High Throughput siRNA Screening Identifies Phosphatidylinositol 3-kinase Class II Alpha as Important for Production of Human Cytomegalovirus Virions

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Running Head: PI3K-C2A is important for HCMV replication

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ABSTRACT

High throughput siRNA screening is a useful methodology to identify cellular factors required for virus replication. Here we utilized a high throughput siRNA screen based on detection of a viral antigen by microscopy to interrogate cellular protein kinases and phosphatases for their importance during human cytomegalovirus (HCMV) replication, and identified the Class II Phosphatidylinositol 3-kinase PI3K-C2A as being involved in HCMV replication. Confirming this observation, infected cells treated with either pooled or individual siRNAs targeting *PI3K-C2A* mRNA produced approximately 10-fold less infectious virus compared to controls. Western blotting and quantitative PCR analysis of infected cells treated with siRNAs indicated that depletion of PI3K-C2A slightly reduced accumulation of late, but not immediate-early or early, viral antigens and had no appreciable effect of viral DNA synthesis. Analysis of siRNA treated cells by electron microscopy and western blotting indicated that PI3K-C2A was not required for production of viral capsids, but did lead to increased numbers of enveloped capsids in the cytoplasm that had undergone secondary envelopment and reduction of viral particles exiting the cell. Therefore, PI3K-C2A is a factor important for HCMV replication and has a role in production of HCMV virions.

43 **IMPORTANCE**

44

45 There is limited information about the cellular factors required for human
46 cytomegalovirus (HCMV) replication. Therefore, to identify proteins involved in
47 HCMV replication we developed a methodology to conduct a high throughput
48 siRNA screen in HCMV infected cells. From our screening data we focused our
49 studies on the top "hit" from our screen, the lipid kinase phosphatidylinositol 3-
50 kinase Class II Alpha (PI3K-C2A), as its role in HCMV replication was unknown.
51 Interestingly, we found that PI3K-C2A is important for the production of HCMV
52 virions and is involved in virion production after secondary envelopment of viral
53 capsids, the encapsidation of HCMV capsids by a lipid bilayer that occurs before
54 virions exit the cell.

55

INTRODUCTION

Identification of factors encoded by the cell that are required for virus replication can illuminate important features of virus-host interactions and identify novel drug targets for therapeutic intervention. Stages of productive human cytomegalovirus (HCMV) replication takes place in both the nucleus and the cytoplasm (1). After replication of the viral DNA genome in the nucleus, newly synthesized viral genomic DNA is packaged into nascent capsids in the nucleus. These capsids then bud through the nuclear membrane and, after accessing a viral assembly compartment in the cytoplasm (2), undergo a process of "secondary envelopment" in the cytoplasm in which capsids gain a lipid bilayer before exiting the cell (1). Many of these processes require the function of cellular factors, a number of which are unknown.

High throughput siRNA screening has been a successful strategy to identify cellular factors important for replication of several viruses (3-9). Many of the factors that have been identified by this strategy are kinases that are involved in a diverse range of cellular processes (3-9). Cellular protein, nucleotide or lipid kinases are involved in many aspects of HCMV replication (1). For example, cellular protein kinases are involved in the intracellular signaling required for activation of viral transcription (10, 11) and many other processes (1). The roles of cellular nucleotide kinases in HCMV replication are less well characterized, but are likely to be important for HCMV DNA synthesis as they are involved in nucleotide metabolism. Another form of kinase protein that must be considered

are lipid kinases, proteins that phosphorylate the inositol ring of phosphatidylinositol (PtdIns). Only a few investigations have examined whether lipid kinases are involved in HCMV replication. Class I Phosphatidylinositol 3-kinase (PI3K) is important for intracellular signaling involved in HCMV replication (12) and class III PI3K Vps34 is required for secondary envelopment to occur (13). However, the role of class II PI3K proteins in HCMV replication is unknown.

High throughput siRNA screens targeting human kinase and metabolic proteins has been performed and have highlighted the involvement of many cellular proteins and pathways in HCMV replication, notably, the cellular metabolic pathways involving 5′-AMP–activated protein kinase (AMPK) (8, 9). However, studies of multiple siRNA screens against a common target demonstrate that different siRNA screens can produce different outcomes due to false positives, false negatives and the efficiency of siRNAs utilized in different experiments (7). Therefore, it is possible that any number of kinase proteins that are required for HCMV replication have yet to be identified.

To identify cellular kinases required for HCMV replication we developed a high throughput siRNA screen based on the detection of a viral antigen in infected cells by microscopy. From this screen, we found that several unrelated kinase proteins scored as hits, including the phosphatidylinositol 3-kinase Class II alpha protein (PI3K-C2A), which we examined further.

100 **MATERIALS & METHODS**

101

102 **Cells and viruses.** Human foreskin fibroblast (HFF) cells (clone Hs29) were
103 obtained from American Type Culture Collection no. CRL-1684 (ATCC,
104 Manassas, VA)). All cells were maintained in complete media: Dulbeccos
105 Modified Eagles Medium (DMEM) (Gibco) containing 5% fetal bovine serum
106 (FBS) (Gibco), plus penicillin and streptomycin. Unless indicated otherwise,
107 HCMV strain AD169 was used**.** HCMV strain Merlin(RCMV1111) (14) was a kind
108 gift from Richard Stanton (Cardiff University).

109

110 **Transfection and infection of cells for high throughput screening.** The
111 Dharmacon SMARTpool kinase-phosphatase collection (catalog number G-
112 003500) comprises 789 siRNA targets (Table S1) and was screened in triplicate
113 at the ICCB-L facility at Harvard Medical School. Each pool contains 4 individual
114 siRNA. Twenty-four hours before transfection 1000 HFF cells were seeded in
115 each well of each Corning 384 plate in complete media with no antibiotics.
116 Unless stated otherwise, liquid was added to wells using a WellMate apparatus.
117 At the time of transfection, an intermediate plate (ThermoScientific 384 well plate
118 no.4309) was prepared to mix siRNA with lipid. Thus, 8.5 l of each siRNA at a
119 concentration of 1 M was added to the intermediate plate along with 7 l of
120 OptiMEM (Gibco). The intermediate plate was incubated at room temperature for
121 5 minutes. During this incubation period a lipid:OptiMEM mix was prepared
122 (0.17 l Dharmafect 2 lipid (Dharmacon) plus 11 l OptiMEM per well) and

123 incubated for no more than 5 mins at room temperature. 11.17 l of lipid:OptiMEM
124 mix was added to each well of the intermediate plate and incubated for a further
125 20 mins at room temperature. Media was removed from plates containing HFF
126 cells with a suction manifold and 8 l of siRNA:lipid mix from the intermediate
127 plate was added to triplicate plates using a liquid handling robot. To the
128 transfected cells 30 l of complete media without antibiotics was added to each
129 well. In each plate, wells used for 6 negative and 6 positive controls were
130 transfected with SMARTpool Non-targeting siRNA #3 (D-001810-03-05) or
131 SMARTpool Human PLK1 (M-003290-01), respectively. Where indicated
132 individual PI3K-C2A SMARTpool siRNAs (LU-006771-00-0005) were used in 4
133 wells of a plate. Control and individual siRNAs were added to intermediate plates
134 by hand. Transfected cells were incubated for 72 hours at 37°C. Cells were then
135 infected with HCMV strain AD169 (MOI 1) in a total volume of 20 l for 2 hours at
136 37°C. The inoculum was removed and cells were incubated for a further 72 hours
137 at 37°C. Plates were then analyzed by microscopy to assess HCMV replication.
138
139 **Preparation of screening plates for high throughput microscopy analysis.**
140 Cell culture media was removed from infected cells and replaced with 20 l
141 Hoecsht 33342 (SIGMA) diluted in phosphate buffered saline (PBS) to a final
142 concentration of 10 g/ml. After incubation for 1 hour at 37°C, 20 l of Deep Red
143 CellMask (Invitrogen) (diluted in PBS to a concentration of 5 g/ml) was added to
144 each well. Cells were incubated for a further 5 mins at 37°C. Cells were then
145 fixed by removing PBS containing Hoescht and Cell Mask and adding 50 l of

