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Title: Inhibition of cellular fatty acid synthase impairs replication of budded virions of *Autographa californica* multiple nucleopolyhedrovirus in *Spodoptera frugiperda* cells

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Highlights

- Fatty acid synthase (FASN) catalyzes the *de novo* biosynthesis of palmitate.
- Transcript level of *fasn* was significantly up-regulated upon AcMNPV infection.
- Inhibition of FASN activity disrupts efficient replication of AcMNPV.
- FASN is not required for efficient entry of AcMNPV into Sf9 cells.

Abstract

Fatty acid synthase (FASN) catalyzes the synthesis of palmitate, which is required for formation of complex fatty acids and phospholipids that are involved in energy production, membrane remodeling and modification of host and viral proteins. Presently, the roles of cellular fatty acid synthesis pathway in *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) infection is not clear. In this study, we found that the transcripts level of *fasn* was significantly up-regulated at the early stage of AcMNPV infection. Treatment of AcMNPV-infected *Spodoptera frugiperda* Sf9 cells with C75, a specific inhibitor of FASN, did not affect the internalization of budded virions into cells, but dramatically reduced the infectious AcMNPV production. Further analysis revealed that the presence of C75 significantly decreased the expression level for two reporter genes, beta-galactosidase and beta-glucuronidase, that were separately directed by the early and late promoter of AcMNPV. Similarly, Western blot analysis showed that, in C75-treated cells, the expression of viral *gp64* was delayed and decreased. Additionally, treatment with C75 also resulted in a significant reduction in the accumulation of viral genomic DNA.

Together, these results demonstrate that the fatty acid synthesis pathway is required for efficient replication of AcMNPV, but it might not be necessary for AcMNPV entry into insect cells.

Keywords: baculovirus; AcMNPV; fatty acid synthase; C75

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1. Introduction

In cellular homeostasis, fatty acids serve as the critical substrates of energy metabolism, lipid synthesis and membrane structure remodeling, signal transduction, and protein modification (Jones and Infante, 2015). The *de novo* fatty acid synthesis pathway initiates the conversion of glucose to pyruvate through glycolysis in the cytoplasm. Pyruvate is then transported to the mitochondria and decarboxylated by the enzyme pyruvate dehydrogenase to generate acetyl coenzyme A (acetyl-CoA), which is the starting component in the Krebs cycle to form citrate. After returning back to the cytoplasm, citrate is converted into acetyl-CoA, which is further catalyzed by acetyl-CoA carboxylase to produce malonyl-CoA. At a final step, Acetyl-CoA and malonyl-CoA are catalyzed by fatty acid synthase (FASN) to produce the 16-carbon saturated fatty acid palmitate. Palmitate is subsequently used to form complex fatty acids and lipids and plays critical roles in energy production, phospholipid synthesis, and protein palmitoylation (Liu et al., 2010).

In the complex interaction between host and viruses, *de novo* fatty acid synthesis pathway contributes to efficient replication of certain mammalian viruses. In hepatitis C virus (HCV) infected cells, the expression of *fasn* was significantly up-regulated and the localization of FASN in HCV infected cells was redirected to the site of viral replication (Nasheri et al., 2013; Yang et al., 2008). Similarly, the dengue virus nonstructural protein NS3 was found to localize FASN to the virus replication site in the cytoplasm (Heaton et al., 2010), and the virus infection dramatically increased the rate of fatty acid synthesis and perturbs intracellular lipid homeostasis (Perera et al., 2012). Increasing the activity of FASN was also found in human cytomegalovirus (HCMV)

infected cells. It has demonstrated that HCMV increased the activity of FASN to up-regulate the synthesis of phospholipids that promote mature virions assembly (Spencer et al., 2011). Recently, potential roles of fatty acid synthesis in virus assembly were also found in human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus infected cells. Inactivation of FASN in cells infected by these two viruses did not disrupt the expression of HIV-1 Gag protein or hepatitis B virus genome replication, however, the progeny virus particles production of both viruses was significantly reduced (Kulkarni et al., 2017; Okamura et al., 2016). Additionally, a number of studies showed that inhibition of FASN significantly reduced viral genomic RNA replication and virus infectivity of rotavirus, respiratory syncytial virus (RSV), and West Nile virus (WNV) (Gaunt et al., 2013; Martin-Acebes et al., 2011; Ohol et al., 2015).

Baculoviruses comprise a large family of invertebrate double-stranded DNA viruses. These enveloped viruses are isolated from infected insects from mainly the Orders Lepidoptera, Diptera, and Hymenoptera (Rohrmann, 2013). Baculoviruses are widely used as biological insecticides and as vectors in protein expression, surface display, and mammalian cells transduction (Haase et al., 2015; Sun, 2015; van Oers et al., 2015). In an infection cycle, baculoviruses usually produce two distinct types of virions: occlusion-derived virions (ODV) and budded virions (BV). ODV and BV share identical genetic DNA content and nucleocapsid structure. However, they differ in their envelopes that are obtained from plasma membrane (BV) or intranuclear microvesicles (ODV). In addition, ODV initiate the virus infection of the midgut epithelial cells, whereas BV is responsible for spreading the viral infection from cell to cell within the infected tissues or within cultured cells *in vitro* (Rohrmann,

2013). *Autographa californica* multiple nucleopolyhedrovirus is the type species of the genus *Alphabaculovirus* of the *Baculoviridae* family (Rohrmann, 2013). The entry of AcMNPV BV is dependent on receptor-mediated endocytosis (Hefferon et al., 1999; Long et al., 2006). During entry, the viral envelope glycoprotein GP64 is necessary for cell surface receptor binding and low-pH triggered fusion of the viral envelope and endosomal membranes (Blissard and Wenz, 1992; Hefferon et al., 1999; Kadlec et al., 2008; Li and Blissard, 2011). After being released into the cytosol, the nucleocapsids are transported to the nucleus via actin filaments (Au and Pante, 2012; Ohkawa et al., 2010). In the nucleus, viral genes transcription and DNA replication occur subsequently (Rohrmann, 2013). Following progeny nucleocapsids assembly, some nucleocapsids bud through the nuclear envelope and are released into the cytoplasm through unknown mechanisms that might involve the function of the endosomal sorting complex required for protein sorting (ESCRT) and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) systems (Fraser, 1986; Guo et al., 2017; Yue et al., 2018). The nucleocapsids are then transported to and bud at the plasma membrane to form BV (Rohrmann, 2013).

