Identification of cellular genes involved in baculovirus GP64 trafficking to the plasma membrane

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Abstract:

The baculovirus envelope protein GP64 is an essential component of the budded virus and is necessary for efficient virion assembly. Little is known regarding intracellular trafficking of GP64 to the plasma membrane, where it is incorporated into budding virions during egress. To identify host proteins and potential cellular trafficking pathways that are involved in delivery of GP64 to the plasma membrane, we developed and characterized a stable *Drosophila* cell line that inducibly expresses the AcMNPV GP64 protein, and used that cell line in combination with a targeted RNAi screen of vesicular protein trafficking pathway genes. Of the 37 initial hits from the screen, we validated and examined six host genes that were important for trafficking of GP64 to the cell surface. Validated hits included Rab GTPases *Rab1* and *Rab4; Clathrin heavy chain* and clathrin adaptor proteins *AP-1-2β* and *AP-2μ*; and *Snap29*. Two gene knockdowns (*Rab5*, *Exo84*) caused substantial increases (up to 2.5-fold) of GP64 on the plasma membrane. We found that a small amount of GP64 is released from cells in exosomes, and that some portion of cell surface GP64 is endocytosed, suggesting that recycling helps to maintain GP64 at the cell surface.

Importance:

While much is known regarding trafficking of viral envelope proteins in mammalian cells, little is known about this process in insect cells. To begin to understand which factors and pathways are needed for trafficking of insect virus envelope proteins, we engineered a *Drosophila melanogaster* cell line and implemented an RNAi screen to identify cellular proteins that aid transport of the model baculovirus envelope protein (GP64) to the cell surface. For this we developed an experimental system that leverages the large array of

tools available for *Drosophila*, and performed a targeted RNAi screen to identify cellular proteins involved in GP64 trafficking to the cell surface. Since viral envelope proteins are often critical for production of infectious progeny virions, these studies lay the foundation for understanding how either pathogenic insect viruses (baculoviruses) or insect-vectored viruses (*e.g.* flaviviruses, alphaviruses) egress from cells in tissues such as the midgut, to enable systemic virus infection.

Introduction:

For enveloped viruses, viral envelope proteins are typically essential for virus entry, playing roles in receptor recognition, binding, and membrane fusion. They may also play critical roles in assembly and production of infectious progeny virions. Virus assembly is a complex process that varies dramatically among different virus groups, depending on the subcellular replication site and strategy utilized for assembly and egress. During replication and assembly, cellular protein transport pathways are critical for delivering viral components to the appropriate subcellular compartments or locations where processes such as budding and egress occur (1). This is especially true for viral envelope proteins, which must associate with newly assembled nucleocapsids and/or other viral and cellular factors during the assembly process. Because different viruses have an assortment of replication and release strategies, the transport of viral envelope proteins to the appropriate sites of viral particle assembly and egress can involve a variety of vesicular protein transport pathways. The virus *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) has a well-studied envelope protein, GP64, that is critical for virion entry and is important for the production of the budded virus (BV)

95 morphotype (2). Production of baculovirus BVs begins when AcMNPV capsids package
96 the viral genome in the nucleus, and the resulting nucleocapsids are transported out of
97 the nucleus by a process that involves actin-mediated propulsion (3). Nucleocapsids then
98 transit to the plasma membrane and bud there, acquiring the GP64 envelope glycoprotein
99 in the final step of the BV assembly process. To begin to understand how the GP64
100 envelope protein is trafficked to the plasma membrane, we have examined the trafficking
101 of GP64 in cultured insect cells. For these studies, we developed an assay based on
102 inducible GP64 expression in a stable cell line, and performed quantitative measurements
103 of the GP64 protein on the cell surface when expression of a single cellular gene was
104 disrupted. This experimental system permits high throughput screening and identification
105 of host proteins required for GP64 trafficking to the plasma membrane. We used cultured
106 *Drosophila* cells, which produce robust RNAi responses following incubation with dsRNAs
107 and have a well-studied genome with readily available resources for interrogating specific
108 pathways or the whole genome (4). Thus, for the current study, we generated a
109 *Drosophila* cell line that expresses the AcMNPV GP64 protein in an inducible manner and
110 performed an RNAi screen targeting a wide variety of genes known to be involved in or
111 associated with vesicular protein transport. Integral membrane proteins such as GP64
112 are transported from the Golgi to the PM via small vesicles and a variety of interconnected
113 endosomal pathways. We found that single gene knockdowns of 6 genes (Rab GTPases
114 *Rab1 and Rab4; Clathrin heavy chain* and clathrin adapter proteins *AP-1-2β* and *AP-2μ*;
115 and *Snap29)* each resulted in significantly reduced GP64 transport to and/or maintenance
116 of GP64 on the cell surface. In addition, we identified two gene knockdowns (*Rab5*,
117 *Exo84*) that each resulted in substantial increases in levels of cell surface GP64. Based

on the predicted functional roles of Rab4, AP-2μ, and Rab5 in membrane protein recycling from the cell surface, we also examined the potential for GP64 recycling. We found that GP64 was endocytosed from the cell surface and that it was internalized and delivered to VPS26 (retromer) containing endosomes, suggesting that the endocytosed GP64 may be recycled back to the plasma membrane.

Methods and materials:

Cell line generation and analysis.

Plasmids. To generate a *Drosophila* cell line for inducible expression of the baculovirus GP64 protein, the AcMNPV *gp64* gene coding region (NCBI NC_00162) was cloned under the transcriptional control of a *metallothionein* promoter, and inserted into the genome of *Drosophila* DL1 cells (https://dgrc.bio.indiana.edu/product/View?product=9), as described below. The AcMNPV *gp64* ORF was subcloned into the EcoRI site of vector pMT-V5-His (Invitrogen) to generate plasmid pMT-GP64 (in which *gp64* is out of frame with the *V5* and *6xHis* codons). Cells. Adherent *Drosophila* DL1 cells and DL1-derived cell lines were grown in Schneider's medium (Gibco, Cat. No. 21720024) supplemented with 8% fetal bovine serum (FBS) (Hyclone). Transfection and selection of a stable cell line. *Drosophila* DL1 cells (5.0 X 10^5 cells in a 35 mm well) were co-transfected (5) with 2 μg of a plasmid mixture containing a 5:1 mass ratio of pMT-GP64:pCoPuro (Addgene # 17533) and incubated for 72 h in the absence of antibiotic. Transfected cells were then selected in growth medium containing 400 ug/ml puromycin (Invitrogen) over 10 days, subculturing every three days into fresh medium containing 400 μg/ml puromycin. The puromycin-selected cells were then induced to express GP64 by incubation in medium containing

500 μM Cu_2SO_4 for 12 h. Cell-surface GP64 was detected by flow cytometry analysis of live cells using the GP64-specific primary antibody, AcV1 (6), and a 555 Alexa-Fluor conjugated donkey anti-mouse secondary antibody (Invitrogen). Approximately 50% of the induced cells displayed GP64 at the cell surface. GP64-positive cells were then isolated (1 cell/well in a 96 well plate) with a BD Biosciences FACSAria Fusion fluorescence-activated cell sorter (FACS), and a single-cell clone was named DL1:mtGP64. After 8 passages of the clonal DL1:mtGP64 cells, GP64 expression was induced (50 μM Cu_2SO_4, 8 h) and GP64 was detected on the surface of 99% of the cells by flow cytometry, using a phycoerythrin-conjugated AcV1 antibody (Thermofisher Cat. no. 12-6991-82). The DL1:mtGP64 clonal cell line was then expanded, frozen in aliquots, and stored in liquid nitrogen. For continuing studies, DL1:mtGP64 cells were grown at 22ºC and used for approximately 50 passages before a new aliquot was resurrected.

Gene-specific dsRNA selection and production:

For RNAi knockdowns of genes potentially involved in trafficking of the GP64 protein, gene-specific dsRNAs were identified and selected from the *Drosophila* RNAi Screening Center (DRSC) database (Harvard Medical School) (7). We selected 213 genes belonging to 13 gene groups (Table 1) and for each, we selected target gene sequences (100 - 1300 nt) that lacked potential off-target effects. Up to three independent dsRNAs specific for each selected gene were identified for the assay, and the resulting 561 target sequences are listed in Table S1, with the DNA primers used for amplifying the target sequences. dsRNAs for RNAi were transcribed from dsDNA templates with T7 RNA polymerase, purified on Qiagen RNEASY columns, adjusted to 50 ng/ul in Nuclease Free Water (GrowCells, Catalog No. NUPW-1000), and arrayed (750 ng in 15 μl per well) into sterile 96 well tissue culture plates (BioOne) by the DRSC (https://fgr.hms.harvard.edu/).

dsRNAs in assay plates were stored sealed at -60ºC. For RNAi knockdown experiments, each assay plate contained 60 wells with dsRNAs, which included 54 individual gene-specific dsRNAs, three wells with the negative control lacZ dsRNA, and three wells with the positive control Rab1 dsRNA (Table S1).

dsRNA treatment of cells:

To initiate RNAi knockdowns in DL1:mtGP64 cells, cells were incubated in culture medium containing a gene-specific or control dsRNA according to the protocol described by the DRSC (7). Briefly, DL1:mtGP64 cells grown to near-confluency were resuspended in serum-free Schneider's medium at 1.67 x 10^6 cells/ml. Cells (50,000 in 30 µl medium) were added to each well of the assay plates which already contained dsRNA (15 ul/well; 50 ng/µl). Plates were incubated at RT for 30 min before addition of 55 ul of Schneider's medium containing 16% FBS to each well. Plates were then incubated at 22ºC for 4 days before induction of GP64 expression. To induce GP64 expression, 50 µl of Schneider's medium (containing 8% FBS and 300 µM Cu_2SO_4) was added to the growth medium in each well by gentle iterative (5x) pipetting with a multichannel pipette for a final concentration of 100 µM Cu_2SO_4.

Optimization of the RNAi assay:

To optimize the RNAi knockdown assay, we assessed the effects of Cu_2SO_4 concentration and incubation time on GP64 cell surface levels in dsRNA-treated DL1:mtGP64 cells. Cells were first incubated in 750 ng (7.5 ng/µl) of either lacZ (negative control) or Rab1 (positive control) dsRNA at 22ºC for 4 days, as described above. Then, the growth medium was replaced with 100 µl of Schneider's medium (with 8% FBS) containing varying Cu_2SO_4 concentrations (two-fold dilutions of Cu_2SO_4 from 12.5 – 400 µM) in each well of the dsRNA-treated cells. After an incubation of 8 h, relative cell surface

levels of GP64 were measured by flow cytometry. After the Cu^{++} induction period, plates were placed on ice for 30 min, then monolayers of cells were washed twice with 200 µl of ice cold PBS (pH 7.5) and then incubated with 50 µl of ice-cold blocking solution (3% BSA, 1% normal donkey serum (NDS) in PBS, pH 7.5) for 30 min. Blocking solution was then replaced with 50 µl of a solution containing a phycoerythrin-conjugated anti-GP64 monoclonal antibody (AcV1, Invitrogen; 1/250 dilution) in 1% BSA and 1% NDS, and incubated on ice in the dark for 2 h. The antibody solution was removed and cells were washed 3x with 100 µl cold PBS (pH 7.5) before resuspension in 100 µl cold PBS with a p200 pipettor. Cells were analyzed for relative GP64 cell surface levels by flow cytometry on an Accuri C6 flow cytometer, using the FLA 2 (585/40) filter. The geometric mean of fluorescence in each well was determined for the entire population of cells. Relative GP64 levels were compared among lacZ dsRNA-treated cells induced with varying Cu_2SO_4 concentrations for 8h to determine effects of Cu_2SO_4 dose on GP64 surface levels. We also compared relative GP64 levels among control lacZ and Rab1 dsRNA treated cells to determine effects of varying Cu_2SO_4 doses and incubation times on the effects of the Rab1 knockdown. Overall, we determined that induction of GP64 expression with 50 - 200 µM Cu_2SO_4 for 8 h provided the most sensitive conditions for analysis of the effects of RNAi knockdowns. A Z-factor was also calculated to assess the feasibility of this 8 h induction scheme to identify genes that, when knocked down, cause reduced transport of GP64 to the cell surface. The total mean cellular fluorescence values of the (negative) lacZ and (positive) Rab1 dsRNA-treated cell controls were used to calculate a Z-factor of 0.67 for this assay. A Z-factor from 0.5 – 1.0 defines an assay that should be capable of identifying hits in an RNAi-based screen (8).

