP3 becomes incorporated into mature HHV-6 virions (15). The
119 interaction of a viral protein with host SNARE system components was also
120 reported in cells infected with human parainfluenza virus type 3 (HPIV3). The
121 HPIV3 phosphoprotein (P) interacts with SNAP-29, and through this interaction,
122 HPIV3 induces incomplete autophagy by inhibiting the interaction of SNAP-29
123 with syntaxin 17 (Syx17), a SNARE associated with autophagosome-lysome
124 fusion (16). Additionally, down-regulation of Syx17 and impairment of
125 autophagosome-lysosome fusion by hepatitis C virus (HCV) are critical for
126 HCV release (17). In another example of the requirement for SNARE protein
127 functions in viral infections, it was found that overexpression of
128 dominant-negative (DN) NSF ($NSF^{E329Q}$) substantially reduced production of
129 infectious human immunodeficiency virus (HIV-1) and this results from reduced
130 levels of Gag at the plasma membrane (18, 19). In addition to the role of
131 SNARE proteins in efficient assembly or egress of viruses, SNARE proteins
132 are also involved in viral entry in many cases. Inhibition of VAMP8 (a SNARE
133 protein that participates in endosomal fusion) significantly decreases influenza
134 A virus and vesicular stomatitis virus (VSV) entry into host cells (20).
135 Inactivation of VAMP3 also results in defects in bunyavirus (Uukuniemi virus)
136 entry (21).

Baculoviruses represent a family of large double-stranded DNA viruses with circular genomes that range from 80 to 180 kb. Many baculoviruses are highly pathogenic to their insect hosts (22). These viruses are widely used as biological insecticides, protein expression vectors, and mammalian cell transduction vectors (23-25). During the infection cycle, baculoviruses produce two types of virions: occlusion-derived virions (ODV) and budded virions (BV). ODV and BV are identical in genomic DNA content and nucleocapsid structure. However, they differ in the source and composition of their envelopes and in their functional roles in the infection cycle in the animal. ODV are acquired orally and initiate the primary infection in the midgut epithelium. Within midgut epithelial cells, BV is produced by budding from the cell surface. BV spread the viral infection from cell to cell within the infected insect. BV obtain the virion envelope from the plasma membrane, whereas ODV assemble in the nucleus and acquire their envelopes from virus-induced intranuclear microvesicles, which are derived from the inner nuclear membrane (22). *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) is the best-studied baculovirus and is the type species of the *Baculoviridae*. BV of AcMNPV enter host cells via clathrin-mediated endocytosis (26). The major viral envelope glycoprotein, GP64, is essential for receptor binding and low-pH triggered membrane fusion (27). During entry, the acidification of endosomes triggers a conformational change in GP64, which then mediates fusion of the viral envelope and endosomal membranes, releasing the nucleocapsid into the cytosol (28). Nucleocapsids are then transported to the nuclear periphery via actin-based motility (29), and enter the nucleus through the nuclear pore complex (22, 30). After the viral genome is released within the nucleus, viral

transcription and DNA replication occur and are subsequently followed by progeny nucleocapsid assembly in a dense region referred to as the virogenic stroma (VS) (22). Some progeny nucleocapsids exit the nucleus. During this egress from the nucleus, nucleocapsids appear to acquire an envelope by blebbing of the nuclear membranes (31). This double envelope (derived presumably from inner and outer nuclear membranes) appears to be lost in the cytosol as naked nucleocapsids are frequently observed in the cytosol and in the process of budding at the plasma membrane (22, 31). Because enveloped virions found within the cytosol represent a virus-induced vesicle, the loss of the double envelope within the cytosol may involve a fusion process that could be mediated by the cellular SNARE system. Intriguingly, several host proteins involved in vesicular transport have been identified in purified budded virions of AcMNPV by mass spectrometry (32).

To investigate whether the host cellular SNARE machinery is required for AcMNPV infection, we first analyzed the transcript levels of the SNARE genes in AcMNPV-infected insect cells (33) and found that most of the SNARE transcripts were up-regulated upon AcMNPV infection. We cloned the NSF gene from Sf9 cells, generated dominant-negative forms of Sf9 NSF, and analyzed the effects of DN NSF proteins on AcMNPV BV entry, viral replication, and BV egress. Our results demonstrated that NSF is required for efficient entry of AcMNPV BV into Sf9 cells, and for egress of BV. In addition, we found that NSF associates with several conserved (core) viral proteins suggesting that NSF associations with viral proteins may be involved in egress or assembly of AcMNPV BV.

## Materials and methods

### Annotation of insect SNARE genes

SNARE genes of the yeast *Saccharomyces cerevisiae* and the orthologs from the human genome were used for searches of insect genomes using BLASTP and TBLASTN programs. BLAST searches were performed using databases of sequenced insect genomes from 6 Orders, including Coleoptera (*Tribolium castaneum;* http://beetlebase.org), Diptera (*Drosophila melanogaster*, *Aedes aegypti*, *Anopheles gambiae*, and *Culex quinquefasciatus*; http://flybase.org and http://www.vectorbase.org), Hemiptera (*Acyrthosiphon pisum*; http://www.aphidbase.com), Hymenoptera (*Apis mellifera, Nasonia vitripennis,* and *Harpegnathos saltator*; http://hymenopteragenome.org), Lepidoptera (*Bombyx mori* and *Danaus plexippus;* http://silkworm.genomics.org.cn and http://monarchbase.umassmed.edu), and Phthiraptera *(Pediculus humanus corporis*; http://www.vectorbase.org). Specific BLAST searches were also carried out at the National Center for Biotechnology Information (NCBI).

### Cells, transfections, and infections

*Spodoptera frugiperda* Sf9, *Trichoplusia ni* Tn5B1-4 (High 5) and Sf9$^{Op1D}$ (a cell line stably expressing Orgyia pseudotsugata (Op)MNPV GP64 (34)) cells were cultured at 27° in TNMFH medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS, Gibco). The cells in 6-well plates ($1\times10^6$ cells per well) or 12-well plates ($2\times10^5$ cells per well) were transfected with plasmid or bacmid DNA or double-strand (ds)-RNA using a $CaPO_4$ precipitation method (28). For virus infections, virus inoculum was added to cells and incubated for 1 h. The cells were washed once in TNMFH. Times postinfection (p.i.) were calculated

211 from the time the viral inoculum was added.

212 **NSF cDNA cloning**

213 Total RNA was extracted from Sf9 cells by using an RNAiso plus kit
214 (TaKaRa). The first-strand DNA complementary to the mRNA (cDNA) was
215 synthesized by using AMV reverse transcriptase and an oligo-dT primer
216 according to the manufacturer's instructions (TaKaRa). Gene-specific primers
217 SfNsfF and SFNsfR, and NsfF and NsfR (Table 1), targeted to the Sf9 NSF
218 ORF and the C-terminus of Sf9 NSF ORF, were designed based on the EST
219 sequences at SPODOBASE database (http://bioweb.ensam.inra.fr/spodobase)
220 (35). The PCR-amplified NSF ORF and a 90 bp fragment of NSF products
221 were separately cloned into pMD18-T vector (TaKaRa) and sequenced with
222 M13-47, M13-48, and NSF-specific primers. The pMD18-T vector containing
223 the ORF of NSF and the small fragment of NSF was designated as
224 NSFpMD18 and NSF90pMD18.

225 **Analysis of the transcription of NSF**

226 Sf9 cells in 6-well plates ($1\times10^6$ cells per well) were infected with wild-type
227 AcMNPV at an MOI of 10. At 1, 3, 6, 12, 18, 24, 36, and 48 h p.i., the infected
228 and uninfected cells were collected and total RNA was extracted by using an
229 RNAiso plus kit (TaKaRa). The genomic DNA elimination and first-strand DNA
230 complementary to mRNA (cDNA) synthesis were performed with
231 PrimeScript™ RT reagent kit with gDNA eraser and an oligo-dT primer
232 (TaKaRa). The transcript of NSF was quantified by real-time PCR (IQ™5
233 Multicolor Real-Time PCR Detection System, Bio-Rad). Each PCR mixture
234 contained 5 µl SYBR® Premix ExTaq II (TaKaRa), 1.25 µM each primer, and
235 500 pg of the cDNA template. Primers NsfF: 5'-

ACCGCCTTAGCCGCTGAACT-3' and NsfR: 5'-AGACTCCGTGAATCCGACCATGT-3' were used to amplify a fragment of 90 bp of Sf9 NSF. Thermal cycling conditions were one cycle of 95° for 3min, followed by 40 cycles of 95° for 10s, and 60° for 45s. A standard curve was generated by a serial dilution of NSF90pMD18. The transcript levels of NSF were expressed as numbers of transcript copies per cell.

**Mutagenesis and construction of plasmids, bacmids, and viruses**

PCR primers and the plasmid constructs are listed in Table 1 and Table 2, respectively. The ORF of NSF containing a translation stop codon mutation (TAA to TAC) was generated by PCR using NSFpMD18 as the template and primers SfNsfXF and SfNsfER, which contain XbaI and EcoRI sites, respectively. The PCR product was digested with XbaI and EcoRI and inserted into the plasmid Vps4-GFPpBlue (36) under the control of the AcMNPV *ie1* immediately early/late promoter to replace Vps4 and yield the C-terminal GFP-tagged NSF transient expression plasmid NSF-GFPpBlue. Two point mutations, E329Q and R385A, were separately introduced into NSF by overlap PCR using NSFpMD18 as a template and primers NsfE329QF, NsfE329QR, NsfR385AF, and NsfR385AR. The PCR products were digested with XbaI and EcoRI and cloned into the plasmid Vps4-GFPpBlue to replace Vps4 and yield $NSF^{E329Q}$-GFPpBlue and $NSF^{R385A}$-GFPpBlue. All of the plasmids were confirmed by restriction enzyme analysis and DNA sequencing. The previously constructed gfppBlue (36) was used as a GFP-expressing control plasmid.

To construct a bimolecular fluorescent complementation (BiFC) system, the ORF of mCherry was separated as two fragments between amino acid positions 159 and 160 as described previously (37). A backbone expression

plasmid pIE was generated by inserting a multiple cloning site (MCS, 5'TCTAGAATGGGATCCACTAGTCCGCGGCCCGGGCTGCAGGATATCGAA TTC3') into the XbaI and EcoRI sites of pBiepA (38) to replace the ORF of GP64. The N- and C-terminal fragments of mCherry (designated as Nm and Cm) containing the linker sequence GTSGGSG and the HA or c-Myc epitope tag were amplified by PCR using mCherryGUS bacmid DNA (36) as template and inserted into the XbaI and BamHI, or the XbaI and EcoRI sites of pIE to generate the BiFC expression plasmids Nm-HApBlue, HA-NmpBlue, Cm-MycpBue, or Myc-CmpBlue (Table 2). The immunoprecipitation vectors pIE-HA-MCS, pIE-Myc-MCS, pIE-MCS-HA, and pIE-MCS-Myc, which contain the HA or c-Myc epitope tag, were constructed in a similar manner (Table 2). The NSF coding region, obtained by digesting NSF-GFPpBlue with XbaI and EcoRI, was inserted into the MCS sites of the BiFC and immunoprecipitation vectors. AcMNPV genes (*ac11*, *ac76*, *ac78*, *ac80 (gp41)*, *ac93*, *ac103*, *ac142*, *ac146*, *lef3*) were amplified by PCR and digested with XbaI and EcoRI or BamHI and EcoRI, and then the coding region of each viral gene was also inserted into the MCS sites of the vectors as described above (Table 2). All of the plasmids were confirmed by DNA sequencing.

Recombinant AcMNPV bacmids expressing GFP, GFP-tagged NSF (WT or with mutations), and c-Myc tagged NSF were constructed by inserting a cassette containing GFP, NSF-GFP, $NSF^{E329Q}$-GFP, $NSF^{R385A}$-GFP, or NSF-Myc under the control of the AcMNPV *ie1* promoter, into a pFastbac plasmid (GUSpFB) that contains a β-glucuronidase (GUS) gene under the control of the AcMNPV *p6.9* late promoter. The resulting pFastbac constructs were each inserted into the polyhedrin locus of an AcMNPV bacmid

(bMON14272) by Tn*7*-mediated transposition (39). The resulting recombinant bacmids were separately named GFPBac, NSF-GFPBac, NSF$^{E329Q}$-GFPBac, NSF$^{R385A}$-GFPBac, and NSF-MycBac. All constructs were verified by DNA sequencing. The *gp64* knockout AcMNPV bacmids, LacZGUS-*gp64*$^{ko}$ and mCherryGUS-*gp64*$^{ko}$, were constructed as described earlier (36). Plasmids and bacmids were purified using a Midiprep kit (Invitrogen). The *gp64* knockout viruses, LacZGUS-*gp64*$^{ko}$ and mCherryGUS-*gp64*$^{ko}$, were grown and titered in Sf9$^{OP1D}$ cells that constitutively express OpMNPV GP64 (34). Wild-type AcMNPV encoding VP39-triple mCherry (AcMNPV-3mC) (29) was kindly provided by Taro Ohkawa and Matthew Welch (University of California, Berkeley).

**Cell viability assay**

Cell viability, upon overexpression of NSF or the NSF DN constructs NSF$^{E329Q}$ and NSF$^{R385A}$, was assessed using the CellTiter96$^{@}$ AQueous One Solution Cell Proliferation Assay (MTS, Promega) according to the manufacturer's recommendations. Briefly, Sf9 cells in 6-well plates were transfected with 3 µg of the plasmid expressing GFP, NSF-GFP, NSF$^{E329Q}$-GFP, or NSF$^{R385A}$-GFP. At 12, 24, and 36 h posttransfection (p.t.), the cells were incubated with CellTiter 96$^{®}$ AQueous One Solution reagent for 2 h at 27° and absorbance at 490 nm was monitored using a 96-well plate reader (Tecan iControl Reader, Mannedorf, Switzerland).

**Infectivity complementation assay**

Sf9 and High 5 cells in 6-well plates were co-transfected with 3 µg of pBieGP64 (38) expressing GP64 and 3 µg of a plasmid expressing GFP, NSF-GFP, NSF$^{E329Q}$-GFP, or NSF$^{R385A}$-GFP (Typically, we observed that about

311 70-80% cells showed GFP signals at 24 h post-transfection). At 12 h p.t., cells
312 were infected with the GP64 knockout virus mCherryGUS-*gp64*$^{ko}$ at a
313 multiplicity of infection (MOI) of 1 or 5. At 24 h p.i., the supernatants and cells
314 were separately collected and virus titers were measured by 50% tissue
315 culture infectious dose ($TCID_{50}$) assays on Sf9$^{OP1D}$ cells. Cell samples were
316 analyzed for the expression of GP64, NSF and its DN mutants by Western
317 blotting.

318 **RNAi assay**

319 The dsRNA-based RNA interference (RNAi) assay was performed as
320 described previously (40) with modifications. A 315 bp or 495 bp fragment of
321 the coding sequence of Sf9 NSF or GFP was amplified by PCR. The PCR
322 primers were designed with the SnapDragon tool
323 (http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl) and each primer
324 contained the T7 RNA polymerase promoter sequence
325 (5'-TAATACGACTCACTATAGGG-3') at the 5'-end (Table 1). The PCR
326 products were purified using a QIAEXII Gel Extraction Kit (Qiagen). The
327 purified PCR products were used as templates to produce dsRNA by using the
328 T7 RiboMAX$^{TM}$ Express RNAi System (Promega). The dsRNA products were
329 purified with RNeasy Mini Kit (Qiagen) and analyzed by 1.2% agarose gel
330 electrophoresis.