Prior studies revealed that cellular fatty acid synthesis pathway is closely related with AcMNPV infection. It was found that phosphatidylserine is the major phospholipid of AcMNPV BV, whereas the major phospholipids in ODV are phosphatidylcholine and phosphatidylethanolamine (Braunagel and Summers, 1994). In AcMNPV-infected *Spodoptera frugiperda* or *Trichoplusia ni* cells, the amount of phosphatidylcholine is increased (Marheineke et al., 1998). Additionally, AcMNPV GP64 was shown to be palmitoylated and the

palmitoylation site of GP64 has been mapped to C503 (Roberts and Faulkner, 1989; Zhang et al., 2003). Also, lipids and fatty acids were found to play important roles in GP64-mediated membrane fusion. Addition of lysolipids in AcMNPV-infected cells significantly inhibited cell-cell fusion and virus infectivity, whereas the addition of oleic and arachidonic acids, or monoolein promoted virus-cell fusion and infectious virus production (Chernomordik et al., 1995).

In this study, we examined whether the host cellular fatty acid synthesis pathway is required for AcMNPV infection. We found that the transcript level of cellular *fasn* was significantly up-regulated in AcMNPV-infected insect cells. Inactivation of FASN with a specific inhibitor, C75 (4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid) (Rendina and Cheng, 2005), dramatically decreased infectious BV production, the expression of viral early and late genes, and viral DNA synthesis. Our findings demonstrate that FASN is involved in efficient AcMNPV replication.

2. Materials and methods

2.1 Cells, viruses, and reagents

Spodoptera frugiperda Sf9 cells were maintained at 27°C in TNMFH medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS, Gibco). Wild-type AcMNPV and the recombinant virus AcMNPV-LacZGUS that contains two reporter genes: LacZ and GUS, which are controlled by the AcMNPV *ie1* early/late promoter and *p6.9* late promoter, respectively (Li and Blissard, 2012), and AcMNPV-3mC that contains mCherry-labeled VP39 (Ohkawa et al., 2010) were propagated in Sf9 cells. Virus infections were performed in 12-well plates (2×10^5 cells per well) and virus inoculum was added to cells and incubated for 1 h. The cells were washed once with TNMFH

medium. Times post infection (p.i.) were calculated from the time the viral inoculum was added. The fatty acid synthase inhibitor C75 was purchased from Sigma Aldrich and dissolved in DMSO (Sigma Aldrich).

2.2 Construction of FASN-spMD18 plasmid

Total RNA was isolated from Sf9 cells by using an RNAiso plus kit (TaKaRa). The first-strand DNA complementary to the mRNA (cDNA) was synthesized by using AMV reverse transcriptase and a random primer according to the manufacturer's instructions (TaKaRa). Gene-specific primers FASN-F: 5'-GAGTGCGCCGAAGTAGAGAGC-3' and FASN-R: 5'-ATGGACACGAAGGAGTTGACGAT-3' were designed based on the EST sequences at SPODOBASE database (<http://bioweb.ensam.inra.fr/spodobase>) (Negre et al., 2006). A 209 bp fragment of FASN product was PCR-amplified and cloned into pMD18-T vector (TaKaRa) and sequenced with M13-47 primer. The pMD18-T vector containing the small fragment of *fasn* was designated as FASN-spMD18.

2.3 Analysis of the transcription of *fasn*

Sf9 cells were infected with wild-type AcMNPV at an MOI of 5. At 1, 3, 6, 12, 18, 24, 36, and 48 h p.i., the infected and mock-infected cells were collected. Total RNA was isolated by using an RNAiso plus kit (TaKaRa). Potential genomic DNA elimination and first-strand DNA complementary to mRNA (cDNA) synthesis were performed with PrimeScript™ RT reagent kit with gDNA eraser and an oligo-dT primer (TaKaRa). The *fasn* transcript was quantified by real-time PCR (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad). Each PCR mixture contained 10 µl SYBR® Premix ExTaq II (TaKaRa), 5 µM of each primer (FASN-F and FASN-R as described above),

and 500 pg of the cDNA template. Thermal cycling conditions were one cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, and 55°C for 45 s. A standard curve was generated by a serial dilution of FASN-spMD18. The transcript levels of *fasn* were expressed as numbers of transcript copies per cell.

2.4 Cell viability assay

Sf9 cells were seeded in 12-well plates overnight. After removing the medium, the cells were cultured in TNMFH medium containing varying concentrations of C75 (5-50 μ M). After 24, 48, 72, and 96 h treatment with C75, the cell viability was assessed using the CellTiter96[®] AQueous One Solution Cell Proliferation Assay (MTS, Promega) according to the manufacturer's recommendations.

2.5 FASN inhibition assay

The FASN inhibition assay was performed as described previously (Perera et al., 2012) with modifications. Sf9 cells in 12-well plates were infected with wide-type AcMNPV or AcMNPV-3mC at an MOI of 5. After adsorption, virus was removed and the cells were washed once with TNMFH medium and cultured in fresh medium containing varying concentrations (5-25 μ M) or 20 μ M of C75. At 24 h or at different times post-infection, the virus supernatants were collected and the virus titers were measured by 50% tissue culture infectious dose (TCID₅₀) assays on Sf9 cells. At different times of addition of C75 assays, three sets of Sf9 cells in 12-well plates were treated with C75 and infected with wild-type AcMNPV (MOI=5): a) Sf9 cells were treated with 20 μ M C75 for 1, 3, or 6 h. After removing the drug and washing the cells once with TNMFH medium, the cells were infected with AcMNPV. b) Sf9 cells were

incubated with TNMFH medium containing AcMNPV and 20 μ M C75 for 1 h. After washing out the virus and C75 with TNMFH medium, the cells were cultured in fresh TNMFH medium. c) Sf9 cells were infected with AcMNPV. At 1, 3, or 6 h p.i., the cultured medium was replaced with fresh TNMFH medium containing 20 μ M C75. The drug was present for the duration of infection. At 24 h p.i., the infectious virus production for all of the treated cells was determined by TCID₅₀ assay on Sf9 cells.