Targeted RNAi screen:

To examine the effects of 213 host gene knockdowns on GP64 transport to the cell surface, we analyzed DL1:mtGP64 cells incubated individually with 561 dsRNAs representing multiple target sites for each of the 213 genes. Each 96-well assay plate contained 54 unique dsRNAs targeting host genes, plus 3 positive and 3 negative control wells. Three plates were prepared and assayed simultaneously, and replicates of the 3 plates were prepared and assayed on each of three separate days (3 biological replicates). Following an 8 h induction of GP64 expression, the geometric mean fluorescence measurement of GP64 detected at the cell surface was calculated for each of the dsRNA-treated cell samples using FCS Express software. The value from each gene-specific dsRNA was assessed on a plate-by-plate basis, by comparison to either a) the geometric mean of values from all wells from the same plate, or b) the geometric mean of the lacZ negative controls on the same plate (Table S2). To identify hits in the two data sets, ANOVA was used for comparisons using either the plate average or the lacZ average, and dsRNA knockdowns that resulted in a ≥40% reduction of GP64 surface levels, with a p value ≤ 0.05 in both calculations were scored as hits (Table S2). Genes identified by this means as having a potential role in GP64 transport to the cell surface are listed in Table S3.

RT-qPCR of *gp64* and *Drosophila* gene transcripts:

To identify gene hits that resulted from effects on GP64 transport to the plasma membrane, and to eliminate hits resulting from pleiotropic effects on *gp64* transcription, the *gp64* mRNA levels of the 37 potential hits were analyzed by reverse transcription-quantitative PCR (RT-qPCR). DL1-mtGP64 cells were first incubated with dsRNAs specific for each of the 37 potential hits (or the lacZ control) as described above for the

initial screen. *gp64* gene expression was then induced by incubation in 100 µM Cu_2SO_4 for 8 h, and total RNA was then isolated from each of three wells of cells in a 96 well plate, using Trizol according to the manufacturer's protocol. Total RNA was resuspended in water and quantified using a Nanodrop spectrophotometer. Each RNA sample (100 ng) was used for reverse transcription with a QScript cDNA synthesis kit (Quantabio) that uses both oligoDT_{18} and random hexamers for reverse transcription. cDNAs were diluted 1:100 in nuclease-free water and 1 µl was used for PCR amplification of *gp64* or *Drosophila rp49*. The *gp64* primers were: (Forward) 5'-GGCAACACGACCTATCACGA-3', and (Reverse) 5'-ATTCGCCTTCAGCCATGGAA-3'. The *rp49* primers used were: (Forward) 5'-GACGCTTCAAGGGACAGTATCTG-3', and (Reverse) 5'-AAACGCGGTTCTGCATGAG-3'. The mean relative *gp64* transcript levels for each of the potential hits and controls were quantified (ΔΔCt) from the three independent dsRNA-treated cellular RNA samples.

Knock down efficiency of select hits:

To verify knockdowns of selected target *Drosophila* gene hits, the same procedure for RT-qPCR described above was followed, but with primers directed at each of the selected target genes: *Rab1* (5'-GTATACGATTGCACGGACCAGG-3' and 5'-AAGTCGCTCTTGTTGCCAACC-3'), *Rab4* (5'-TTGAAGAGGCCTTTCTCAAGTGC-3' and 5'-AGCTCCGCCGTACTGAATACC-3'), *AP-1-2β* (5'-ACGTGTCCGCCCTCTTCC-3' and 5'TCGCAGTCCTTCACGAACG-3'), *AP-2µ* (5'-GGCGGCTGTGACCAAGC-3' and 5'-GCCGAAGTAGGATTGCATCACC-3'), *Chc* (5'-AAGGCTGTCGATGTCTTCTTTCCG-3' and 5'-TATATGCACGTGGCCGTCTCC-3'), *GAPsec* (5'-CCTGCAAGATCATCCGCTGAG-3' and 5'-AATCGGTGGGCTGCTGG-3'), CG16896 (5'-CTCAATTCTTGGGGTCGACGG-3' and 5'-GCTGCCGAAACGCATCAGAG-3'), and

Snap29 (5'-GCAGAACAAACGGACGGGAG-3' and 5'-TTCTCAGCATAGGCCAGTCG-3'). The mean relative transcript levels for each of the target gene hits were quantified (ΔΔCt) and normalized to that quantified (ΔΔCt) for the corresponding transcript from lacZ dsRNA-treated cells.

Immunolocalization of GP64 and organelles in dsRNA-treated cells:

To examine GP64 localization in the presence of selected gene knockdowns, GP64 immunolocalization was performed in cells following dsRNA-mediated knockdowns and induction of GP64 expression. Briefly, DL1:mtGP64 cells were incubated with the selected dsRNAs in a well of a 96-well plate as described above. After 4 days of incubation, cells were resuspended in PBS (pH 7.5, RT) and seeded onto zones (limited with a liquid-blocking PAP-pen) on sterile, poly-L-lysine-coated coverslips in 6-well plates (6 for each gene knockdown). GP64 expression was induced by the addition of Cu_2SO_4 (200 μM) for 16 h at 22ºC. Alexa647-conjugated concanavalin A (Thermofisher C21421) was used to identify cell boundaries. Cells were incubated with the concanavalin A-Alexa647 (100 μg/ml in PBS, pH 7.5) on ice for 30 min in the dark, then rinsed 3x with cold PBS (pH 7.5) and fixed in the dark with paraformaldehyde (4% in PBS, pH 7.5) at 4ºC for 30 min. The fixative was removed and coverslips were rinsed 3x (5 min/rinse) with PBS (pH 7.5) at RT. Cells were permeabilized by incubating in 0.1% Triton X-100 (in PBS, pH 7.5) for 10 min at RT, then incubated in blocking solution (3% BSA, 1% NDS, 0.1% Triton X-100 in PBS pH 7.5) for 30 min at RT. Cells were incubated with primary antibodies overnight (16 h) at 4ºC in the dark. GP64 and organelles were stained with specific antibodies (see below) in antibody staining solution (1% BSA, 1% NDS, 0.1% Triton X-100 in PBS, pH 7.5). The primary antibody solution was removed and cells were rinsed with PBS (3x for 10 min at RT), then cells were then incubated with a secondary

Alexa dye conjugated antibody (each diluted 1:1000 in antibody staining solution) for 4 h at RT in the dark. Nuclei were stained by addition of DAPI (5 mM in PBS, pH 7.5) for 30 min then excess DAPI and secondary antibodies were removed and cells washed 3x for 10 min with PBS (pH 7.5) before coverslips were mounted in a glycerol-based anti-fade buffer (Citifluor^{TM}). Cells were photographed using a SP2 Zeiss confocal microscope (750 TLM) using 63x oil immersion lens and a 2.5x digital zoom.

Analysis of extracellular vesicles

For isolation of extracellular vesicles (EVs), DL1 or DL1:mtGP64 cells (2.5 x 10^7) were plated in 100 mm culture dishes in Schneider's growth medium with 8% FBS. Cell monolayers were washed 3x with 10 ml RT PBS (pH 7.5) then growth medium containing 200 µM Cu_2SO_4 was added to induce GP64 expression. Uninduced DL1:mtGP64 cells were similarly washed, but were incubated in fresh Schneider's medium without Cu_2S0_4. Cells were then incubated for 8, 16, or 24 h, followed by collection of cells and supernatant. For control DL1:mtGP64 cells lacking Cu_2SO_4 treatment and for control DL1 cells exposed to Cu_2SO_4-containing medium, cells and corresponding growth medium supernatants were collected separately after 24 h. EVs were isolated from the cell culture medium by the following procedure: The medium was first centrifuged at 3000 x g for 10 min at RT to pellet floating cells or large debris. The supernatant was then collected and centrifuged at 10,000 x g for 30 min at 4°C to pellet additional cellular debris. The clarified medium (approximately 11 ml) was then underlaid with 1 ml 25% sucrose and EVs pelleted at 100,000 x g for 2h at 4ºC in a Beckman Optima XE-90 ultracentrifuge using a SW-41 rotor (12.5 ml tubes). The resulting EV pellet was resuspended in 12 ml PBS (pH 7.5) then re-pelleted (100,000 x g, 2 h, 4ºC). The EV pellet was subsequently

resuspended in 50 ul PBS (pH 7.5) and stored at -20ºC until analysis by immunoblotting. Eleven ml of the Cu_2S0_4-containing Schneider's growth medium was used as a control for possible EVs derived from the FBS supplement. For analysis of cell lysates, the cells from each 100 mm plate were resuspended in PBS (pH 7.5), then pelleted by centrifugation (1000 x g, 10 min, RT) and lysed in 200 ul of lysis buffer (1% triton X-100, 100 mM NaCl, 10 mM tris, pH 7.5) supplemented with a protease inhibitor cocktail (Roche cOmplete™, EDTA-free Protease Inhibitor Cocktail). EV preparations or cell lysate samples were mixed with an equal volume of 2x SDS-PAGE loading buffer and heated to 95ºC for 10 min, then proteins were resolved on a SDS-12% polyacrylamide gel. For comparisons of relative levels, cell lysate samples were serially diluted 2-fold in 1x SDS PAGE loading buffer. For immunoblotting, 5% of each total EV pellet and 0.5% of each total cell lysate was loaded onto each lane of an SDS-PAGE gel.

Sucrose gradient fractionation of EVs: To identify EVs from the supernatants of GP64-expressing DL1:mtGP64 cells, twelve 178 mm culture plates were seeded with approximately $1x10^7$ DL1:mtGP64 cells, and cells were grown to approximately 70% confluence (3-4 days). Cells were washed 3x with PBS (pH 7.5, RT) then incubated at 22ºC in 17 ml/plate of Schneider's growth medium containing Cu_2S0_4 (200 µM). After a 24 h incubation period, EVs were purified from the culture medium by the protocol described above, but using 6 tubes (35 ml/tube) in a Beckman SW-32 rotor. The resulting six EV pellets were resuspended in a total of 600 µl PBS (pH 7.5) and applied to a 12 ml sucrose step gradient (3ml 80%, 2.5 ml 60%, 2.5 ml 45%, 2.5 ml 30%, 2 ml 15% sucrose) and centrifuged to equilibrium (100,000 x g for 18 h at 4ºC). Twelve 1 ml fractions were collected from the top to bottom of the gradient. EVs were purified from 0.5 ml of each 1

ml fraction by mixing with 1 ml of PBS (pH 7.5) and pelleting at 100,000 x g for 2 h at 4ºC, in capped conical 1.5 ml tubes (Beckman) using a TLA 55 rotor (Beckman Optima TLX ultracentrifuge). Each EV pellet was resuspended in 20 µl of 1x SDS-PAGE loading buffer and 5 µl was loaded onto a 0.75 mm, 12% polyacrylamide SDS-PAGE gel. The resolved proteins were then electroblotted onto polyvinylidene fluoride membrane for Western blot analysis.