331 Sf9 cells in 12-well plates were transfected with 7.5 μg of dsRNA targeting
332 NSF, or 7.5 μg of the GFP dsRNA as a negative control. At 24, 48, and 72 h p.t.,
333 cell viability was determined as described above. NSF knock-down efficiency
334 was determined by transfecting Sf9 cells with 2 μg of the plasmid
335 NSF-MycpBlue that expresses Myc-tagged NSF, or co-transfecting Sf9 cells

with 2 μg of NSF-MycpBlue and either a) 7.5 μg of dsRNA targeting NSF or b) 7.5 μg dsRNA targeting GFP. At 24 and 48 h p.t., the transfected and co-transfected cells were collected and the expression of NSF-Myc was determined by Western blot analysis. Quantities of proteins on Western blots were estimated by using Quantity One software. For analysis of virus infection, Sf9 cells were transfected with 7.5 μg of dsRNA targeting NSF, or 7.5 μg of the GFP dsRNA. At 48 h p.t., the cells were infected with wild-type (WT) AcMNPV at an MOI of 5. At 24 h p.i., the supernatants were collected and virus titers were measured by $TCID_{50}$ assays on Sf9 cells.

**Analysis of viral gene expression and DNA replication**

To determine the effects of dominant-negative NSF proteins on viral gene expression, Sf9 cells in 6-well plates were co-transfected with 3 μg of pBieGP64 expressing GP64 and 3 μg of a plasmid expressing NSF-GFP, $NSF^{E329Q}$-GFP, $NSF^{R385A}$-GFP, or the control GFP protein. At 12 h p.t., the cells were infected with the *gp64* knockout virus LacZGUS-$gp64^{ko}$ at an MOI of 5. At 6 and 24 h p.i., the infected cells were lysed with 0.5% NP-40 in PBS (pH7.4) and the β-galactosidase or β-glucuronidase activities were measured using the substrate Chlorophenol red-β-D-galactopyranoside (CPRG, Roche Diagnostics GmbH) or 4-Nitrophenyl β-D-glucuronide (PNPG, Sigma-Aldrich) by absorbance at 570 nm (CPRG) or 405 nm (PNPG).

To evaluate the effects of DN NSF proteins on viral DNA replication, Sf9 cells were co-transfected with the same plasmids described above (3 μg of each), then infected at 12 h p.t. with *gp64* knockout virus LacZGUS-$gp64^{ko}$ (MOI=5). At 24 h p.i., total DNA was extracted from infected cells using a DNeasy blood tissue kit (Qiagen). Viral genomic DNA was quantified by

real-time PCR (IQ™5 Multicolor Real-Time PCR Detection System, Bio-Rad). Each PCR mixture contained 5 µl SYBR® Premix ExTaq II (TaKaRa), 1.25 µM each primer, and 500 pg of the DNA template. The primers, ODV-e56F: 5'-GATCTTCCTGCGGGCCAAACACT-3' and ODV-e56R: 5'-AACAAGACCGCGCCTATCAACAAA-3', were used to amplify a fragment of 183 bp of the AcMNPV ODV-e56 gene as described previously (36). Thermal cycling conditions were one cycle of 95° for 3min, followed by 40 cycles of 95° for 10s, and 60° for 45s. A standard curve was generated by a serial dilution of ODV-e56pGEM, which contains the ODV-e56 ORF (36). AcMNPV genomic DNA was expressed as numbers of viral DNA copies per cell.

**Analysis of virus entry**

Sf9 cells in 6-well plates were transfected with 3 µg of the plasmid GFP-pBlue, NSF-GFPpBlue, $NSF^{E329Q}$-GFPpBlue or $NSF^{R385A}$-GFPpBlue. At 12 h p.t., the cells were pre-chilled at 4° for 30 min and then incubated with mCherry-labeled virions of AcMNPV-3mC (MOI=10 or 20 $TCID_{50}$) at 4° for 1 h. After removing the virus inoculum, cells were washed twice with cold TNMFH medium and shifted to 27° for 60 min. One set of cells (which infected with an MOI of 10) were used for extraction of total DNA and viral genomic DNA was quantified by real-time PCR as described above. The other set of cells (which infected with an MOI of 20) was fixed with 3.7% paraformaldehyde in PBS (pH 7.4) and analyzed by confocal microscopy as described below.

**Analysis of virus egress**

To examine the effects of DN NSF on infectious budded virion production, Sf9 cells in 6-well plates were transfected with 3 µg of recombinant AcMNPV bacmid GFP-Bac, NSF-GFPBac, $NSF^{E329Q}$-GFPBac, or $NSF^{R385A}$-GFPBac,

which express NSF or control genes under an *ie1* promoter. At 24 h p.t., GFP-positive cells were counted under an epifluorescence microscope (Nikon Eclipse Ti) to evaluate the transfection efficiency. Transfected cells were also solubilized with 0.5% NP-40 in PBS (pH 7.4) and GUS activities were measured as described above. The cell supernatants were collected and infectious virus titers were determined by $TCID_{50}$ assays on Sf9 cells.

**Confocal microscopy and imaging**

Transfected and/or infected Sf9 cells were prepared on glass coverslips and fixed with 3.7% paraformaldehyde in PBS (pH 7.4) for 10 min and permeabilized with 0.05 % Triton X-100 in PBS (pH 7.4) for 1 min. The nuclei were then stained with 1 µg/ml Hoechst 33258 (Invitrogen) for 8 min. After washing four times with PBS (pH 7.4), the cells were mounted on slides in Fluoromount-G reagent (Southern Biotech). Images were collected on a Nikon A1R confocal microscope (Nikon Instruments Inc., Melville, NY, USA) with a 60× oil immersion objective (NA 1.4). GFP was excited with a blue argon ion laser (488 nm), and emitted light was collected between 480 and 520 nm. mCherry was excited with an orange helium-neon laser (594 nm), and emitted light was collected from 580 to 650nm. Hoechst 33258 was excited with UV light at approximately 350 nm, and emitted light was collected from 400 nm to 450 nm. GFP and mCherry signals were collected separately from the Hoechst 33258 signal and later superimposed. Images were processed using Nikon NIS-Elements AR software (version 4.0), and Adobe Photoshop CC (version 14.0) (Adobe Systems).

**cELISA, immunofluorescence, and syncytium formation assay**

Sf9 cells in 12-well plates were transfected with 2.5 µg of the bacmid

(GFP-Bac, NSF$^{E329Q}$-GFPBac, or NSF$^{R385A}$-GFPBac), or 0.5-2.5 μg NSF-GFPBac. At 24 h p.t., the supernatant was collected and the infectious BV titers were determined by $TCID_{50}$ assay. Two sets of transfected cells were separately fixed with either 0.5% glutaraldehyde or 3.7% paraformaldehyde, and analyzed by cELISA or immunofluorescence using anti-GP64 monoclonal antibodies AcV5 or AcV1, respectively. Another set of cells was treated with PBS (pH 5.0) for 3 min to induce syncytium formation. The cells in syncytia (containing ≥5 nuclei) were scored. ELISA, immunofluorescence, and syncytium formation assays were carried out as described previously (38).

**Transmission electron microscopy**

Sf9 cells in 6-well plates were transfected with 6 μg of each bacmid (NSF-GFPBac, NSF$^{E329Q}$-GFPBac, or NSF$^{R385A}$-GFPBac). At 48 h p.t., the cells were harvested by centrifugation (500 *g*, 10 min) and fixed with 2.5% glutaraldehyde in PBS (pH 7.4) overnight at 4°. Cells were then washed five times with PBS buffer (0.1 M, pH 7.2) and stained with 1% osmium tetroxide in PBS buffer (0.2 M, pH 7.2) for 2 h at 4°. After rinsing five times in PBS buffer (0.1 M, pH 7.2), the samples were dehydrated stepwise with a gradient of ethanol from 30% to 100%. The samples were then embedded in Epon-812 and dried for about 48 h at 55°. Ultrathin sections were prepared and stained with lead citrate and uranyl acetate. Images were collected with a HT7700 transmission electron microscope (Hitachi, Ltd. Japan).

**Coimmunoprecipitation**

Sf9 cells in 12-well plates were transfected or co-transfected with the plasmids expressing HA-tagged GP41 and/or c-Myc tagged GP41 or Lef3, or c-Myc tagged NSF and/or HA-tagged viral proteins (2 μg of each plasmid). At

36 h p.t., the cells were lysed in RIPA buffer (0.1% SDS, 50mM Tris pH 8.0, 150 mM NaCl, 5mM EDTA, 0.5% Sodium deoxycholate, 1% NP-40) containing protein inhibitor cocktail (Roche) at 4° for 10 min. Then, the supernatant was collected and debris removed by centrifugation (15,000 *g*, 15 min, 4°). For immunoprecipitation, the lysate supernatants were mixed with anti-HA monoclonal antibodies for 4 h at 4°, then the mixture was incubated with Protein G agarose beads (Pierce) overnight at 4°. After pelleting and washing twice with RIPA buffer, the agarose beads were resuspended with 1x SDS-PAGE gel loading buffer (2% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.02% bromophenol blue, 0.05 M Tris, pH 6.8), heated at 100° for 5 min, and analyzed in 10% or 15% SDS-PAGE and Western blot.

**Bimolecular fluorescence complementation (BiFC) assay**

Sf9 cells in 12-well plates were co-transfected with the BiFC plasmid pairs (2 μg of each plasmid) expressing the specific protein fused with the N- or C-terminus of mCherry (Nm or Cm, respectively). At 36 h p.t., bimolecular fluorescent complementation was examined by observing mCherry fluorescence in transfected cells with a Nikon Eclipse Ti epifluorescence microscope. In five randomly selected representative fields, the numbers and percentages of mCherry-positive cells were calculated for each pair of constructs. The protein pair associations were estimated by the ratio of the number of fluorescent cells in the field compared to the total number of cells in that field as described previously (41). Expression of the target proteins was confirmed in transfected cells by Western blot analysis.

**BV purification**

The purification of budded virions (BV) was performed as described

previously (36). Briefly, Sf9 cells in 6-well plates were transfected with 6 μg DNA of NSF-MycBac. At 72 h post transfection (p.t.), the infected cell supernatants were collected and cell debris removed by centrifugation at 4° for 10 min at 3,000 *g*. The supernatant was loaded onto a 25% (wt/vol) sucrose cushion, and centrifuged (4°, 90 min, 28000 rpm, Himac P28S rotor). Virus pellets were resuspended in PBS (pH 6.2) and overlaid onto a 30-55% (wt/vol) continuous sucrose gradient, and centrifuged (4°, 90 min, 28000 rpm, Himac P40ST rotor). The virus fraction was collected and diluted (1:10) with PBS (pH 6.2), and centrifuged at 28000 rpm for 90 min at 4° (Himac P40ST rotor). BV pellets were resuspended in PBS (pH 6.2) with protease inhibitor cocktail (Roche) and subjected to Western blot analysis.

**Western blot analysis**

Virion and cell lysates were separated on 6%, 10% or 15% reducing or non-reducing polyacrylamide gels and transferred to PVDF membrane (Millipore) as described previously (42). GFP, GFP-tagged proteins, c-Myc-tagged proteins, and AcMNPV VP39 were separately detected with anti-GFP (GenScript), anti-Myc (EarthOx, L.L.C.), or anti-VP39 polyclonal antibodies (43), HA-tagged proteins, β-glucuronidase (GUS), or actin were detected with anti-HA (EarthOx, L.L.C.), anti-GUS (BGI), or anti-β-actin monoclonal antibodies (Abbkine). Immunoreactive proteins were visualized using alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG antibody and nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP, Promega).

**Accession number**

The *Spodoptera frugiperda* NSF gene was deposited under GenBank

accession number KY548397.

## Results

### Expression profiles of SNARE genes upon AcMNPV infection

As a first step, we performed a comprehensive comparison of SNARE protein components in yeast, humans, and insects. We found that most of the components of the cellular SNARE machinery are evolutionarily conserved across these eukaryotic species (Table 3). Insect genes encoding SNARE components appear to more closely mirror the yeast genome in terms of SNARE gene numbers. In contrast to the rather large expansion of SNARE genes observed in the human genome, we identified only one SNARE gene (the ortholog of yeast Sec17) that was expanded in the six insect Orders (Table 3). In addition, several yeast SNARE genes were not identified in insect genomes, and these include Sft1, Vam3, Vam7, and Snc1/Snc2. In a recent transcriptome analysis of AcMNPV-infected *Trichoplusia ni* cells (Tnms42), expression profiles were generated for host genes throughout the AcMNPV infection cycle (33). We therefore analyzed the expression profiles of host SNARE gene orthologs (Fig. 1 and Table S1) in uninfected and AcMNPV-infected cells. Upon AcMNPV infection, more than 70% of the SNARE genes (17/23) were up-regulated (>1-fold change in transcript abundance upon AcMNPV infection). Of these genes, the expression levels of Bet1, Sec20, Sec22, SNAP-29, Syb, and Use1 were increased >2 fold in AcMNPV infected cells. Overall, we found that in the early stages of AcMNPV infection, most of the SNARE genes were either up-regulated or maintained their expression levels (Fig. 1, Table S1).

In eukaryotic cells, NSF forms a regulatory complex with α-SNAP to unfold

and recycle SNARE proteins (9). Although the α-SNAP transcript is up-regulated in the very early stage of AcMNPV infection (1-6 h p.i.) in *T.ni* cells, the transcript level of NSF remained stable, and slightly decreased by 6 h p.i. (Fig. 1E, Table S1). To determine the transcript levels of NSF in AcMNPV-infected Sf9 cells, we first identified the Sf9 NSF mRNA (from Spodobase) and used quantitative real-time PCR to measure NSF transcript levels from uninfected and infected Sf9 at various times post infection. As shown in Figure 2, AcMNPV infection significantly up-regulated the transcript levels of NSF at 1 and 3 h p.i. Similar to observations in AcMNPV-infected Tnms42 cells, the transcript levels of NSF were substantially decreased at 6 h p.i. Combined, these transcript data suggest that the cellular SNARE system may be important in AcMNPV infection or that specific SNARE components may play important roles.

**Analysis of NSF from Sf9 cells**

Because the SNARE system may be important for successful AcMNPV infection, we asked whether NSF, a key regulator of SNARE activity, is required for AcMNPV replication. To isolate the NSF gene from Sf9 cells, we designed gene-specific primers targeting the 5' and 3' ends of the NSF ORF based on partial EST sequences of *S. frugiperda* NSF obtained from Blast searches. We then amplified and cloned the NSF ORF from Sf9 cells. The Sf9 NSF gene contains a 2241 bp ORF encoding a 746 amino acid protein with a predicted molecular weight of 82.6 kDa. Sf9 NSF had highest amino acid sequence identity to NSF of *B. mori* (92.25%) and is highly conserved with orthologs from other insect species (71.64%-78.51%) and other eukaryotes (44.77% similar to yeast NSF and 63.19% similar to human NSF). Mammalian

536 NSF proteins contain several functional domains: an amino-terminal domain
537 (NSF-N), followed by two homologous domains termed D1 and D2. These
538 functional domains were predicted to be present in NSF of *Spodoptera*
539 *frugiperda* (Sf9 cells) and other insects (Fig. S1).

540 **Transient expression of WT and DN NSF in Sf9 cells**

541 To generate dominant-negative (DN) forms of Sf9 NSF proteins, two
542 previously characterized point mutations (E329Q and R385A) that each
543 abolish ATP hydrolysis activity in human NSF (10, 12) were introduced into Sf9
544 NSF (Fig. 3A and S1, $NSF^{E329Q}$ and $NSF^{R385A}$). To confirm the expression and
545 subcellular localization of NSF constructs, wild-type (WT) and DN NSFs were
546 fused with GFP at the C-terminus and inserted into a plasmid under the control
547 of an AcMNPV *ie1* promoter. These constructs were transiently expressed in
548 Sf9 cells (Fig. 3B-C). A plasmid expressing GFP under the same promoter
549 (gfppBlue) was used as a control. Transient expression of each construct was
550 confirmed by Western blot analysis with an anti-GFP antibody (Fig. 3B) and by
551 epifluorescence and confocal microscopy (Fig. 3C). GFP-tagged WT NSF was
552 distributed diffusely throughout the cytoplasm. In contrast, $NSF^{E329Q}$-GFP and
553 $NSF^{R385A}$-GFP showed a generally cytoplasmic punctate distribution in
554 transfected Sf9 cells (Fig. 3C) similar to that observed previously with human
555 NSF DN constructs (44). To determine potential general effects of
556 $NSF^{E329Q}$-GFP and $NSF^{R385A}$-GFP on the proliferation of Sf9 cells, the viability
557 of cells expressing each construct was measured. At 12, 24, or 36 h
558 post-transfection (p.t.), the viability of Sf9 cells transiently expressing DN NSF
559 proteins was similar to that of cells expressing WT NSF or GFP (Fig. 3D).
560 These data suggest that transient expression of DN NSF proteins over this

short time period did not result in a measurable negative effect on the viability of Sf9 cells.