146 3.5% formaldyhyde (SIGMA) in PBS to each well. After incubating at room
147 temperature for 10 mins, fixative was removed and 50 l of PBS containing 0.5%
148 TritonX-100 was added per well to permeablize cells. After 10 mins incubation at
149 room temperature, PBS containing detergent was removed, and cells were
150 washed once with PBS. PBS was removed and replaced with 20 l MAb P207
151 recognizing pp28 (Virusys) (dilution 1:1000) and anti-mouse secondary antibody
152 conjugated to flurophore Alexa488 (Molecular Probes) (dilution 1:1000). Plates
153 were incubated at 37°C for 1 hour. After incubation, PBS containing antibodies
154 was removed and replaced with 50 l of PBS. Plates were then analyzed by
155 microscopy.
156
157 **Microscopy analysis of screening plates.** Infected cells stained with antibody
158 to detect pp28 were imaged on an Image Express Micro (IXM) microscope
159 (Molecular Devices) at 10x magnification to detect 3 wavelengths; 488 nm to
160 detect antibody recognizing pp28, 568nm to detect Deep Red CellMask and 350
161 nm to detect Hoescht 33342 stain bound to DNA. Three images were captured
162 from each wavelength in each well of 384-well plates. The number of cells
163 positive for all 3 wavelengths and percentage of pp28 positive cells in each well
164 was determined by analyzing the presence of signal from each wavelength using
165 the Metamorph Multiwavelength Cell Scoring software (Molecular Devices).
166 Typically, the average number of pp28 positive cells found when cells were
167 treated with non-targeting siRNA was 30-40% (data not shown).
168

Analysis of screening results. To assess the quality of data that could be returned from the screening protocol we calculated the Z'-factor (15, 16) derived from the positive (PLK1 siRNA treated infected cells) and negative (Non-targeting siRNA #3 infected cells) controls. The screening controls returned Z'-factors of greater than or equal to 0.5, indicating a robust separation of difference in the data derived from positive and negative controls. Thus, the screening protocol could be reliably used to screen the siRNA collection.

After screening of the siRNA collection data was analyzed to address siRNA cytotoxicity. siRNAs were judged to have a cytotoxic effect when the number of cells stained with Hoescht 33342 in a well fell below 2-fold of the mean of the number of cells in each well of the plate. Data from those wells containing cytotoxic siRNA was discarded. The data from the remaining wells from each plate was converted to a z-score (15, 16) and the average z-score from data in triplicate plates was determined. Images chosen at random were visually inspected throughout image capture and analysis to ensure raw data was consistent with z-scores.

GESS and Haystack analysis of siRNA binding. The on-line version of GESS (17, 18) was used. For Haystack (19) analysis of top screening "hits", Haystack was downloaded from http://rnai.nih.gov/haystack/. siRNA sequences were compared to 3' untranslated regions (3' UTRs) of human mRNA provided by Haystack. A list of viral 3'UTRs (300 nucleotides) was generated from the HCMV

191 AD169 genome and compared to siRNA sequences. All siRNA sequences were
192 supplied by Dharmacon.
193
194 **Kinase inhibitors.** Compound C was purchased from Merck, and resuspended
195 in dimethyl sulfoxide (DMSO).
196
197 **Preparation of cell lysates for western blotting.** In experiments to detect
198 PI3K-C2A, $1x10^5$ HFF cells were washed twice with ice cold PBS and
199 immediately scraped into 100 ml of lysis buffer (50 mM Trizma, 150 mM NaCl,
200 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 10% glycerol, 1 mM
201 sodium fluoride, 2.5 mM sodium pyrophosphate, 50 mM β-glycerophosphate, 1
202 mmol/L sodium orthovanadate, 40 mg/L phenylmethylsulfonyl fluoride, plus 1
203 Halt Protease Inhibitor tablet (ThermoSci #1860932)/100ml). Lysate was
204 incubated on ice for 30 mins, then centrifuged for 5 mins at 12,700 rpm.
205 Supernatant was removed and diluted 1:1 in 2x Laemmli buffer containing 5% β-
206 mercaptoethanol. Samples were incubated to 60°C for 10 mins.
207 All other samples were prepared for western blotting by washing cells
208 once in PBS, the suspending cells directly in 2x Laemmli buffer containing 5% β-
209 mercaptoethanol before incubating at 95°C for 5 mins.
210
211 **Western blotting.** Western blotting of proteins separated on 8% or 10% or
212 polyacrylamide gels was carried out as described elsewhere (20), using
213 antibodies recognizing IE1/2, UL44, pp28, (all Virusys, 1:1000 dilution), β-actin

(SIGMA, 1:5000 dilution), UL86 (a kind gift from Wade Gibson, Johns Hopkins University, 1:1000 dilution), gB (F3-11E, from the NIH AIDS Reagents Program, 1:1000 dilution) and PI3K-C2A (BD Bioscience #611046, 1:1000 dilution). All primary antibodies were incubated overnight at 4^{o}C and detected using anti-mouse- or anti-rabbit-horseradish peroxidase (HRP) conjugated antibodies (Southern Biotech). Chemiluminescence solution (GE Healthcare) (or when assaying for PI3K-C2A, Fempto detection reagent (Thermo)) were used to detect secondary antibodies on film. Where indicated in the text, relative band intensities were calculated using ImageJ software provided by the National Institutes of Health, USA.

Transfection of siRNA into HFF cells. Briefly, 1 x 10^5 HFF per well were seeded in 12-well plates 24 hours before transfection in DMEM+5%FBS with no antibiotics. Per well, 113 µl of 1 µM siRNA and 2 µl Dharmafect2 (Dharmacon) were diluted in 93 µl and 146 µl Optimem (Invitrogen), respectively. After 5 mins at room temperature, both solutions were combined. After 20 mins, media was removed from each well and replaced with the siRNA/Dharmafect mixture and 500 µl of DMEM+5%FBS with no antibiotics was added to each well. Transfected cells were incubated at 37^{o}C for 72 hours then either prepared for western blotting or infected with 1 x 10^5 plaque forming units (p.f.u./ml) of AD169.

Determination of viral titer. Titers were determined by serial dilution of viral supernatant onto HFF monolayers which were then covered in DMEM containing

5% FBS and 0.6% methylcellulose. Cultures were incubated for 14 days, cells were stained with crystal violet and plaques were counted.

Real time quantitative PCR analysis of viral DNA synthesis in siRNA treated cells. HFF cells were treated with siRNA and infected as outlined above in triplicate. DNA was isolated from infected cells using the NucleoSpin Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions. Viral genomes were quantified with a primer pair (pp549s and pp812as) to *UL83* (21) and the number of viral genomes was normalized to cellular copies of *adipsin* (22). Unknown sample values were determined on the basis of standard curves of known copy numbers of *UL83* (pcDNAUL83, a kind gift from Jeremy Kamil, Louisiana State University) and *adipsin* (from uninfected cell DNA). PCRs for *UL83* and *adipsin* were carried out on a BioRadCFX96 machine using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) as per the manufacturer's instructions. Linear regression analysis of *UL83* and *adipsin* standards in triplicate yielded R^2 values of 0.997 and 0.996. The mean number of copies of the viral gene *UL83* per copy of the cellular gene *adipsin* was calculated from the triplicate samples assayed.

Immunofluorescence. $5x10^4$ HFF cells were plated on glass coverslips. Cells were uninfected or infected with AD169 (MOI 1). Cells were fixed in ice-cold methanol at time points indicated in the text. After washing in Dulbecco's phosphate-buffered saline (DPBS), samples were permeabilized with 0.1% Triton X-100 dissolved in DPBS at room temperature (RT) for 10 min. The cells were

washed again with DPBS and incubated in 1% bovine serum albumin (BSA) dissolved in DPBS for 1 h at RT. Primary antibodies in 0.5% BSA dissolved in DPBS were applied and incubated for 1 h at 37°C. Antiserum was removed by washing cells once in 0.5% Tween dissolved in DPBS and twice with DPBS, each time for 5 min with rocking. This procedure was repeated for the secondary antibodies. DAPI (4′,6-diamidino-2-phenylindole) was applied in the last 10 min of the secondary antibody incubation. Coverslips were mounted on microscope slides with ProLong Antifade (Invitrogen-Molecular Probes). All imaging experiments were performed at the Nikon Imaging Center at Harvard Medical School, using a Nikon Ti microscope with a spinning disk confocal laser at a magnification of ×100. Images shown were obtained by acquiring sequential optical planes in the *z* axis, using the MetaMorph program.

All primary antibodies recognizing UL53 (23) or PI3K-C2A (BD Bioscience #611046) were used at a dilution of 1:100. All fluorescently labeled secondary antibodies (Alexa Fluor 488 or Alexa Fluor 594) were obtained from Molecular Probes and used at a dilution of 1:1000.