GP64 Endocytosis assay

To examine endocytosis of GP64 from the cell surface, DL1:mtGP64 cells (approximately 2 x 10^5) were seeded onto zones (limited with a liquid-blocking PAP-pen) on sterile, poly-L-lysine-coated coverslips in 6-well plates. Growth medium (2.5 ml) containing Cu_2SO_4 (200 µM) was added to cells to induce GP64 expression, and cells were incubated at 22 °C for 12 h. Plates were then placed on ice for 30 min and coverslips were washed 3x with 3 ml cold PBS (pH 7.5) to inhibit endocytosis. Control coverslips were then incubated with 2.5 ml ice-chilled 1x trypsin-EDTA (0.25%, Cat. No 25200056) on ice for 30 min, and washed 3x with 3 ml cold PBS, while other coverslips were maintained on ice in PBS. Coverslips (trypsin treated and untreated) were incubated with an anti-GP64 antibody (AcV1; diluted 1:100 in cold PBS, pH 7.5) on ice for 2 h, then the antibody solution was removed and cells washed 3x with 3 ml cold PBS (pH 7.5). After washing, coverslips were either retained on ice to block endocytosis, or transferred to growth medium in prewarmed (27ºC) 6-well plates and incubated for 30 min to permit endocytosis of GP64:AcV1 antibody complexes. After incubation at 27ºC for 30 min, cells on coverslips transferred to 27ºC were fixed for 30 min in 2.5 ml of 4% paraformaldehyde in PBS at RT. Cells retained on ice were fixed with 2.5 ml cold 4% paraformaldehyde in PBS on ice for 30 min. Cells were permeabilized with Triton X-100 (0.1% in PBS) for 10

min at RT then blocked with 3% BSA in PBS with 0.1% triton-X 100. Cells were then incubated with an anti-VPS26 antibody (a gift from Hugo Bellen) at 4ºC overnight. After washing 3x with PBS, cells were incubated with a solution of Alexa555-conjugated donkey anti-mouse antibody (for detection of AcV1-GP64 complexes) and Alexa488-conjugated goat anti-guinea pig antibody (for detection of VPS26).

Immunoblotting

Protein samples were mixed with an equal volume of 2x SDS-PAGE loading buffer (4% SDS, 20% glycerol, 200 mM β-mercaptoethanol, 0.01% bromophenol blue, 0.1 M Tris HCl, pH 6.8) and denatured at 95ºC for 10 min. For dilutions, denatured samples were diluted in 1x SDS-PAGE loading buffer. Proteins were resolved on 12% polyacrylamide SDS-PAGE gels, electroblotted to polyvinylidene fluoride membrane, then blots were blocked in 5% skim milk in TBST (20 mM tris, 150 mM NaCl, 0.05% Tween-20). Primary and secondary antibodies were diluted in 1% skim milk in TBST, and incubated with blots at 4ºC overnight or for 2h at RT. Blots were washed 3x for 10 min per wash with TBST. Signal from the HRP-conjugated secondary antibodies was detected using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermofisher) and a BioRad ChemiDoc Imaging System.

Antibodies

For flow cytometry and endocytosis assays, GP64 was detected with monoclonal antibody AcV1. AcV1 used for the RNAi screen was conjugated with phycoerythrin (Thermofisher Cat. no. 12-6991-82). For immunoblots, GP64 was detected with monoclonal antibody AcV5, and for confocal microscopy of fixed cells, GP64 was detected with either AcV5 or with a rabbit polyclonal anti-GP64 antibody (SinoBiological 40496-RP01). Syntaxin 1A was detected on immunoblots with monoclonal antibody 8C3

(DSHB; from S. Benzer and N. Colley). Organelles were identified in fixed cells using the following antibodies: goat polyclonal anti-Golgin245 and goat polyclonal GMAP (DSHB; S. Munro) used together to label the Golgi; mouse monoclonal anti-Calnexin 99A (DSHB; S. Munro; Product Cnx99A 6-2-1) to label the endoplasmic reticulum; mouse monoclonal anti-Rab7 (DSHB; S. Munro; Hybridoma Product Rab7) to label early/late endosomes; rabbit polyclonal anti-cathepsin L/MEP antibody (Abcam ab58991) to label lysosomes, mouse monoclonal anti-lamin DmO (DSHB; P. A. Fisher; Hybridoma Product ADL67.10) to label the nuclear envelope, mouse monoclonal anti-Rab11 (BD Biosciences #610656) to label recycling endosomes; and guinea pig polyclonal anti-VPS26 (a gift from Hugo Bellen) to label the retromer complex.

Results

Generation of a *Drosophila* cell line expressing GP64

To begin to understand the roles of cellular proteins and pathways in trafficking of the baculovirus envelope glycoprotein GP64 to the plasma membrane in insect cells, we engineered a *Drosophila* cell line to inducibly express GP64 and used that cell line for a targeted RNAi screen of host genes associated with vesicular protein trafficking of membrane proteins (Fig. 1A,B and 2). First, *Drosophila* DL1 cells that inducibly express the baculovirus GP64 envelope protein were generated by co-transfecting DL1 cells with two plasmids: one encoding the WT AcMNPV GP64 protein under the control of the inducible *Drosophila melanogaster metallothionein* promoter, and a second containing a Puromycin resistance gene under a constitutive *D. melanogaster copia* promoter. Following transfection, cells were selected on puromycin, then GP64 expression was

induced by addition of Cu_2SO_4 to the medium. Cells displaying GP64 at the cell surface were identified by immunolabeling (with an anti-GP64 monoclonal antibody) cells at 4ºC, and single cells identified as expressing GP64 were isolated by FACS and single-cell cloned. A clonal cell line that inducibly expresses GP64 was named DL1:mtGP64. DL1:mtGP64 cells were further examined to verify efficient trafficking of GP64 to the plasma membrane (Fig. 1A,B). DL1:mtGP64 cells did not express detectable GP64 in the absence of Cu_2SO_4 induction, but GP64 was detected on the surface of cells after an 8 h incubation in Cu_2SO_4 (Fig. 1A; DL1:mtGP64 vs DL1:mtGP64+Cu_2SO_4).

GP64 trafficks predominantly to the cell surface of DL1 cells

Comparisons of GP64 detected from non-permeabilized and permeabilized cells (Fig. 1B) indicated that the majority of GP64 was trafficked to and displayed on the cell surface, while relatively little GP64 was detected in-transit. Because some viral envelope proteins are also released from the cell in extracellular vesicles (EVs) (9-14), we examined the potential for GP64 release from the cell via EVs. We first compared relative levels of GP64 detected in EVs vs. whole cells after 8, 16, and 24 h of copper-induced GP64 expression. EVs in the culture supernatant were isolated by low-speed centrifugation to remove cells and cell debris, followed by pelleting through a 25% sucrose cushion. GP64 in the EV fraction was then compared to that in whole-cell lysates at each timepoint (Fig. 1C). While GP64 was detected in the EV fraction from cell culture supernatant, cellular GP64 levels appeared to be >100 fold higher than that from the culture supernatant EVs (Fig. 1C, EVs vs. Cells), based on estimates from western blots. We next examined the type of extracellular vesicle (*i.e.* microvesicles or exosomes) that contained the extracellular GP64. EVs from the cell culture supernatant were analyzed by pelleting through a 25% sucrose cushion, followed by fractionation on a sucrose

gradient. Our results indicate that extracellular GP64 was found in exosomes since the distribution of GP64 in the gradient was coincident with that of Syntaxin 1A, a protein enriched in exosomes (15, 16)(Fig. 1D). Extracellular GP64 co-fractionated with Syntaxin 1A in sucrose densities of 1.1659 – 1.2052 g/L, densities in which exosomes are typically recovered (17). We did not further assess whether GP64 was present in larger, plasma membrane derived microvesicles, so it is unclear whether GP64 may also be released from cells in that manner. Overall, these results show that the majority of GP64 expressed in the cell is trafficked to the plasma membrane and that only a minor fraction of GP64 exits the cell by exocytosis.

Optimization of GP64 cell surface levels for detecting the effects of host gene knockdowns.

The strategy and workflow of experiments to identify cellular factors involved in GP64 trafficking to the cell surface are illustrated in Figure 2. To maximize sensitivity in identifying quantitative changes in GP64 surface levels resulting from trafficking defects from host gene knockdowns (Fig. 2A), GP64 cell surface levels should be measured at a relatively early time following induction, and should be robust but not saturating. Because relatively robust GP64 surface levels were detected at 8 h post induction in our preliminary studies, we optimized induction and the effects of RNAi knockdowns at that time point. First, GP64 surface levels were measured after 8 h of incubation in increasing concentrations of Cu_2SO_4 (Fig. 3A). We identified Cu_2SO_4 concentrations (100-800 μM) that resulted in GP64 surface levels that were robust but not saturating, and within a linear range of the dose-response curve. This range of Cu_2SO_4 concentrations elicited increasing levels of detectable GP64, while concentrations above 800 μM resulted in less consistent levels of GP64 induction and/or detection.

To optimize the assay for measuring the effects of RNAi knockdowns, we next measured GP64 surface levels resulting from increasing concentrations (12.5-400 μM) of Cu_2SO_4, in the presence of an RNAi knockdown (Fig. 3B-D). For the RNAi knockdown, we selected Rab1 as a positive control for inhibition of trafficking to the cell surface. Rab1 is important for regulating vesicular protein trafficking from the ER to Golgi (18) and the Rab1 knockdown should therefore inhibit protein trafficking to the cell surface. As expected, we found that dsRNA-mediated knockdown of Rab1 in DL1:mtGP64 cells, followed by induction of GP64 expression by Cu_2SO_4, resulted in dramatically lower levels of cell surface GP64, when compared with negative control cells treated with lacZ dsRNA (Fig. 3B). Typical cell surface localization of GP64 was observed for the control lacZ dsRNA-treated cells, but was disrupted in cells with the Rab1 knockdown (Fig. 3C, lacZ vs. Rab1). To optimize the detection of the effects of RNAi knockdowns on GP64 trafficking, we next examined the effect of a Rab1 knockdown in the context of varying concentrations of Cu_2SO_4 (12.5-400 μM). By comparisons to RNAi knockdowns with the negative control lacZ dsRNA, we identified a range of Cu_2SO_4 concentrations (50-400 μM) that resulted in substantial GP64 cell surface differences between the Rab1 knockdown and the negative control (lacZ) (Fig. 3D). Thus, these conditions provide a sensitive measure of the effect of the Rab1 RNAi knockdown on GP64 surface levels. Based on the differential effects of negative control lacZ and positive control Rab1 knockdowns on GP64 cell surface detection, we calculated a Z-factor of 0.67 for our assay (Z-factors between 0.5 and 1.0 signify an excellent high throughput assay for detecting hits (8)), indicating that the assay parameters we developed are capable of identifying gene knockdowns that modulate GP64 trafficking and surface display. We therefore

based our RNAi screen (see below) on these induction parameters established using Rab1.

Overview of screen results:

To identify genes and pathways necessary for GP64 trafficking to the cell surface, we selected 213 cellular genes for dsRNA knockdowns in this targeted RNAi screen. Families of genes examined in the screen included members of approximately 13 groups of genes associated with membrane protein trafficking (Table 1). For RNAi knockdowns of each gene, up to 3 unique gene regions (containing no identified off-target effects) were selected for generation of dsRNAs. This resulted in a total of 561 dsRNAs in each replicate of the RNAi knockdown experiment (Table S2), which was performed 3 times (3 biological replicates).

We used the following method to identify genes as hits for our immediate follow-up analysis. Genes were selected as hits when a knockdown resulted in significantly lower GP64 surface levels relative to 2 controls: 1) the lacZ knockdown controls on each assay plate, and 2) the average value from all wells on each assay plate.

From the 213 genes targeted in the RNAi screen, 37 gene knockdowns resulted in GP64 surface levels that were reduced by ≥40% as compared with the controls. The 37 knockdowns (Fig. 4A; Table S3) included genes encoding 4 Arf GTPases, 3 ESCRT proteins, 10 Clathrin/adaptor proteins, 2 Kinesins, 2 P24 Transporters, 5 RAB GTPase effectors, 5 RAB GTPases, 2 SNARE Proteins, 1 SNAP Protein, and 3 VPS Proteins.

Validation of genes required for GP64 transport to the plasma membrane:

qPCR analysis of GP64 mRNA

In addition to direct effects of knockdowns on protein transport, reduced GP64 surface levels from some knockdowns could have resulted from indirect effects on other

cellular systems affecting *gp64* gene expression. Therefore, to determine whether some knockdowns affected *gp64* gene expression, we examined *gp64* transcript levels. For each of the 37 hits (Fig. 4A, Table S3), we used RT-qPCR to quantify GP64 transcript levels following RNAi knockdown of the target host gene, and Cu_2SO_4 induction of GP64 expression. The analysis of *gp64* transcript levels revealed that *gp64* transcripts were substantially affected (reduced to ≤50%) for 24 of the 37 gene knockdowns (Fig. 4B, red bars), indicating indirect effects on GP64 expression. The remaining 13 gene knockdowns (Fig. 4B, black bars) had *gp64* transcript levels exceeding 50% of the control knockdown (lacZ). Ten of these genes (*Rab1, Rab4, Rab26, GAPsec, CG16896, CG1695, Snap29, Chc, AP-1-2β, AP-2μ*) were selected for further analysis, in part selected because they resulted in the most severely depleted cell surface GP64 levels and also due to their importance in Golgi-to-PM transport of integral membrane proteins.

