



Published in final edited form as:

*Virology*. 2007 June 5; 362(2): 421–427. doi:10.1016/j.virol.2007.01.003.

## A host cell membrane protein, golgin-97, is essential for poxvirus morphogenesis

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### Abstract

Acquisition of the membrane and genome encapsidation is an important step in the replication of enveloped viruses. The biogenesis of the poxviral primary membrane and the core as well as the mechanisms of their maturation are poorly understood. Using RNA interference approach we demonstrate that a cellular trans-Golgi network membrane protein, golgin-97 is essential for virus replication. Analysis of the virion morphology in the cells depleted of golgin-97 shows that the protein is required for the virus morphogenesis and, in particular, for the formation of the first infectious virus form, mature virus, but not its precursor, immature virus. This suggests that golgin-97 may be involved in the maturation of the virus core and, potentially, the virus membrane.

### Keywords

poxvirus; vaccinia; IMV; MV; golgin-97; TGN; RNAi

### Introduction

Many animal viruses possess a lipid envelope derived from cellular membranes that protects the nucleocapsid and aids virus entry to the host cell. Poxviruses, the largest and most complex of the viruses sequentially acquire two envelopes during the morphogenesis and produce four structurally and functionally distinct infectious virion particles. The primary membrane is initially acquired during assembly of the un Infectious immature virus (IV) (Condit, Moussatche, and Traktman, 2006; Smith and Law, 2004). Maturation of this membrane accompanying by the virus core assembly and/or proteolytic processing and condensation transforms IV into the first infectious virus form, the intracellular mature virus or mature virus (MV) according to recently introduced new terminology (Byrd and Hruby, 2006; Condit, Moussatche, and Traktman, 2006; Moss, 2006; Smith and Law, 2004). Wrapping of the IMV by the secondary membrane leads to formation of the other three virion types, intracellular enveloped/wrapped virus (WV), cell-associated enveloped/extracellular virus (CEV), and extracellular enveloped/unattached extracellular virus (EV) (Condit, Moussatche, and Traktman, 2006; Moss, 2006; Smith and Law, 2004). It is generally accepted that the secondary membrane derives from budding of MV particles through the trans-Golgi network (TGN) and/or early endosomes (Tooze et al., 1993; van Eijl et al., 2002), whereas the source of the MV membrane and the mechanisms of its biogenesis are actively debated. At the present time there

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are three hypotheses for the mechanisms of the primary membrane generation. First, since the composition of the membrane seems to be different from that of the cellular membranes, researchers hypothesized that the primary membrane is synthesized *de novo* during viral infection (Dales and Mosbach, 1968). Second, based on the experiments showing accumulation of MV envelope proteins at the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) (Krijnse-Locker et al., 1996; Salmons et al., 1997) as well as labeling of the membranes surrounding MV precursor, IV particles with markers from the same compartment (Sodeik et al., 1993), it was suggested that the membrane originates from the ERGIC. In order to test this hypothesis, a dominant-negative mutant of Sar1, a small GTPase involved in coatamer protein (COP) II mediated transport from the ER to the ERGIC and other post-ER compartments was transiently expressed in vaccinia virus (VV)-infected cells (Husain and Moss, 2003). Although overexpression of the mutant protein was able to block protein transport from the ER, it did not interfere with IV and MV assembly indicating that the primary membrane may originate in the ER compartment itself. Further investigation of this problem has demonstrated that a pathway between the ER and assembling primary membranes exists at least for one of the viral membrane proteins, A9L (Husain, Weisberg, and Moss, 2006). All attempts to detect either ER or ERGIC marker proteins in purified MV particles and confirm one of the models have been unsuccessful (Krauss et al., 2002). Recently, using VV and cowpox virus as model organisms we have demonstrated that a host protein residing at the TGN membrane, golgin-97 (97kDa) becomes associated with the insoluble virion core proteins and incorporates into MV particles (Alzhanova and Hruby, 2006). These findings were surprising and unexpected because previous experiments with brefeldin A have shown that intact Golgi complex and COPI mediated transport pathway from the Golgi to the ER is not required for MV assembly (Ulaeto, Grosenbach, and Hruby, 1995). Consistent with its ability to assume a coiled-coil stick-like conformation, golgin-97 seems to penetrate MV envelope and protrude from the virion surface. In the infected cell, golgin-97 was shown to re-locate to the virus replication and assembly factories between 6 and 24 hours post infection (hpi) (Alzhanova and Hruby, 2006). These data led to a new hypothesis, based on which we proposed that as a membrane-associated protein, golgin-97 may play a role in MV membrane biogenesis by facilitating delivery of the TGN membranes and, possibly, associated protein factors required for its maturation to the sites of virion assembly.

