Title: Granularity screening identifies candidate genes involved in vaccinia virus induced LC3 lipidation

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Abstract:

Autophagy is a catabolic process used for the degradation of organelles and proteins. Macroautophagy involves the formation of autophagosomes and subsequent fusion with lysosomes to mediate cargo degradation. It also functions as a cellular defence mechanism, known as xenophagy, during infection. Previous studies show that different viruses manipulate the autophagy pathway of the host cell to assure successful replication and/or virion assembly. Vaccinia virus (VACV), the prototypic poxvirus, replicates exclusively in the cytoplasm of host cells. It is known that VACV infection causes LC3 lipidation and prevents autophagosome formation, yet the double membrane vesicles formed during autophagy do not serve as the source of the mature VACV membrane. To date the viral protein(s) causing increased LC3 lipidation have not been identified. Here we developed an image-based screening approach based on LC3 granularity to identify candidate VACV genes affecting its lipidation. We identify several candidate viral membrane proteins as effectors of LC3 lipidation, suggesting that the interplay between VACV and autophagy is more directed than previously thought.

Introduction:

Vaccinia virus (VACV) is a double stranded DNA virus that replicates exclusively in the cytoplasm of its host cell. It was used as vaccine agent to successfully eradicate smallpox. However, in 2022 a closely related virus – monkeypox (mpox) caused a global outbreak with 91,000 confirmed human cases in 115 countries(Lansiaux et al., 2022; Moss, 2024). With a mortality rate of up to 15%, understanding the biology of mpox and other clinically relevant pox viruses continues to be crucial for treating human disease(Gessain et al., 2022). Additionally, VACV and several VACV derived poxvirus strains are used as vehicles for immunotherapy in cancer research and vaccine delivery. This necessitates continued research to further understand the interactions between VACV, its host and cellular immune responses.

An important part of the cellular immune response to pathogens residing in the cytoplasm, such as VACV, is autophagy. Macroautophagy (hereafter referred to as autophagy) provides protective immunity by capturing pathogens in autophagosomal compartments which later fuse with lysosomes to mediate the degradation of pathogens and other unwanted cytoplasmic content(Vargas et al., 2023).

ATG8 (in yeast) and ATG8-family proteins (in mammals) are crucial for functional autophagy. In mammals, at least seven ATG8 orthologs exist. The most studied ATG8 ortholog is LC3b (hereafter LC3), which is critical for the execution of autophagy and often used as a marker for autophagic activity and flux (Klionsky et al., 2021).

Two essential ubiquitin-like conjugation systems drive autophagy to covalently conjugate ATG8-family proteins to the phagophore membrane. The first complex crucial for this process is the ATG12-ATG5-ATG16L1 complex. The carboxy-terminal glycine of the small ubiquitin-like protein ATG12 is activated by transient linkage first to the E1-like enzyme ATG7 and then to the E2-like enzyme ATG10, before becoming covalently attached to ATG5(Mizushima et al., 1998; Tanida et al., 2001). This ATG12-ATG5 complex non-covalently associates with the protein ATG16L, forming the ATG12-ATG5-ATG16L1 complex that participates in the second ubiquitin-like conjugation reaction(Dooley et al., 2014). The second conjugation step results in the covalent addition of the lipid phosphatidylethanolamine (PE) to LC3. This process, known as ATG8/LC3 lipidation, is mediated by the E1-like ATG7 and E2-like ATG3 enzymes. The carboxy-terminal amino acids of LC3 are cleaved by the cysteine protease ATG4 presenting a conserved glycine residue. Cleaved LC3 can then be transiently linked to ATG7, then to ATG3, and finally to phosphatidylethanolamine (PE). The role of an E3-like ligase is filled by the ATG12-ATG5-ATG16 complex(Feng et al., 2014).

Although a complete lack of autophagosome formation is found in in VACV-infected cells, previous research showed that VACV infection leads to LC3 lipidation independent of the presence of ATG5 or ATG7(Moloughney et al., 2011). While ATG5 and ATG7 independent autophagy exists, and has been shown to mediate autophagic degradation of Shigella in intestinal cells(Ra et al., 2016), it does not typically involve LC3 lipidation(Arakawa et al., 2017; Nishida et al., 2009). Mologhney *et al*., also observed that VACV infection causes ATG12-ATG3 conjugation in the absence of ATG5 and ATG7, and that these ATG12-ATG3 conjugates cluster with viral DNA(Moloughney et al., 2011). They proposed a model whereby ATG12-ATG3 complexes drive aberrant LC3 lipidation due to spatial dislocation from the phagophore, resulting in a failure of autophagosome formation. The viral protein causing this complex formation has not been identified.