Confirmation of cellular gene mRNA knockdowns

To verify the knockdown of mRNAs of each of the 10 candidate cellular genes, we used RT-qPCR to confirm the depletion of each of the target mRNAs by dsRNA-mediated RNAi. Of these 10 knockdowns, we verified the mRNA knockdown for 6 genes (Fig. 4C, green bars). Assessments of mRNA levels for two of these genes (*Rab26* and *CG1695*) were difficult in both control lacZ dsRNA-treated cells, as well as Rab26 and CG1695 dsRNA-treated cells. Our results suggested that neither of these genes was expressed in our DL1-derived cell line (Fig. 4C, asterisks), and modENCODE Cell Line Expression Data available at the Drosophila Genomics Resource Center indicate that neither gene is expressed in DL1 (aka S1) or many other Drosophila cell lines (https://dgrc.bio.indiana.edu/cells/TilingSearch). The dsRNA knockdowns of *GAPsec* and *CG16896* resulted in a reduction of only approximately 30% relative to control mRNA

levels (Fig. 4C, yellow bars). While this modest knockdown level may be responsible for the modest reduction in GP64 transport, we did not further examine these genes. Therefore, we considered the 6 remaining gene knockdowns that resulted in more dramatic mRNA depletions (Fig. 4C, green bars). The 6 gene knockdowns that were fully validated in reducing GP64 trafficking to the cell surface included genes encoding 3 clathrin adaptor proteins or clathrin (AP-1-2β, AP-2μ, and Chc or Clathrin heavy chain), 2 Rab GTPase proteins (Rab1 and Rab4) and a SNAP protein associated with vesicular trafficking (Snap29) (Fig. 4C).

Microscopic analysis of GP64 localization in cells with each of the 6 validated gene knockdowns

Our flow cytometry-based RNAi screen identified 37 gene knockdowns (hits) that resulted in substantially reduced GP64 levels at the cell surface. By eliminating hits that may have resulted from indirect effects (pleiotropic effects on *gp64* transcription or mRNA levels; or effects on non-target genes), we subsequently narrowed the validated hits to a subset of 6 gene knockdowns. To further detail the effects of these knockdowns, we used confocal microscopy to examine the localization of GP64 following knockdown of each of the 6 identified genes (Fig. 5). To identify GP64 accumulation in organelles or cellular compartments following these target gene knockdowns, we immunostained GP64 and counterstained for markers of a variety of cellular compartments. Specific antibodies or reagents used to identify the following compartments (by specific marker proteins) were: plasma membrane (concanavalin A), nuclear envelope (lamin A), endoplasmic reticulum (calnexin 99A), Golgi apparatus (Golgin 245 and Golgi microtubule-associated protein), Retromer (Vps26), recycling endosomes (Rab11), late endosomes (Rab7), and lysosomes (cathepsin L). For studies of GP64 accumulation, GP64 localization was

examined in the presence of each of the 6 knockdowns and compared with GP64 localization in control cells treated with lacZ dsRNA. As with untreated cells, (Fig. 1A/B), we found that GP64 was localized predominately at the plasma membrane in control cells treated with lacZ dsRNA (Fig. 5, lacZ). Only small amounts of GP64 were detected in the perinuclear region and in a few very small, faint cytoplasmic puncta in the control cells. For cells with knockdowns from each of the 6 hits in the screen, we confirmed the substantial reductions of GP64 at the plasma membrane (Fig. 5: lacZ vs. Chc, AP-1-2β, AP-2μ, Rab1, Rab4, and Snap29). Furthermore, we identified substantial intracellular GP64-specific staining implying GP64 accumulation in cellular compartments due to dysregulated trafficking.

Clathrin-related gene knockdowns

Clathrin-coated vesicles (CCVs) produced from the TGN, endosomes or at the plasma membrane are each involved in distinct trafficking routes (19, 20). The coat proteins (clathrin light and heavy chains) form triskelions that themselves do not bind directly to lipid bilayers. Rather, they require accessory proteins (adaptor proteins) to be recruited to specific organelle membranes (21-24) and therefore dictate the composition and location of distinct CCVs (25). Each CCV subtype is composed of four discrete adaptor protein subunits (22-24), which provides specificity to the CCV protein complexes, likely because CCVs are used in many different trafficking routes amongst several organelles. The adaptor proteins (adaptins) are involved in recruiting cargo proteins (*e.g.* GP64) into their vesicles. In the current study, knockdown of *clathrin* and related adaptin genes (*Chc*, *AP-1-2β*, *AP-2μ*) resulted in severely depleted GP64 cell surface levels (Fig. 4A) while *gp64* mRNA levels were either increased (*AP-1-2β* and *AP-2μ*) or only moderately reduced (*Chc*) (Fig. 4B). For knockdowns of *Chc*, *AP-1-2β*, and

AP-2μ, we detected a substantial accumulation of GP64 in the perinuclear area, and also frequently in large cytoplasmic puncta. GP64 accumulation appeared most notable for the knockdown of *Chc* (Fig. 5; LacZ vs. Chc, AP-1-2β, and AP-2μ). The perinuclear GP64 accumulation colocalized with the nuclear envelope marker (Lamin A) indicating accumulation in the nuclear envelope when expression of clathrin or clathrin adaptors was disrupted. Cytoplasmic accumulation of GP64 (large puncta) was observed colocalized with Golgi (Golgin245), lysosome (Cathepsin L) and late-endosome (Rab7) markers, suggesting that clathrin and related adaptors are involved at different levels of GP64 intracellular trafficking. Cells with a *Chc* knockdown often contained bright GP64 ring-shaped puncta decorated with several adjacent Retromer/Vps26-stained puncta highlighting that GP64 trafficking is likely stalled in endosomes due to disruption of CCV assembly, tethering and/or GP64 cargo recruitment.

Rab knockdowns

Rab GTPases are important regulators of vesicular transport, and many Rab GTPases (and Rab effectors) were identified in the 37 hits initially identified from the RNAi screen. Following further analysis and validation by monitoring *gp64* mRNA levels and target gene mRNA knockdown efficiencies, we narrowed the hits to 2 Rab GTPases, Rab1 and Rab4. Rab1 is generally known as a factor required for both ER-Golgi vesicular trafficking and Golgi maintenance, but it also has roles in ER tubulation and/or initiation of autophagy (26). Cells with the *Rab1* knockdown had GP64 surface levels that were reduced by approximately 50%. In those *Rab1* knockdowns, we observed increased perinuclear GP64 (Fig. 5), and detected large cytoplasmic GP64 puncta. The GP64-positive perinuclear ring co-localized with the Lamin A stained nuclear envelope. However, the GP64 puncta, that were mostly perinuclear, did not overlap with either the

593 Lamin A or ER (Calnexin 99A) stains. Large GP64 puncta colocalized with markers for
594 Golgi, Retromer, lysosomes, and late endosomes.

595 Rab4 localizes to sorting/recycling endosomes and is important for targeting proteins
596 to the cell membrane. Both Rab4 and Rab11 are associated with sorting/recycling
597 endosomes; Rab11 as part of the so-called “fast” route and Rab4 alternatively, as part of
598 the “slow” route. The result from the knockdown of Rab11, although substantial was not
599 significant based on our selection criteria. On the other hand, the Rab4 knockdown was
600 significant (Fig. 4C) and resulted in an approximately 50% reduction of surface GP64
601 levels (Fig. 4A). Dramatic accumulation of perinuclear GP64 (that overlapped with Lamin
602 A staining) was also observed in cells with a Rab4 knockdown. We observed large GP64-
603 positive puncta that were localized both centrally and peripherally in cells, but they did not
604 overlap with the ER marker. In the Rab4 knockdown cells, GP64 appeared to colocalize
605 with Golgi and recycling endosomes (Fig. 5). Thus, GP64 accumulation in recycling
606 endosomes in the Rab4 knockdown suggests that GP64 may be transported to the cell
607 surface via sorting/recycling endosomes containing Rab4. Alternatively, GP64 may be
608 transported more directly to the plasma membrane, then endocytosed and recycled to the
609 cell surface. Further experiments are needed to understand GP64 accumulation in Rab4-
610 depleted cells.

611 *Snap29* was also identified as important for GP64 cell surface localization (Fig. 4).
612 Snap29 is a multifunctional protein, with roles related to autophagy as well as endocytic
613 and exocytic trafficking of proteins (27). The *Snap29* knockdown resulted in an
614 approximately 50% reduction in surface GP64 levels in the screen. In Snap29 depleted
615 cells, GP64 accumulated dramatically in the perinuclear region and colocalized with

Lamin A. Notably, GP64 accumulated strongly in the Golgi, and was also associated with Retromer complexes (Fig. 5; Snap29). We also noted that GP64 accumulation in Golgi stained structures appeared at substantially higher levels than in Golgi from control cells, or cells with most other gene knockdowns. Although reduced levels of surface localized GP64 due to *Snap29* knockdown was documented from the screen, microscopy revealed a strong GP64 signal at the cell periphery, and colocalization with the plasma membrane marker (concanavalin A). This may suggest that GP64 accumulates below or very near the plasma membrane in the presence of the *Snap29* knockdown.

Gene knockdowns that resulted in increased GP64 cell surface levels

The RNAi screen was initially designed to identify cellular proteins that are important for GP64 transport to the cell surface, and thus to identify gene knockdowns that resulted in a reduction of GP64 levels at the cell surface. Surprisingly, we also identified several gene knockdowns that significantly increased GP64 surface levels. The results from 3 gene knockdowns (*Rab5*, *Rab7*, and *Exo84*) that led to substantial increases in GP64 surface levels are shown in Figure 6A (left panel). In the presence of each of these knockdowns, *gp64* mRNA levels were similar to those of the control lacZ dsRNA treated cells (Fig. 6A, center panel), suggesting increased efficiency of GP64 trafficking or retention at the plasma membrane. Cells treated with Exo84 or Rab5 dsRNA had highly efficient target mRNA depletion (12% or 29% of the control, respectively), whereas *Rab7* mRNA depletion was less efficient (86% of control) (Fig. 6A, right panel). However, it is notable that two independent Rab7-specific dsRNAs yielded the same approximate levels of increased cell surface GP64, suggesting that the phenotype is reproducible and results from depletion of Rab7. Rab5 functions in early endosomes but it is also detectable at the plasma membrane (28), compatible with its known roles in CCV formation, fusion with

early endosomes, and homotypic fusion between early endosomes (29, 30). Rab7 regulates transport from early to late endosomes and lysosomes, acting downstream of Rab5 (31, 32) in addition to playing an important role in autophagy (31, 33). Thus, Rab5 and Rab7 are both involved in post-endocytic trafficking whereas Exo84 is well established as a component of the exocyst complex that is required for targeting of vesicles to the cell membrane for protein exocytosis and other events involving the plasma membrane (cytokinesis, cell growth, ciliogenesis).