**Effects of DN NSF expression or NSF knock-down on infectious AcMNPV production**

To determine whether NSF is required for productive AcMNPV infection, we first used a viral complementation assay to examine AcMNPV replication in the presence of DN NSF proteins. Because all cells do not become transfected and express the DN constructs in transient transfection assays, the complementation assay insures that productive viral replication can occur only in cells that are productively transfected and express both the DN NSF construct and WT GP64 (which complements the *gp64* knockout and permits production of infectious BV). In this assay, Sf9 cells were co-transfected with two plasmids: one expressing GP64 and another expressing either GFP, NSF, or DN NSF constructs ($NSF^{E329Q}$-GFP or $NSF^{R385A}$-GFP). At 12 h p.t., the transfected cells were infected with the *gp64* knockout virus (mCherryGUS-$gp64^{ko}$) at an MOI of 1 or 5. At 24 h p.i., the cell culture supernatants were harvested and the infectious virus titers were determined on a GP64-expressing cell line ($Sf9^{Op1D}$) that complements the *gp64* knockout virus. (Note: Virus produced from the transfected cells should contain GP64 produced from the plasmid, but the virus cannot spread from cell to cell in an endpoint titration assay. Thus the $Sf9^{Op1D}$ cell line is used for the $TCID_{50}$ assay, to detect virions produced in the transfection-infection assay.). As shown in Fig. 4B, the production of infectious AcMNPV is significantly reduced (>97% reduction at an MOI of 5) in the presence of either DN NSF construct, $NSF^{E329Q}$-GFP or $NSF^{R385A}$-GFP. In contrast, the expression of WT NSF had

no effect on infectious virus production compared with that in cells expressing GFP. A similar result was observed when cells were infected at an MOI of 1. In these co-transfected and infected Sf9 cells, both GP64 and each of the NSF protein constructs were expressed at substantial levels (Fig. 4A). Additionally, in parallel experiments a similar reduction of infectious AcMNPV production was also observed in another lepidopteran cell line, *T. ni* High 5 cells, in the presence of DN NSF proteins (data not shown).

To extend our observations, we used a dsRNA-based RNAi approach to evaluate the effect of an NSF knock-down on the production of infectious AcMNPV. Sf9 cells were mock transfected or transfected with a dsRNA specific for Sf9 NSF or a dsRNA targeting a GFP control gene. Knock-down efficiencies for NSF were approximately 16.2% (24 h p.t.) and 74.6% (48 h p.t.) (Fig. 4C), and transfection with the dsRNA targeting NSF or GFP resulted in no significant change in the viability of Sf9 cells at 24, 48, and 72 h p.t. (data not shown). Similar to the results from DN NSF expression, we found that depletion of NSF also resulted in a dramatic reduction in the production of infectious AcMNPV (Fig. 4D). Together, these results suggest that functional NSF appears to be important for the efficient replication of AcMNPV.

**Effects of DN NSF on early stages of AcMNPV infection**

Budded virions of AcMNPV enter host cells via clathrin-mediated endocytosis (26), which is regulated in part, by the coordinated action of several SNARE proteins (Table 3) (4). The negative effects of the DN NSF and the NSF knock-down on AcMNPV BV production could result from disruption of the viral infection cycle at an early stage of infection, possibly by interfering with the transport of virions within endosome during entry. To examine this

possibility, Sf9 cells were co-transfected with two plasmids expressing: a) GP64 and b) one of the following proteins: NSF-GFP, NSF$^{E329Q}$-GFP, NSF$^{R385A}$-GFP, or GFP. At 12 h p.t., the cells were infected with a *gp64* knockout virus (LacZGUS-*gp64*$^{ko}$) that contains two reporter genes: LacZ and GUS, which are controlled by the AcMNPV *ie1* early/late promoter and *p6.9* late promoter, respectively. At early and later times in the infection cycle, the transfected and infected cells were lysed and relative β-Gal and GUS activities were measured. As shown in Figures 5A and B, the activities of both β-Gal and GUS were similar in cells expressing control protein GFP or NSF-GFP. However, in the presence of the two DN NSF proteins (NSF$^{E329\text{-}GFP}$ or NSF$^{R385A}$-GFP), both β-Gal and GUS activities were significantly reduced when compared with the controls (Fig. 5A and B). In addition, viral genomic DNA was isolated from transfected-infected cells (under the same conditions), and viral genomic DNA replication efficiency was measured by quantitative real-time PCR detection of genomic DNA. The results showed that the transient expression of DN NSF proteins (NSF$^{E329}$-GFP or NSF$^{R385A}$-GFP) resulted in a significantly reduced level of viral genomic DNA (Fig. 5C). Because early and late gene expression and viral DNA replication were all reduced, these results suggest that the presence of DN NSF proteins inhibit viral infection at a step prior to early gene expression, possibly inhibiting trafficking of the entering virion from the cell surface to the nucleus.

**Effects of DN NSF on internalization and transport of AcMNPV BV during entry**

Several SNARE proteins and NSF (which regulates their function) play important roles in endocytosis by mediating vesicle fusion (4, 45). To examine

636 whether viral entry by endocytosis involved SNARE-mediated fusion events,
637 we examined mCherry-tagged BV entry in the presence of WT or DN NSF
638 proteins. We first transfected Sf9 cells with a plasmid expressing either WT
639 NSF, a DN-NSF, or GFP. After a 12 h period of expression, cells were
640 incubated with mCherry-labeled AcMNPV virions (AcMNPV-3mC) at 4° for 60
641 min to permit virus adsorption at the cell surface. After removing the viral
642 inoculum and washing the cells twice with cold medium, the temperature was
643 raised to 27° and cells incubated for 60 min. Internalized virus was quantified
644 by qRT-PCR measurements of viral genomic DNA. Internalized viral DNA was
645 dramatically reduced in cells expressing DN NSF proteins (61.8% and 65.0%
646 reduction in $NSF^{E329Q}$-GFP and $NSF^{R385A}$-GFP expressing cells, respectively)
647 as compared with cells expressing the WT NSF protein or the control GFP (Fig.
648 6A). In addition to qRT-PCR, the transport of virions was also analyzed by
649 confocal microscopy (Fig. 6B). In control cells expressing WT NSF or GFP, the
650 mCherry labeled nucleocapsids were observed as small but discrete foci of
651 fluorescence distributed more evenly throughout the cytoplasm, and some
652 were observed within the nucleus. In contrast, the mCherry-labeled
653 nucleocapsids in cells expressing DN NSF proteins ($NSF^{E329Q}$-GFP and
654 $NSF^{R385A}$-GFP) were observed aggregated in the cytoplasm in discrete foci
655 which likely represent endosomes. Interestingly, the aggregated
656 mCherry-tagged nucleocapsids were not colocalized with GFP-tagged DN
657 NSF proteins (Fig. 6B, lower panels, GFP vs. mCherry). Thus, while qPCR
658 results suggested that binding and entry were affected by DN NSF expression,
659 confocal microscopy show that transport of AcMNPV nucleocapsids was also
660 disrupted. Overall, these data suggest that NSF is required for efficient

internalization and intracellular transport of AcMNPV BV during entry.

**Is NSF is required for efficient egress of infectious AcMNPV?**

In the studies described above, we found that expression of DN NSF proteins in Sf9 cells led to inefficient internalization and transport of AcMNPV BV. Therefore, to study the role of NSF in virion egress and to circumvent the negative effects on virus entry, we initiated infections by transfecting Sf9 cells with AcMNPV bacmid DNA. Each AcMNPV bacmid DNA expressed a WT or DN NSF protein (tagged with GFP) plus a reporter GUS protein. NSF constructs were expressed from the viral genome under the control of the AcMNPV *ie1* early/late promoter, and the GUS reporter gene was expressed from the *p6.9* late promoter. Thus DN-NSF proteins were expressed in the early phase of infection prior to egress, which occurs during the late phase after assembly of progeny nucleocapsids. Because NSF is a homo-hexamer (9) that is continually assembling and disassembling, DN NSF expressed early should inactivate the function of pre-existing wild-type NSF. At 24 h post-transfection, GFP-positive cells were scored to evaluate transfection efficiency and expression of the NSF-GFP fusions. In parallel, late promoter-driven GUS activity was measured to monitor progression of the viral infection. As shown in Figure 7, the percentage of GFP-positive cells from transfections with different bacmids (GFPBac, NSF-GFPBac, $NSF^{E329Q}$-GFPBac, $NSF^{R385A}$-GFPBac) varied from 35.7% to 38.4% (Fig. 7A). Also, relative GUS activities measured from the transfected cells lysates from cells transfected with the same bacmid DNAs were also at similar levels (Fig. 7B). These results indicate that, in cells transfected with the different bacmids (expressing GFP, NSF-GFP, or DN NSF constructs), transfection efficiencies

were approximately equivalent, and in all cases virus infection progressed into the late phase. Virus titers in supernatants from cells expressing NSF-GFP or the control GFP protein were both approximately $2.5\times10^5$ $TCID_{50}$/ml. In contrast, no infectious AcMNPV BV was detected in supernatants (24 h p.t.) from cells expressing DN NSF constructs $NSF^{E329Q}$-GFP or $NSF^{R385A}$-GFP (Fig. 7C). Thus, these data suggest that NSF is required for the egress and production of infectious AcMNPV BV.

**Is cell surface expression of functional GP64 dependent on NSF?**

Since the viral envelope glycoprotein GP64 is necessary for production of infectious BV (42) and is trafficked through the secretary pathway, the SNARE system may be important for GP64 trafficking. Therefore, we asked whether DN NSF proteins affected expression, transport, and/or cell surface localization of GP64 in the context of a viral infection. To address this question, we transfected Sf9 cells with AcMNPV bacmid DNA expressing either WT NSF or DN NSF proteins (both tagged with GFP) as described above. At 24 h p.t., the expression of GP64 in these bacmid-transfected Sf9 cells was examined by Western blot analysis under reducing and non-reducing conditions for SDS-PAGE. Under non-reducing conditions, GP64 is found as disulfide-linked trimers of GP64 monomers. Two forms of GP64 trimers (known as Trimer I and Trimer II) are typically observed in infected cells (46-48). These typical forms of GP64 were detected in cells expressing DN NSF proteins ($NSF^{E329Q}$-GFP or $NSF^{R385A}$-GFP), and in cells expressing control proteins: WT NSF-GFP and GFP (Fig. 8A). The intensities of the trimeric and monomeric GP64 bands were similar for all bacmid-transfected cells, suggesting that the DN NSF proteins did not substantially alter the expression, stability, or oligomerization of GP64.

711 Cell surface localized GP64 was examined by cELISA with the AcV5 antibody
712 and by immunofluorescence analysis with the MAb AcV1, which recognizes
713 the native neutral-pH conformation of GP64 (49) (Fig. 8B and C). In cells
714 expressing DN NSF proteins, the cell surface levels of GP64 were reduced to
715 approximately 40% of that from cells expressing WT NSF or GFP (Fig. 8B).
716 Although GP64 cell surface levels were significantly reduced in the presence
717 of DN NSF, the recognition of surface localized GP64 by MAb AcV1 indicates
718 that GP64 is in the native conformation. To determine whether the presence of
719 DN NSF proteins affected the fusion activity of GP64 expressed in those cells,
720 we measured fusion activity of GP64 on cells transfected with bacmids
721 expressing WT or DN NSF proteins. Because cell surface levels of GP64
722 varied between the cells expressing WT and DN NSF proteins, and surface
723 GP64 levels may affect the detection of membrane fusion activity, we initially
724 generated a standard curve of cell surface levels of GP64 by transfecting Sf9
725 cells with decreasing quantities of the bacmid that expresses wild-type NSF,
726 and performing cELISA analysis (Fig. 8B). A parallel standard curve of
727 pH-triggered membrane fusion levels was generated by transfecting cells with
728 decreasing quantities of the same bacmid (Fig. 8D, NSF-GFP) and measuring
729 fusion activity as percentages of cells in syncytial masses. Comparisons of
730 GP64-mediated fusion activity in cells expressing DN NSF constructs
731 ($NSF^{E329Q}$-GFP and $NSF^{R385A}$-GFP) with fusion from WT NSF expressing cells
732 in which GP64 was present at the same levels, show that there was no
733 apparent effect on GP64-mediated fusion efficiency in the presence of DN NSF
734 proteins (Fig. 8B, D). Thus, while expression of the DN NSF proteins caused a
735 moderate decrease in surface levels of GP64, there was no effect on the

function of the surface-localized protein.

We also examined infectious BV produced from cells transfected with bacmids expressing WT or DN NSF proteins. When cells were transfected with the bacmid expressing WT NSF (NSF-GFPBac, 2.5 µg) or expressing a control GFP protein, we detected infectious BV titres of approximately 1.5 x $10^5$ $TCID_{50}$/ml. However, in supernatants from cells transfected with DN NSF constructs $NSF^{E329Q}$-GFPBac and $NSF^{R385A}$-GFPBac, no infectious BV was detected (Fig. 8E). Thus while NSF may play a minor role in intracellular transport and cell surface localization of GP64, the defect in infectious AcMNPV BV production in the presence of DN NSF proteins was dramatic, and was not explained by the modestly lower cell surface levels of GP64.

**Effects of DN NSF on nucleocapsid egress from the nuclear membrane**

To examine the stage of virion egress that was affected by DN NSF proteins, we first used transmission electron microscopy (TEM) of bacmid-transfected cells, to compare the transport of nucleocapsids in control cells with that in cells expressing DN NSF proteins. In cells expressing WT NSF-GFP, a typical AcMNPV infection is observed. We observed the typical presence of an electron-dense virogenic stroma (VS) in the nucleus, rod-shaped nucleocapsids at the edges of the VS, intranuclear microvesicles (IM) and nucleocapsids budding from the nuclear membrane (NM) and from the plasma membrane (PM) (Fig. 9A-D). Cells expressing DN NSF proteins ($NSF^{E329Q}$-GFP and $NSF^{R385A}$-GFP) also showed the VS, abundant rod-shaped nucleocapsids, numerous IM, and nucleocapsids associated with IM in the nuclei (Fig. 9E, F, I, J). However, no progeny nucleocapsids were observed budding from the plasma membrane (Fig. 9G and K). Interestingly, in

761 cells expressing DN NSF proteins, some progeny nucleocapsids were
762 observed budding through the inner nuclear membrane (INM) but the INM
763 enclosed nucleocapsids appeared to be trapped within a perinuclear space
764 formed by the inner nuclear membrane and the deformed outer nuclear
765 membrane (Fig. 9G, H, K, L). Combined with the absence of BV production in
766 the presence of DN NSF, these results suggest that functional NSF could be
767 important directly or indirectly, for transit of progeny nucleocapsids across the
768 nuclear membrane.

769 **Association of NSF with viral proteins**

770 Viruses in the family *Baculoviridae* contain a set of about 37 “core” genes
771 that are conserved in most or all sequenced baculovirus genomes (22). Recent
772 studies reported that some of these baculovirus core proteins and some of the
773 highly conserved viral proteins are important for the release of progeny
774 nucleocapsids from nuclei (50-57). These proteins include Ac11, Ac76, Ac78,
775 Ac80 (GP41), Ac93, Ac103, Ac142, and Ac146. A similar defect in infectious
776 AcMNPV production, combined with the aberrant nuclear structures observed
777 at the nuclear membrane (both caused by DN NSF proteins) suggests the
778 possibility that NSF may also be involved in egress of nucleocapsids from the
779 nucleus, perhaps associating with some of the above baculovirus highly
780 conserved or core proteins. To test this hypothesis, an immunoprecipitation
781 assay was used to examine the potential association between NSF and each
782 of the above viral proteins. We selected AcMNPV GP41 and Lef3 as control
783 proteins, as it was previously demonstrated that GP41 interacts with itself but
784 does not interact with Lef3 (58). Sf9 cells were co-transfected with two
785 plasmids: one expressing an HA-tagged viral protein, and another expressing

NSF-Myc. At 36 h p.t., the cells were lysed and proteins were immunoprecipitated with an anti-HA MAb. Expression of the HA-tagged and Myc-tagged proteins was confirmed by Western blot using appropriate antibodies (Fig. 10). Of the 8 viral proteins examined, we found that 5 co-immunoprecipitated NSF, suggesting that viral proteins Ac76, Ac78, GP41, Ac93, and Ac103 may either interact with NSF or may be found in a complex that includes NSF (Fig. 10).