Electron Microscopy. Cells were transfected with siRNA and infected as outlined above. Infected cells were incubated for 1 hour at room temperature in fixative (2.5% glutaraldehyde 1.25% paraformaldehyde and 0.03% picric acid in 0.1 M sodium cacodylate buffer (pH 7.4)) at the indicated time points post infection. Cells were provided to the Harvard Medical School Electron Microscope Facility and washed in 0.1M sodium cacodylate buffer (pH 7.4), then

283 postfixed for 30 min in 1% Osmium tetroxide (OsO4)/1.5% potassium
284 ferrocyanide (KFeCN), washed in water 3x and incubated in 1% aqueous uranyl
285 acetate for 30min followed by 2 washes in water and subsequent dehydration in
286 ethanol (5min each; 50%, 70%, 95%, 2x 100%). Cells were removed from the
287 dish in propyleneoxide, pelleted at 3000 rpm for 3 min and infiltrated for 2hrs in a
288 1:1 mixture of propyleneoxide and TAAB Epon (Marivac Canada Inc. St. Laurent,
289 Canada). The samples were subsequently embedded in TAAB Epon and
290 polymerized at 60^{o}C for 48 hrs. Ultrathin sections (about 60nm) were cut on a
291 Reichert Ultracut-S microtome, picked up on to copper grids stained with lead
292 citrate. All samples were examined and images were recorded using a JEOL
293 1200EX Transmission electron microscope and an AMT 2k CCD camera,
294 respectively.
295
296 **Preparation of virions from siRNA treated cells for western blotting.** HFF
297 cells were transfected with either Ctrl or PI3K-C2A siRNA and infected as
298 outlined above. At 96 hours post infection viral supernatant from four Ctrl or four
299 PI3K-C2A siRNA transfections was collected (4ml in total). Supernatants were
300 clarified by centrifugation (13,000g, 5 mins, 4^{o}C) to remove cells and cell debris.
301 Virions were then pelleted from supernatants by ultracentrifugation (20,000rpm, 1
302 hour, 4^{o}C). Pellets were resuspended in 20 l of phosphate buffered saline
303 (Gibco). To test for protection from protease digestion, ten l of each
304 resuspended pellet was mixed with 10 l of trypsin (Gibco) and incubated for 1
305 hour at either 4^{o}C or 37^{o}C. After incubation, 20 l of 2x Laemmli buffer containing

306 5% β-mercaptoethanol was added to each samples and incubated at 95°C for 5
307 mins. For each western blot, 10 l of each sample was analyzed.
308
309

310 **RESULTS**

311

312 **Development of a high throughput screening methodology.** To
313 identify siRNAs that can affect HCMV replication, we sought to develop a high
314 throughput screening methodology. In our initial studies, we attempted to
315 establish a screen in which plates of cells were treated with siRNAs and infected
316 with viruses expressing reporters such as green fluorescent protein (GFP) or
317 luciferase (data not shown). However, we found that reporter expression was not
318 sufficient to be readily detected in our assay (data not shown) and we found
319 considerable well-to-well variation in the numbers of GFP positive cells, which
320 may be due, in part, to production of particles that initiate abortive infections.
321 These issues were found in all cell lines tested including human foreskin
322 fibroblasts (HFFs) and U373-MG cells, where additional issues arose due to at
323 least ten-fold lower titers (data not shown). Therefore, we chose not pursue a
324 screen based on detection of a reporter protein expressed from a recombinant
325 HCMV virus. Instead, we decided to establish an automated high throughput
326 screening methodology based on the detection of a viral antigen by microscopy,
327 similar to siRNA screens created by Koyuncu and co-workers or Brass and co-
328 workers to interrogate what cellular factors are required for replication of HCMV
329 and a variety of other viruses (3-7, 9). In preliminary experiments we found that
330 antibody staining of HFF cells to detect that viral antigen pp28 was a convenient,
331 reproducible and statistically robust methodology to detect HCMV replication
332 that, unlike our preliminary experiments, could be readily detected and did not

exhibit well-to-well variation that would make data interpretation problematic. As pp28 is expressed late in infection, this allowed our screen to identify factors affecting all stages of virus replication through to late gene expression, including virus attachment, entry, transcriptional activation and genome replication.

High throughput screening of siRNAs. We then carried out an siRNA screen, as outlined in Fig. 1A. Briefly, human foreskin fibroblasts (HFFs) were transfected in triplicate with siRNAs from the Dharmacon kinase-phosphatase collection (listed in Table S1). This collection of 779 pools of 4 siRNA targets mRNA encoding kinases (including nearly all protein kinases, 7 nucleoside kinases and 15 lipid kinases) and kinase related proteins (including 9 dual specificity phosphatases and proteins that interact with kinases, such as CD4). As negative and positive controls for siRNA treatment 6 wells in each plate were treated with either Dharmacon non-targeting siRNA #3 (Crtl siRNA) or siRNA targeting *polo-like kinase 1* (PLK-1) mRNA, respectively. PLK-1 is routinely used as a positive control in siRNA screening as depletion of PLK-1 induces apoptosis. Under conditions used here we found that treatment with PLK-1 siRNA inhibits HCMV replication without a statistically significant decrease in the number of cells per well. Thus, even though this treatment is cytotoxic, it fulfilled the conditions of the screen to serve as a positive control (see Materials & Methods and below). Each control and screened siRNA used contained a pool of 4 individual siRNAs. HFF were incubated with siRNA for 72 hours. In preliminary experiments 72 hour incubation was required to deplete proteins to near

356 undetectable levels by western blotting after treatment with a number of siRNAs
357 in the Dharmacon kinase-phosphatase collection (data not shown and Figs. 3A
358 and 3C). After incubation, siRNA transfected HFF cells were infected with HCMV
359 strain AD169 at a multiplicity of infection of 1 (MOI1). In preliminary experiments
360 this MOI provided the most statistically robust data (data not shown). At 72 hours
361 post infection (h.p.i.) cells were stained with Hoescht 33342 to detect nuclear
362 DNA and CellMask to identify the cell cytoplasm, plus treated with antibodies to
363 detect pp28. An automated microscopy system was then used to assay the
364 number of cells in each well and the number of cells containing pp28. An image
365 of infected cells treated as described above and captured using automated
366 microscopy is shown in Figure 1B. DNA and cells stained with Hoescht 33342
367 and CellMask are shown in blue and red, respectively. The cytoplasmic
368 localization of pp28 in viral assembly compartments is shown in green.
369 We then processed the screening data. Specifically, the mean number of
370 cells in each well per plate was determined by counting the number of Hoechst
371 stained nuclei. Where the number of cells in any well was less than 2-fold below
372 the mean number of cells of the plate, the siRNA in that well was judged to be
373 grossly cytotoxic. Five hundred and seventy two siRNAs (listed in Table S2) did
374 not pass this test and were not analyzed further. Data from the remaining 207
375 wells on triplicate plates were converted to a z-score (the number of standard
376 deviations from the mean of the data (15, 16)) to demonstrate the positive or
377 negative effect of siRNA on the number of pp28 positive cells detected. These

data were then combined to find the mean z-score of each pool of siRNA (Fig. 1C and listed in Table S3).

Analysis of siRNA that have positive and negative effects on HCMV replication. We adjudged any siRNA that produced a z-score between 1 to -1 to have had little or no effect on HCMV replication, whereas those with z-scores of -1 to -2 and 1 to 2 had modest negative or positive effects on HCMV replication, respectively. However, siRNAs with z-scores of less than -2 or more than 2 were deemed to have strong negative or positive effects on HCMV replication, respectively. Twenty five siRNAs were found to have modest positive effects on HCMV replication and 26 siRNAs had modest negative effects on HCMV replication. No siRNA was found to have a strong positive effect on HCMV replication, whereas 4 siRNAs (siRNAs targeting mRNA encoding PI3K-C2A, CD4, EXOSC10 and WNK4) had strong negative effects on HCMV replication (Fig. 1C and listed in Table S3).

Bioinformatics analysis of siRNA screening results to assay for off-target effects of siRNA treatment. False positive results can be found in siRNA screens due to off-target binding of siRNAs. Off-target siRNA binding is most likely to occur in the 3' untranslated region (3' UTR) of mRNA (24, 25) where siRNA exhibit microRNA-like properties upon binding of a limited number of bases, akin to a microRNA seed region, to mRNA (24-26). Indeed, it has been proposed that the data returned from some screens is the result of unintentional

401 screening of partial seed sequence matches and not on-target binding of siRNA
402 (26). Therefore, using genome-wide enrichment of seed sequences matches
403 (GESS) (17, 18) we investigated if the siRNAs screened in this study (all 4
404 siRNAs that constitute the pools of siRNAs used) are enriched with seed
405 sequences that could bind either human 3'UTRs or full human mRNA transcripts.
406 We found no statistical evidence that siRNAs screened here are enriched with
407 seed sequences that would bind to any human mRNA sequence (data not
408 shown). The binding of siRNA to the sequence of the entire HCMV AD169
409 genome was also assayed and it was found that there was no statistically
410 significant evidence for enrichment of seed sequences in the siRNAs assayed
411 that would bind to any sequence in the viral genome (data not shown).

412 We sought to confirm this analysis by analyzing the binding of siRNAs
413 from the top 4 "hits" from our screen (siRNAs targeting mRNA encoding PI3K-
414 C2A, CD4, EXOSC10 and WNK4, Fig.1C) using Haystack (19), another
415 bioinfomatic analysis that searches for statistically significant matches of siRNA
416 sequences with 3'UTR transcripts. We found no statistically significant matches
417 between the siRNAs tested with human or HCMV 3'UTRs (data not shown).