Cell surface GP64 is Endocytosed

Rab5 and *Rab7* knockdowns resulted in a 1.5x – 1.75x increase in cell surface GP64 compared with control cells, and considering that the major roles of Rab5 and Rab7 are in post-endocytic trafficking, this suggested that GP64 may be continually endocytosed and transported through the endocytic pathway. To determine whether surface localized GP64 is internalized into cells by endocytosis, we labeled cell surface GP64 with a GP64-specific antibody at 4ºC (to inhibit endocytosis), then shifted the temperature to 27ºC for 30 min and assessed internalization of the GP64:Ab complexes after the inhibition of endocytosis was released. When cells were then fixed, permeabilized, and incubated with a secondary Alexa55-conjugated anti-mouse antibody to detect GP64:Ab complexes, we detected intracellular GP64:Ab complexes. However, if cells were treated with trypsin to remove surface GP64 prior to binding of the primary anti-GP64 antibody, no internal GP64 was detected. The results demonstrated GP64 endocytosis from the cell surface. To gain insight into the potential fate of the endocytosed GP64, we also co-stained for a component of the Retromer complex (Vps26). The major function of Retromer is in retrieval of cargo proteins from the endolysosomal system back to the ER/Golgi or sorting/recycling endosomes, for recycling back to the plasma membrane (34). We

observed colocalization of the endocytosed GP64:Ab complexes with the marker for Retromer complex (Vps26) (Fig. 6B, green) suggesting that endocytosed GP64 may be recycled back to the plasma membrane, as is known to occur for viral envelope proteins from Nipah virus (35), respiratory syncytial virus (36), vesicular stomatitis virus (37), and varicella-zoster virus (38). We cannot eliminate the possibility that some of the GP64 endocytosed from the cell surface may also be trafficked through the endolysosomal system and eventually degraded. However, the observation that Rab5 and Rab7 depletion leads to increased cell surface levels of GP64 and that we observed endocytosis of GP64 indicates that GP64 is not static at the cell surface once delivered there. Thus, at least a portion of the GP64 delivered to the cell surface appears to be endocytosed and is likely recycled back to the cell surface.

Discussion

To examine the requirements for trafficking of the viral GP64 envelope protein in insect cells, we generated a cell line that inducibly expresses GP64 and used it to measure the effects of a variety of RNAi knockdowns on trafficking of GP64 to the plasma membrane. We selected *D. melanogaster* cells as an experimental system because of the plethora of genetic and molecular tools available for this system, and *in vivo* tools and models available for extended future studies. We first developed an inducible cell line for expression of the GP64 protein and demonstrated that induction of GP64 expression was tightly regulated and robustly activated upon incubation of cells in Cu_2SO_4. Following GP64 induction, most of the GP64 was displayed on the cell surface, and only a small fraction of GP64 was found within the cell or released from cells in exosomes. Using the

688 engineered cell line, we developed and performed an RNAi screen that targeted 213
689 cellular genes that included 13 groups of genes involved in vesicular trafficking. We
690 identified 37 gene knockdowns that reduced GP64 surface levels by 40-90%, and thus
691 initially appeared to affect trafficking. To eliminate hits that may have resulted from
692 indirect or pleiotropic effects on the cell (and not on GP64 trafficking), we selected hits
693 with ≥50% of the control *gp64* mRNA levels, and found that 13 of the original 37 hits met
694 this criterion. The 13 hits included genes that encode Rab GTPases (and Rab effectors),
695 clathrin and associated adaptors, and a SNAP protein. Among the 13 hits, we further
696 assessed and confirmed the target gene knockdowns, and selected 6 genes for further
697 analysis. We then examined the cellular effects on GP64 trafficking from knockdowns of
698 Rab GTPases (*Rab1* and *Rab4*), clathrin and associated adaptors (*Chc*, *AP-2μ*, *AP-1-*
699 *2β*) and *Snap29*.

700 Rab GTPases are regulators of vesicular trafficking that control vesicular
701 interactions and tethering of vesicles to discrete organellular membranes. Rab1 serves a
702 variety of roles or functions, and is largely associated with the ER, Golgi and intermediate
703 compartments (ERGIC) whereas Rab4 is localized to post-Golgi sorting/recycling
704 endosomes (39, 40). The individual knockdown of *Rab1* or *Rab4* disrupted transport of
705 GP64 to the cell surface indicating that GP64 trafficking requires Rab1 assisted ER to
706 Golgi trafficking, as well as Rab4 mediated post-Golgi sorting (Fig. 7). GP64 glycosylation
707 in the Golgi is necessary for proper folding and trimerization of the envelope fusion
708 protein. It is understandable then that disrupted Rab1-mediated ER-Golgi transport
709 should result in reduced GP64 transport within post-Golgi organelles, and accordingly,
710 we saw that increased ER/Golgi GP64 accumulation reflected the reduced amount of cell

surface GP64 with Rab1 depletion. The observation that Rab4 depletion also reduced GP64 trafficking to the cell surface suggests a necessary post-Golgi GP64 quality control or sorting step in Rab4 positive endosomes. Some proteins are transported directly from the Golgi to the PM, while others may traffic through sorting or recycling endosomes, and some proteins may use both routes to the PM (41). Proteins can be transported directly from the Golgi to the PM in a Rab8/Rab10-dependent manner. Otherwise proteins may transit via either Rab11-positive or Rab4-positive endosomes and/or vesicles *en route* to the PM. In addition, proteins delivered to the cell surface may be subsequently endocytosed and then trafficked through either Rab11-positive (slow) or Rab4-positive (fast) endosomes back to the cell surface. Because we found that cell surface GP64 is endocytosed, and that the endocytosed GP64 associates with Retromer, a molecular hub for recycling of endocytosed membrane proteins, this suggests that GP64 that is endocytosed might then be recycled back to the cell surface. Thus, it is unclear whether the requirement for Rab4 is for initial trafficking to the cell surface (via Rab4-positive sorting endosomes), or for maintenance at the cell surface via efficient recycling from the cell surface and back via Rab4 recycling endosomes. It is also possible that both are true, with Rab4 recycling endosomes involved in both the initial trafficking of GP64 to the cell surface, and subsequently endocytosis and recycling back to the cell surface. It is also notable that a knockdown of Rab11 (another Rab GTPase known to localize to and function in slow recycling endosomes), also reduced GP64 surface levels but the reduction was less severe ($\leq$ 30% reduction)(Table S2). More detailed studies should better define the role of endosomes in the route(s) of GP64 transport and possibly recycling.

734 The knockdown of *clathrin heavy chain* (*chc*) and the *AP-2μ* and *AP-1-2β* clathrin
735 adaptors comprised 3 of the 6 genes validated as important for GP64 transport. It is also
736 notable that of these 6 selected genes, *chc* and *AP-1-2β* represented the most severe
737 reductions of GP64 plasma membrane levels (Fig. 4A). Formation of clathrin coated
738 vesicles (CCVs) occurs at many potential organellular membranes, but certain clathrin/AP
739 complexes (5 known, 3 well described) are known to assemble at distinct membranes
740 and have defined roles in directional subcellular transport: AP-1 CCVs assemble at the
741 trans-Golgi and traffic to recycling endosomes or the PM; AP-2 CCVs form at the PM and
742 traffic to early, recycling or sorting endosomes; AP-3 CCVs form from Golgi membranes
743 and transit toward late endosomes (42). AP complexes are heterotetrameric, each being
744 composed of two large (one of γ/α/δ/ε/ζ and β1-5), a medium (μ) and a small (α) subunit.
745 In mammals, there are also multiple isoforms of some subunits, but a single gene for each
746 subunit exists in *D. melanogaster* and other invertebrates. In *Drosophila*, the AP-1 and
747 AP-2 complexes share the AP-1-2β subunit (43) that was identified and validated as an
748 important gene in our screen. Because AP-1-2β has functional roles in both AP complex
749 1 (secretory) and AP complex 2 (endocytosis), it is difficult to discern at which step GP64
750 transport is affected when AP-1-2β is depleted. RNAi of other members of the AP-1 or
751 AP-2 complexes in our screen also did not elucidate further the requirement of either
752 complex for GP64 trafficking. (Knockdowns of AP-1μ or AP-1σ had only modest effects,
753 and knockdowns of AP-2α or AP-2σ had modest or inconsistent effects, respectively, on
754 GP64 surface levels. See Table S2.) It is also noted that we found that surface localized
755 GP64 was endocytosed and routed to Retromer labeled endosomes, implying that GP64
756 was recycled back to the PM. Thus, while the precise role(s) of the AP-1-2β subunit is not

757 clearly distinguished, overall it appears that both AP-1 and AP-2 CCVs contribute to GP64
758 trafficking, with roles in post-Golgi anterograde trafficking and retrograde recycling from
759 the PM, respectively.

760 Specificity of the AP complexes 1-3 to transport cargo proteins lies in the amino acid
761 motifs present on the cytoplasmic domains of cargo proteins they bind. AP complex
762 binding to cargo protein motifs is attributed to either the μ subunits, which binds tyrosine-
763 based (YXXØ) motifs, or through the combination of the γ–σ1, α–σ2, and δ–σ3 subunits
764 (of AP complexes 1-3, respectively) which bind dileucine-based [DE]XXXL[LI] motifs.
765 GP64 has a YXXØ motif in its cytoplasmic tail but no sequence that resembles the
766 dileucine motif. YXXØ motifs are bound by the μ1, μ2 and μ3 subunits (of the AP-1, AP-
767 2 and AP-3 complexes, respectively) (42, 44). A YXXØ motif in a cargo protein, when
768 bound by the μ1 of an AP-1 complex in the Golgi and post-Golgi vesicles, typically
769 promotes its transport to the PM, although additional motifs may modify the precise
770 trafficking pathway. The mammalian AP-1μA (mammals have 1μA and 1μB homologs) is
771 required for basolateral transport of YXXØ-containing membrane proteins. The
772 *Drosophila* AP-1μ ortholog, although much less well characterized, is also implicated in
773 basolateral protein trafficking since Sanpodo (an AP-1 dependent cargo protein trafficked
774 to basolateral membranes) accumulates apically in *Drosophila* sensory organ precursor
775 cells with depleted/mutant AP-1μ (45). This suggests that *Drosophila* AP-1μ, like its
776 mammalian ortholog AP-1μA, is responsible for basolateral transport of YXXØ-containing
777 cargo. In our screen, we detected only modest reductions (<20%) in GP64 surface levels
778 for three independent dsRNAs targeting *AP-1μ*, but we did not confirm GP64 mRNA
779 levels or *AP-1μ* knockdown efficiency in cells exposed to those dsRNAs. YXXØ motifs on

780 cell surface membrane proteins, through interaction with μ2 of AP-2 complexes, promote
781 internalization of the YXXØ-harboring proteins via clathrin-dependent endocytosis.
782 Although it has yet to be functionally characterized in detail, the GP64 YXXØ motif is a
783 likely candidate for directing the initial PM targeting of GP64 and also its subsequent
784 internalization by endocytosis, as observed in DL1:mtGP64 cells. In prior studies in which
785 the TM and cytoplasmic domains of AcMNPV GP64 were deleted or substituted with
786 sequences that do not contain a YXXØ motif, the cell surface localization of GP64 was
787 dramatically reduced (46, 47) suggesting that this motif plays a role in the initial targeting
788 of GP64 to the PM or its maintenance there. Currently, the fate of endocytosed GP64 is
789 unclear. Post-endocytic trafficking could lead to lysosomal degradation, or the
790 endocytosed GP64 may be recycled to the cell surface or released from cells by
791 exocytosis, as we observed (Fig. 1C,D).