To extend the results from co-immunoprecipitation studies, we further examined the possible associations of NSF and viral proteins using a bimolecular fluorescence complementation assay (BiFC) in living cells. Sf9 cells were co-transfected with two plasmids, separately expressing NSF and one of the viral proteins, with each as a fusion with the N- or C-terminal domain of mCherry (referred to as Nm or Cm, respectively). Initially, to verify the specificity of the mCherry-based BiFC system, we also selected AcMNPV GP41 and Lef3 as candidate bait and prey proteins. By co-expressing GP41-HA-Nm with either GP41-Myc-Cm or Lef3-Myc-Cm (Nm and Cm represent N- and C-terminal fragments of mCherry, respectively), we observed mCherry fluorescence complementation in approximately 50% of the cells co-transfected with GP41-HA-Nm and GP41-Myc-Cm plasmids (Fig. 11A, lower right panels). In contrast, fluorescence was not detected in cells co-expressing GP41-HA-Nm and Lef3-Myc-Cm, even though both of the fusion proteins were expressed (Fig. 11A, lower right panels). In cells co-expressing NSF-HA-Nm in combination with Cm-tagged viral proteins, BiFC fluorescence was observed between combinations of NSF and Ac76, Ac78, GP41, Ac93, or Ac103, the same 5 proteins identified in Co-IP studies. The

811 percentage of fluorescent cells was highest (40-50%) in cells co-expressing
812 NSF-HA-Nm with GP41-Myc-Cm or Ac93-Myc-Cm (Fig. 11B). Lower
813 percentages of fluorescent cells (5-18%) were observed when NSF-HA-Nm
814 was co-expressed with Ac76-Myc-Cm, Ac78-Myc-Cm, or Ac103-Myc-Cm.
815 While Western blot analysis showed that all of the fusion proteins were
816 expressed in co-transfected Sf9 cells, we observed no BiFC fluorescence in
817 cells co-expressing NSF-HA-Nm with constructs Ac11-Myc-Cm,
818 Ac142-Myc-Cm, and Cm-Myc-Ac146. (Note: Two different size bands were
819 detected in Western blots for Ac76-Myc-Cm and Cm-Myc-Ac146 and this
820 phenomenon has been described previously (53, 54)). Similar BiFC
821 fluorescence results were observed by reciprocal protein fusions of Nm fused
822 to viral proteins and Cm fused with NSF (data not shown). Thus, our initial
823 Co-IP results were confirmed in BiFC experiments in which associations occur
824 and are detected within live cells. While these data may suggest
825 protein-protein interactions, BiFC interactions can occur at distances of up to 7
826 nm and therefore are not definitive evidence of direct protein-protein
827 interactions. Thus, combined with effects of DN NSF on virus egress, these
828 Co-IP and BiFC studies suggest that the baculovirus conserved (core)
829 structural proteins (Ac76, Ac78, GP41, Ac93, and Ac103) are closely
830 associated with NSF, and complexes of these proteins with cellular NSF may
831 be involved in AcMNPV egress.

832 **Association of NSF with AcMNPV BV**

833 Because NSF is important for efficient entry and egress of AcMNPV BV,
834 and is closely associated with conserved viral structural proteins, this
835 suggested that NSF might be assembled into BV during the maturation

836 process. To examine this possibility, Sf9 cells were transfected with AcMNPV
837 bacmid DNA expressing c-Myc-tagged NSF and a GUS reporter (NSF-MycBac)
838 and BV preparations were probed for the presence of NSF-Myc by Western
839 blot analysis. While a negative control (β-glucuronidase, GUS) was not
840 detected in BV, Myc-tagged NSF was detected in both purified BV and cell
841 lysates (Fig. 12, NSF). Positive controls GP64 and VP39 were also detected in
842 both cell lysates and BV. A role for NSF in BV is not known but it is possible
843 that NSF in the virion could play a role during entry, or that NSF may be
844 assembled into the BV fortuitously during virion egress.

845 **Discussion**

846 The infection of insect cells by baculoviruses involves a complex interplay
847 between cell and virus (32, 33, 59-61). Analysis of transcriptome data from
848 AcMNPV-infected *Trichoplusia ni* Tmns42 cells (33), showed that early in the
849 infection, many host genes are up-regulated immediately following virus
850 inoculation, and among these are a number of SNARE-encoding genes (Fig. 1,
851 Table 3). SNARE proteins function as protein complexes that mediate fusion
852 between intracellular transport vesicles and membrane-bound compartments
853 and they are important in endocytosis, ER and Golgi trafficking, and the
854 secretary pathway (1). The activity of the SNARE system is regulated by a
855 complex that is formed by NSF and α-SNAP (9). In AcMNPV infected Tnms42
856 cells, α-SNAP and a number of SNARE-related genes are slightly to
857 moderately up-regulated early after infection and then decline as infection
858 progresses (Fig. 1). The levels of NSF transcripts are not increased but
859 decrease gradually after infection. For comparison, we examined the transcript
860 levels of NSF in AcMNPV-infected Sf9 cells and found that at 1 and 3 h p.i.

NSF transcripts were moderately elevated, but had decreased by 6 h p.i. (Fig. 2). It is unclear whether these cell line differences are significant. However, because of their critical roles in cells, we asked whether they may also be important for productive viral infection. During entry, AcMNPV nucleocapsids are typically trafficked to the nucleus within approximately 1 h after inoculation (29, 62). During this time, the cellular SNARE system components should be fully intact and functional. We found that general disruption of the SNARE machinery in insect cells (by overexpressing DN NSF or by down-regulating NSF using RNAi) resulted in dramatically reduced infectious AcMNPV production. To understand how viral replication was constrained, we examined the roles of NSF in both entry and egress.

Budded virions of AcMNPV enter host cells via clathrin-mediated endocytosis (26, 63). Endosomes are then trafficked within the cell, and during this process they are gradually acidified. The decreasing pH within endosomes triggers a conformational change in the major viral envelope glycoprotein (GP64) which mediates membrane fusion and release of the nucleocapsid into the cytosol (28). Prior studies (64) measuring AcMNPV virion internalization from the cell surface and transit time within the endosome estimated that virus entry required approximately 12.5 min from binding until entry into the endosome, and another approximately 12.5 min before virions become insensitive to an inhibitor of endosome acidification, indicating release from the endosome. After endosome release, nucleocapsids are transported to the nucleus by a mechanism that involves actin polymerization (29). Using DN NSF constructs, we found that virus entry required a functional SNARE system. We initially observed that expression of DN NSF resulted in reduced reporter

886 gene expression, reduced viral DNA replication, and lower production of
887 infectious progeny virus (Fig. 4, 5). Because those results were consistent with
888 an entry or early phase defect, we examined virion entry more directly, by
889 tracking the entry of virions containing mCherry-tagged nucleocapsids. In the
890 presence of DN NSF, we found that mCherry-labeled nucleocapsids were
891 aggregated in the cytoplasm, indicating that they were not efficiently
892 transported to the nucleus and were perhaps trapped within endosomes (Fig.
893 6B).

894 In mammalian cells, the homotypic fusion of early endosomes is driven by a
895 SNARE complex consisting of an R-SNARE (VAMP4) and Q-SNAREs (Syx6,
896 Syx13, and Vti1a). Late endosome or endosome–lysosome fusion is mediated
897 by SNARE complexes consisting of three Q-SNAREs (Syx7, Syx8 and Vti1b)
898 and one R-SNARE (VAMP7 or VAMP8) (1). (Note: we did not find orthologs of
899 Syx13 or VAMP8 in sequenced insect genomes, and orthologs of Syx8,
900 VAMP4, and Vti1b were not found in Lepidoptera insect genomes (Table 3)).
901 AcMNPV virion entry by receptor mediated endocytosis may require the
902 function of these or other SNARE complexes. Analysis of gene expression of
903 SNARE components in AcMNPV-infected *T. ni* cells shows that the
904 components of these SANRE complexes are either initially up-regulated during
905 infection or are maintained at constant levels for the first few hours of infection
906 (Fig. 1). Because AcMNPV BV entry appears to be relatively rapid, preexisting
907 levels of SNARE complex proteins are likely to be sufficient for BV entry and
908 changes associated with infection may represent either cellular defensive
909 reactions to infection, or virus-induced changes associated with requirements
910 for SNARE complexes at later stages in the infection cycle. The importance of

911 SNARE proteins in entry for some other viruses was demonstrated for certain
912 negative-stranded RNA viruses, including influenza virus and VSV (20). For
913 influenza virus and VSV, VAMP8 (which forms the SANRE complex with
914 endosomal Q-SNAREs Syx7, Syx8 and Vti1b) is recruited to the
915 virus-containing endosome and promotes virus entry (20). Presently, the series
916 of events associated with trafficking of baculovirus BV through endosomes
917 during entry has received little attention, and further studies of the
918 requirements and/or interactions of cellular factors such as the SNARE
919 complex proteins should provide new and valuable mechanistic detail on
920 baculovirus entry.

921 Substantial budding of infectious AcMNPV BV can be detected from
922 infected cells by 24 h p.i. (22). Early TEM studies showed that progeny
923 nucleocapsids that assembled in the nucleus, appeared to exit the nucleus by
924 budding through the nuclear membrane (31). Enveloped nucleocapsids (often
925 with double membranes presumably derived from inner and outer nuclear
926 membranes) were observed in the cytoplasm. The observation of free
927 nucleocapsids in the cytoplasm suggested that these nucleocapsids were
928 de-enveloped by an unknown mechanism prior to transport to the plasma
929 membrane, where free nucleocapsids bud through the plasma membrane (31,
930 65). Recent studies (66) indicate that nucleocapsid trafficking during egress
931 requires functional kinesin suggesting that either enveloped or free
932 nucleocapsids move along microtubules during egress. Actin may also be
933 involved in nucleocapsid egress as actin has been shown to be involved in
934 nucleocapsid trafficking during entry (29). Expression of DN NSF caused a
935 modest but significant reduction in surface levels of GP64 (Fig. 8B) and a

dramatic reduction in infectious BV production (Fig. 8E). However, GP64 produced in the presence of DN NSF was oligomerized and transported to the cell surface, and appeared fully functional as a fusion protein (Fig. 8), suggesting that the modest effects of DN NSF proteins on GP64 trafficking does not explain the defect in infectious AcMNPV production. We therefore used TEM to further examine the effects of DN NSF proteins on egress. When examining the nuclear membranes in the late phase of infection, we found that the presence of DN NSF proteins resulted in large perinuclear spaces formed by the INM and a deformed outer nuclear membrane (Fig. 9 G, H, K, and L). Interestingly, it is known that the SNARE system participates in nuclear membrane remodeling in *Xenopus laevis* eggs (67). The observation that AcMNPV nucleocapsids are found in large aberrant perinuclear structures of the nuclear membranes, suggests that NSF and the SNARE system may be directly involved in virus-induced remodeling of nuclear membranes during nucleocapsid egress from the nucleus. Further studies to immunolocalize NSF and SNARE complexes within infected cells should shed light on whether the observed phenomena at the nuclear membrane results from direct or indirect effects. As the mechanism of nuclear egress is not know, it is possible that SNARE complexes may play a direct role in the pinching off of vesicles containing progeny nucleocapsids during nuclear egress, or in the release of nucleocapsids into the cytoplasm after nuclear egress.

Because a number of recent studies found that certain highly conserved or core AcMNPV genes (*ac11*, *ac76*, *ac78*, *ac80 or gp41*, *ac93*, *ac103*, *ac142*, and *ac146*), are required for infectious BV production, and knockouts of these genes result in reduced nucleocapsid egress from the nucleus (50-54, 56, 57,

68), we examined their potential associations with NSF. We found that five of these conserved (core) proteins (Ac76, Ac78, GP41, Ac93, and Ac103) associated directly or indirectly (perhaps in larger complexes) with NSF. Predicted structure analysis indicated that, different from the predominant β-sheet composition of Ac11, Ac142, and Ac146, the other five examined viral proteins only contains 3-6 α-helixes (data not shown). The potential structure difference might imply the interaction specificity of these viral proteins with NSF. As such, we might speculate that these viral proteins may associate in a possible “egress complex” on nuclear membranes. The associations of these viral proteins with NSF may contribute to the detection of NSF at what appear to be low levels, in BV preparations (Fig. 12). While NSF was not detected in BV of AcMNPV in a prior proteomics analysis (32), NSF was identified in BV of another baculovirus, Helicoverpa armigera (Ha)NPV (59). Because of the low levels of NSF detected in BV, further studies will be required to determine whether NSF may play a possible functional role in BV.

Our studies reveal the importance of the cellular SNARE system in AcMNPV infection and the critical role of NSF in efficient BV entry, and in nuclear egress. A hypothetical model (Fig. 13) illustrates the potential bottlenecks to viral entry and egress and points of potential inhibition by DN NSF. Further studies will be necessary to define the precise roles of specific SNARE proteins in AcMNPV infection and to better characterize the interactions between components of the SNARE system and viral proteins. Overall, these studies should shed light on understanding the complex interplay between baculoviruses and the host cells trafficking pathways and proteins during viral entry and egress.

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1177 **Figure legends**

1178 **Figure 1**

1179 **Expression profiles of the components of the cellular SNARE system**
1180 **before and during AcMNPV infection**. Transcription data for SNARE system
1181 components were retrieved from a transcriptome analysis of AcMNPV infected
1182 Tnms42 cells (33) and are grouped into panels A-F according to the SNARE
1183 genes that are involved in a specific pathway or complex. Measurements of
1184 transcript levels (RPKM, reads per kilobase per million reads) from samples
1185 from control (C, uninfected) or infected cells at various time points post
1186 infection (h p.i., hours post infection) are plotted. The RPKM values (total
1187 RPKM<5) for γ-SNAP and Syx17 were cutoff in the transcriptome analysis (33).
1188 The ortholog of Syx4 was not found in the transcriptome database. Error bars
1189 represent standard deviations from the mean of three replicates. Host gene
1190 abbreviations are detailed in the legend to Table 3.

1191 **Figure 2**

1192 **Transcript levels of NSF in AcMNPV-infected Sf9 cells**. Sf9 cells were
1193 infected with wild-type AcMNPV at an MOI of 10 and total RNAs were isolated
1194 from uninfected (C, control) cells or infected cells at various time points post
1195 infection (1-48 h p.i.) and transcribed into cDNA. Transcript levels of NSF were
1196 measured by quantitative real-time PCR. Error bars represent standard
1197 deviations from the mean of three replicates. h p.i., hours post infection.

1198 **Figure 3**

1199 **Transient expression of GFP-tagged wild-type (WT) and dominant**
1200 **negative (DN) NSF proteins in Sf9 cells**. (A) Schematic representation of
1201 domain structure and GFP fusions of WT and DN NSF constructs (NSF-GFP,

1202 NSF$^{E329Q}$-GFP, NSF$^{R385A}$-GFP). NSF domains are abbreviated as: N, NSF-N
1203 domain; D1, the first ATP-binding domain; D2, the second ATP-binding domain.
1204 (B) Western blot analysis of GFP-tagged NSF proteins transiently expressed in
1205 Sf9 cells. GFP and NSF-GFP fusions were detected with an anti-GFP
1206 polyclonal antibody. The anti-β-actin blot served as a loading control. (C)
1207 Subcellular localization of WT and DN NSF-GFP fusion proteins in transfected
1208 cells by epifluorescence microscopy (left panels, Epi) and confocal microscopy
1209 (right panels, Confocal). (D) Viability of transfected cells expressing WT or DN
1210 NSF proteins was measured at various times post-transfection (12-36 h) using
1211 a tetrazolium-based cell viability assay as described in the Materials and
1212 Methods section. Error bars represent standard deviations from the mean of
1213 three replicates.