2.6 Analysis of virus entry

The virus entry assay was performed as described previously (Yue et al., 2018). Briefly, Sf9 cells in 12-well plates were pre-chilled at 4°C for 0.5 h and then were incubated with the medium containing wild-type AcMNPV (MOI=5) at 4°C for 1 h. After removing the virus inoculums, cells were washed twice with cold TNMFH medium and changed to warm medium (27°C) containing 20 μ M C75 and continue to be cultured at 27°C for 0.5, 1, and 1.5 h. Total DNA was extracted using a DNeasy blood tissue kit (Qiagen). Viral genomic DNA was measured by quantitative real-time PCR (CFX96 Touch™ Real-Time PCR Detection System, Bio-Rad). Each PCR mixture contained 10 μ l SYBR® Premix ExTaq II (TaKaRa), 5 μ M of 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 (Li and Blissard, 2012). Thermal cycling conditions were one cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 45 s. A standard curve was generated by a serial dilution of ODV-e56pGEM, which contains the ODV-e56 open reading frame (ORF) (Li and Blissard, 2012).

AcMNPV genomic DNA was expressed as numbers of viral DNA copies per cell.

2.7 Analysis of viral gene expression and DNA replication

To evaluate the effects of inhibition of FASN on viral gene expression, Sf9 cells in 12-well plates were infected with the virus AcMNPV-LacZGUS at an MOI of 5. Following adsorption, the cells were washed once with medium and then overlaid with TNMFH medium containing 20 μ M C75. At different times post infection (3-24 h p.i.), the infected cells were collected. Two sets of the cells were lysed with 1% Triton-X 100 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). The other set of infected cells were analyzed for the expression of GP64 by Western blotting.

To determine the effects of inhibition of FASN activity on viral DNA replication, Sf9 cells were infected with AcMNPV-LacZGUS as described above. After removing the virus inoculums, the cells were washed once and cultured in TNMFH medium containing 20 μ M C75. At 18 and 24 h p.i., total DNA was extracted from infected cells and viral genomic DNA was quantified by real-time PCR as described above.

2.8 Western blot analysis

Infected Sf9 cells were lysed in NET buffer [20 mM Tris (pH7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 1.0% Nonidet P-40, 1mM EDTA] containing the complete protease inhibitor cocktail (Roche Applied Science). Cell lysates were separated on 10% reducing polyacrylamide gels and transferred to PVDF

membrane (Millipore) as described previously (Li and Blissard, 2008). AcMNPV GP64 and actin were separately detected with anti-GP64 (AcV5, Santa Cruz) and anti- β -actin (Abbkine) monoclonal antibodies. Immunoreactive proteins were visualized using alkaline phosphatase-conjugated anti-mouse IgG antibody and nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP, Promega).

3. Results

3.1 Up-regulation of the transcription of *fasn* upon AcMNPV infection

In a recent transcriptome analysis of AcMNPV-infected *Trichoplusia ni* cells (Tnms42), expression profiles were generated for host genes throughout the AcMNPV infection cycle (Chen et al., 2014). As a first step, we performed an analysis of the expression profile of host fatty acid synthase (Fig. 1A) in uninfected and AcMNPV-infected Tnms42 cells. Upon AcMNPV infection, the expression level of *fasn* was increased (>2-fold change in transcript abundance upon AcMNPV infection) at 1 h p.i. and slightly decreased by 6 h p.i. (Fig. 1A).

To determine the transcript levels of *fasn* in AcMNPV-infected Sf9 cells, we first identified the EST sequences of Sf9 *fasn* (from SPODOBASE) and used quantitative real-time PCR to measure *fasn* transcript levels from uninfected and infected Sf9 at various times post infection. As shown in Figure 1B, AcMNPV infection significantly up-regulated the transcript levels of *fasn* at 1-3 h p.i. Similar to observations in AcMNPV-infected Tnms42 cells, the transcript levels of *fasn* were slightly decreased at 6 h p.i. Combined, these transcript data suggest that the cellular FASN may play important roles in AcMNPV infection.

3.2 Effects of C75 on infectious AcMNPV production

To determine whether the activity of FASN is required for AcMNPV propagation, we examined the effects of C75, an irreversible inhibitor of FASN (Rendina and Cheng, 2005), on infectious AcMNPV production. First, to assess the potential general effects of C75 on the proliferation of Sf9 cells, the viability of cells treated with 5-50 μ M C75 was measured. As shown in Figure S1, the viability of Sf9 cells treated with 5-25 μ M C75 at different time points was similar to that of the control (DMSO-treated cells). However, treatment of Sf9 cells with 50 μ M C75 significantly reduced the viability of cells about 40-60%. These data suggest that C75 treatment at a lower concentration (\leq 25 μ M) did not result in a measurable negative effect on the viability of Sf9 cells.

To identify the requirement of FASN for productive AcMNPV infection, we infected Sf9 cells with AcMNPV-3mC virus, which expresses mCherry-tagged VP39, and then treated the cells with 5-25 μ M C75. At 24 h p.i., the number of infected cells were slightly decreased in treatment with 5 and 10 μ M C75 (Fig. 2A), and the infectious virus production in these two treatments was reduced about 4-5 fold (Fig. 2B). However, dramatically fewer cells showed evidence of infection with increasing concentrations of C75 (15-25 μ M) (Fig. 2A). The infectious virus titers were reduced 13, 104, and 113 fold when the cells were treated with 15, 20, and 25 μ M C75, respectively (Fig. 2B), suggesting a dose-dependent effect of C75 on BV production. One step growth curve analysis showed that the production of infectious AcMNPV budded virions was significantly decreased at different time points (24-96 h p.i.) in the presence of 20 μ M C75 (Fig. 3). It is worth to note that the decrease of virus infectivity was less at later in infection with only a 10 fold difference in virus yield at 96 h p.i.,

compared to that of about 100 fold at 24 h p.i. (Fig. 3). Together, these results suggest that functional FASN appears to be important for the efficient replication of AcMNPV.