418 Therefore, the effects of siRNAs judged to be toxic or have negative or
419 positive effects on HCMV replication in our analysis may not be be due to off
420 target binding of siRNA seed matches to human or HCMV mRNA transcripts.

421 We then compared hits with z-scores of less than -1 and greater than 1 to
422 a proteomic dataset that lists all viral and cellular proteins detected in HCMV
423 infected HFF cells (27) (Table S4). We found that a number of proteins

424 purportedly depleted by siRNAs in our screen were not found in HCMV infected
425 HFF cells (8 of 30 hits and 12 of 25 hits with negative and positive effects of
426 HCMV replication, respectively.) Therefore, although GESS and Haystack
427 indicates that off-target siRNA binding does not occur, several false positives in
428 our screening data could be identified.
429
430 **PI3K-C2A siRNAs deplete PI3K-C2A and specifically reduce numbers**
431 **of pp28-infected cells.** We then chose to focus our studies on one of the top
432 "hits" in our screen. PI3K-C2A and EXOSC10, but not CD4 or WNK4, have been
433 found in HFF cells infected with HCMV (27) (Table S4). Therefore, CD4 and
434 WNK4 were excluded from further analysis. siRNAs targeting *PI3K-C2A* mRNA
435 had the greatest negative effect in our screen, and a role for PI3K-C2A in HCMV
436 replication had not, to our knowledge, been previously reported. We, therefore,
437 decided to focus on the role of PI3K-C2A in HCMV replication. We first examined
438 the effects of each of the 4 individual PI3K-C2A siRNAs (PI3KC2A-1 to -4) from
439 the PI3K-C2A siRNA pool was tested for its ability to inhibit HCMV replication by
440 assaying cell number and pp28 expression using the scheme shown in Fig. 1A.
441 Compared to cells treated with Ctrl siRNA, treatment of cells with each PI3K-C2A
442 siRNA had no statistically significant effect on cell number (+/- 10% of DMSO
443 control, Fig. 2A) and resulted in a statistically significant decrease in the number
444 of pp28 expressing cells by at least 45% (Fig. 2B). This confirmed that PI3K-C2A
445 is involved in HCMV replication and implied that the effect of the PI3K-C2A

446 siRNA pool used in our screen was due to depletion of PI3K-C2A, not result of an
447 off-target effect of any one siRNA in the siRNA pool.
448
449 **Effect of PI3K-C2A siRNA on production of infectious HCMV.** We then
450 assayed the effects of PI3K-C2A siRNA on production of infectious virus. HFF
451 cells were treated with either Ctrl siRNA, PI3K-C2A siRNA or the 4 individual
452 PI3K-C2A siRNAs from the PI3K-C2A siRNA pool (PI3KC2A-1 to -4) and infected
453 with HCMV. At various time points, supernatants were collected for titration of
454 infectious virus and infected cells were prepared for western blotting. Compared
455 to treatment of cells with Ctrl siRNA, treatment of cells with PI3K-C2A siRNA or
456 individual PI3K-C2A siRNAs resulted in a clear decrease in accumulation of
457 PI3K-C2A in infected cells (Figs. 3A and 3C), and a decrease in production of
458 infectious virus over time (9- and 7-fold with PI3K-C2A at 72-96 h.p.i.,
459 respectively, and 3- to 12-fold with individual siRNAs, Figs. 3B and 3D,
460 respectively). In these and subsequent western blots the amount of *β*-actin in
461 each sample was also assayed, which demonstrated equivalent loading of
462 samples in each lane. Therefore, depletion of PI3K-C2A in infected cells was
463 associated with a defect in the production of infectious HCMV virus. Also, a
464 decrease in production of virus from siRNA treated cells similar to that seen in
465 Figure 3B was observed from HFF cells treated with either Ctrl or PI3K-C2A
466 siRNA when infected with HCMV strain Merlin (data not shown). Thus, PI3K-C2A
467 is required for replication of at least two different HCMV strains.
468

Examination of viral protein and DNA production in cells treated with siRNA. We next sought to understand how HCMV replication was inhibited by the loss of PI3K-C2A. Therefore, western blotting was used to analyze the accumulation of immediate-early (IE1/IE2), early (UL44) and late (pp28) HCMV proteins and PI3K-C2A in lysate of HFF cells treated with either Ctrl or PI3K-C2A siRNA and infected with HCMV (Fig. 4). Treatment of HFF cells with PI3K-C2A siRNA resulted in a loss of PI3K-C2A accumulation in HCMV infected cells (Fig. 4A). However, we found no obvious difference in the accumulation of any viral protein (Fig. 4A). We reasoned that this may be due to high levels of protein produced late in virus replication saturating the signal from our western blots. Therefore, we created dilution series from the samples taken at 72 and 96 h.p.i. in Figure 4A and subjected these samples to western blotting (Fig. 4B). We found no obvious difference in accumulation of either IE1/2 or UL44. However, consistent with the data in Figures 1 and 3, we observed a ~2-fold difference in accumulation of pp28 in cells treated with PI3K-C2A siRNA compared to Ctrl siRNA (Fig. 4B). A similar reduction in the expression of the late viral antigen UL86 was observed (data not shown). However, loss of viral production (Fig. 3) in the presence of PI3K-C2A siRNA is greater than the modest decrease in pp28 expression that we observe in Fig. 4B. Therefore, depletion of PI3K-C2A is likely to inhibit the production or function of viral or cellular factors important for productive replication other than pp28.

As HCMV late gene expression is related to viral DNA synthesis (1), we hypothesized that a decrease in pp28 protein might reflect a defect in genome

replication. Therefore, we treated HFF with Ctrl or PI3K-C2A siRNA and after infection used quantitative real-time PCR to assay the number of HCMV genomes in infected cells at 72 h.p.i. The number of viral genomes present in each Ctrl or PI3K-C2A siRNA treated sample was determined by normalizing the copy number of a viral locus (*UL83*) to the copy number of a cellular locus (*adipsin*). We found no obvious decrease in the accumulation of viral DNA between infected cells that had been treated with either Ctrl or PI3K-C2A siRNA (1100 and 1200 copies *UL83*/copies *adipsin*, respectively). Therefore, the defect in virus replication and loss of pp28 expression in the absence of PI3K-C2A was unlikely to be due to a defect in viral DNA replication.

Analysis of HCMV capsid and virion production in cells treated with PI3K-C2A siRNA. We then employed electron microscopy (EM) analysis to further investigate how PI3K-C2A is involved in HCMV replication. We treated cells with either Ctrl or PI3K-C2A siRNA and after infection submitted cells for EM analysis and counted the number of viral capsids present in the nucleus and cytoplasm in whole cell sections of five cells (Figs. 5A and 5B, respectively). HCMV capsids can be found in 3 forms: A capsids; nonproductive forms thought to result from failed packaging of viral genomes, B capsids; forms that contain a scaffolding protein but no DNA, and C capsids; assembled forms in which the scaffolding protein has been removed and replaced with viral DNA. We, therefore, also counted the number of each form of HCMV capsid found in siRNA treated cells (Fig 5A and 5B). In both Ctrl and PI3K-C2A siRNA treated cells

515 approximately 3-fold more capsids were found in the nucleus compared to the
516 cytoplasm and we found no obvious difference in the number of any form of the
517 HCMV capsid in either the nucleus or the cytoplasm. Therefore, the loss of PI3K-
518 C2A did not affect the production of capsids, including genome containing C
519 capsids that lead to infectious virions, or the movement of capsids from the
520 nucleus to the cytoplasm.

521 However, compared to cells treated with Ctrl siRNA, we noted that in cells
522 treated with PI3K-C2A siRNA there was an ~3-fold increase in the number of
523 cytoplasmic capsids surrounded by lipid bilayer envelopes in the cytoplasm (Fig.
524 5C). Such particles are thought to have undergone secondary envelopment, a
525 late step in virion maturation prior to egress from the cell. Examples of such
526 enveloped capsids in cells treated with PI3K-C2A siRNA are shown in Fig. 5E(i)).
527 Therefore, depletion of PI3K-C2A produced an accumulation of capsids that had
528 undergone secondary envelopment in the cytoplasm, which may reflect a defect
529 in the ability of these particles to egress from the cell.

530 We noted that in our EM analysis more extracellular virions leaving the cell
531 were found in cells treated with Ctrl siRNA compared to those treated with PI3K-
532 C2A siRNA (Fig. 5D). An example of a virion leaving a cell treated with Ctrl
533 siRNA is shown in Fig. 5E(ii). Although this difference was not statistically
534 significant there was a clear trend toward a defect in virion production upon PI3K-
535 C2A depletion. A confounding factor in this analysis could be that it does not
536 account for the number of virions that have left infected cells.