1214 **Figure 4**

1215 **Effects of DN NSF expression (A, B) and NSF knock-down (C, D) on**
1216 **AcMNPV production**. (A, B) Sf9 cells were co-transfected with two plasmids
1217 expressing a) GP64 and b) either WT NSF (NSF-GFP), DN NSF
1218 (NSF$^{E329Q}$-GFP or NSF$^{R385A}$-GFP), or GFP. At 12 h p.t., cells were infected with
1219 the *gp64*-knockout virus (mCherryGUS-*gp64*$^{ko}$) at an MOI of 1 or 5. At 24 h p.i.,
1220 expression of GP64 and GFP-tagged WT and DN NSF in transfected-infected
1221 cells was confirmed by Western blot analysis of cell lysates (A). Effects of DN
1222 NSF proteins were analyzed by measurements the titers of infectious BV
1223 released from transfected-infected cells (B). (C) Sf9 cells were transfected with
1224 a NSF-MycpBlue plasmid or co-transfected with a NSF-MycpBlue plasmid and
1225 the dsRNA targeting NSF or GFP (dsNSF, dsGFP). At 24 and 48 h p.t., the
1226 transfected cells were collected and the expression of Myc-tagged NSF was

detected by Western blot with an anti-Myc polyclonal antibody. The anti-β-actin blot served as a loading control. (D) Sf9 cells were mock transfected (Mock) or transfected with the dsRNA targeting NSF or GFP (dsNSF, dsGFP). At 48 h p.t., the transfected cells were infected with wild-type AcMNPV at an MOI of 5. At 24 h p.i., the cell culture supernatants were collected and the virus titers were determined by $TCID_{50}$ assays. Error bars represent standard deviations from the mean of three replicates. ***, $p<0.0005$; ****, $p<0.00005$ (by unpaired *t* test).

**Figure 5**

**Effects of DN NSF on early stages of AcMNPV infection**. The effects of DN NSF proteins on early and late viral gene expression and viral DNA replication were examined. Sf9 cells were co-transfected with two plasmids expressing a) GP64 and b) either WT NSF (NSF-GFP) or DN NSF ($NSF^{E329Q}$-GFP or $NSF^{R385A}$-GFP), or GFP. At 12 h p.t., the tranfected cells were infected with a *gp64*-knockout virus (LacZGUS-$gp64^{ko}$). Early gene expression was monitored by analyzing AcMNPV *ie1* early/late promoter driven β-Gal activity at 6 h p.i. (A). Late phase gene expression was monitored by analyzing AcMNPV *p6.9* late promoter driven GUS activity at 24 h p.i. (B). Viral DNA replication was monitored by quantitatively real-time PCR analysis of viral genomic DNA extracted from transfected-infected cells at 24 h p.i. (C). Error bars represent standard deviations from the mean of three replicates. **, $p<0.005$; ***, $p<0.0005$ (by unpaired *t* test).

**Figure 6**

**Effects of DN NSF on AcMNPV entry**. Sf9 cells were transfected with plasmids expressing either WT NSF (NSF-GFP), DN NSF ($NSF^{E329Q}$-GFP or

NSF$^{R385A}$-GFP), or GFP. At 12 h p.t., the cells were infected at 4° for 60 min with an AcMNPV virus containing mCherry-tagged VP39 capsid protein. After raising the culture temperature to 27° for 60 min, the internalization and transport of entering virions were analyzed by quantitative real-time PCR analysis of viral DNA from cell lysates (A) and by confocal microscopic detection of mCherry-tagged capsids (B). Error bars represent standard deviations from the mean of three replicates. Scale bar, 10 μm; *, $p<0.05$ (by unpaired *t* test).

**Figure 7**

**Effects of DN NSF proteins on production of infectious budded virions**. Sf9 cells were transfected with bacmid DNA expressing either WT NSF (NSF-GFP), DN NSF (NSF$^{E329Q}$-GFP or NSF$^{R385A}$-GFP), or GFP. Transfection activity was monitored by analysis of GFP-positive cells (A). Percentages of GFP positive cells at 24 h p.t. are indicated below each micrograph of GFP epifluorescence. Late phase gene expression was monitored by analysis of GUS activity from a *p6.9* late promoter driven GUS gene at 24 h p.t. (B). Production of infectious BV was measured by $TCID_{50}$ assay of cell culture supernatants collected at 24 h p.t. (C). Error bars represent the standard deviations from the mean of three replicates.

**Figure 8**

**Effects of DN NSF on cell surface levels of GP64**. Sf9 cells were transfected with 2.5 μg bacmid DNA expressing GFP-tagged NSF proteins (WT or DN) or GFP. To generate a standard curve for GP64 surface levels and for cell-cell fusion analysis, cells were transfected with 0.5-2.5 μg bacmid DNA expressing GFP-tagged WT NSF. At 24 h p.t., the expression, cell-surface localization,

and fusion activity of GP64, and infectious virus production were analyzed. (A) Western blot analysis of the expression and oligomerization of GP64 on reducing (R) and non-reducing (NR) gels. The anti-β-actin blot served as a loading control. (B, C) The transfected cells were fixed and the cell surface localization of GP64 was detected by cELISA using AcV5 antibody (B), or by indirect immunofluorescence using AcV1 antibody (C). (D) Transfected cells were treated with PBS at pH 5.0 to induce GP64-mediated cell-cell syncytium formation. The percentages of nuclei in syncytia (defined as more than five nuclei per syncytium) in five fields was calculated. (E) Infectious BV production from transfected cells expressing WT or DN NSF proteins, or GFP was determined by TCID50 assays. Error bars represent the standard deviations from the mean of three replicates. *, $p<0.05$; **, $p<0.005$ (by unpaired *t* test).

**Figure 9**

**TEM analysis of AcMNPV-infected Sf9 cells expressing WT or DN NSF proteins**. Sf9 cells were transfected with AcMNPV bacmids expressing either wild-type NSF (A-D), or dominant negative NSF proteins $NSF^{E329Q}$-GFP (E-H) or $NSF^{R385A}$-GFP (I-L). At 48 h post-transfection, the bacmid-transfected cells were fixed and analyzed by transmission electronic microscopy. Electron-dense virogenic stroma (VS) and intranuclear microvesicles (IM) were observed in cells expressing both WT NSF and DN NSF (A vs. E or I). White and black triangles show intranuclear microvesicles (IM) and nucleocapsids associated with the membranous vesicular structures, respectively (Panels B vs. F vs. J). Progeny nucleocapsids (white arrows) appear to be budding into the nuclear membrane (NM, black arrows) (C) and free in the cytoplasm (D) in cells expressing WT NSF. In contrast, nucleocapsids (white arrows) were

found primarily in large perinuclear space or vesicular structures (black triangles) associated with the nuclear membrane in cells expressing DN NSF (G, H, K, and L). Plasma membrane (PM); Scale bar, 500 nm.

**Figure 10**

**Co-immunoprecipitation analysis of NSF and AcMNPV proteins**. Sf9 cells were transfected or co-transfected with two plasmids separately expressing NSF-Myc and a HA-tagged viral protein. At 36 h p.t., the cells were lysed and the proteins were immunoprecipitated with anti-HA monoclonal antibody and protein-G agarose. The precipitates (Co-IP) were detected on Western blots with an anti-Myc polyclonal antibody (right panel in each group). The cell lysates (Lysate Input) were also examined on Western blots with an anti-HA monoclonal antibody (Lysate Input, top panels) or an anti-Myc polyclonal antibody (Lysate Input, bottom panels).

**Figure 11**

**BiFC analysis of NSF and AcMNPV protein associations**. Sf9 cells were co-transfected with a pair of BiFC plasmids: one expressing NSF-HA-Nm (NSF-Nm) and the other expressing a viral protein fused with Myc-Cm (Ac#-Cm or Cm-Ac146). As controls, cells were transfected with plasmid pairs expressing GP41-HA-Nm and GP41-Myc-Cm, or GP41-HA-Nm and Lef3-Myc-Cm. At 36 h p.t., cells were examined for fluorescence complementation by epifluorescence microscopy (A) and the percentage of mCherry-positive cells in five fields was calculated (B). The expression of each construct in co-transfected cells was confirmed by Western blot analysis with anti-Myc antibody (A, panels a) or anti-HA antibody (A, panels b). Error bars represent the standard deviations from the mean of three replicates. *, $p<0.05$;

**, p<0.005; ****, p<0.00005 (by unpaired *t* test).

**Figure 12**

**Detection of NSF in budded virions of AcMNPV**. Sf9 cells were transfected with bacmid NSF-MycBac, which expresses a Myc-tagged WT NSF protein. At 72 h p.t., cell supernatants were collected and the budded virions were purified by sucrose density gradient centrifugation. The transfected cells and the purified virions were analyzed on Western blots using anti-GP64, anti-VP39, anti-Myc, and anti-GUS antibodies. BV, budded virions. Typical and long (left and right, respectively) exposures of Western blots are shown, illustrating the presence of NSF at low quantities in BV preparations.

**Figure 13**

**A hypothetical model for the role of cellular NSF protein in the infection cycle of AcMNPV**. Steps at which NSF is potentially required for trafficking during budded virion entry and progeny nucleocapsid egress are indicated as dashed lines. BV, budded virions; CCV, clathrin-coated vesicles; EE, early endosome; ER, endoplasmic reticulum; LE, late endosome; MVB, multivesicular bodies; NC, nucleocapsid; NPC, nuclear pore complex; NSF, N-ethylmaleimide-sensitive factor; VS, virogenic stroma.

**Table 1**. PCR primers

| Primer | Sequence (5' to 3') | Description |
|---|---|---|
| SfNsfF | atgtctgcaatgcggatgaag | Amplification of the |
| SfNsfR | ttattgtacattggtgcctaggtc | ORF of Sf9 NSF |
| SfNsfXF | aattctagaatgtctgcaatgcggatgaag | Amplification of the |
| SfNsfER | aatgaattctgattgtacattggtgcctaggtc | ORF of Sf9 NSF |
| NsfF | accgccttagccgctgaact | Amplification of 90 bp |
| NsfR | agactccgtgaatccgaccatgt | of Sf9 NSF |
| E329QF | cagatcgacgctatttgcaaagc | Construction of E329Q |
| E329QR | gcaaatagcgtcgatctggtcaaagatgataatgtgga | and R385A mutants |
| R385AF | gctcccggtcgtctcgaggttc | |
| R385AR | ctcgagacgaccgggagccatcaacgcctcgtcaatcat | |
| HANmF | aatgaattctacccatacgatgttccagattacgctgggacgtcgggtggaag cggtatggtgagcaagggcgagga | Construction of HA-NmpBlue |
| NmFt | cgagcggatgtaccccgaggactaaatgtaataataaaaattgtatca | |
| NmRt | tgatacaatttttattattacatttagtcctcggggtacatccgctcg | |
| 64pAR | attaagcttcacactcgctatttggaacat | |
| MycCmF | aatgaattcgaacaaaaactcatctcagaagaggatctggggacgtcgggt ggaagcggtggcgccctgaagggcgagatc | Construction of Myc-CmpBlue |
| CmFt | cggcatggacgagctgtacaagtagatgtaataataaaaattgtatca | |
| CmRt | tgatacaatttttattattacatctacttgtacagctcgtccatgccg | |
| 64pAR | attaagcttcacactcgctatttggaacat | |
| NmHAF | aattctagaatggtgagcaagggcgaggag | Construction of |
| NmHAR | attggatccagcgtaatctggaacatcgtatgggtaaccgcttccacccgacg tcccgtcctcggggtacatccgctcg | Nm-HApBlue |
| CmMycF | aattctagaatgggcgccctgaagggcgagatc | Construction of |
| CmMycR | aatggatcccagatcctcttctgagatgagtttttgttcaccgcttccacccgac gtccccttgtacagctcgtccatgccg | Cm-MycpBlue |
| nMycF | ctagaatggaacaaaaactcatctcagaagaggatctgaatg | Construction of |
| nMycR | gatccattcagatcctcttctgagatgagtttttgttccatt | pIE-Myc-MCS |
| nHaF | ctagaatgtacccatacgatgttccagattacgctg | Construction of |
| nHaR | gatccagcgtaatctggaacatcgtatgggtacatt | pIE-HA-MCS |
| cMycF | ataagaattcgaacaaaaactcatctcagaagaggatctgaattagatgtaa taataaaaattgtatca | Construction of pIE-MCS-Myc |
| 64pAR | attaagcttcacactcgctatttggaacat | |
| cHAF | ataagaattctacccatacgatgttccagattacgcttagatgtaataataaaa attgtatca | Construction of pIE-MCS-HA |
| 64pAR | attaagcttcacactcgctatttggaacat | |
| NSFiF | ggatcctaatacgactcactatagggactcgcagccctaacaaaga | Amplification of the |
| NSFiR | ggatcctaatacgactcactatagggcttcagtagcccttgcttg | dsDNA of Sf9 NSF |
| GFPiF | ggatcctaatacgactcactataggacgtaaacggccacaagttc | Amplification of the |
| GFPiR | ggatcctaatacgactcactataggtgttctgctggtagtggtcg | dsDNA of GFP |

**Table 2**. Plasmids constructed in this study

| Purpose | Construct name |
|---|---|
| Expression and subcellular localization of NSF and its mutants | NSF-GFPpBlue |
| | E329Q-GFPpBlue |
| | R385A-GFPpBlue |
| Real-time PCR | NSF90pMD18 |
| Co-immunoprecipitation | GP41-MycpBlue |
| | Lef3-MycpBlue |
| | NSF-MycpBlue |
| | Ac11-HApBlue |
| | Ac76-HApBlue |
| | Ac78-HApBlue |
| | GP41-HApBlue |
| | Ac93-HApBlue |
| | Ac103-HApBlue |
| | Ac142-HApBlue |
| | HA-Ac146pBlue |
| Bimolecular fluorescence complementation (BiFC) assay | NSF-HA-NmpBlue* |
| | Ac11-HA-NmpBlue |
| | Ac76-HA-NmpBlue |
| | Ac78-HA-NmpBlue |
| | GP41-HA-NmpBlue |
| | Ac93-HA-NmpBlue |
| | Ac103-HA-NmpBlue |
| | Ac142-HA-NmpBlue |
| | Nm-HA-Ac146pBlue |
| | NSF-Myc-CmpBlue$ |
| | Ac11-Myc-CmpBlue |
| | Ac76-Myc-CmpBlue |
| | Ac78-Myc-CmpBlue |
| | GP41-Myc-CmpBlue |
| | Ac93-Myc-CmpBlue |
| | Ac103-Myc-CmpBlue |
| | Ac142-Myc-CmpBlue |
| | Lef3-Myc-CmpBlue |
| | Cm-Myc-Ac146pBlue |

Note: *Nm and $Cm represent the N- and C-terminus of mCherry, respectively.