In this work, we set out to identify candidate viral genes involved in increased LC3 lipidation employing an image-based siRNA screen of 80 early VACV genes. To quantify $LC3^{+}$ autophagosomes, we used a readout based on single-cell granularity(Carpenter et al., 2006; Luc Vincent, 2000, 1992). The core methodology involves a pipeline starting with cell nuclei and cytoplasm segmentation(Carpenter et al., 2006), followed by single-cell granularity analysis(Luc Vincent, 2000,

63 1992) employing dsRed-LC3 fluorescence. We identified five VACV genes that up regulate and nine
64 genes that down regulate LC3 lipidation. Conformational siRNA depletion coupled with immunoblot
65 analysis for LC3 lipidation showed that H3, A14, L5 and E8 influence LC3 lipidation during infection.
66 This study delivers the first systematic analysis of VACV genes intersecting with LC3 lipidation and
67 provides the starting point for detailed follow-up analysis of VACV mediated modulation of LC3
68 lipidation.

69 **Results:**

70 VACV infection leads to the accumulation of LC3^{+} puncta

71 To confirm the previous finding that VACV induces LC3 lipidation, we monitored a 24 hr infection
72 time course. VACV infection led to the accumulation of LC3^{+} puncta indicative of an enrichment of
73 autophagosomes. We observed a slight increase in LC3^{+} puncta during VACV infection. While at late
74 time points post infection viral DNA replication sites appeared to show some minor overlap with
75 cytoplasmic LC3, we did not observe direct co-localization between LC3 and newly formed virions.
76 Interestingly accumulation of LC3^{+} puncta occurred very early in infection and was already visible at
77 1 hpi (**Figure 1a**).

78 VACV infection causes increased LC3 lipidation

79 Moloughney *et al.* described an increase in LC3-II during VACV infection at 24 hpi assuming an
80 increase in lipidated LC3(Moloughney et al., 2011). To validate these findings and determine the flux
81 of LC3 lipidation, we performed an infection kinetics assay with subsequent western blot analysis.
82 BafilomycinA1- and Torin1-treated samples were used as positive controls and 3-MA treated
83 samples as a negative control for LC3 lipidation. Consistent with Moloughney *et al.,* LC3-II was
84 increased in VACV infected cells. Quantification showed the increase to occur gradually over time
85 with 5-fold more 14 kDa LC3 being present in cells 24 hpi compared to uninfected control samples
86 (**Figure 1b**).

87 However, when visualizing LC3 protein using western blotting, two bands at 16 kDA and 14 kDa can
88 be observed. These bands are indicative of three different states of LC3: pro-LC3, LC3-I and LC3-II. To
89 be lipidated, pro-LC3 (14 kDA) is converted to LC3-I (16 kDa) through cleavage of carboxy-terminal
90 amino acids by ATG4. PE is then linked to the 16kDa LC3-I converting it into LC3-II (14 kDa). As pro-
91 LC3 and LC3-II both migrate at 14 kDa by western blot, an increase in 14 kDa LC3 can be attributed
92 to either an increase in LC3-II formation or accumulation of pro-LC3 caused by a block in cleavage.

93 To differentiate between pro-LC3 and LC3-II during VACV infection, we utilized a Phospholipase D
94 (PLD) assay(Agrotis et al., 2019). This assay relies on the ability of PLD to cleave LC3-II, but not pro-
95 LC3. Incubating cell lysate with PLD shifts LC3-II from 14 kDa to 16 kDa, while pro-LC3 remains
96 unaffected. PLD-cleaved LC3 cannot be lipidated again due to the different cleavage sites of ATG4b
97 and PLD, thus a shift of LC3 from 14kDa to 16kDa in presence of PLD indicates that the increased
98 14kDA LC3 is due to LC3-II formation.

99 The PLD cleavage assay was performed at two different time points to differentiate between a pro-
100 cleavage block and LC3-II formation during both early and late stages of VACV infection. The earliest
101 time point with an increase in lower band LC3 sufficient for visualization was at 8 hpi. While 24 hpi
102 was chosen as the late time point representing the peak of VACV-mediated LC3 modification in WB-
103 based time course experiments. While incubation at 37 °C led to some degradation of LC3, enough
104 remained to be used as functional readout. ATG4b which cleaves both LC3-II and pro-LC3 was used
105 as positive control. An additional control used in this assay was uninfected lysate treated with
106 BafilomycinA1 and Torin1 which causes LC3-II resulting in a band shift to LC3-I upon PLD incubation.
107 Importantly, PLD treatment of either VACV infected or BafilomycinA1 + Torin1 treated samples
108 displayed an LC3 band shift with the lower band shifting upward, indicating that the lower LC3 band
109 mainly consists of LC3-II. This was documented for both time points at 8 and 24 hpi during VACV
110 infection (**Figure 2a&b**). This shows that VACV causes LC3 lipidation throughout the course of
111 infection rather than blocking pro-LC3 cleavage.