In the reported work, we use a RNA interference approach (RNAi) in order to test our hypothesis and investigate the role of golgin-97 protein in VV replication. Our results demonstrate that depletion of the protein significantly affected virus replication. Electron microscopic analysis revealed that the protein is essential for virus morphogenesis at the IV maturation stage, but not for acquisition of the primary membrane surrounding IV particles.

## Results and Discussion

To study the potential role of golgin-97 in VV morphogenesis and replication we employed an RNAi approach to suppress golgin-97 expression inside the infected cell. To induce RNAi we tested two double-stranded (ds) oligoribonucleotides, dsRNA-1 and dsRNA-2 with non-overlapping target sites within golgin-97 mRNA (Fig 1A). In order to analyze RNAi dose response and optimize the conditions, each dsRNA was titrated. As a negative control for potential dsRNA-induced side effects we used double-stranded oligoribonucleotides with minimized sequence homology to vertebrate transcripts. Fig 1B demonstrates that golgin-97 expression was significantly repressed by both dsRNAs at relatively low concentrations. Decreasing protein levels corresponded well with increasing amounts of each dsRNA and reached a minimum at 30 pmol (10%) and 20 pmol (18%) for dsRNA-1 and dsRNA-2, respectively (Fig 1B,C). Anti-actin antibodies used as a control for non-specific RNAi effect on expression of unrelated proteins showed that inhibition of golgin-97 expression did not interfere with actin accumulation (Fig 1C). Confirming the immunoblotting results,

immunofluorescence experiments (Fig 2, supplementary Fig S1, S2) showed striking difference in levels of golgin-97 accumulation and redistribution between the negative RNAi control and each dsRNA.

To test whether golgin-97 is required for VV replication, HeLa cells were transfected with either dsRNA-1 or dsRNA-2 and infected with VV Western Reserve (WR) strain at a multiplicity of infection (MOI) of 5.0 plaque forming units (pfu) per cell. Surprisingly, VV replication was significantly affected by both dsRNAs. Reduction in virus titers estimated as 85% (dsRNA-1) and 73% (dsRNA-2) correlated with the loss of golgin-97 expression (Table 1) implying that the protein is essential for virus replication.

To decipher the inhibitory effects of golgin-97 depletion on VV replication the infected cells were analyzed by immunofluorescence. Repression of the protein expression did not appear to interfere with either virus entry into the cell or its ability to establish replication factories (Fig 2, supplementary Fig S1, S2). Golgin-97 accumulated normally inside the factories in the presence of the negative RNAi control and as expected was absent in dsRNA-1 and dsRNA-2 treated samples (Fig 2, supplementary Fig S1, S2). These results suggested that golgin-97 may act at the late steps of virus infection such as virion assembly/maturation or virion translocation within the infected cell. This interpretation would be consistent with the fact that golgin-97 is a membrane protein (Barr, 1999; Gleeson et al., 2004; Munro and Nichols, 1999) and that it was shown to play a role in exocytosis, the pathway used by many enveloped viruses to exit the cell (Lu, Tai, and Hong, 2004). To test this hypothesis we examined virion morphology at 24 hpi by electron microscopy. In the presence of the negative RNAi control, virion assembly and maturation were not affected (Fig 3A,D). In contrast, depletion of golgin-97 resulted in the loss of MV particles and dramatic increase in the number of IVs (Fig 3B, C, E-I). To rule out the possibility of a delay in virus morphogenesis, we compared the data of virus replication assays at 24hpi and 48hpi. There was no significant difference or noticeable increase in relative efficiency of virus replication for 48hpi-samples (data not shown). Interestingly, some of the examined cells also contained variable amounts of IV with nucleoid (IVn), an intermediate form in IV maturation stage that normally can be seen at early hours in infection cycle (Fig 3B, E, G-I) and abnormal looking particles that are likely to be an intermediate between IVn and MV (Fig 3E,F,I; IVn'). This observation could be explained by incomplete suppression of golgin-97 expression and indicate that the protein may act at IVn formation, although we can not exclude that it may be important for the next stage, IVn-MV as well.