3.3 Is FASN required for efficient entry of AcMNPV?

Since budded virions of AcMNPV enter host cells via clathrin-mediated endocytosis (Long et al., 2006), the inhibitory effects of C75 on infectious AcMNPV production could result from interfering with the entry step of virions. To address this possibility, we detected the efficiency of AcMNPV entry in the presence of 20 μ M C75. Sf9 cells were incubated with AcMNPV virions (AcMNPV-3mC) at 4°C for 1 h to permit virus adsorption at the cell surface. After removing the viral inoculum and washing the cells twice with cold medium, the temperature was raised to 27°C and cells incubated with fresh TNMFH medium containing 20 μ M C75 for 0.5, 1, and 1.5 h. The amount of virus that entered cells was quantified by real-time PCR measurements of viral genomic DNA. As showed in Figure 4, treatment with C75 at different times during virions entry did not significantly reduce internalized viral genomic DNA as compared with that of cells treated with DMSO only. To extend our observations, we determined infectious virus production in the presence of C75 at different times of infection: a) Sf9 cells were pre-treated with C75 for 1, 3, or 6 h, the drug was removed prior to infection. b) Sf9 cells were treated with C75 during the virus inoculation. After inoculation of the virus for 1 h, the virus and drug were both removed. c) Sf9 cells were infected with AcMNPV. At 1, 3, or 6 h p.i., the infected cells were treated with C75 for the rest time of infection. As shown in Figure 5, pre-treatment of Sf9 cells or treatment of cells during virus adsorption with 20 μ M C75 did not inhibit production of infectious budded

virions. In contrast, when C75 was added to the medium at 1, 3, or 6 h post infection, a significant reduction of infectious BV production (more than 100 fold) was observed (Fig. 5). Combined together, these data suggest that FASN is not required for efficient entry of budded virions of AcMNPV.

3.4 Effects of inhibition of FASN on viral gene expression and viral DNA synthesis

In a life cycle, the expression of AcMNPV genes during the life cycle can be divided to three distinct stages: early, late, and very late phases. The early genes are usually transcribed before viral DNA replication and rely on host RNA polymerase, whereas late and very late genes are transcribed by viral RNA polymerase and are dependent on viral genomic DNA replication (Rohrmann, 2013). To further identify the roles of FASN in AcMNPV infection, we examined the effects of C75 on viral gene expression and viral DNA replication. Sf9 cells were infected with a recombinant virus (AcMNPV-LacZGUS) that contains two reporter genes: LacZ and GUS, which are controlled by the AcMNPV *ie1* early/late promoter and *p6.9* late promoter, respectively (Li and Blissard, 2012). After removing the viral inoculum, the infected cells were treated with 20 μ M C75. At various times post infection (3-24 h p.i.), the infected cells were lysed and relative β -Gal and GUS activities were measured. As shown in Figure 6A, no significant difference of the activity of β -Gal was detected in C75-treated cells compared with that of cells treated with DMSO at 3 h p.i. However, at 6-24 h p.i., 2~6-fold reduction of the activity of β -Gal was observed in C75-treated cells. In contrast, in the presence of C75, the activity of GUS was reduced about 2 fold at 3-12 h p.i., whereas it was reduced more than 100 fold at 18 and 24 h p.i. when compared with the

controls (Fig. 6B). Western blot analysis showed that the expression of GP64, which is directed by its early/late promoter, was delayed and was significantly decreased (Fig. 6C). To determine whether viral DNA replication is dependent on FASN, the infected Sf9 cells were treated with 20 μ M C75 at 1 h p.i and the amount of viral genomic DNA was measured by quantitative real-time PCR. As shown in Figure 6D, AcMNPV propagated in C75-treated Sf9 cells showed about 100-fold and 25-fold reduction of viral DNA production at 18 and 24 h p.i., respectively. Taken together, these results suggest that the activity of cellular FASN is required for viral genes expression and viral DNA synthesis.

4. Discussion

In the infection cycle of AcMNPV, modification of viral proteins by palmitoylation (Roberts and Faulkner, 1989; Zhang et al., 2003), formation of large amounts of intranuclear microvesicles induced by viral core proteins (Hu et al., 2010; Shi et al., 2018; Yuan et al., 2011), and the remodeling of plasma and nuclear membranes to envelope progeny nucleocapsids (Fraser, 1986; Shi et al., 2015) are closely related with the cellular fatty acid synthesis pathway. Prior studies revealed that many host genes including those of ESCRT and SNARE systems are up-regulated immediately following virus inoculation (Chen et al., 2014; Guo et al., 2017). In AcMNPV-infected *Trichoplusia ni* Tmns42 cells (Chen et al., 2014) and *S. frugiperda* Sf9 cells, *fasn* transcripts were significantly increased at the early stage of infection (Fig. 1). In *de novo* fatty acid synthesis pathway, FASN is responsible for catalyzing the formation of palmitate, which is the key component for energy production, and formation of long-chain fatty acids and phospholipids formation (Jones and Infante, 2015). Because of the critical roles of FASN in cellular metabolism, we

asked whether FASN may also be necessary for productive AcMNPV infection. We found that inhibition of the activity of FASN in *S. frugiperda* Sf9 cells by using C75 (an irreversible inhibitor of FASN) (Rendina and Cheng, 2005) resulted in a dramatic reduction of infectious AcMNPV BV production. To understand how viral replication was constrained, we examined the roles of FASN in virus entry, viral genes expression, and viral genomic DNA replication.