537 To further investigate virion production from infected cells we used
538 western blotting to assay the presence of virion proteins in supernatant from
539 infected cells that had been treated with either Ctrl or PI3K-C2A siRNA (Fig. 5F).
540 Viral supernatants were collected and to remove cells and cellular debris
541 supernatants were first clarified with low speed centrifugation. Virions were then
542 pelleted using ultracentrifugation and pellets were treated with trypsin to remove
543 protein not protected by virion membranes. Thus, HCMV glycoprotein gB which
544 is found on the exterior of virions, on cells or in cell debris or shed into the
545 supernatant could be found in the samples treated with trypsin at 4^{o}C (Fig. 5F,
546 lanes 1 and 3), but not 37^{o}C (Fig. 5F, lanes 2 and 4). The similar amounts of gB
547 found in the supernatant of cells treated with either Ctrl or PI3K-C2A siRNA is
548 consistent with our observations from Figure 4, wherein depletion of PI3K-C2A
549 has only a modest effect on production of late viral proteins. The major HCMV
550 virion protein UL86, which is a component of virions and protected from trypsin
551 digestion by the virion membrane, was found in all samples. However, notably
552 less UL86 was found pellets from cells treated with PI3K-C2A siRNA (Fig. 5F,
553 lanes 3 and 4) compared to cells treated with Ctrl siRNA (Fig. 5F, lanes 1 and 2).
554 Using ImageJ software we found a 4-fold (Fig. 5F, lanes 1 and 3) and 9-fold (Fig.
555 5F, lanes 2 and 4) decrease in relative band intensity of UL86 blots of samples
556 treated a 4^{o}C and 37^{o}C, respectively. Therefore, consistent with data presented
557 in Figs. 5A-5E, depletion of PI3K-C2A was associated with a defect in virion
558 production from infected cells.

559 The accumulation of capsids that have undergone secondary
560 envelopment and a lack of virion production inferred that there was an
561 accumulation of infectious intracellular virus in infected cells treated with PI3K-
562 C2A. We examined this possibility by treating cells with Ctrl or PI3K-C2A siRNA
563 and, after infection, assaying the amount of infectious virus released from
564 infected cells and the amount of intracellular virus at 96 h.p.i.. To release
565 intracellular virus infected cells were lysed by three sequential freeze/thaw cycles
566 to -80 ^{o}C. In cells treated with Ctrl or PI3K-C2A siRNA we observed a 10-fold
567 decrease in titre of infectious virus released from infected cells ($8x10^5$ and $8x10^4$
568 p.f.u./ml, respectively) and a less than a 2-fold difference in the amount of
569 infectious intracellular virus produced ($1x10^3$ and $2x10^3$ p.f.u./ml, respectively).
570 Similar results were observed when infected cells were lysed by passing cells
571 three times through a needle (data not shown). Therefore, we found no obvious
572 increase in the amount of infectious intracellular virus in infected cells treated
573 with PI3K-C2A siRNA, which implied that the capsids that had undergone
574 secondary envelopment in infected cells treated with PI3K-C2A siRNA were not
575 infectious.

576 **DISCUSSION**

577

578 Here we provide a further demonstration that high throughput screening of
579 siRNAs is a valid methodology to identify cellular factors required for viral
580 replication. We identified a range of cellular kinase proteins involved in HCMV
581 replication and focused our studies on how PI3K-C2A might be required for
582 HCMV replication. These studies indicated that PI3K-C2A is involved in
583 production of late viral proteins and production of infectious virus. However, in
584 infected cells treated with PI3K-C2A siRNA we observed a greater defect in
585 virion production compared to viral protein production. Thus, PI3K-C2A most
586 likely plays a more prominent role in the production of infectious HCMV virions,
587 than the production of HCMV proteins. Our data indicates that PI3K-C2A is
588 involved in processes that lead to egress from the cell of capsids that have
589 undergone secondary envelopment.

590 Our screening methodology allows us to survey siRNAs that affect nearly
591 all facets of HCMV replication and, as we demonstrate here, we can identify
592 siRNAs that have a fairly modest effect on pp28 expression, but a greater effect
593 on virus replication. An added advantage is that the cytoplasmic localization of
594 pp28 in viral assembly compartments can be dictated by the virally encoded
595 kinase UL97 (28), which is required for function of known anti-HCMV drugs (29)
596 and is itself a major drug target (30). Therefore, in future experiments our
597 methodology can be converted to a high throughput/high content screening
598 approach wherein the number of pp28 positive cells and localization of pp28

599 within infected cells can identify factors affecting UL97 function and assembly
600 compartment morphology. Conversely, this screening approach does not allow
601 us to assess to what degree any protein is depleted by siRNA within the screen
602 and the use of antibodies to detect viral replication limits the screen to only the
603 detection of HCMV replication. Furthermore, the MOI1 infections used during the
604 screening process typically result in 30-40% of cells infected in wells treated with
605 Ctrl siRNA (data not shown). Therefore, the transfection process may limit HCMV
606 infection and further optimization of our methodology may be required.

607 In our screen depletion of a range of kinase proteins had positive or
608 negative effects on HCMV replication. Each of these proteins may act alone or in
609 concert with other factors to facilitate HCMV replication. No phosphatase proteins
610 were found to have any effect. Identification of pathways involved in viral
611 replication has been possible from the results of whole genome siRNA screens.
612 Bioinformatics analysis of our screening results using STRING analysis, a
613 bioinformatics application that identifies known and predicted protein-protein
614 associations (31), did not identify obvious pathways that might be involved in
615 HCMV replication (data not shown). These pathways may only reveal themselves
616 in the context of a whole genome siRNA screen. However, these approaches
617 must be applied cautiously as bioinformatics analysis may draw together protein-
618 protein associations that may be the result of false-positive or false-negative
619 screening hits or that have only very modest effects on viral replication.

620 Previously, a screen of siRNA targeting the human kinome was conducted
621 by Terry and co-workers based in on detection of virus produced from siRNA

622 treated cells and implicated AMPK as being important in HCMV replication (8). In
623 agreement with Terry et al., we have found that treatment of infected cells with
624 the AMPK inhibitor compound C decreases accumulation of HCMV proteins and
625 production of infectious HCMV virus (data not shown). However, we observed
626 very little overlap in the identification of siRNAs that had either a positive or
627 negative effect on HCMV replication when comparing the results of Terry *et al.*
628 and our own. For example, in the work by Terry et al. siRNA targeting PI3K-C2A
629 had little or no effect on HCMV replication and the siRNA targets involved or
630 implicated in AMPK function during HCMV replication differ from those found in
631 our study. It is likely that differences in screening methodologies and siRNA
632 reagents reflect the differences in our data sets. It is widely accepted that
633 different siRNA reagents will display different false-positives and false-negatives
634 in different screens against the same pathogen (7, 32). To combat this issue
635 several siRNA datasets using orthologous RNAi reagents must be integrated and
636 refined to identify factors that have positive or negative effects with high certainty
637 (7, 32). Therefore, it is likely that several more siRNA datasets will have to be
638 generated before we can fully understand the effects of siRNAs on HCMV
639 replication.

640 Moreover, screening data can be further interrogated with the use of
641 bioinformatics tools such as GESS and Haystack to eliminate off target effects of
642 the siRNA seed sequence binding to mRNAs. However, it must be stressed that
643 the statistical power of these analyses increases with the number of siRNAs
644 assayed. Therefore, siRNA collections of the size used here are not often

assayed to find siRNA seed matches (Eugen Buehler, personal communication). Thus, while the GESS and Haystack analysis used here suggests that no obvious off-target binding effects occur in our screen, combining the data presented here with other siRNA screening data sets may reveal as yet unappreciated off-targets effects that will influence our interpretation of siRNA function in HCMV infected cells.

Furthermore, several of the targets of siRNA in our screening data were not found in a proteomic study of HFF cells infected with HCMV (27). Therefore, analysis of siRNA off-target binding alone may not be sufficient to exclude siRNAs from analysis. Indeed, analysis of siRNA screening data can include comparison of screening hits with gene expression profiles to identify false positives (7). Further analysis of siRNA screening data from HCMV infected cells should benefit from comparison of siRNA screening hits with proteins known to be expressed in HCMV infected cells (27), as we have preformed here. It remains unknown that RNAs are targeted by the siRNAs that we judge to be false positives in our screening data. Further analysis of this question should identify viral or cellular RNA transcripts involved in HCMV replication.