Based on the data described here and the results of our previous experiments (Alzhanova and Hruby, 2006) we propose a model of the MV biogenesis (Fig 4) and suggest a host cell protein, golgin-97 as a key player in this process. Although the mechanisms and the functions of the protein are yet to be determined, one of the possible scenarios may be that when attached to the TGN membrane at the C-terminus, golgin-97 delivers its fragments along with associated protein factors required for maturation of the virus core and, potentially, virus membrane to the virion assembly sites upon its relocation inside the factories. Supporting this hypothesis, a processed form of transiently expressed C-terminal but not N-terminal FLAG: golgin-97 fusion protein was detected inside the virions (Alzhanova and Hruby, 2006). In this process, golgin-97 is likely to associate with the proteins comprising insoluble core fraction and incorporate into IVn particles while they are being assembled. Alternatively, golgin-97 may be attached to the nascent viral membranes via a viral or cellular protein co-factors in a manner similar to its association with the TGN membrane (Lu and Hong, 2003; Panic et al., 2003; Setty et al., 2003; Wu et al., 2004) and become passively incorporated during IV/IVn assembly. In this case, the protein may simply play a role of a structural protein: its packaging may contribute to the formation and/or stabilization of the condensed core structure. In addition to this, the surface exposed part of the protein may be involved in the transportation of the assembled MV particles to the TGN compartment where they acquire the secondary membrane and mature

into WV. The latter would support the existing hypothesis about the TGN as the origin of the secondary membrane. Further investigation of golgin-97 functions in virus morphogenesis and the experimental verification of the proposed model will undoubtedly provide new insights on the mechanisms of host-pathogen interactions, revealing yet another example of how viruses exploit cellular proteins and pre-existing pathways to serve their needs. As to continuous efforts in developing smallpox vaccines and discovery of anti-poxvirus drugs, these data have identified new potential targets, virus proteins that facilitate golgin-97 delivery inside the virus factories and its incorporation into the virions, as well as golgin-97 itself.

## Materials and Methods

### Cells and viruses

HeLa cells were grown in minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). Vaccinia virus Western Reserve (WR) strain was propagated and purified as described previously (Hruby, Guarino, and Kates, 1979).

### Double-stranded oligoribonucleotides

Stealth siRNA 25bp-long duplex oligoribonucleotides targeting golgin-97 mRNA sequence, GOLGA1-HSS104275 (5'-AAGAGCUGUUGUCUGGUAUCCUCCA-3') and GOLGA1-HSS104276 (5'-UCUCCAGGGUGUUA AUGCCUAGC-3') referenced as dsRNA-1 and dsRNA-2, respectively, were purchased from Invitrogen. As RNAi negative control, we have used Stealth RNAi Negative control Duplex oligoribonucleotide (Invitrogen) with medium GC content (48%).

### Antibodies

Anti-human golgin-97 mouse monoclonal antibodies and rabbit anti-actin (AA20-33) antibodies were purchased from Invitrogen and Sigma (Sigma-Aldrich, Inc., Saint Louis, MO), respectively. Rabbit polyclonal anti-I3L antibodies (5) were kindly provided by J. Krijnse-Locker (EMBL, Heidelberg, Germany).

### RNAi induction and virus replication assay

HeLa cells were plated on glass coverslips in 6 well plates at 20% confluency. The next day, the cells were transfected with double-stranded oligoribonucleotides using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. For RNAi dose response experiment double-stranded oligoribonucleotide concentrations were 5 pmol, 10 pmol, 20 pmol, 30 pmol, and 40pmol. For all other experiments, the cells were transfected with 30 pmol of a double-stranded oligoribonucleotide. After 72 h incubation, the cells were either harvested and processed for immunoblot analysis or infected with VV WR strain at a multiplicity of infection (MOI) of 5.0 plaque forming units (pfu) per cell and incubated for 48 h. Titration of the virus was carried out according to previously described protocol (Hruby, Guarino, and Kates, 1979). The infected cells were harvested and centrifuged at  $700 \times g$ ,  $4^{\circ}\text{C}$ . The pellet was resuspended in phosphate-buffered saline (PBS) and subjected to three cycles of freeze/thaw in liquid nitrogen and  $37^{\circ}\text{C}$  water bath. The samples were titrated on BSC-40 cells and stained with 0.1% crystal violet (Sigma-Aldrich, Inc.) in 30% ethanol.