The entry of budded virions of AcMNPV is dependent on clathrin-mediated endocytosis (Long et al., 2006). The acidification of endosomes during trafficking triggers the conformational change of the major viral envelope glycoprotein GP64 which mediates the fusion of the endosomal membrane and viral envelope to release the nucleocapsid into the cytosol (Blissard and Wenz, 1992; Kadlec et al., 2008; Li and Blissard, 2011). Prior studies indicated that AcMNPV virions internalization and transit within the endosome, and transport of nucleocapsids to the nucleus usually occur within 1 h after virus uptake (Hefferon et al., 1999; Ohkawa et al., 2010). Treatment of Sf9 cells with C75 during virus entry or prior to infection have no effect on virus internalization and infectious virus production (Fig. 4, 5), suggesting that FASN is not necessary for efficient entry of AcMNPV BV into Sf9 cells. The transcript level changes of *fasn* in AcMNPV-infected cells may be related to virus-induced host resistance response or requirement for FASN at later stages in the infection cycle.

In AcMNPV-infected cells, the viral genes expression and viral DNA synthesis are temporally regulated. The proteins encoded by viral early genes serve mainly in transcriptional regulation. Viral DNA synthesis is dependent on early genes expression, whereas the expression of late and very late genes

rely on viral DNA synthesis (Rohrmann, 2013). We observed that, at the early stage of infection (3-12 h p.i.), inhibition of FASN with C75 resulted in a moderate reduction or delayed expression of viral genes. However, at late stage of infection (18-24 h p.i.), dramatic reductions of viral gene expression and accumulation of viral DNA were observed. These results suggest that the late stage of AcMNPV infection, especially viral genomic DNA synthesis and late gene expression, is relatively more sensitive to FASN activity. The involvement of cellular FASN in efficient virus replication has been demonstrated for certain mammalian viruses, including vaccinia virus (Greseth and Traktman, 2014). Inactivation of FASN in vaccinia virus-infected cells moderately suppressed viral DNA replication and viral protein synthesis. However, the virus assembly efficiency was dramatically reduced in the presence of the inhibitors of FASN. It was found that palmitate, the primary product of the *de novo* fatty acid biosynthesis pathway, contributes to mainly ATP production that is required for viral protein and DNA synthesis, and the assembly of progeny virions (Greseth and Traktman, 2014). The energy requirement for AcMNPV infection is not yet clear. However, replication and incorporation of newly synthesized viral genomic DNA into nucleocapsids are both energy requirement processes (Rohrmann, 2013). Inactivation of FASN with C75 might directly reduce the efficiency of viral DNA generation and/or packaging, which in turn leads to the decrease of viral gene expression at the late stage of infection. Intriguingly, the viral core proteins, such as Ac75, Ac76, and Ac93, induced large amounts of intranuclear microvesicles formation and remodeling of the nuclear membrane (Hu et al., 2010; Shi et al., 2018; Yuan et al., 2011) should require elevated fatty acid synthesis to provide additional

phospholipids. Suppression of FASN activity might also result in the lack of intracellular phospholipids and disturb progeny nucleocapsids egress from the nuclear membrane and/or plasma membrane. Additionally, even though the palmitoylation of GP64 was found not to be required for GP64 synthesis, cell surface localization, and fusion activity (Zhang et al., 2003), modification of other viral proteins by palmitoylation has not been precisely characterized. Overall, the necessity of FASN in AcMNPV infection promotes us to propose that the *de novo* fatty acid biosynthesis pathway may be required for one or up to all of these processes related with AcMNPV infection. Further studies will address these possibilities and such studies should shed light on the complex interaction of baculoviruses and host cells.

Declarations of interest: none

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References

- Au, S., Pante, N., 2012. Nuclear transport of baculovirus: revealing the nuclear pore complex passage. *Journal of Structural Biology* 177(1), 90-98.
- Blissard, G.W., Wenz, J.R., 1992. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *Journal of Virology* 66(11), 6829-6835.
- Braunagel, S.C., Summers, M.D., 1994. *Autographa californica* nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: structural proteins, antigens, lipid and fatty acid profiles. *Virology* 202(1), 315-328.
- Chen, Y.R., Zhong, S., Fei, Z., Gao, S., Zhang, S., Li, Z., Wang, P., Blissard, G.W., 2014. Transcriptome responses of the host *Trichoplusia ni* to infection by the baculovirus *Autographa californica* multiple nucleopolyhedrovirus. *Journal of Virology* 88(23), 13781-13797.
- Chernomordik, L., Leikina, E., Cho, M.S., Zimmerberg, J., 1995. Control of baculovirus gp64-induced syncytium formation by membrane lipid composition. *Journal of Virology* 69(5), 3049-3058.
- Fraser, M., 1986. Ultrastructural observations of virion maturation in *Autographa californica* nuclear polyhedrosis virus infected *Spodoptera frugiperda* cell cultures. *Journal of Ultrastructure and Molecular Structure Research* 95, 189-195.
- Gaunt, E.R., Cheung, W., Richards, J.E., Lever, A., Desselberger, U., 2013. Inhibition of rotavirus replication by downregulation of fatty acid synthesis. *The Journal of General Virology* 94(Pt 6), 1310-1317.
- Greseth, M.D., Traktman, P., 2014. De novo fatty acid biosynthesis contributes significantly to establishment of a bioenergetically favorable environment for vaccinia virus infection. *PLoS Pathogens* 10(3), e1004021.
- Guo, Y., Yue, Q., Gao, J., Wang, Z., Chen, Y.R., Blissard, G.W., Liu, T.X., Li, Z., 2017. Roles of cellular NSF protein in entry and nuclear egress of budded virions of *Autographa californica* multiple nucleopolyhedrovirus. *Journal of Virology* 91(20), pii: e01111-17.
- Haase, S., Sciocco-Cap, A., Romanowski, V., 2015. Baculovirus insecticides in Latin America: historical overview, current status and future perspectives. *Viruses* 7(5), 2230-2267.
- Heaton, N.S., Perera, R., Berger, K.L., Khadka, S., Lacount, D.J., Kuhn, R.J., Randall, G., 2010. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. *Proceedings of the National Academy of Sciences of the United States of America* 107(40), 17345-17350.
- Hefferon, K.L., Oomens, A.G., Monsma, S.A., Finnerty, C.M., Blissard, G.W., 1999. Host cell receptor binding by baculovirus GP64 and kinetics of virion entry. *Virology* 258(2), 455-468.
- Hu, Z., Yuan, M., Wu, W., Liu, C., Yang, K., Pang, Y., 2010. *Autographa californica* multiple nucleopolyhedrovirus ac76 is involved in intranuclear microvesicle formation. *Journal of Virology* 84(15), 7437-7447.
- Jones, S.F., Infante, J.R., 2015. Molecular pathways: Fatty acid synthase. *Clinical Cancer Research* 21(24), 5434-5438.
- Kadlec, J., Loureiro, S., Abrescia, N.G., Stuart, D.I., Jones, I.M., 2008. The postfusion structure of baculovirus gp64 supports a unified view of viral fusion machines. *Nature Structural & Molecular Biology* 15(10), 1024-1030.