We chose to focus our study on the involvement of PI3K-C2A in HCMV replication. The role of a least one class I and class III PI3K protein has been investigated (12, 13), however, the role of class II PI3K proteins in HCMV replication is unknown. PI3K-C2A is found in endosomes, the trans-golgi network and clathrin-coated vesicles (33, 34). Like other PI3K proteins, PI3K-C2A phosphorylates PtdIns at the D3 position of the inositol ring producing 3-

phosphorylated PtdIns (PtdIns3*P)*, lipids involved membrane specification and dynamics (33-36). Other phosphorylated lipids involved in membrane trafficking can be produced by this lipid kinase (37). PI3K-C2A and the phosphorylated lipids it produces are associated with a number of processes that involve intracellular membranes including exocytosis, endocytosis, and autophagy (33, 34, 38), although the function of PtdIns3*P* produced by PI3K-C2A is largely unclear. Also, PI3K-C2A can be found in the nucleus where it appears to associate with cellular RNA splicing factors (39, 40).

Our observations point to a role for PI3K-C2A in the production of HCMV virions at a step after secondary envelopment of capsids. HCMV secondary envelopment is very poorly defined and our understanding of this process largely relies on observations made using related viruses such as herpes simplex virus. Briefly, secondary envelopment occurs upon budding of capsids into cytoplasmic membranes related to the Golgi (including cis-Golgi, trans-Golgi network and endosomes) and is associated with changes in cytoplasmic membranes that occur during development of viral assembly compartments (2, 41). PI3K-C2A is known to be enriched in the aforementioned cytoplasmic membranes (33, 34, 38), and in preliminary immnoflorescence experiments we observe PI3K-C2A in the cytoplasm of HCMV infected cells (data not shown). Therefore, it is plausible that PI3K-C2A could be associated with secondary envelopment. Furthermore, PI3K-C2A is reported to be involved in exocytosis (33, 34, 38), which is thought to be involved in movement of enveloped herpesvirus particles to the plasma membrane (41). Therefore, we suggest that PI3K-C2A functions in exocytosis in

HCMV infected cells, possibly linking the completion of secondary envelopment with virion egress. Interestingly, in HCMV infected cells the function of Class III PI3K protein Vps34 appears to be separable from PI3K-C2A as these proteins are required for processes before and after secondary envelopment, respectively (13). A further point to consider is our observation that intracellular virus that accumulates in infected cells treated with PI3K-C2A may not be infectious. This would imply that the secondary envelopment of capsids we observe in cells depleted of PI3K-C2A is somehow defective or that there are further, as yet unrecognized, functions of PI3K-C2A that are required for the production of infectious virus.

It is worth considering whether PI3K-C2A might be a future anti-HCMV drug target. To our knowledge there is currently no compound that potently and specifically inhibits PI3K-C2A function, or the function of any other class II PI3K protein. Also, it has been reported that well characterized inhibitors of class I PI3K proteins, wortmannin and LY294002, have little or no effect on PI3K-C2A function (42). Therefore, an original approach is required to discover inhibitors of PI3K-C2A.

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FIGURE LEGENDS

Figure 1 High throughput screening of siRNA. (A) Diagram of high throughput screening process. (B) A representative example of a microscopy image from an Image Express Micro microscope of HFF cells treated with Ctrl siRNA and infected with AD169. Cells were then treated with Hoecsht 33342 (blue), Deep Red Cell Mask (Red) and primary and secondary antibodies to detect HCMV pp28 (green). The large white box is an enlarged image of the area identified in the small white box. (C) z-scores from the siRNA screen. Each data point that represents the z-score for PI3K-C2A siRNA is indicated. A full list of siRNAs with z-scores is shown in Table S1.

Figure 2 Analysis of individual PI3K-C2A siRNAs. (A and B) HFF were treated with siRNA and infected, then analyzed by automated microscopy. The number of (A) cells treated with siRNA (no. of Hoecsht 33342 positive cells) and (B) the percentage of those cells that are pp28 positive are shown. The mean and standard deviation of each data from 4 wells treated with siRNA are shown. The values above horizontal black bars in each Figure indicate the p-value derived from a two-tailed (unpaired) students t-test calculated using PRISM software (**=p less than or equal to 0.05, ns= no statistical difference).

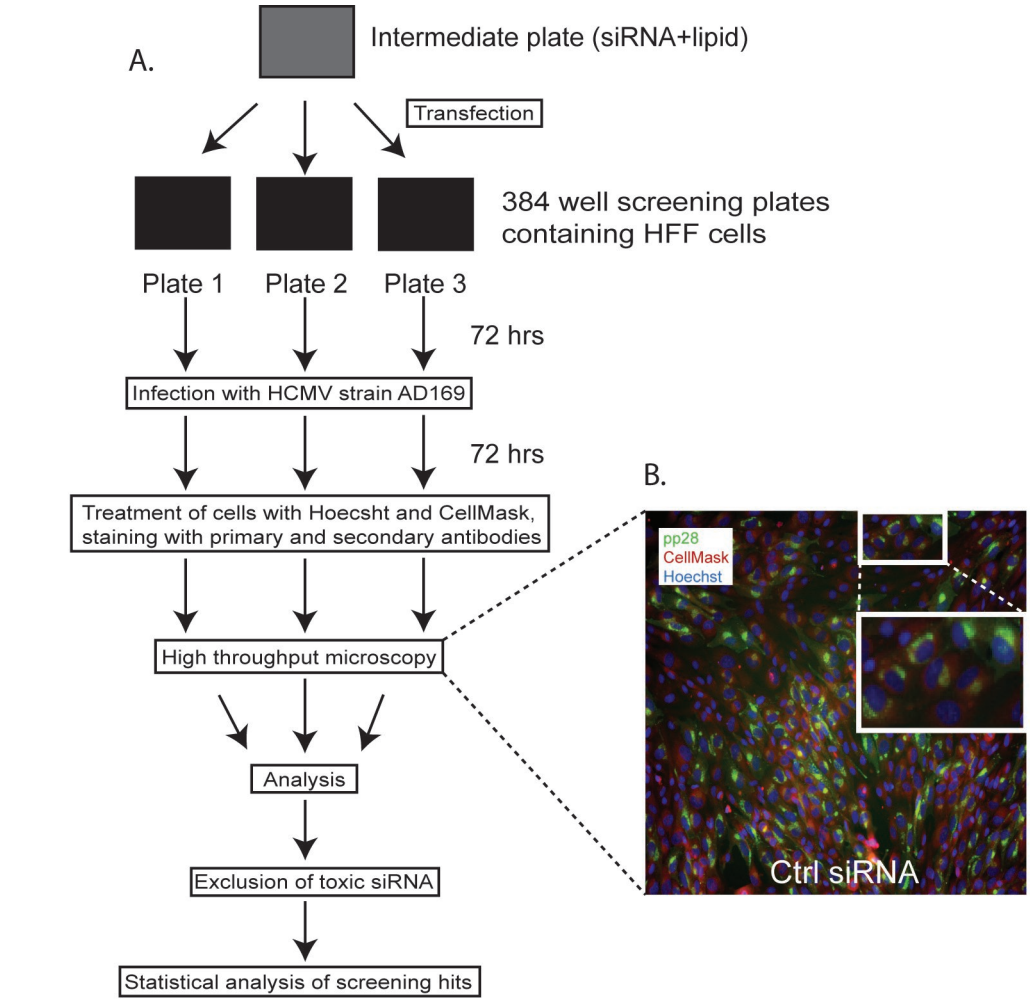
Figure 3 Production of HCMV from cells treated with PI3K-C2A siRNA. HFF cells were treated with Ctrl, PI3K-C2A siRNA or siRNAs PI3KC2A-1 to -4 and

infected with HCMV. (A and B) At the time points indicated in the figure (hours post infection (h.p.i.)) viral supernatant was harvested and lysates were prepared for western blotting from infected cells treated with PI3K-C2A siRNA. The data in Figure 3B is representative of two experiments. (C and D) Cells treated with siRNAs PI3KC2A-1 to -4 were infected with HCMV and viral supernatant was harvested at 72 h.p.i., plus uninfected cell lysate was prepared at the time of infection. In (A) and (C) proteins recognized by the antibodies used in each experiment are indicated to the right of each figure. The positions of molecular weight markers (kDa) are indicated to the left of each figure. In (B) and (D) viral titre is expressed at plaque forming units/ml (p.f.u./ml).

Figure 4 Western blotting of viral and cellular proteins from siRNA treated cells. (A) HFF cells were treated with either Ctrl or PI3K-C2A siRNA then infected with AD169. Cell lysates were prepared for western blotting at the time points (hours post infection (h.p.i.)) indicated above the figure. Uninfected cells harvested at the time of infection are shown as 0 h.p.i.. Panels showing signals from Ctrl or PI3K-C2A siRNA treated cells are from the same exposure of the same blot. (B) A 2-fold dilution series was created from samples of infected cells treated with either Ctrl (C) or PI3K-C2A (P) siRNA harvested at 72 or 96 h.p.i. from figure A. The siRNA and dilution factor are indicated above the figure. In both figures proteins recognized by the antibodies used in each experiment are indicated to the right of each figure. The positions of molecular weight markers (kDa) are indicated to the left of each figure.