### Immunofluorescence staining

HeLa cells were plated on glass coverslips in 24 well plates at 20% confluency. The next day, the cells were transfected with 12 pmol of the double-stranded oligoribonucleotide using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. The cells were infected with vaccinia virus strain WR at a MOI of 5.0 pfu/cell after 72 h incubation. At the 24hpi, the

cells were fixed with 100% methanol for 10 min. The coverslips were washed three times with PBS, blocked in P- bovine serum albumin (BSA) (PBS-2% BSA, pH 7.4) for 30 min and incubated with primary anti-I3L (1:2000) and anti-golgin-97 (1µg/ml) diluted in P-BSA for 1 h. The cells were washed three times with PBS and once with P-BSA and then incubated with anti-rabbit (Southern Biotechnology Associates, Inc, Birmingham, AL) and anti-mouse (Invitrogen) antibodies conjugated to TRITC and Alexa Fluor 488, respectively. The coverslips were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen) and analyzed with Zeiss LSM 510 Meta confocal laser scanning microscope. The channels were collected in multi track mode. Alternatively, the cells were examined at 1000x magnification using a Leika fluorescence microscope. The images were collected with SPOT digital camera system (Diagnostics Instruments, Inc., Sterling Heights, MI).

### Electron microscopy

HeLa were plated at 6-well plates at 20% confluency. The next day, the cells were transfected with either Negative RNAi Control or dsRNA-1 or dsRNA-2 as described above. The cells were infected with VV WR at a MOI of 5.0 after 72 h incubation. At 24 hpi, the cell were scraped from the plates, resuspended in culture medium and pelleted by centrifugation at  $700 \times g$ ,  $4^{\circ}C$ . The cells were fixed in 1% paraformaldehyde, 0.5% glutaraldehyde, 200mM cacodylate buffer (pH 7.4) for 4h. Embedding in LR White resin and sectioning was performed by personnel of the Oregon State University EM laboratory. The thin sections were placed on uncoated gold 300 mesh grids and stained with lead citrate and uranyl acetate. The samples were examined on a Philips EM 300 electron microscope.

### Immunoblot analysis

Harvested cells were pelleted by centrifugation at  $700 \times g$ ,  $4^{\circ}C$  and resuspended in 10mM Tris (pH 8.0). The cells were lysed by freeze-thawing and cleared by centrifugation at  $1500 \times g$ ,  $4^{\circ}C$ . The samples were normalized based on measured OD  $A_{280}$ . Proteins were separated by sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF-membrane (Immobilon-P<sup>SQ</sup>, Millipore, Billerica, MA). The membrane was blocked in 3% gelatin in TTBS (0.05% Tween 20– 150mM NaCl - 50mM TrisHCl, pH 7.4) and incubated with primary anti-golgin-97 (1 µg/ml) or anti-actin antibodies (1:1000) in 1% gelatin - TTBS overnight. The membrane was washed three times in TTBS and incubated with secondary anti-mouse (Invitrogen) or anti-rabbit (Promega, San Luis Obispo, CA, USA) antibodies conjugated to horse reddish peroxidase (HRP). The proteins were detected with a chemiluminescence kit (West-Pico; Peirce Biotechnology, Inc., Rockford, IL).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

We thank Jacomine Krijnse-Locker (EMBL, Heidelberg, Germany) for providing anti-I3L-antibodies, Michael Nesson (Electron Microscopy lab, Oregon State University) for assistance with electron microscopy, and Cliff Gagnier for help with virus purification. This study was supported by NIH grant number AI21335 and in part by NIH grant number 1S10RR107903-01. We would like to acknowledge the Confocal Microscopy Facility of the Center for Gene Research and Biotechnology and the Environmental and Health Sciences Center at Oregon State University.