792 We demonstrated endocytosis of cell surface GP64 and its subsequent association
793 with Retromer, suggesting that GP64 may be recycled to the cell surface. However, if the
794 endocytosed GP64 is not returned to sorting/recycling endosomes or the Golgi it could
795 follow the endocytic route to late endosomes/multivesicular bodies and be released from
796 cells in exosomes. We confirmed that DL1:mtGP64 cells produce exosomes containing
797 GP64, albeit at very low levels in the context of our assay kinetics (8 hr induction period).
798 The μ3 subunit of AP-3 complexes also binds YXXØ motifs and directs transport of
799 YXXØ-containing cargo proteins from the Golgi to late endosomes/multivesicular bodies.
800 We cannot determine from our current data whether AP-3 mediated trafficking of GP64
801 to late endosomes/multivesicular bodies occurs. Furthermore, we do not know what
802 proportion, if any, of the endocytosed GP64 is recycled to sorting/recycling endosomes

803 or the Golgi. Therefore, further studies will be required to determine which pool(s) of GP64
804 in cells is affected by knockdown of *Chc*, *AP-2μ*, *AP-1-2β* or *Rab4*.
805 Although the RNAi screen was developed to identify gene knockdowns that reduce
806 GP64 transport to the PM, we also detected significantly increased (0.5 - 2.5 fold) GP64
807 cell surface levels that resulted from several gene knockdowns. The most dramatic GP64
808 increases were observed from knockdowns of *Exo84* (2.5 fold), *Rab5* (1.75 fold) and
809 Rab7 (0.5 fold). We confirmed normal *gp64* mRNA levels in cells with each of these
810 knockdowns, and a robust depletion (<20%) of *Exo84* and *Rab5* mRNAs, as well as a
811 minor depletion of *Rab7* (86%). We did however note that two independent Rab7 dsRNA
812 knockdowns yielded similarly increased GP64 surface levels. Rab5 and Rab7 localize to
813 and have roles in sequential post-endocytic trafficking organelles (early endosome, EE;
814 late endosome, LE; multivesicular body, MVB; lysosome, LYS), so the observed increase
815 of surface GP64 levels from these knockdowns could result from trafficking defects in the
816 same trafficking pathway. When cell surface proteins (*i.e.* GP64) are endocytosed, the
817 internalized endocytic vesicles that are pinched off have Rab5 on their cytosolic surface.
818 Endocytic vesicles containing Rab5 fuse with EEs, a process that begins with Rab5
819 interacting with early endosome antigen 1 (EEA1) of EEs. Rab5-positive endosomes
820 containing the cargo protein (GP64) transition to Rab7-positive LEs in a manner involving
821 a co-regulated Rab5-Rab7 exchange. For this, Rab5 stimulates endosome acquisition of
822 Rab7 by recruiting the Rab7 GTPase exchange factor (GE; the Mon1/Ccz complex), and
823 Rab7 in turn brings in the GTPase activating proteins (GAP) for Rab5 (48). Because we
824 found that GP64 was endocytosed from the cell surface, it is possible that the Rab5 and
825 Rab7 knockdowns resulted in a reduction in endocytosis and thus an accumulation of

826 GP64 at the surface. There also exists the possibility that bifurcated trafficking of the
827 endocytosed GP64 within in Rab5-positive endosomes occurs (Fig. 7). Rabaptin-5 (a Rab
828 effector) binds both Rab5 and Rab4, via distinct binding sites, promoting cargo (GP64)
829 transfer from early endosomes to Rab4-positive recycling endosomes (49). At least a
830 minor portion of the GP64 is trafficked to LEs/MVBs because GP64 is present in
831 exosomes presumably derived from MVBs (Fig. 1, 7). However, we do not know the
832 original source of GP64 present in exosomes since GP64 may be transported to
833 LEs/MVBs by either the a) PM endocytosis-Rab5-Rab7 pathway or b) the AP-3 complex
834 that assists sorting and transport of YXXØ-containing cargo proteins (such as GP64) from
835 the Golgi to LEs/MVBs. It should be noted that in our screen we also observed reductions
836 in GP64 surface levels for knockdowns of two of the three AP-3 components (cm and rb).
837 For the YXXØ-binding μ3 ortholog (cm) and the β3 homolog (rb) we saw moderate to
838 substantial reductions (Fig. 4A and Table S2) but only for a single gene-specific dsRNA.
839 Further studies will be required to understand the precise roles of Rab5 and Rab7 and
840 the pathways involved in GP64 trafficking.

841 Sorting/recycling endosomes are also known to sometimes play important roles in
842 basolateral trafficking in polarized epithelia. Proteins targeted to basolateral membranes
843 can traffic either directly from the TGN (50) or traffic first through sorting/recycling
844 endosomes before surface delivery (41) (Fig. 7). During the primary phase of the
845 infection, GP64 traffics to basal membranes of polarized lepidopteran midgut epithelial
846 cells, facilitating basolateral release of infectious budded viral progeny into the hemocoel
847 (2). In the subsequent systemic infection, many cell types are infected and those cells
848 may have divergent states of polarity and thus trafficking pathways. It is possible that

GP64 may be transported via several trafficking pathways as a means of efficient virus assembly and budding from different cell types that are infected in the animal. In the current study, we used the power of a relatively broad screen in *Drosophila* cells to identify host proteins and to provide clues to the specific pathways utilized for trafficking of this important viral envelope protein. In addition to further studies to refine the results of the current study, it will also be important in future studies to examine determinants of polarized GP64 protein transport in cells of critical tissues such as the midgut epithelium, and in an insect model. Understanding viral trafficking in tissues that are critical for successful infection will have relevance not only to insect-pathogenic viruses such as baculoviruses, but also to arboviruses and plant viruses that are transmitted by insects, and that must also navigate cells of the polarized midgut epithelium. We are currently continuing these studies in a *Drosophila*-based *in vivo* model system.

Acknowledgments

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Figure 6: Validation and analysis of gene knockdowns that increased cell surface GP64 levels. A) Identification and validation of several host genes for which dsRNA treatment resulted in increased cell surface GP64 levels. Left panel: RNAi knockdowns of Rab7, Rab5, and Exo84 resulted in approximately 1.5 - 2.5 fold increases in GP64 cell surface levels (black bars), compared with control lacZ dsRNA as determined by flow cytometry as in Figure 4 (dashed red line indicates the relative control lacZ value). Middle panel: *gp64* mRNA levels (red bars) in the presence of each of the RNAi knockdowns were determined by RT-qPCR and were similar to that from control cells treated with lacZ dsRNA (1.0 indicates control RNAi against lacZ). Right panel: mRNAs from RNAi knockdowns of target host genes (*Rab5*, *Rab7*, and *Exo84*) were measured by RT-qPCR and compared with levels of the same mRNAs in the presence of a control lacZ dsRNA knockdown (1.0). *Rab5* and *Exo84*, but not *Rab7* target mRNA levels were substantially depleted (>50%) after 4 days of treatment with the corresponding dsRNA. B) Endocytosis of cell surface GP64. Cell surface GP64 was labelled with a GP64-specific monoclonal antibody (AcV1) by incubation at 4ºC. As a control, trypsin was used to remove cell surface GP64 prior to GP64 immunolabelling (+Trypsin). Cells were shifted to 27ºC and allowed to endocytose GP64:Ab complexes for 30 min before fixation, permeabilization, and counterstaining with an anti-VPS26 antibody, and analysis by confocal microscopy as described in the Materials and Methods section. GP64 (red) that was endocytosed was observed associated with VPS26 (Retromer, green) labeled organelles (white arrowheads). Colocalization is indicated by yellow.

Figure 7: A model of GP64 trafficking in insect cells. The roles of cellular pathways, protein complexes, and individual proteins are illustrated based on known functions and localization patterns, and from experimental data. Trimeric GP64 is synthesized, folded, and post-translationally modified in the endoplasmic reticulum (ER) and Golgi. The AP-1/clathrin complex mediates transport of select membrane proteins from the Golgi to the

plasma membrane (PM) either directly in Rab8/Rab10 positive Golgi-derived secretory vesicles, or indirectly through Rab4 (fast) or Rab11 (slow) containing recycling endosomes. Individual depletion of Rab4 or Rab11 reduced PM levels of GP64, although the effect of the Rab11 depletion (dashed line) was only moderate. The AP-2/clathrin complex is involved in clathrin-mediated endocytosis of PM-localized proteins. GP64 displayed on the PM was subsequently endocytosed and localized to retromer-containing endosomes, suggesting recycling of endocytosed GP64 to the PM. Recycling to the PM may occur either directly, or via the Golgi or recycling endosomes. AP-1-2β depletion resulted in a dramatic decrease in GP64 levels on the PM. In Drosophila, both the AP-1 and AP-2 adaptor complexes contain AP-1-2β. Thus, it is unclear whether lack of either AP-1 or AP-2 complex function (or both) are the cause of reduced GP64 PM levels. Depletion of either Exo84, Rab5 or Rab7 resulted in increased levels of GP64 at the PM, which may have resulted from disruptions in GP64 endocytosis or exocytosis.

Figure 1

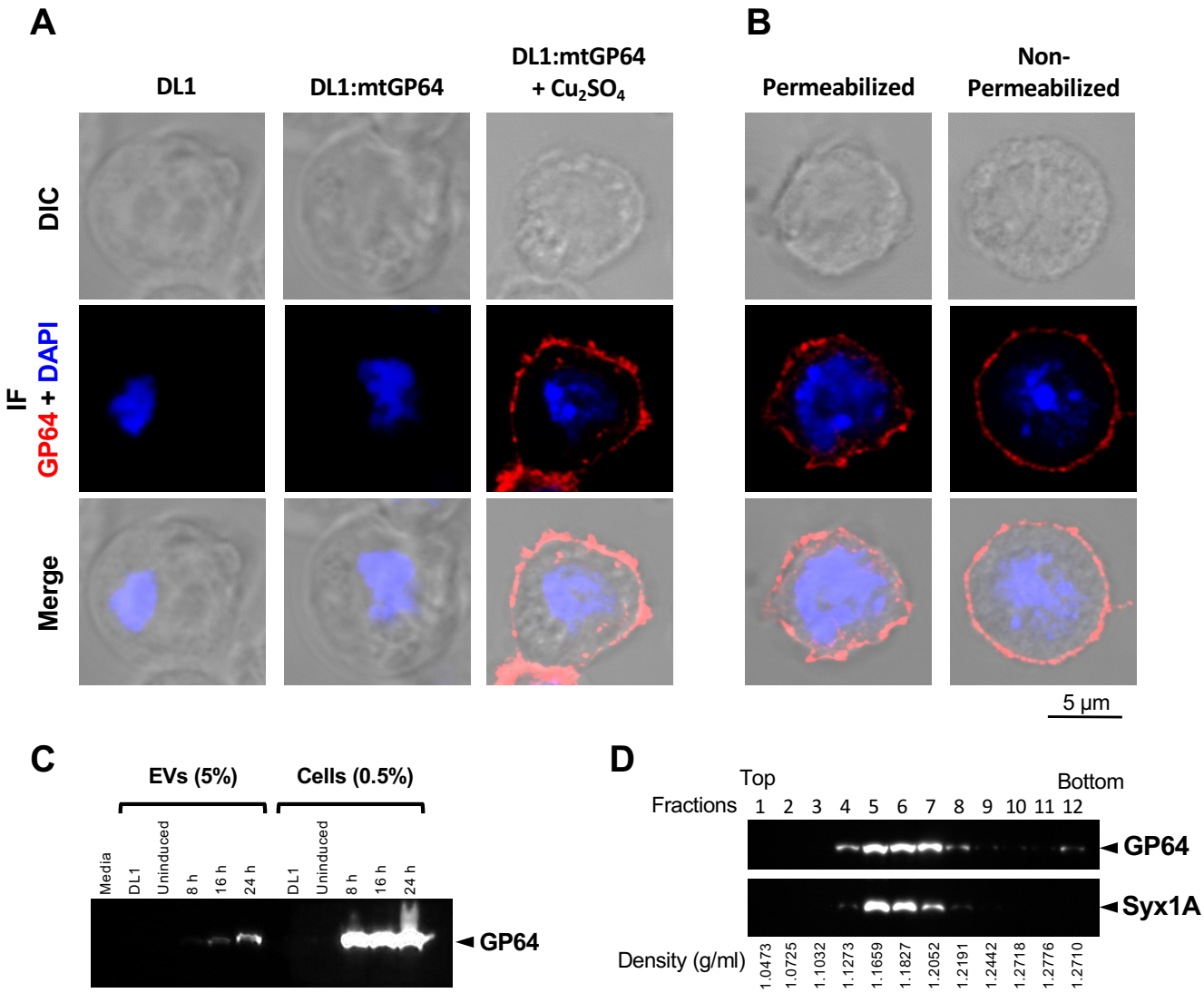

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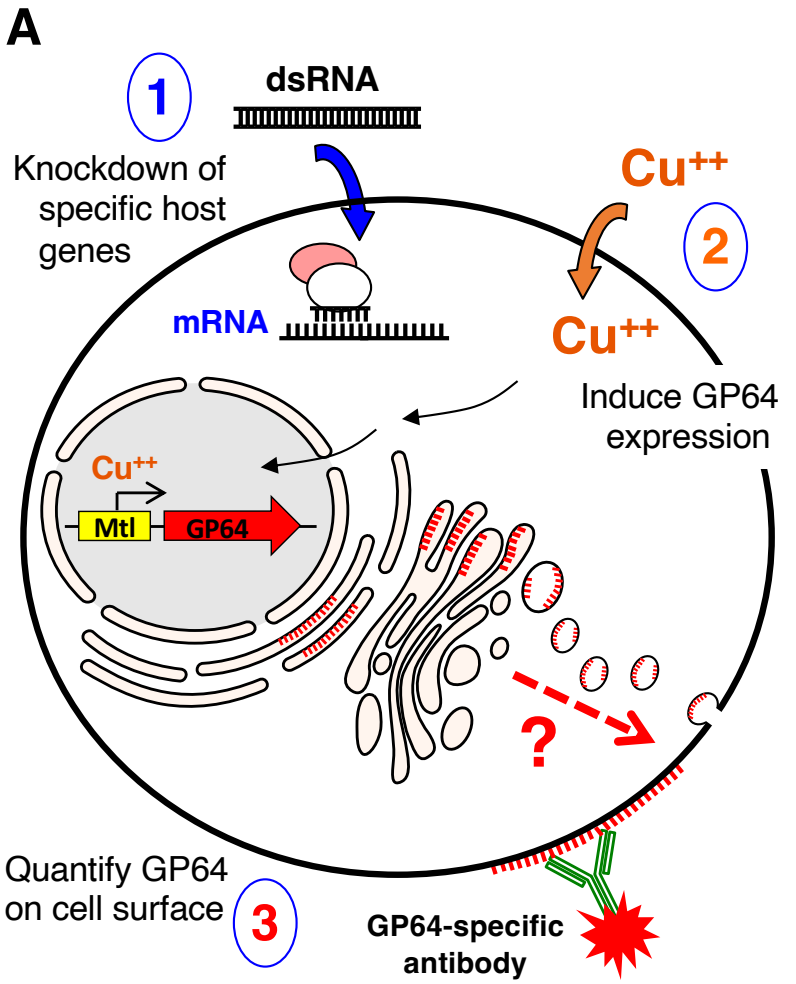
Figure 2