Figure 5 Analysis of capsid localization and virion production. (A)-(D) Electron microscopy analysis. HFF cells were treated with either Ctrl or PI3K-C2A siRNA then infected with AD169. At 96 hours post infection cells were prepared for analysis by electron microcopy. Images that cover the entire area of a 5 infected cells from each condition chosen at random were captured at a magnification of ×9,600. The total number of A, B, and C capsids in the (A) nuclei and (B) cytoplasm of infected cells were counted, as were (C) the number of C capsids undergoing secondary envelopment and (D) virions leaving the cell. Thin horizontal black bars indicate the mean value of each group. The values above thick horizontal black bars indicate the p-value derived from a two-tailed (unpaired) students t-test calculated using PRISM software (**=$p<0.05$, ns= no statistical difference). (E)(i) Examples of capsids undergoing secondary envelopment (indicated by white arrows) in a PI3K-C2A siRNA treated cell. (ii) A virion leaving a cell treated with Ctrl siRNA. (F) Western blotting of viral proteins in supernatant of infected cells treated with siRNA. HFF cells were treated with either Crtl or PI3K-C2A siRNA and infected with AD169. Cell supernatant was collected at 96 hours post infection. Supernatant was clarified with low speed centrifugation, then virions were pelleted by ultracentrifugation. Pelleted virions were treated with trypsin at 4^{o}C or 37^{o}C and prepared for western blotting. Proteins recognized by the antibodies used in each experiment are indicated to the right of each figure. The positions of molecular weight markers (kDa) are

977 indicated to the left of the figure. A non-specific band recognized by the gB
978 antibody is marked with an asterisk. The position of gB is indicated with an arrow.
979