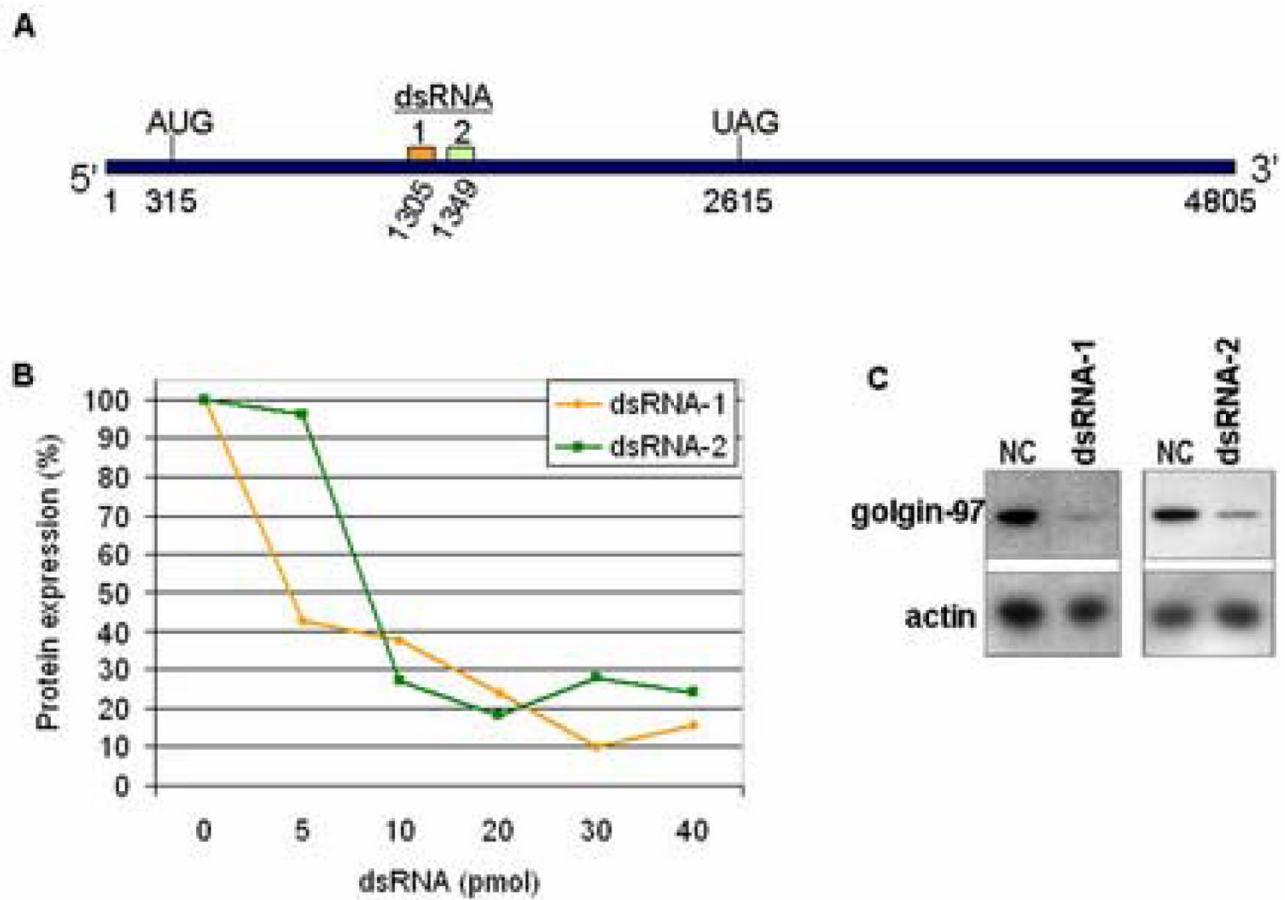
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