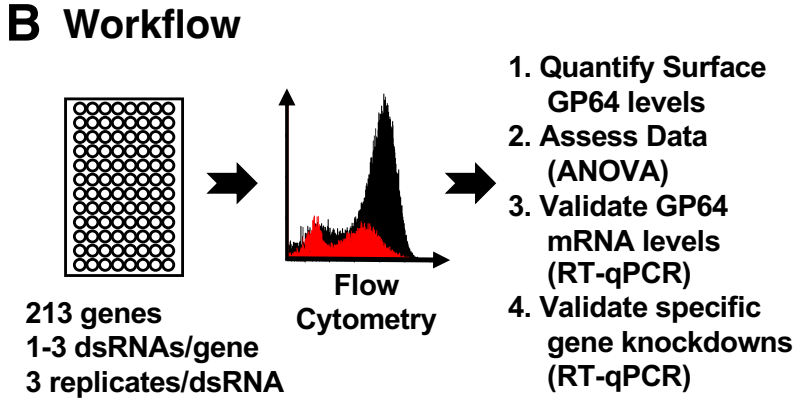


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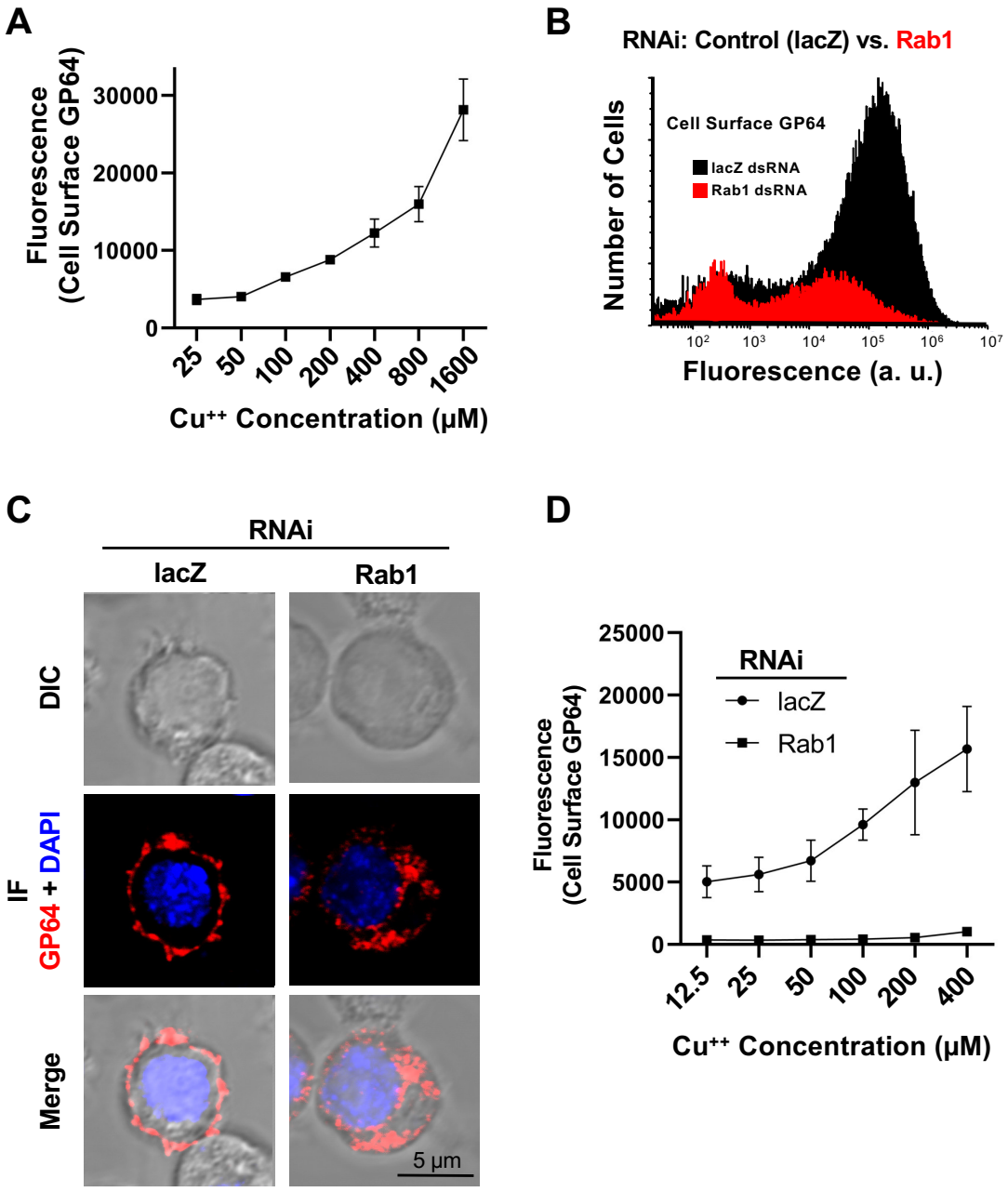
Figure 3



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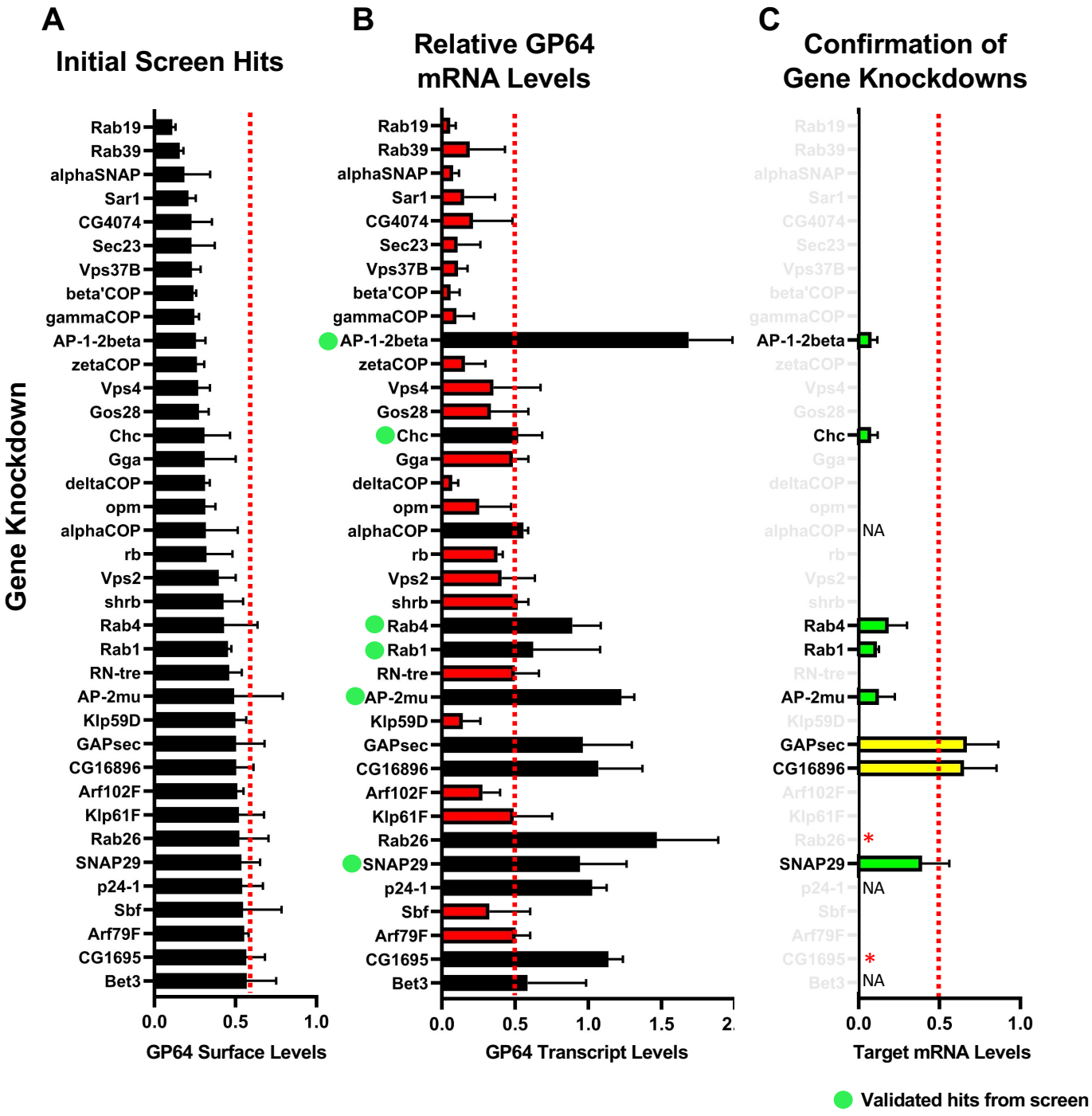