**Table 3**. The SNARE proteins in yeast, human, and insects

| Function sites | Classification | Species | | |
|---|---|---|---|---|
| | | Yeast | Human | Insects[$] |
| Endosome /Lysosome | Qa | Pep12 | Syx7 | + |
| | | Vam3 | - | - |
| | | - | Syx13 | - |
| | | - | Syx17 | +[1] |
| | | - | Syx20 | +[2] |
| | Qb | Vti1 | Vti1a<br>Vti1b | Vti1[3] |
| | Qc | Syx8 | Syx8 | +[4] |
| | | Tlg1 | Syx6 | + |
| | | - | Syx10 | - |
| | | Vam7 | - | - |
| | R | Nyv1 | VAMP7 | + |
| | | - | Endob/VAMP8 | - |
| | | - | VAMP4 | +[5] |
| Endoplasmic reticulum | Qa | Ufe1 | Syx18 | + |
| | Qb | Sec20 | Sec20 | + |
| | Qc | Slt1/Use1 | Use1 | + |
| | R | Sec22 | Sec22a<br>Sec22b<br>Sec22c | Sec22 |
| | | - | | |
| | | - | | |
| Golgi apparatus | Qa | Sed5 | Syx5 | + |
| | | Tlg2 | Syx16 | + |
| | Qb | Bos1 | Memb | + |
| | | Gos1 | Gos28 | + |
| | Qc | Bet1 | Bet1 | + |
| | | Sft1 | Gos15 | - |
| | R | Ykt6 | Ykt6 | + |
| Polarization | R | - | Amisyn | - |
| | | - | Tom1<br>Tom2 | Tom |
| | | Sro7/Sro77 | Lgl1<br>Lgl2 | Lgl |
| Regulatory complex | | Sec17 | α - SNAP | + |
| | | | β - SNAP | - |
| | | | γ - SNAP | + |
| | | Sec18 | NSF | + |
| Secretion pathway | Qa | Sso1/Sso2 | Syx1a<br>Syx1b | Syx1 |
| | | - | Syx2 | - |
| | | - | Syx3 | - |
| | | - | Syx4 | +[6] |
| | | - | Syx11 | - |
| | | - | Syx19 | - |
| | Qbc* | Sec9 | SNAP-25a<br>SNAP-25b | SNAP-25 |
| | | SPO20 | | |
| | | - | SNAP-23 | - |
| | | - | SNAP-29 | + |
| | | - | SNAP-47 | - |
| | R | Snc1/Snc2 | Syb1<br>Syb2<br>Syb3 | Syb |
| | | - | Myob | - |

**Note:** [$]Insects represent sequenced insect genomes, which including those of *Acyrthosiphon pisum*, *Aedes aegypti*, *Anopheles gambiae*, *Apis mellifera*, *Bombyx mori*, *Culex quinquefasciatus*, *Danaus plexippus*, *Drosophila melanogaster*, *Harpegnathos saltator*, *Nasonia vitripennis*, *Pediculus humanus corporis*, *Tribolium castaneum*. *Qbc represents the SNARE proteins possess one Qb-SNARE motif and one Qc-SNARE motif.

+[1]: absent in *Culex quinquefasciatus*, *Pediculus humanus corporis*;

+[2]: absent in *Acyrthosiphon pisum*;

+[3]: Two orthologs of Vti1 were found in *Acyrthosiphon pisum, Anopheles gambiae, Apis mellifera, Culex quinquefasciatus, Drosophila melanogaster, Harpegnathos saltator, Tribolium castaneum*;

+[4]: absent in *Bombyx mori*, *Danaus plexippus*;

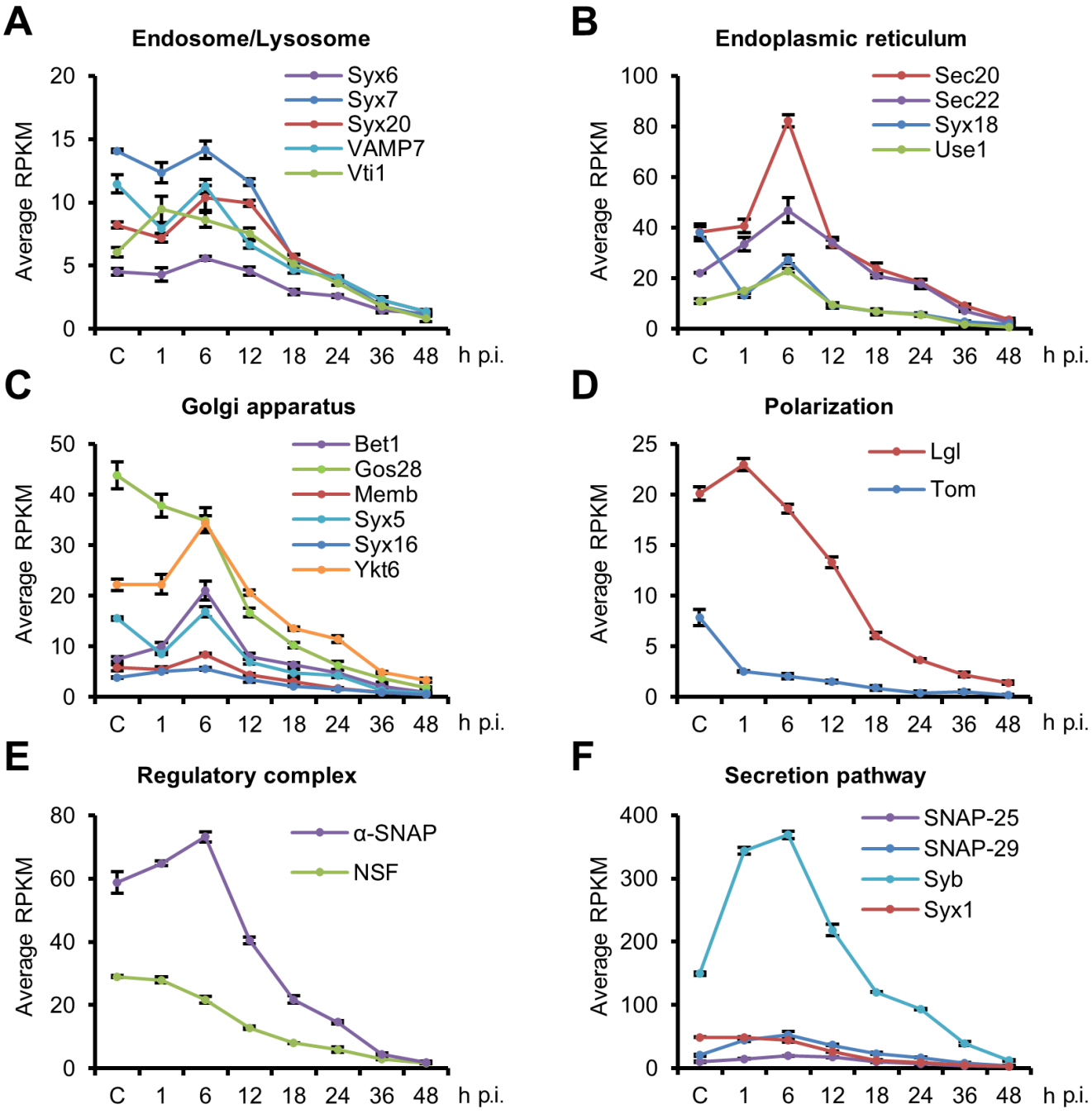
+[5]: only present in *Apis mellifera*, *Nasonia vitripennis*;

+[6]: absent in *Harpegnathos saltator*, *Pediculus humanus corporis*;

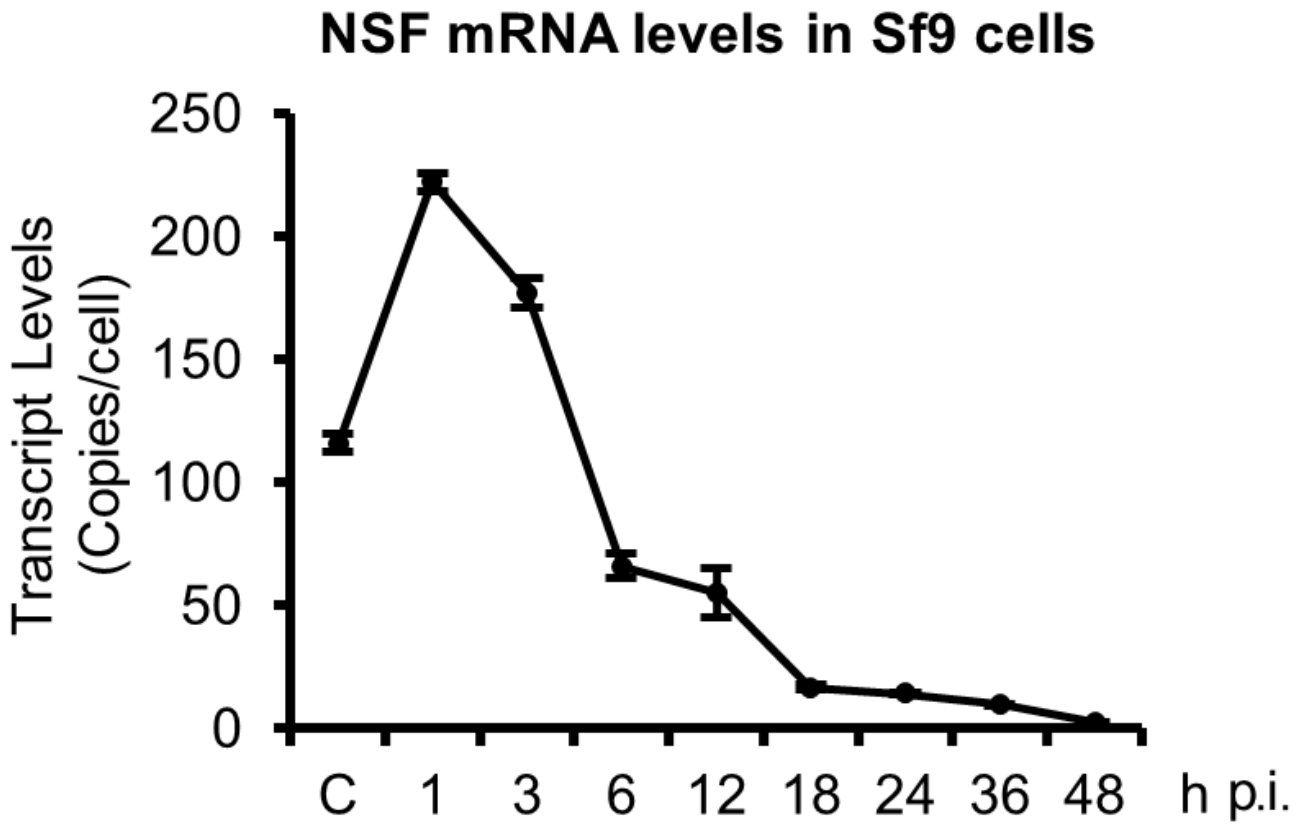
Abbreviations: α-SNAP, α-soluble NSF attachment protein; Bet1, blocked early in transport; Bos1, Bet1 suppressor 1; Endob, endobrevin; Gos#, Golgi SNARE protein with a size of # kDa; Lgl#, lethal giant larvae #; Memb, membrin; Myob, myobrevin; NSF, N-ethylmaleimide sensitive factor; Nyv1, new yeast v-SNARE; Pep12, carboxypeptidase Y-deficient; Sec#, secretory mutant # protein; Sed5, suppressor of Erd2 deletion; Sft1, suppressor of Sed5; Slt1, SNARE-like tail-anchored protein 1; Snc1/Snc2, suppressor of the null allele of CAP; SNAP-#, synaptosome-associated protein of # kDa; SPO20, SPOrulation 20; Sro7/Sro77, suppressor of Rho3; Sso1/Sso2, supressor of Sec1; Syb#, synaptobrevin #; Syx#, syntaxin #; Tlg, T-snare affecting a late Golgi compartment; Tom, tomosyn; Ufe1, unknown function essential 1; Use1, unconventional SNARE in the ER; Vam#, vacuolar morphogenesis #; VAMP#, vesicle-associated membrane protein #; Vti1, Vps10 interacting protein. Ykt6, YKL196c-encoding protein.

# Figure 1

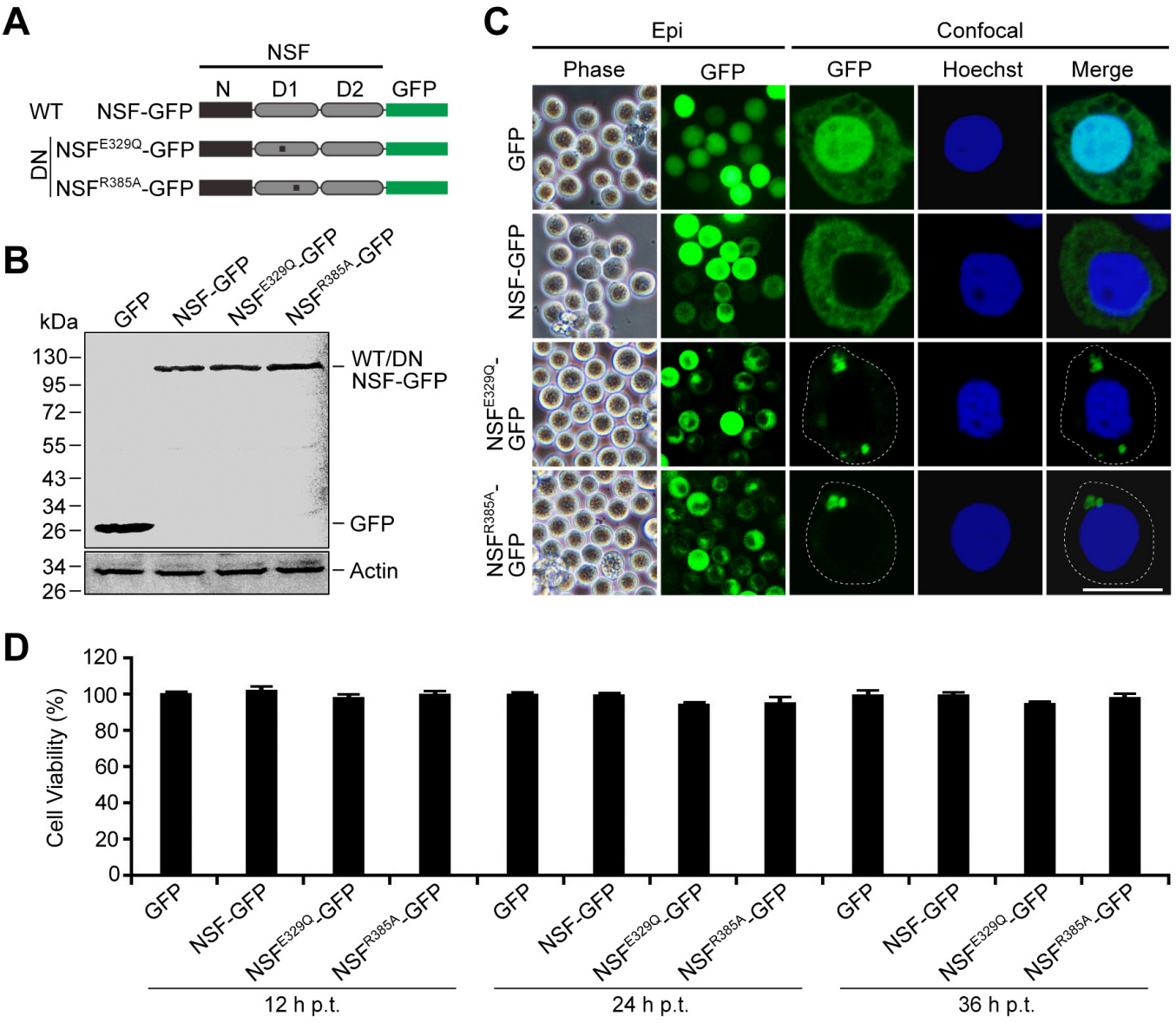
**RNA-seq analysis of SNARE mRNAs in AcMNPV-infected *T.ni* cells**