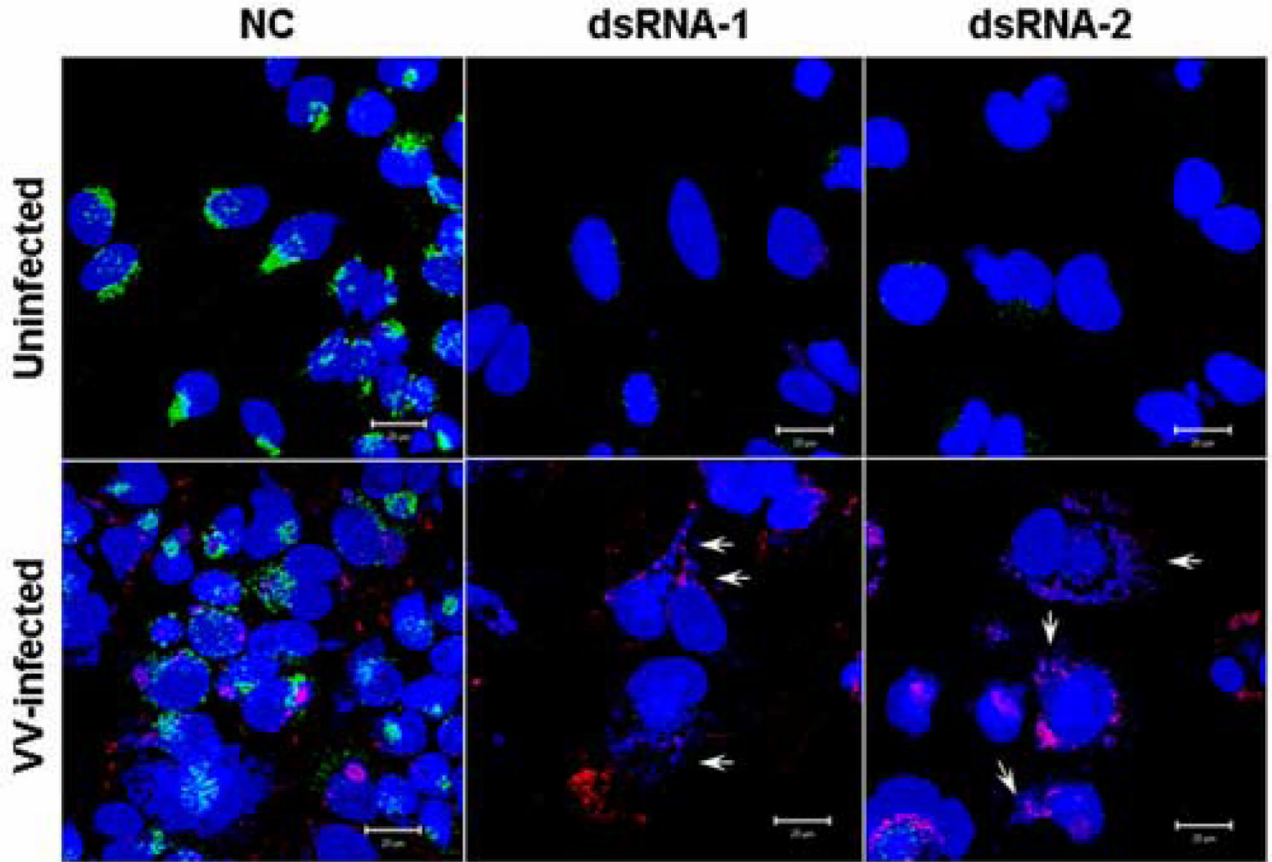
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**Fig 1.**