- Kulkarni, M.M., Ratcliff, A.N., Bhat, M., Alwarawrah, Y., Hughes, P., Arcos, J., Loisel, D., Torrelles, J.B., Funderburg, N.T., Haystead, T.A., Kwiek, J.J., 2017. Cellular fatty acid synthase is required for late stages of HIV-1 replication. *Retrovirology* 14(1), 45.
- Li, Z., Blissard, G.W., 2008. Functional analysis of the transmembrane (TM) domain of the *Autographa californica* multicapsid nucleopolyhedrovirus GP64 protein: substitution of heterologous TM domains. *Journal of Virology* 82(7), 3329-3341.
- Li, Z., Blissard, G.W., 2011. *Autographa californica* multiple nucleopolyhedrovirus GP64 protein: roles of histidine residues in triggering membrane fusion and fusion pore expansion. *Journal of Virology* 85(23), 12492-12504.
- Li, Z., Blissard, G.W., 2012. Cellular VPS4 is required for efficient entry and egress of budded virions of *Autographa californica* multiple nucleopolyhedrovirus. *Journal of Virology* 86(1), 459-472.
- Liu, H., Liu, J.Y., Wu, X., Zhang, J.T., 2010. Biochemistry, molecular biology, and pharmacology of fatty acid synthase, an emerging therapeutic target and diagnosis/prognosis marker. *International Journal of Biochemistry and Molecular Biology* 1(1), 69-89.
- Long, G., Pan, X., Kormelink, R., Vlaskovits, J.M., 2006. Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. *Journal of Virology* 80(17), 8830-8833.
- Marheineke, K., Grunewald, S., Christie, W., Reilander, H., 1998. Lipid composition of *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Tn) insect cells used for baculovirus infection. *FEBS Letters* 441(1), 49-52.
- Martin-Acebes, M.A., Blazquez, A.B., Jimenez de Oya, N., Escibano-Romero, E., Saiz, J.C., 2011. West Nile virus replication requires fatty acid synthesis but is independent on phosphatidylinositol-4-phosphate lipids. *PloS One* 6(9), e24970.
- Nasheri, N., Joyce, M., Rouleau, Y., Yang, P., Yao, S., Tyrrell, D.L., Pezacki, J.P., 2013. Modulation of fatty acid synthase enzyme activity and expression during hepatitis C virus replication. *Chemistry & Biology* 20(4), 570-582.
- Negre, V., Hotelier, T., Volkoff, A.N., Gimenez, S., Cousserans, F., Mita, K., Sabau, X., Rocher, J., Lopez-Ferber, M., d'Alencon, E., Audant, P., Sabourault, C., Bidegainberry, V., Hilliou, F., Fournier, P., 2006. SPODOBASE: an EST database for the lepidopteran crop pest *Spodoptera*. *BMC Bioinformatics* 7, 322.
- Ohkawa, T., Volkman, L.E., Welch, M.D., 2010. Actin-based motility drives baculovirus transit to the nucleus and cell surface. *The Journal of Cell Biology* 190(2), 187-195.
- Ohl, Y.M., Wang, Z., Kemble, G., Duke, G., 2015. Direct inhibition of cellular fatty acid synthase impairs replication of respiratory syncytial virus and other respiratory viruses. *PloS One* 10(12), e0144648.
- Okamura, H., Nio, Y., Akahori, Y., Kim, S., Watashi, K., Wakita, T., Hijikata, M., 2016. Fatty acid biosynthesis is involved in the production of hepatitis B virus particles. *Biochemical and Biophysical Research Communications* 475(1), 87-92.
- Perera, R., Riley, C., Isaac, G., Hopf-Jannasch, A.S., Moore, R.J., Weitz, K.W., Pasa-Tolic, L., Metz, T.O., Adamec, J., Kuhn, R.J., 2012. Dengue virus infection perturbs lipid homeostasis in infected mosquito cells. *PLoS Pathogens* 8(3), e1002584.
- Rendina, A.R., Cheng, D., 2005. Characterization of the inactivation of rat fatty acid synthase by C75:

- inhibition of partial reactions and protection by substrates. *The Biochemical Journal* 388(Pt 3), 895-903.
- Roberts, T.E., Faulkner, P., 1989. Fatty acid acylation of the 67K envelope glycoprotein of a baculovirus: *Autographa californica* nuclear polyhedrosis virus. *Virology* 172(1), 377-381.
- Rohrmann, G., 2013. *Baculovirus Molecular Biology: Third Edition* [Internet]. National Center for Biotechnology Information (US), Bethesda (MD).
- Shi, A., Hu, Z., Zuo, Y., Wang, Y., Wu, W., Yuan, M., Yang, K., 2018. *Autographa californica* multiple nucleopolyhedrovirus ac75 is required for the nuclear egress of nucleocapsids and intranuclear microvesicle formation. *Journal of Virology* 92(4), pii: e01509-17.
- Shi, Y., Li, K., Tang, P., Li, Y., Zhou, Q., Yang, K., Zhang, Q., 2015. Three-dimensional visualization of the *Autographa californica* multiple nucleopolyhedrovirus occlusion-derived virion envelopment process gives new clues as to its mechanism. *Virology* 476, 298-303.
- Spencer, C.M., Schafer, X.L., Moorman, N.J., Munger, J., 2011. Human cytomegalovirus induces the activity and expression of acetyl-coenzyme A carboxylase, a fatty acid biosynthetic enzyme whose inhibition attenuates viral replication. *Journal of Virology* 85(12), 5814-5824.
- Sun, X., 2015. History and current status of development and use of viral insecticides in China. *Viruses* 7(1), 306-319.
- van Oers, M.M., Pijlman, G.P., Vlak, J.M., 2015. Thirty years of baculovirus-insect cell protein expression: from dark horse to mainstream technology. *The Journal of General Virology* 96(Pt 1), 6-23.
- Yang, W., Hood, B.L., Chadwick, S.L., Liu, S., Watkins, S.C., Luo, G., Conrads, T.P., Wang, T., 2008. Fatty acid synthase is up-regulated during hepatitis C virus infection and regulates hepatitis C virus entry and production. *Hepatology* 48(5), 1396-1403.
- Yuan, M., Huang, Z., Wei, D., Hu, Z., Yang, K., Pang, Y., 2011. Identification of *Autographa californica* nucleopolyhedrovirus ac93 as a core gene and its requirement for intranuclear microvesicle formation and nuclear egress of nucleocapsids. *Journal of Virology* 85(22), 11664-11674.
- Yue, Q., Yu, Q., Yang, Q., Xu, Y., Guo, Y., Blissard, G.W., Li, Z., 2018. Distinct roles of cellular ESCRT-I and ESCRT-III proteins in efficient entry and egress of budded virions of *Autographa californica* multiple nucleopolyhedrovirus. *Journal of Virology* 92(1), pii: e1636-17.
- Zhang, S.X., Han, Y., Blissard, G.W., 2003. Palmitoylation of the *Autographa californica* multicapsid nucleopolyhedrovirus envelope glycoprotein GP64: mapping, functional studies, and lipid rafts. *Journal of Virology* 77(11), 6265-6273.

Figure legends

Figure 1

Transcript profiles of *fasn* in AcMNPV-infected cells. (A) Transcription data for *fasn* (RPKM, reads per kilobase per million reads) was retrieved from a transcriptome analysis of control (C, uninfected) or AcMNPV-infected Tnms42 cells at various time points post infection (1-48 h). Error bars represent standard deviations from the mean of three replicates. (B) Transcript levels of *fasn* in uninfected (C, control) or AcMNPV-infected Sf9 cells at various time points post infection (1-48 h) were measured by quantitative real-time PCR. The control or uninfected cells were collected at the same time as other cells were infected. Error bars represent standard deviations from the mean of three replicates.

Figure 1

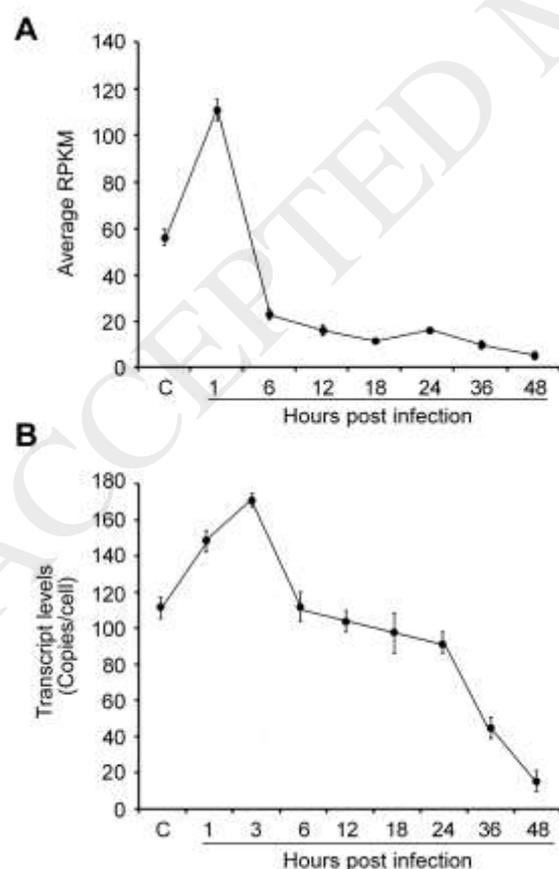
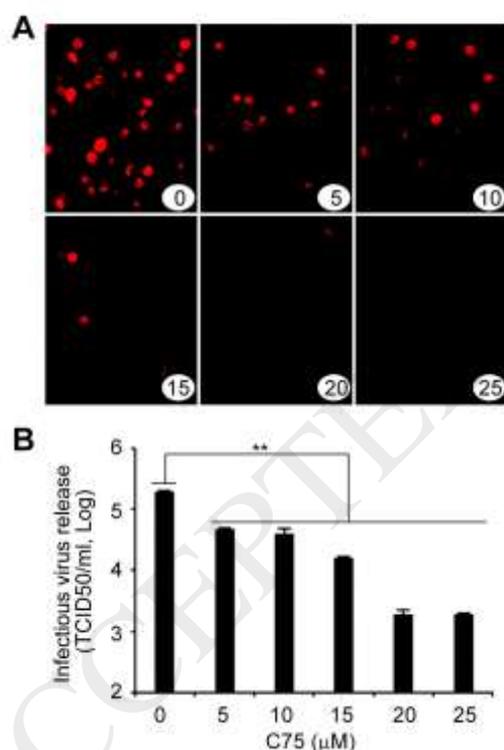


Figure 2

Dose-dependent effect of C75 on AcMNPV production. Sf9 cells were infected with AcMNPV-3mC at an MOI of 5. Following inoculation of the virus, the indicated concentrations of C75 (5-25 μ M) were added to the cells. A control of DMSO only was also included. At 24 h post infection, the infected cells indicated by mCherry-tagged viral VP39 protein were visualized by epifluorescence microscopy (A) and the infectious virus production was measured by TCID₅₀ assay (B). Error bars represent standard deviations from the mean of three replicates. **, $P < 0.005$ (by unpaired *t* test).