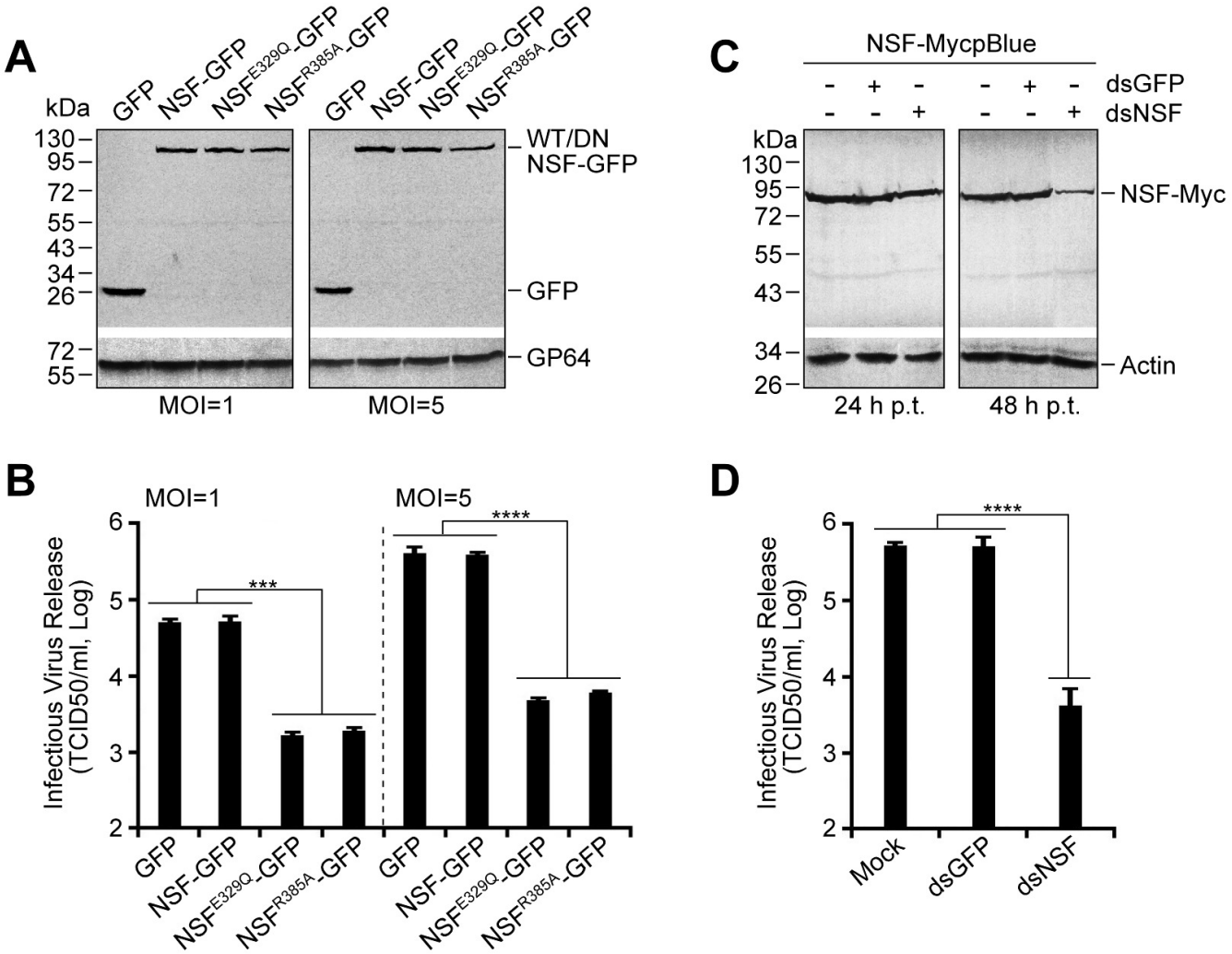
# Figure 2



# Figure 3



# Figure 4



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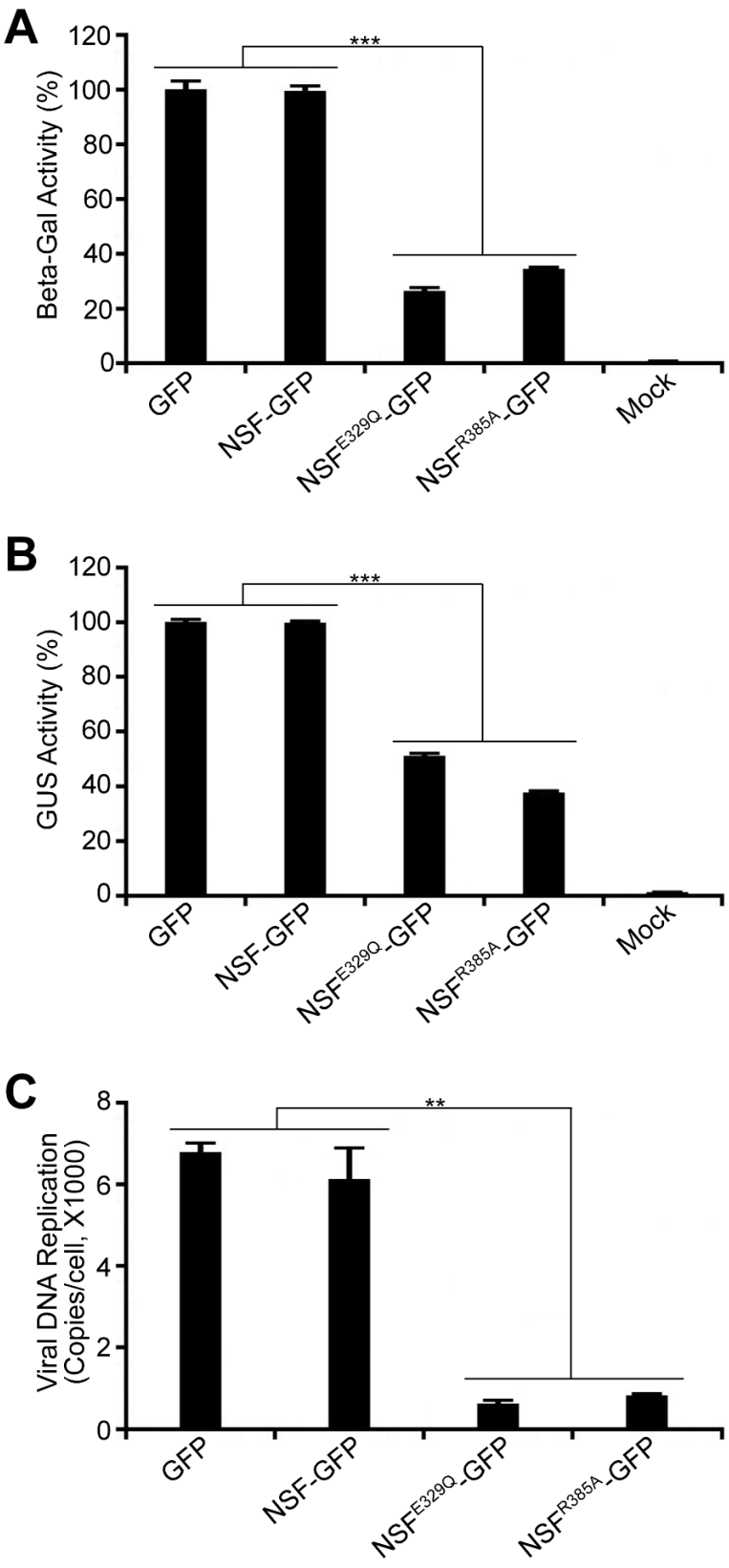

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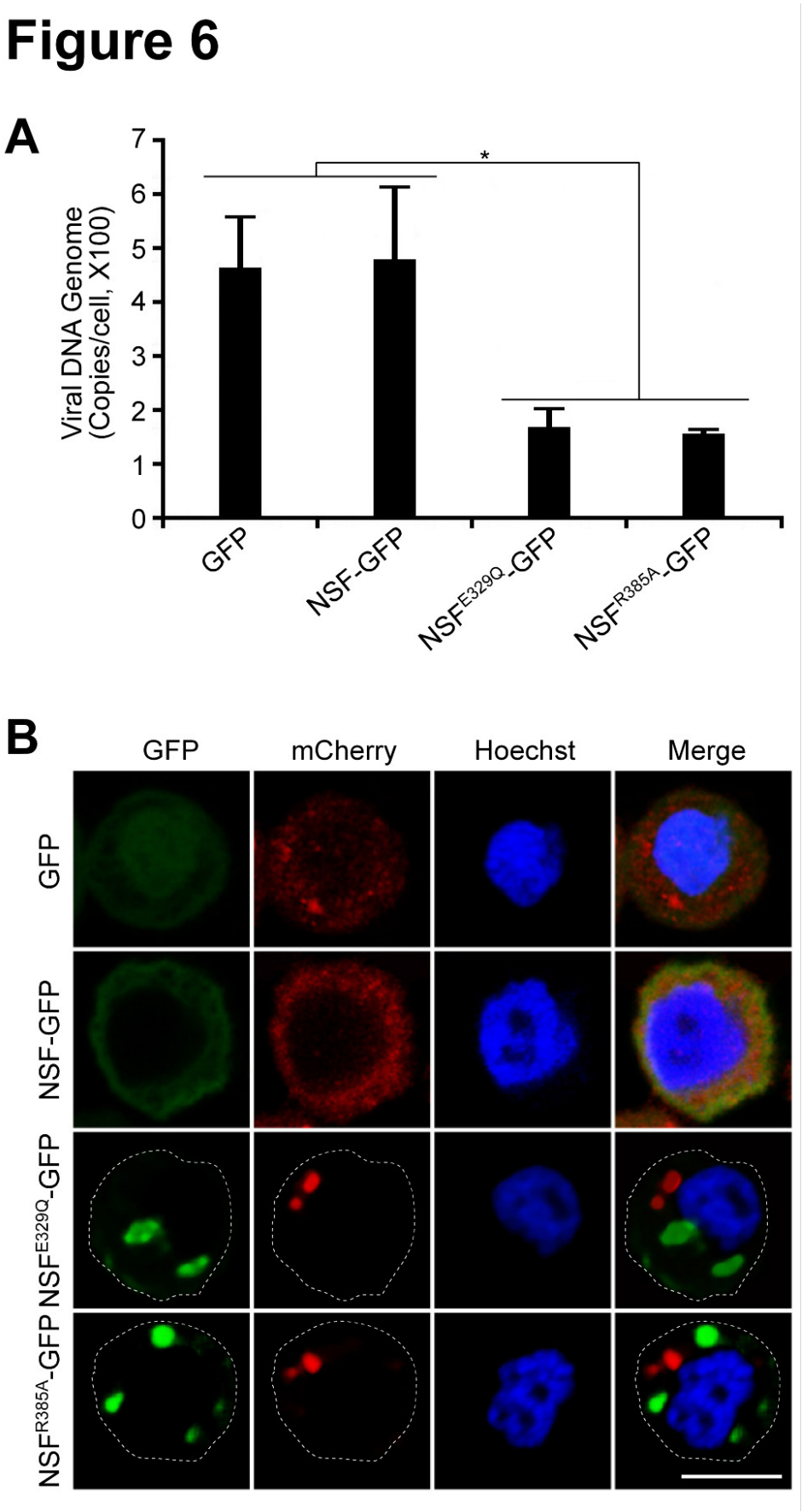

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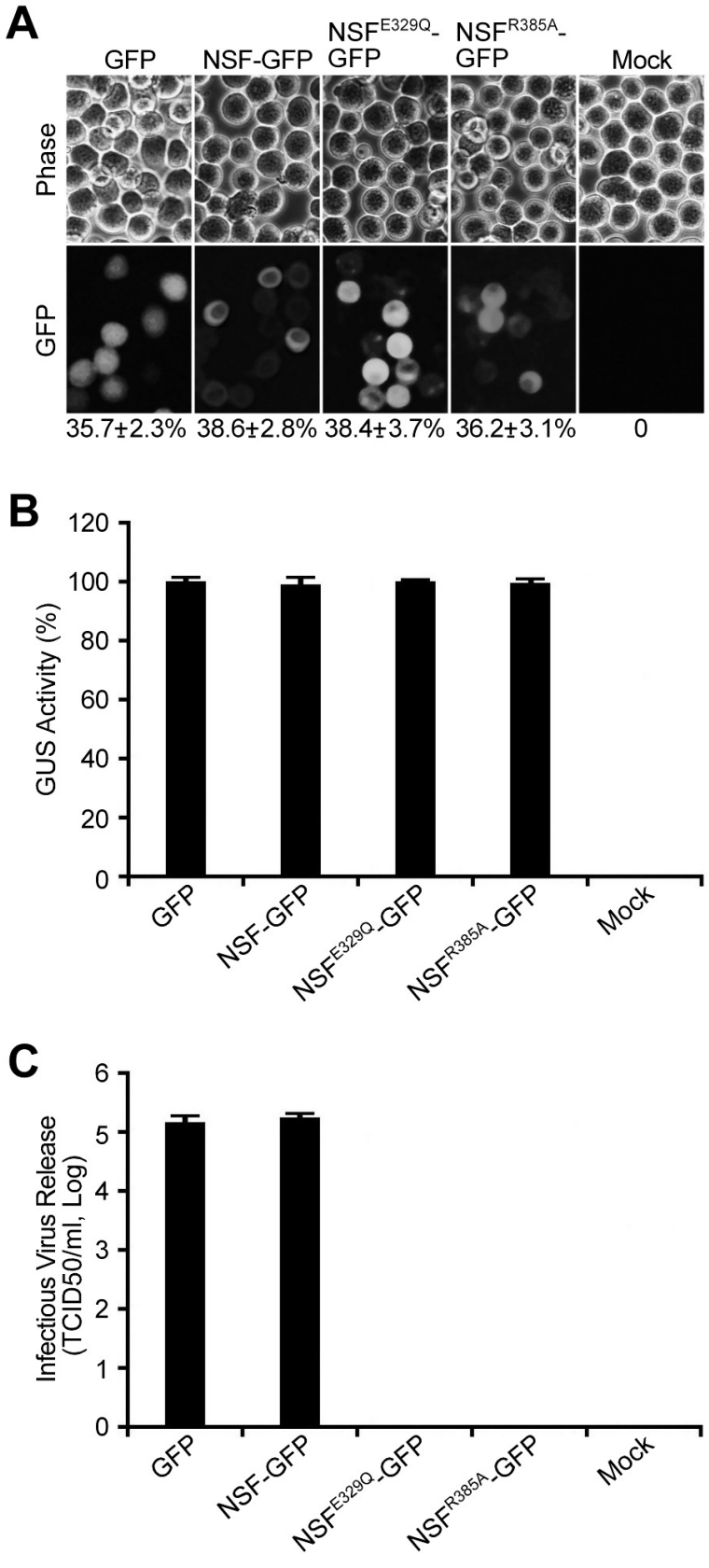
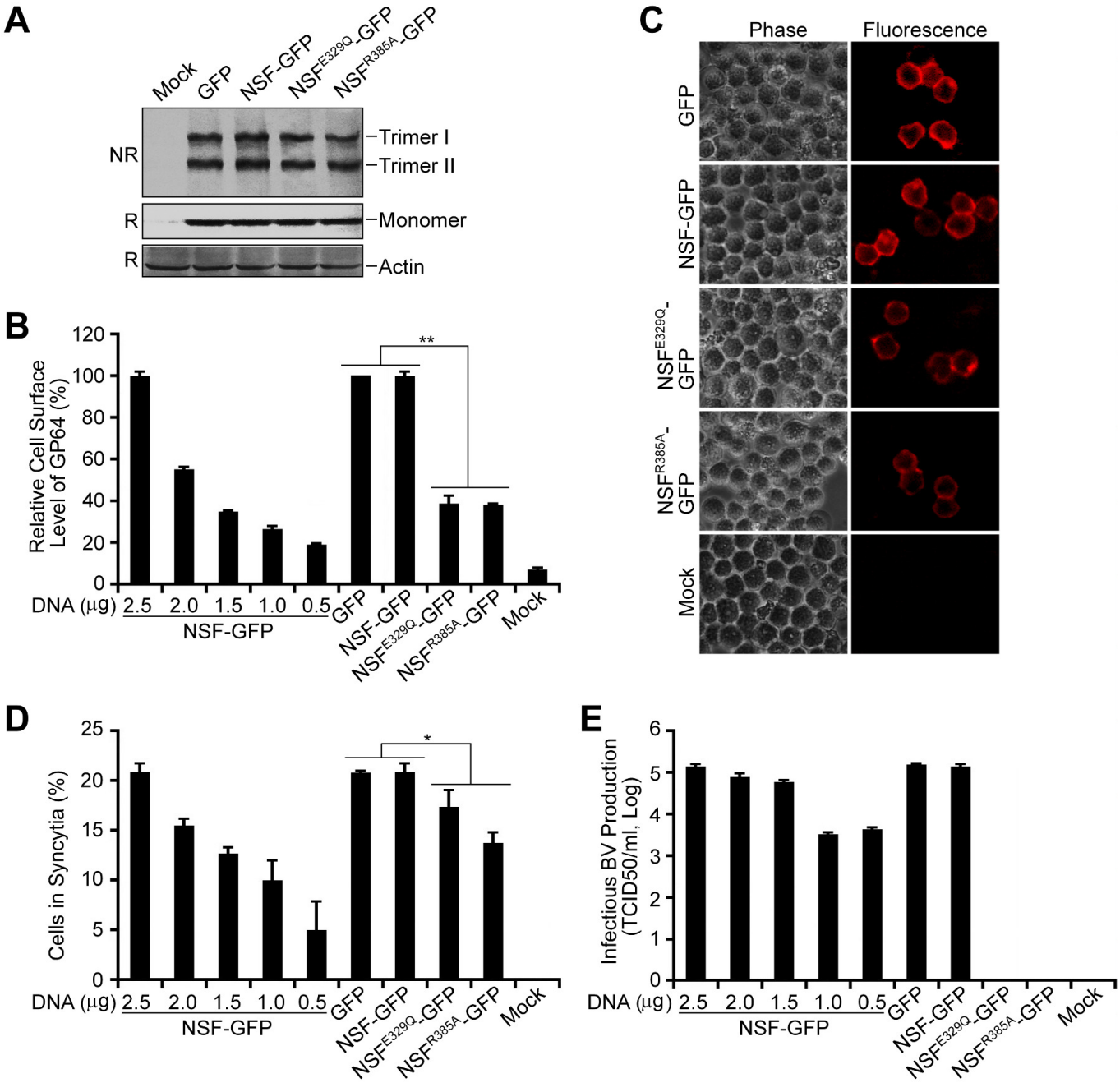
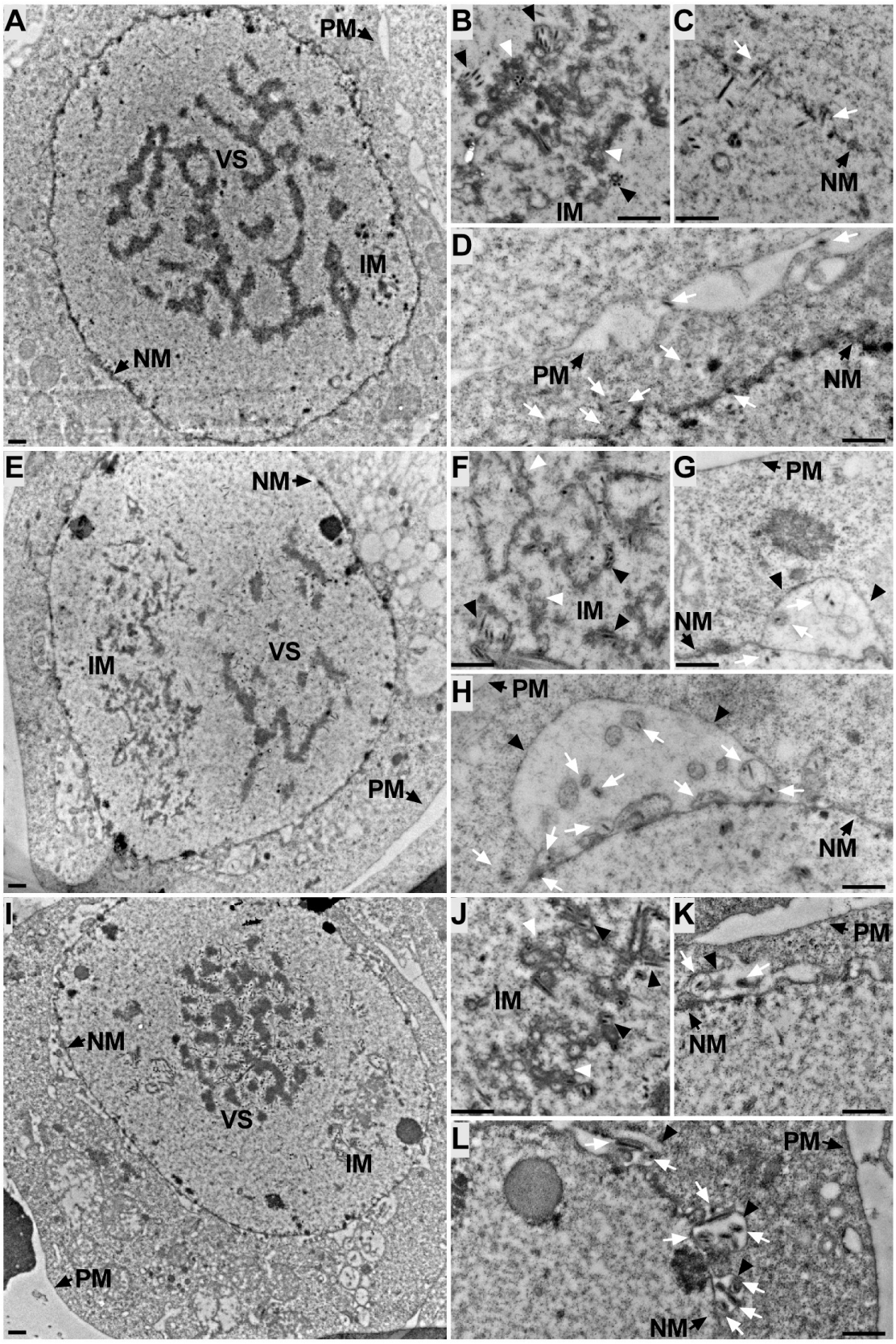