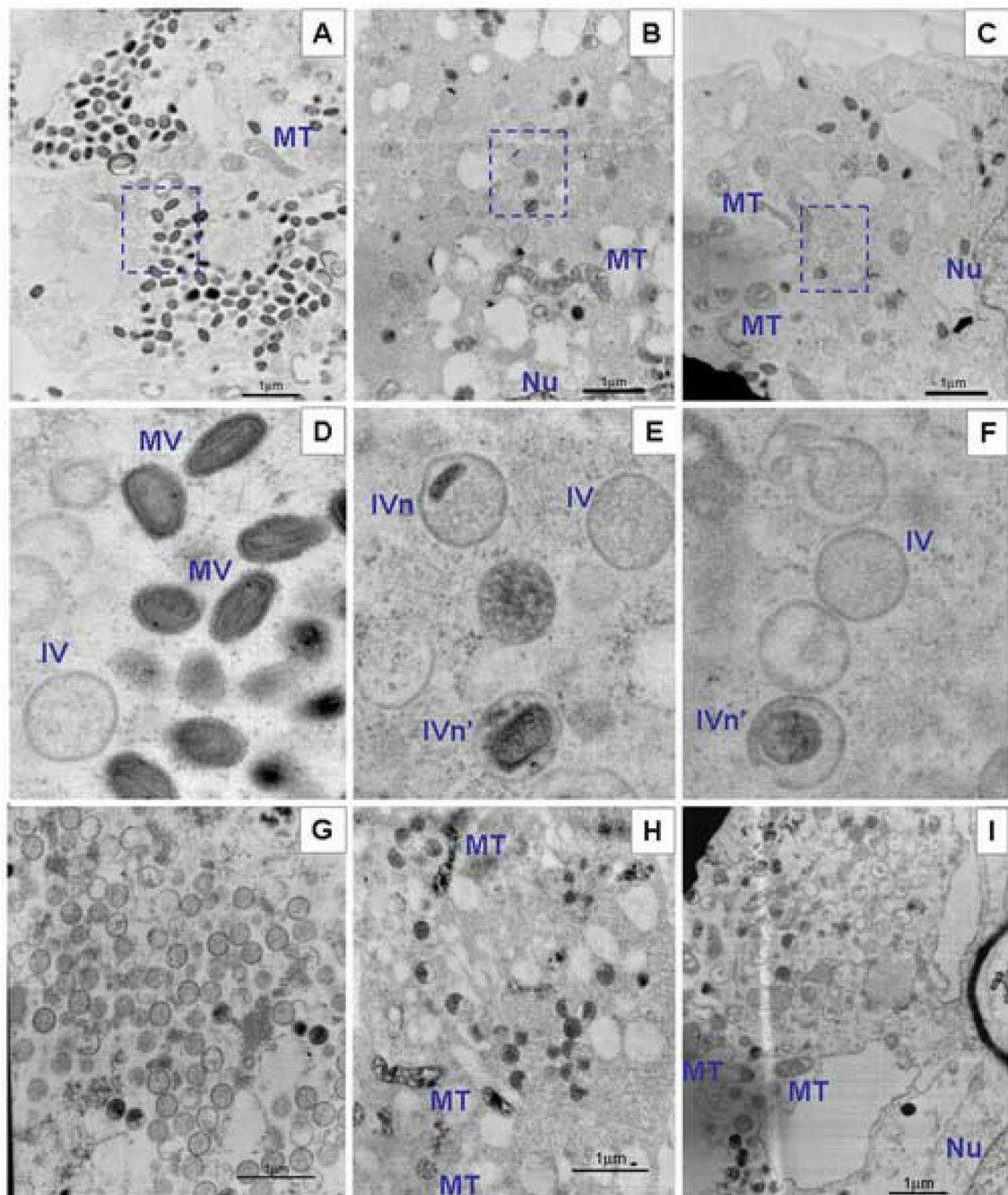
(A) dsRNA-1 and dsRNA-2 target sites within golgin-97 mRNA. (B) RNAi dose response curve. HeLa cells were transfected with indicated amounts of dsRNA-1, dsRNA-2, or the negative RNAi control. The relative levels of golgin-97 expression, “dsRNA-1” or “dsRNA-2” versus “Negative RNAi control” were evaluated by immunoblot analysis. (C) Immunoblot analysis of golgin-97 expression in the presence of golgin-97 targeting dsRNAs. Normalized samples transfected with 30 pmol of dsRNA-1 and the negative RNAi control (“NC”) or 20 pmol of dsRNA-2 and the negative RNAi control (“NC”) were separated on SDS-PAGE, blotted, and incubated either with anti-golgin-97 antibodies or anti-actin antibodies used as a control for non-specific RNAi effect on expression of unrelated proteins.