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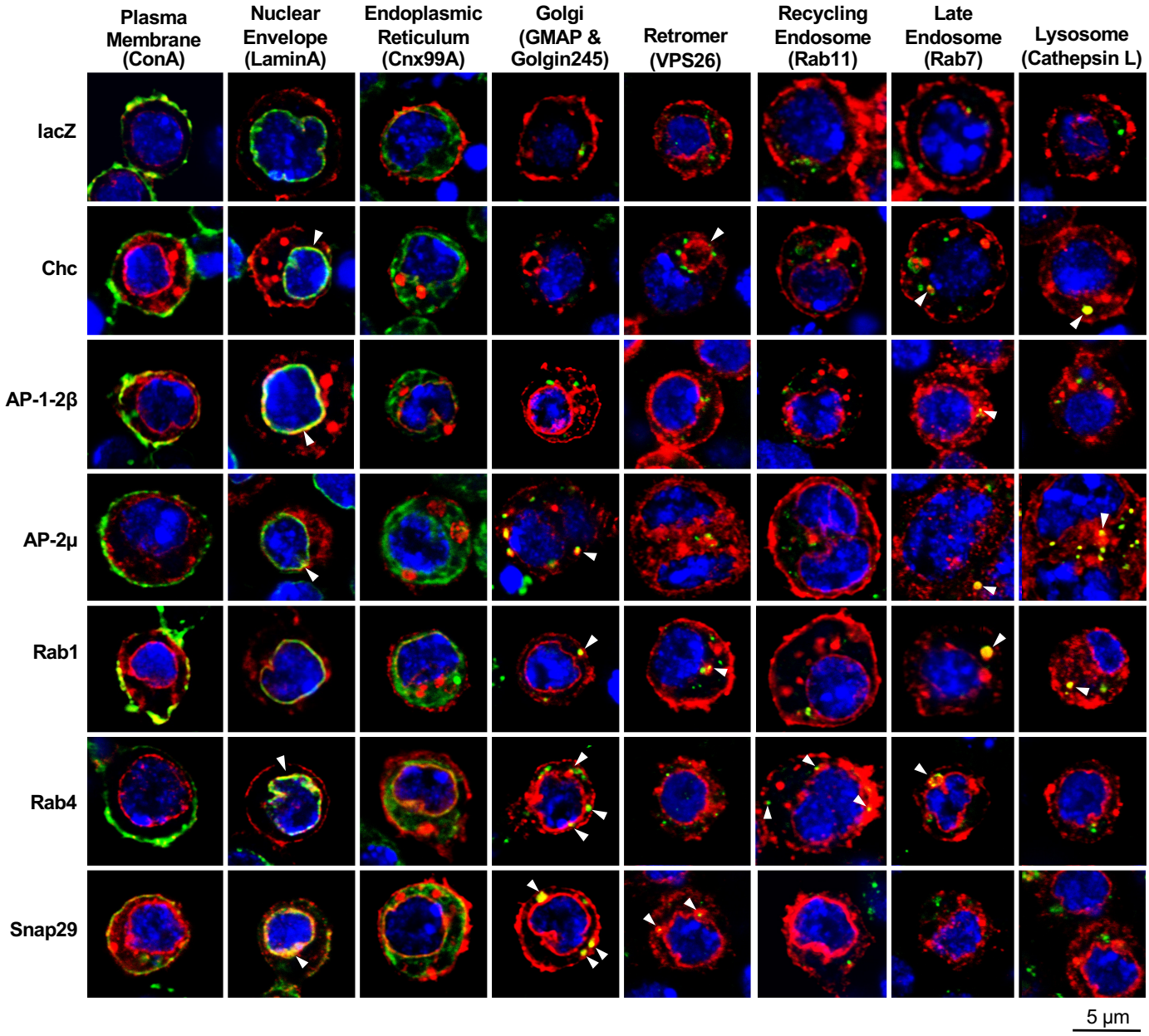

Figure 5: Cellular localization of GP64 in cells with RNAi knockdowns of validated target genes. Cells were exposed to each indicated dsRNA for 4 days, then seeded onto sterile coverslips. GP64 expression was induced by incubation in 200 μM Cu_2SO_4 for 16 h. Cells were permeabilized with Triton x-100 and co-stained for GP64 (red), DAPI (blue), and the indicated organelle protein markers (green) and imaged with a 63x oil immersion objective of a confocal microscope. RNAi knockdowns are indicated on the left of each row, and cellular compartment marker staining is indicated above each column. Examples of colocalization of GP64 with compartment-specific markers are indicated by white arrowheads.

Figure 6

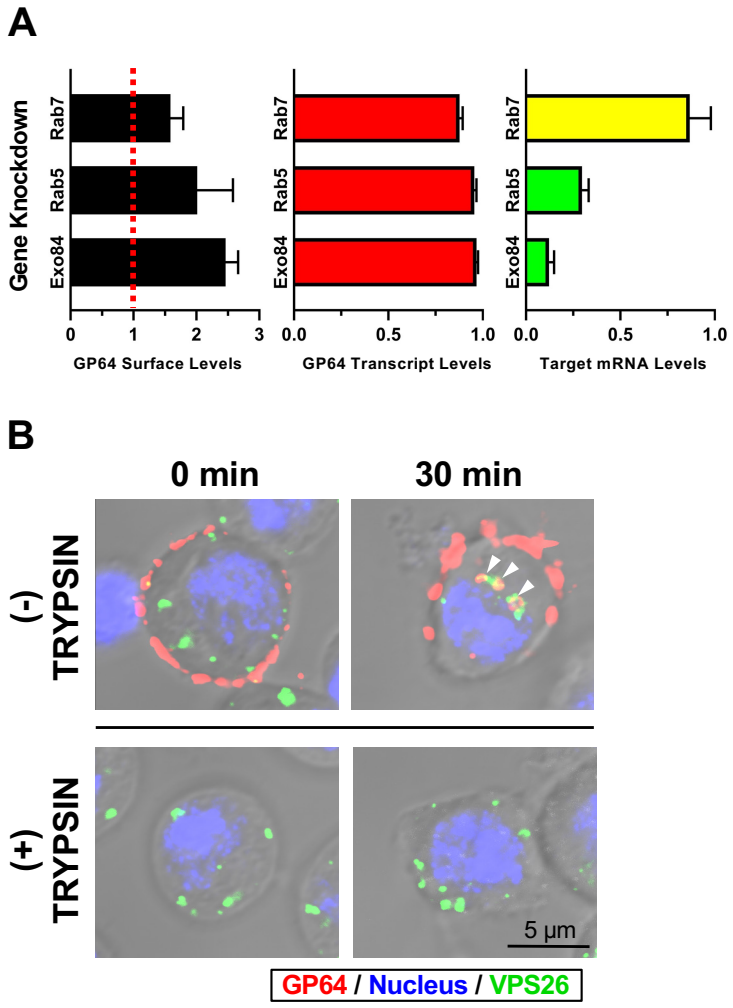

Figure 6: Validation and analysis of gene knockdowns that increased cell surface GP64 levels. A) Identification and validation of several host genes for which dsRNA treatment resulted in increased cell surface GP64 levels. Left panel: RNAi knockdowns of Rab7, Rab5, and Exo84 resulted in approximately 1.5 - 2.5 fold increases in GP64 cell surface levels (black bars), compared with control lacZ dsRNA as determined by flow cytometry as in Figure 4 (dashed red line indicates the relative control lacZ value). Middle panel: *gp64* mRNA levels (red bars) in the presence of each of the RNAi knockdowns were determined by RT-qPCR and were similar to that from control cells treated with lacZ dsRNA (1.0 indicates control RNAi against lacZ). Right panel: mRNAs from RNAi knockdowns of target host genes (*Rab5*, *Rab7*, and *Exo84*) were measured by RT-qPCR and compared with levels of the same mRNAs in the presence of a control lacZ dsRNA knockdown (1.0). *Rab5* and *Exo84*, but not *Rab7* target mRNA levels were substantially depleted (>50%) after 4 days of treatment with the corresponding dsRNA. B) Endocytosis of cell surface GP64. Cell surface GP64 was labelled with a GP64-specific monoclonal antibody (AcV1) by incubation at 4º. As a control, trypsin was used to remove cell surface GP64 prior to GP64 immunolabelling (+Trypsin). Cells were shifted to 27 ºC and allowed to endocytose GP64:Ab complexes for 30 min before fixation, permeabilization, and counterstaining with an anti-VPS26 antibody, and analysis by confocal microscopy as described in the Materials and Methods section. GP64 (red) that was endocytosed was observed associated with VPS26 (Retromer, green) labeled organelles (white arrowheads). Colocalization is indicated by yellow.

Figure 7

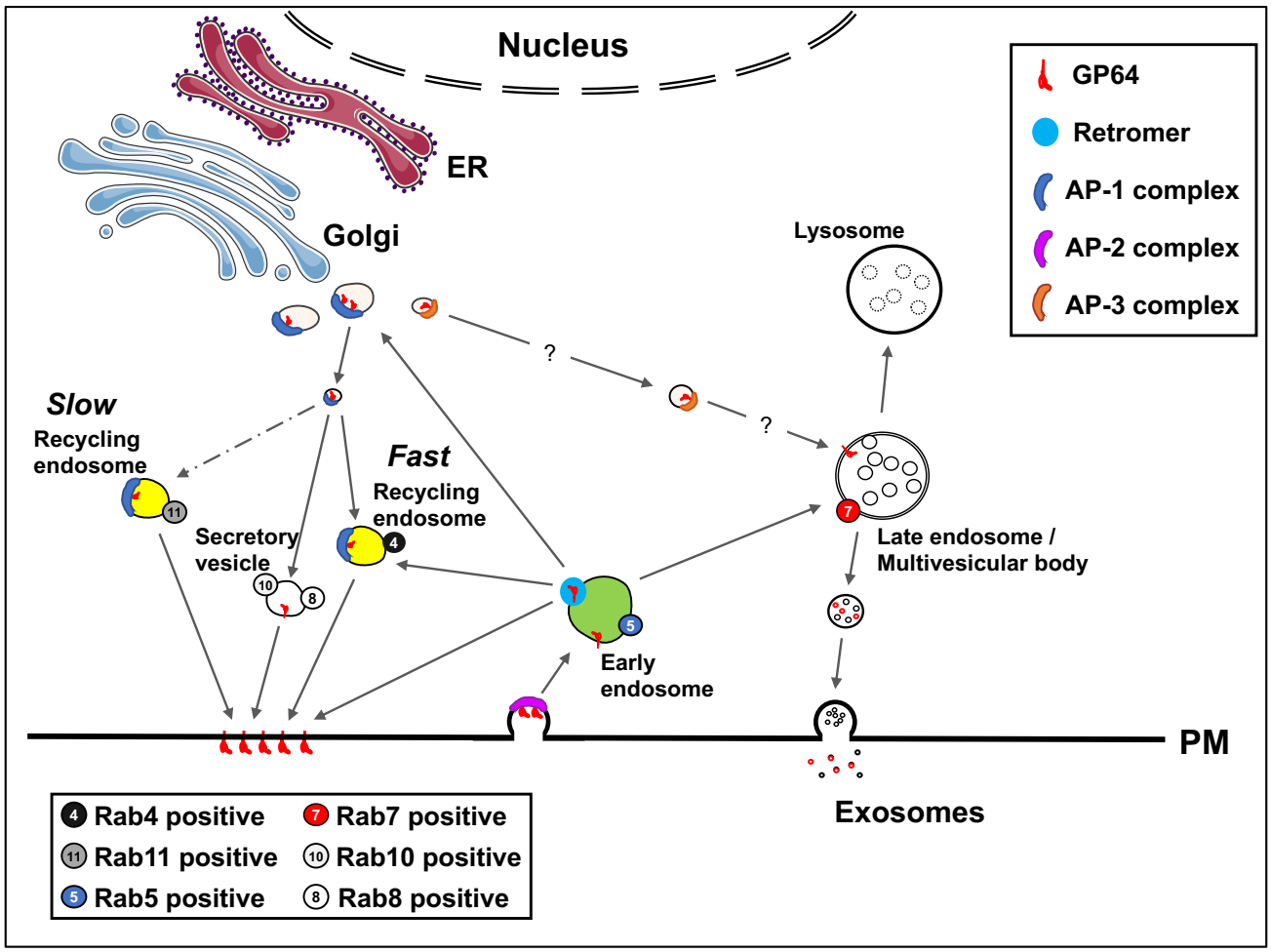

Figure 7: A model of GP64 trafficking in insect cells. The roles of cellular pathways, protein complexes, and individual proteins are illustrated based on known functions and localization patterns, and from experimental data. Trimeric GP64 is synthesized, folded, and post-translationally modified in the endoplasmic reticulum (ER) and Golgi. The AP-1/clathrin complex mediates transport of select membrane proteins from the Golgi to the plasma membrane (PM) either directly in Rab8/Rab10 positive Golgi-derived secretory vesicles, or indirectly through Rab4 (fast) or Rab11 (slow) containing recycling endosomes. Individual depletion of Rab4 or Rab11 reduced PM levels of GP64, although the effect of the Rab11 depletion (dashed line) was only moderate. The AP-2/clathrin complex is involved in clathrin-mediated endocytosis of PM-localized proteins. GP64 displayed on the PM was subsequently endocytosed and localized to retromer-containing endosomes, suggesting recycling of endocytosed GP64 to the PM. Recycling to the PM may occur either directly, or via the Golgi or recycling endosomes. AP-1-2β depletion resulted in a dramatic decrease in GP64 levels on the PM. In Drosophila, both the AP-1 and AP-2 adaptor complexes contain AP-1-2β. Thus, it is unclear whether lack of either AP-1 or AP-2 complex function (or both) are the cause of reduced GP64 PM levels. Depletion of either Exo84, Rab5 or Rab7 resulted in increased levels of GP64 at the PM, which may have resulted from disruptions in GP64 endocytosis or exocytosis.