# Figure 8



# Figure 9



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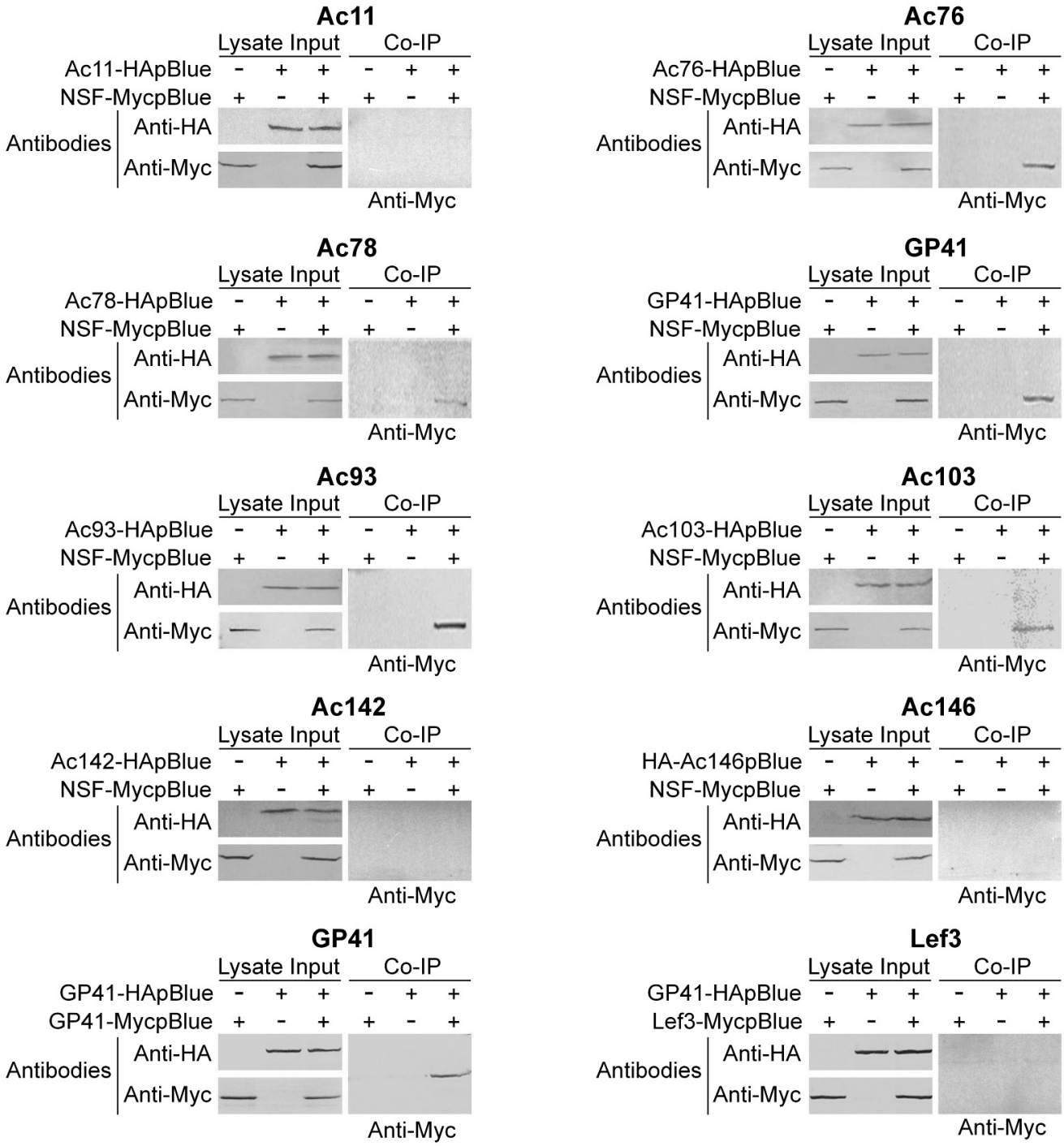

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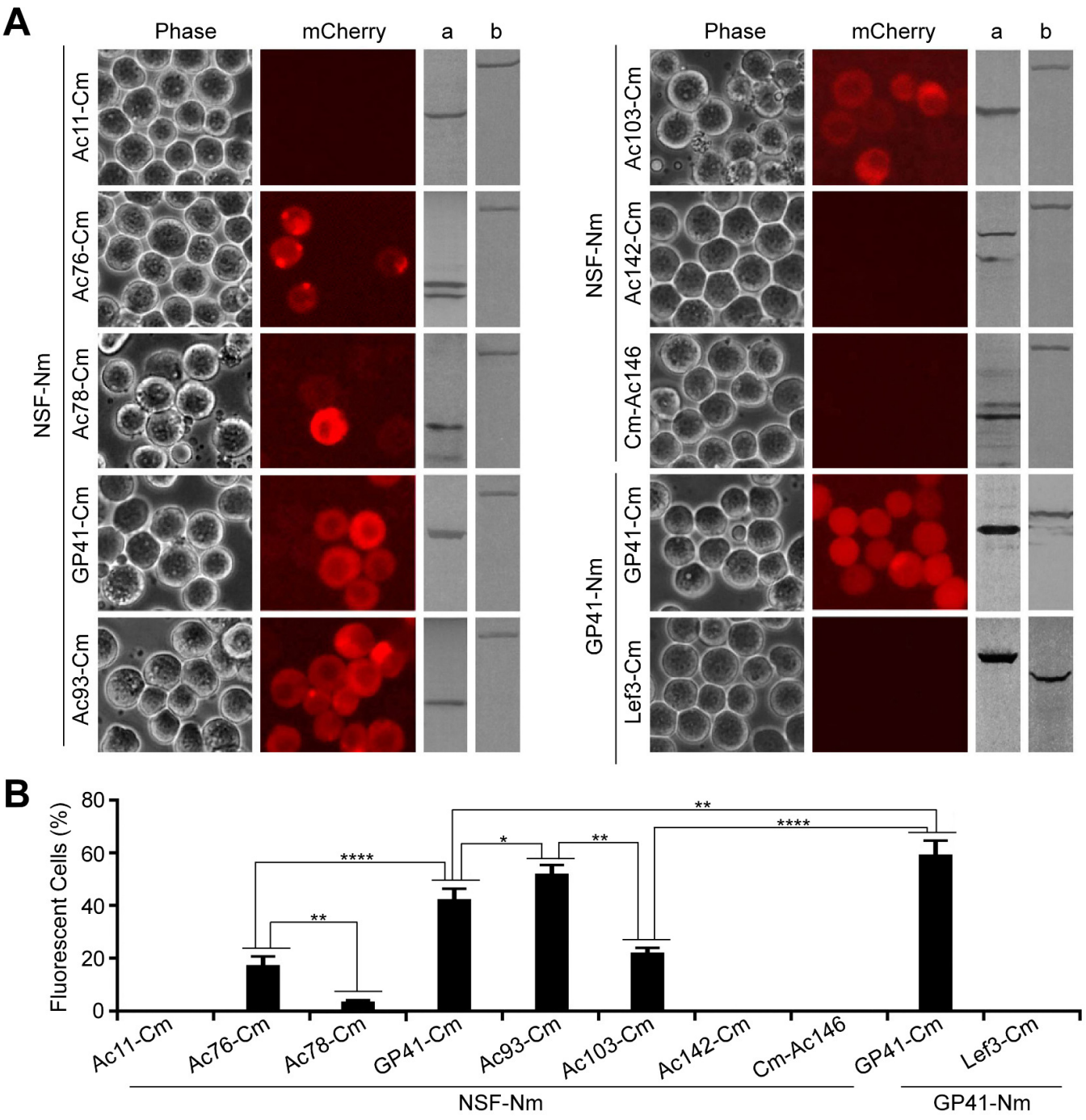

# Figure 12

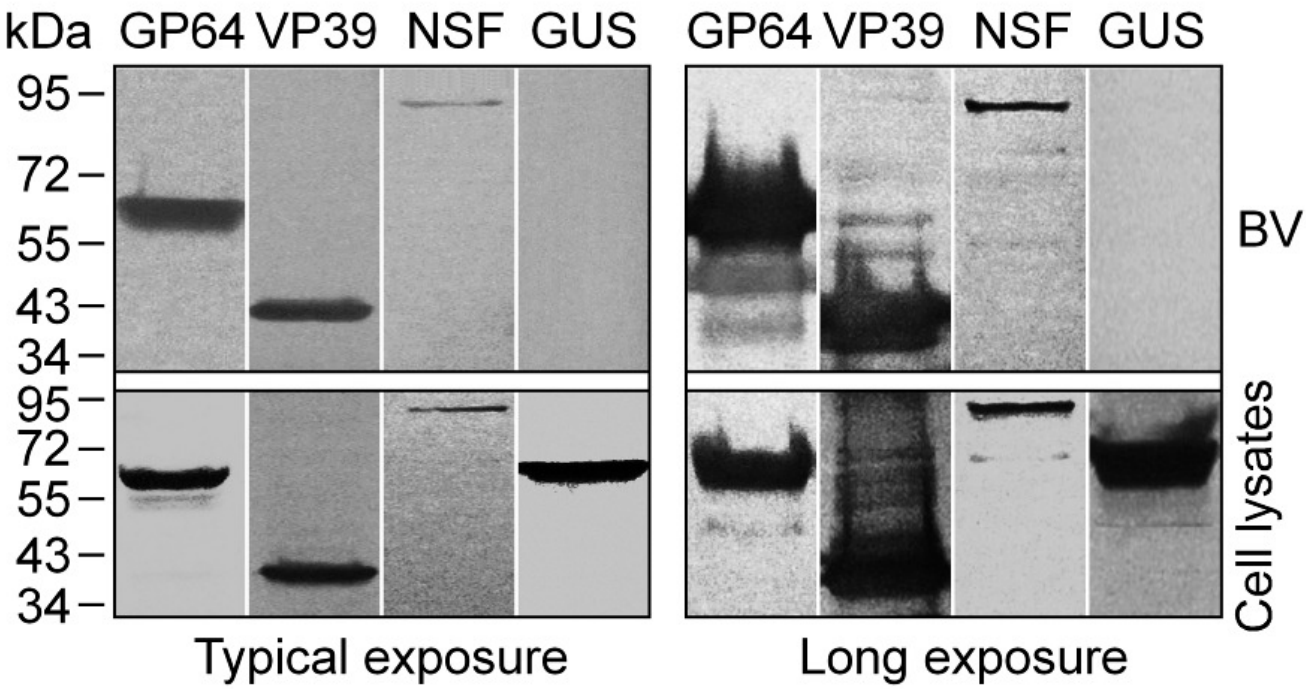

**Figure 13**