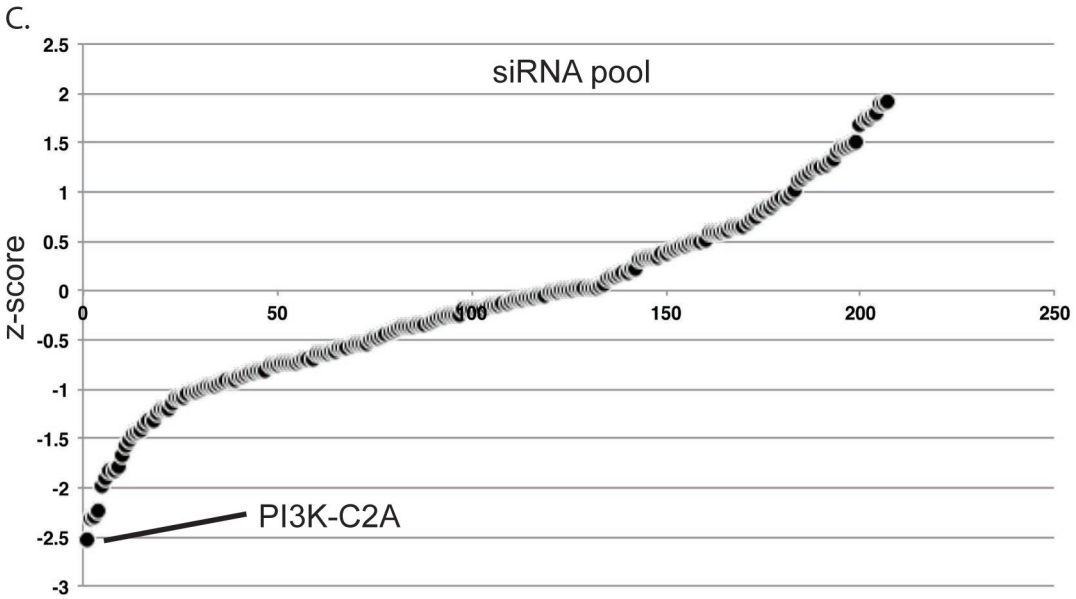


Figure 1

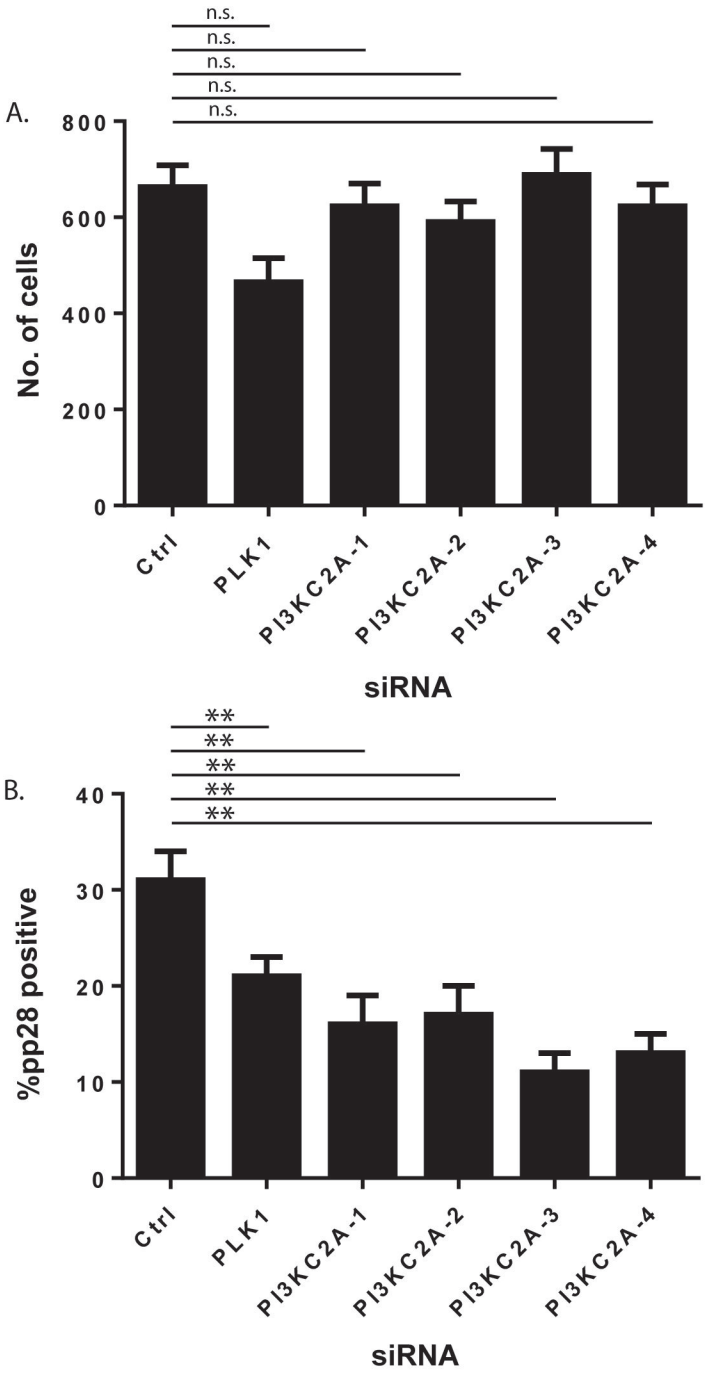


Figure 2

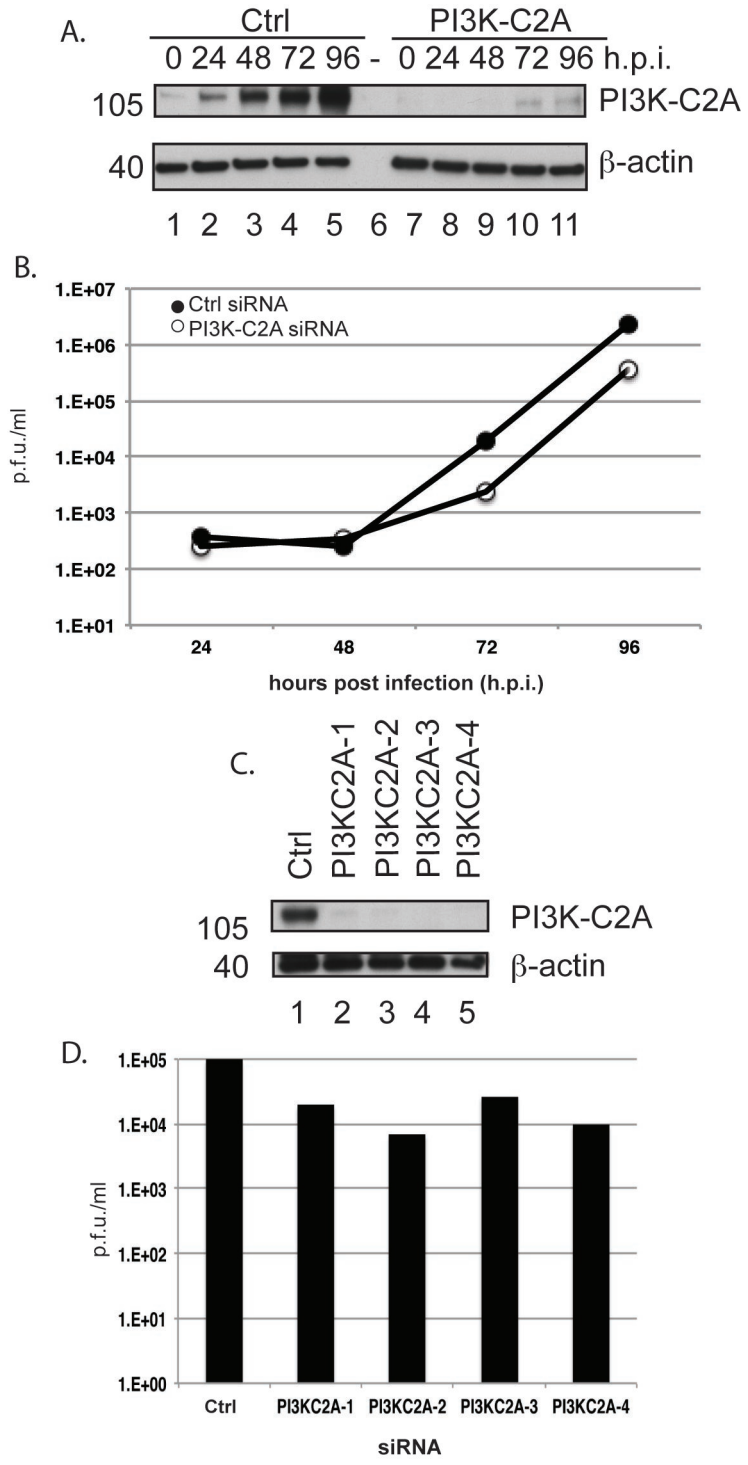


Figure 3

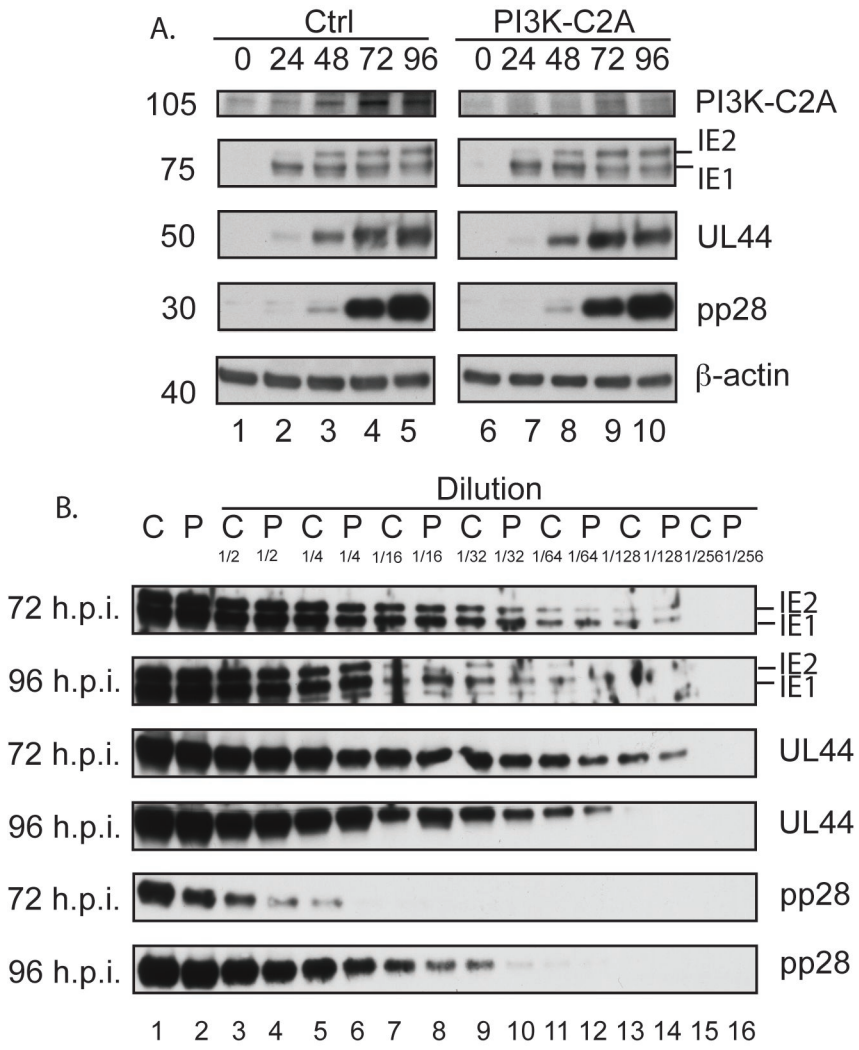


Figure 4

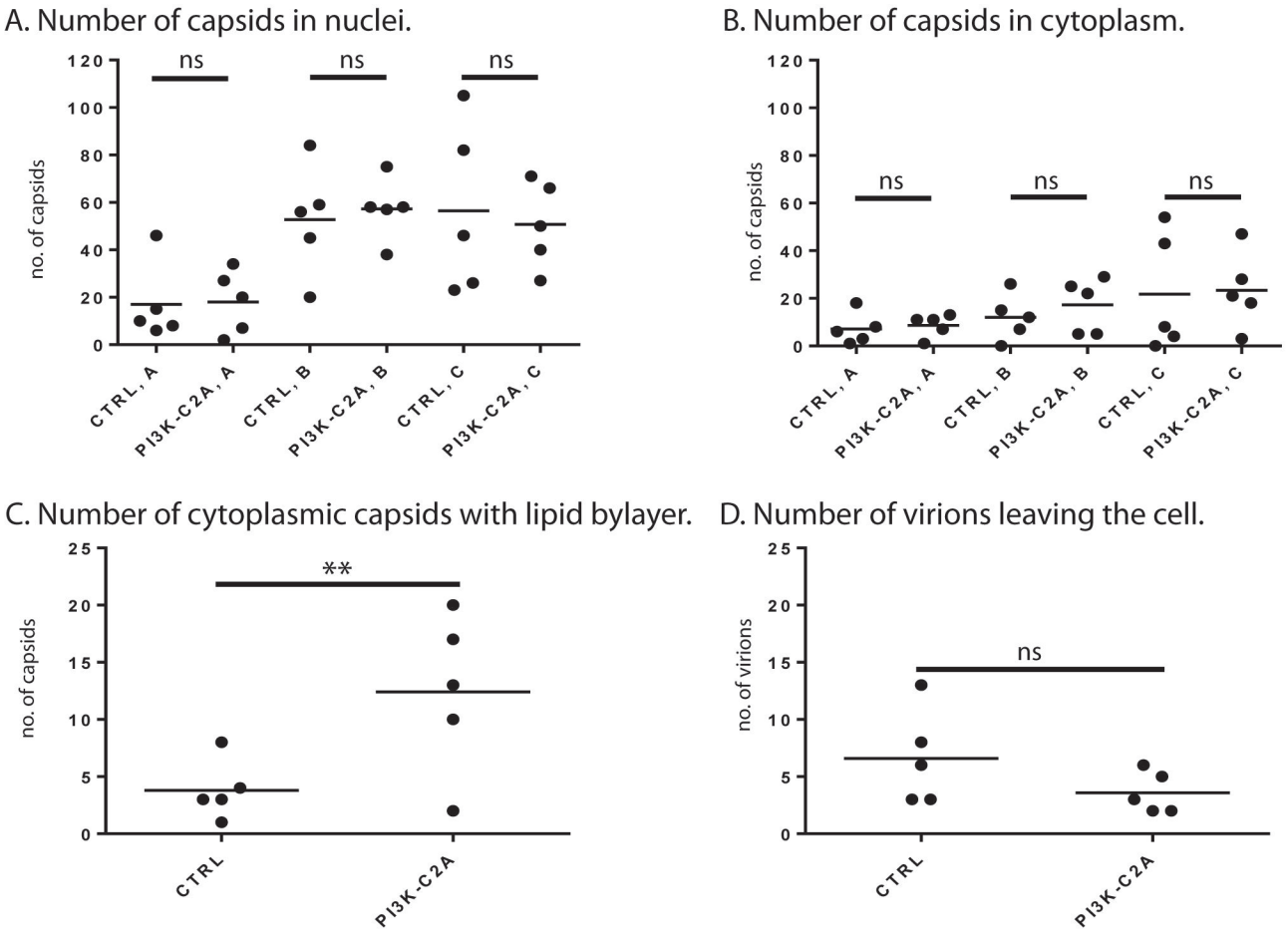


Figure 5