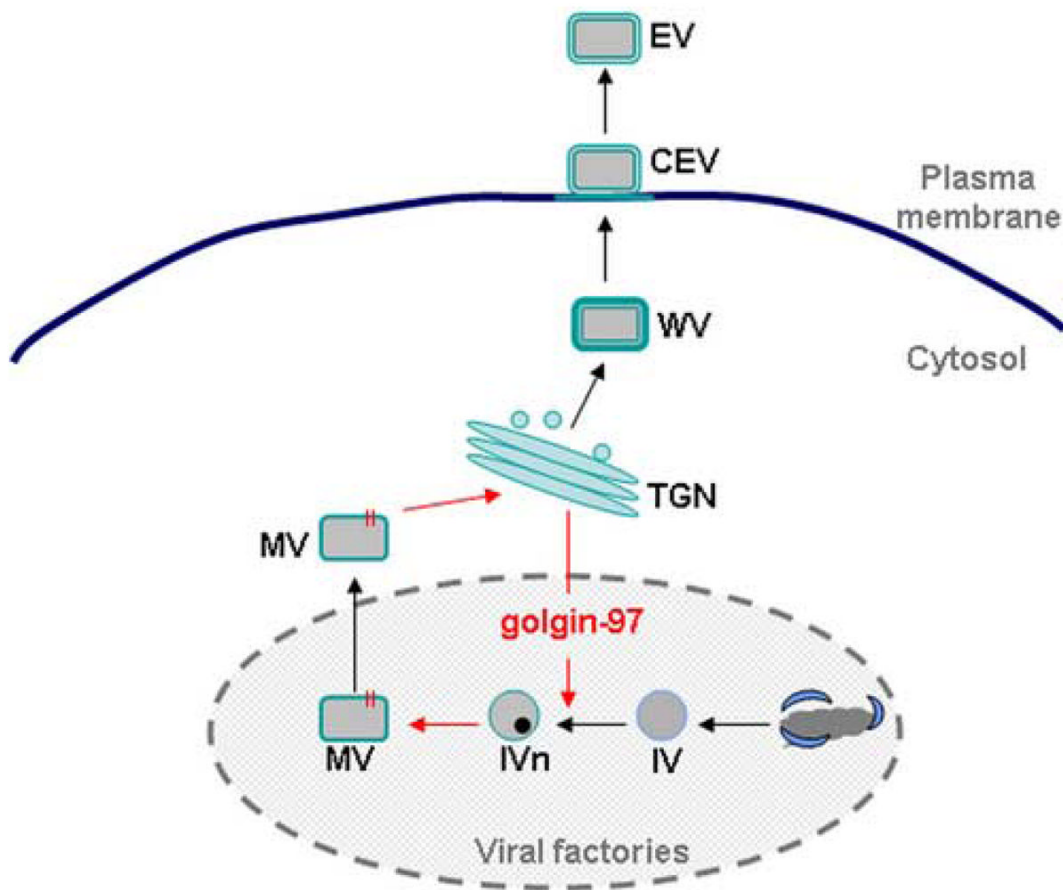
**Fig 2.**

VV replication in golgin-97 depleted cells by confocal laser scanning microscopy. HeLa cells transfected with dsRNA-1, dsRNA-2 or negative RNAi control (“NC”) were infected with VV WR at a MOI of 5.0 pfu/cell and fixed at 24 hpi. The cells were stained with primary antibodies to golgin-97 and VV protein I3L used as a marker for infected cells and secondary antibodies conjugated to Alexa fluor 488 (green channel) and TRITC (red channel), respectively. Host and viral DNA were stained with DAPI (blue channel). All panels show merged 3-D images. The size of each bar is 20  $\mu$ m. White arrows show viral factories organized in the absence of golgin-97.



**Fig 3.**

VV morphogenesis in golgin-97 depleted cells by electron microscopy of ultrathin sections. HeLa cells transfected with negative RNAi control (A and D), dsRNA-1 (B, E, G-I) or dsRNA-2 (C and F) were infected with VV WR at an MOI of 5.0 pfu/cell. At 24 hpi the cells were fixed and processed for electron microscopy analysis. MT and Nu refer to mitochondria and nuclei, respectively. Immature virions, immature virions with nucleoid, abnormal virions (IVn-MV stage), and mature virions are labeled as IV, IVn, IVn', and MV, respectively. The size of each bar is 1 μm.



**Fig. 4.** Working model. During poxvirus infection, a host cell protein, golgin-97 translocates into the viral factories. Being anchored to the TGN membrane, the protein may transport the membrane fragments and associated protein factors important for virus maturation to the sites of virion assembly. Via its association with viral core proteins, golgin-97 incorporates into the virion and aids IV<sub>n</sub>/MV assembly. Alternatively, the protein may be attached to the nascent viral membrane via a viral or cellular protein cofactors and incorporate into virions during IV/IV<sub>n</sub> assembly, in this case, playing a role of a structural protein only. In addition to this, being exposed at the virion surface golgin-97 may traffic assembled IMV particles to the TGN compartment where they become wrapped by the secondary membrane.

Relative reduction in golgin-97 expression and virus replication efficiency in the presence of dsRNA-1 and dsRNA-2. HeLa cells transfected with dsRNA-1, dsRNA-2 or negative RNAi control were infected with VV WR at an MOI of 5.0 pfu/cell. At 48 hpi the progeny virus was titrated by plaque assay.

**Table 1**

Target site (nt)	Relative reduction in protein expression (%)	Efficiency of virus replication (pfu/ml)		Relative reduction in virus replication (%)	p-value
		golgin-97 dsRNA	Negative RNAi control		
dsRNA-1 1305-1329	90	$4.79 \times 10^8 \pm 1.89 \times 10^8$	$3.30 \times 10^9 \pm 1.44 \times 10^9$	85	$0.5 \times 10^{-7}$
dsRNA-2 1349-1373	82	$9.50 \times 10^8 \pm 4.50 \times 10^8$	$3.51 \times 10^9 \pm 1.94 \times 10^9$	73	$0.3 \times 10^{-7}$