Figure 2**Figure 3**

One-step growth curves of AcMNPV in the presence of C75. Sf9 cells were infected with wild-type AcMNPV at an MOI of 5. Following inoculation of the virus, the cells were treated with 20 μ M C75 or DMSO only. At different time

points post infection, the infectious virus production was determined by TCID₅₀ assay. Error bars represent standard deviations from the mean of three replicates.

Figure 3

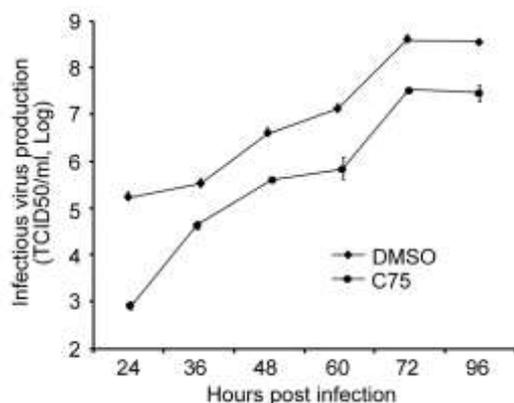
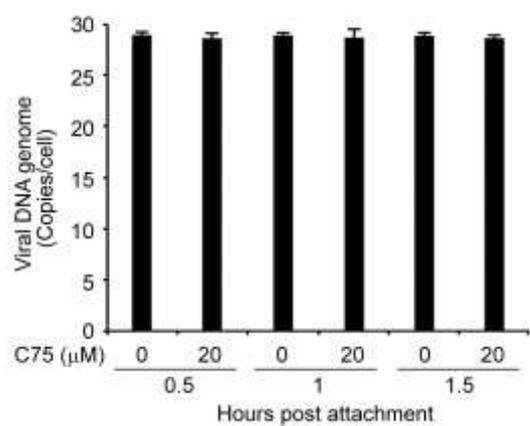


Figure 4

Effects of C75 on AcMNPV entry. Sf9 cells were infected with wild-type AcMNPV (MOI=5) at 4°C for 1 h. After the attachment of the virus, the cells were incubated with fresh TNMFH medium containing 20μM C75 and the culture temperature was raised to 27°C for 0.5, 1 or 1.5 h. The internalization of entering virions was analyzed by quantitative real-time PCR analysis of viral DNA from cell lysates. Error bars represent standard deviations from the mean of three replicates.

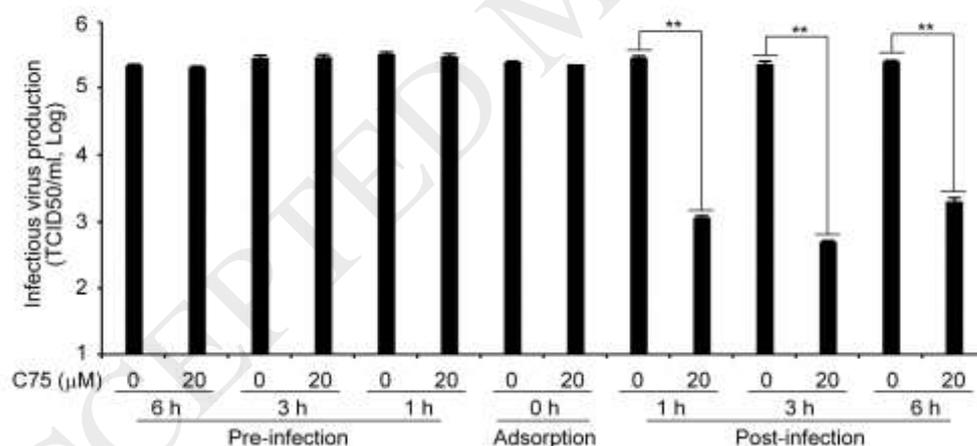
Figure 4



ACCEPTED MANUSCRIPT

Figure 5

Effects of different time treatment with C75 on production of infectious budded virions. Sf9 cells were infected with wild-type AcMNPV at an MOI of 5. Three set of Sf9 cells were treated with 20 μ M C75: a) Sf9 cells were treated with C75 at 1, 3, or 6 h prior to infection, then the drug was removed and the cells were infected. b) Sf9 cells were treated with C75 during the virus adsorption. After 1 h of adsorption, the virus and drug were both removed. c) At 1, 3, or 6 h post infection, Sf9 cells were treated with C75 and the drug was present during the rest time of infection. At 24 h p.i., the infectious virus production for all of the treated cells was determined by TCID₅₀ assay. Error bars represent the standard deviations from the mean of three replicates. **, $P < 0.005$ (by unpaired *t* test).

Figure 5**Figure 6**

Effects of C75 on viral gene expression and viral DNA replication. Sf9 cells were infected with AcMNPV-LacZGUS at an MOI of 5. After inoculation of virus, the cells were treated with 20 μ M C75 or DMSO. At different time points post infection, viral gene expression and viral DNA replication were evaluated. (A, B) Viral early and late gene expression was separately monitored indirectly

by analyzing AcMNPV *ie1* early/late promoter driven β -Gal activity (A) and *p6.9* late promoter driven GUS activity (B). Values represent the means from triplicate treatments and are normalized to that of cells treated with DMSO at 24 h p.i. Error bars represent the standard deviations from the means. (C) Western blotting analysis of the expression of GP64. The anti- β -actin blot served as a loading control. C, control (uninfected cells). (D) Accumulation of viral DNA was measured by real-time PCR analysis of viral genomic DNA extracted from infected cells at 18 and 24 h p.i. Error bars represent standard deviations from the mean of three replicates. *, $P < 0.05$; **, $P < 0.005$ (by unpaired *t* test).

Figure 6