112 One or more early VACV genes facilitate LC3 lipidation

113 To narrow down the search for the viral gene(s) initiating LC3-II formation, we looked at VACV-
114 induced LC3 lipidation in the presence of three drugs known to interfere with the VACV life cycle.
115 Cells were infected with WT VACV (MOI 3) in the presence or absence of cycloheximide (CHX;
116 50 µM), MG-132 (25 µM), or Cytosine arabinoside (AraC; 10 µM), all of which are used in VACV
117 research for temporal inhibition of the VACV life cycle at distinct stages. CHX prevents early viral
118 protein synthesis without impacting virus entry and core release. MG132 prevents viral uncoating
119 through proteasome inhibition, while allowing for viral early gene expression(Kilcher et al., 2014;
120 Satheshkumar et al., 2009; Schmidt et al., 2013). AraC allows for uncoating and early gene
121 expression, but blocks intermediate and late gene expression by interfering with viral DNA
122 synthesis(Furth and Cohen, 1968).

123 HeLa cells were infected with VACV WR WT, treated with CHX, MG132 or AraC, and LC3-II formation
124 was analysed by WB. MG132 and AraC did not prevent LC3 lipidation during infection. CHX however,
125 completely abrogated LC3-II formation suggesting an early viral gene product was responsible
126 (**Figure 2c**).

127 Assessing LC3-II lipidation during VACV infection using a dsRed-LC3-GFP marker cell line

128 While flow cytometry provides information regarding intensity and cell granularity, an image-based
129 read-out can provide additional single cell information including the number, size, and shape of LC3
130 puncta and, thus, is a preferable readout. To visualize the formation of $LC3^+$ autophagosomes, we
131 constructed a modified dsRed-LC3-I-GFP HeLa cell line expressing fluorescent LC3 generated using a
132 pQCXI-Puro-dsRed-LC3-GFP construct(Sheen et al., 2011) which is a modified version of another LC3
133 construct(Kabeya et al., 2000).

134 Here, LC3 bears a n-terminal dsRed tag and a c-terminal eGFP tag. As mentioned, during LC3
135 processing the c-terminus is modified by ATG4 cleaving the protein at the location indicated in
136 **Figure 3a** leading to removal of the GFP tag. This cleavage process is necessary to attach PE to LC3
137 and to conjugate it to the autophagosome. Thus, LC3-II appears only as red, making it screen-able in
138 both IF and flow cytometry-based readout systems. Similar constructs have been published and used
139 for various applications in autophagy research such as to screen for autophagy inducing drugs with
140 LC3 as readout for autophagic flux(Gump and Thorburn, 2014; Hundeshagen et al., 2011).

141 Cells were either treated with autophagy modulating drugs or infected with VACV. While only few
142 red autophagosomal puncta were present in untreated and uninfected cells, treatment with a
143 combination of BafilomycinA1 and Torin1 drastically increased the number of $LC3^+$ autophagosomes.
144 Additionally, we observed a steady increase in $LC3^+$ puncta during a time-course of VACV infection
145 peaking at 24 hpi (**Figure 3b**). These results confirmed that VACV infection induces LC3 lipidation,
146 and indicated that an image-based screening using this cell line as a readout for LC3-II formation is
147 feasible.

148 To screen for the early viral gene(s) impacting LC3-II formation during VACV infection in the dsRed-
149 LC3-I-GFP HeLa cell line mentioned above, we utilized a VACV early gene siRNA library(Kilcher et al.,
150 2014) which targets 80 VACV early genes with three individual siRNAs per gene.

151 Development of a screening system measuring LC3-II granularity

152 The VACV early gene - LC3 lipidation screen was carried out by transfecting HeLa dsRed-LC3-I-GFP
153 cells with siRNAs directed against 80 early VACV genes(Caroline Martin et al. 2023; Kilcher et al.,
154 2014). Prior to infection cells were reverse transfected with siRNAs in 96-well plates and incubated
155 for 16 hrs, which is sufficient for early VACV gene depletion(Beerli et al., 2019; Kilcher et al., 2014;
156 Valderrama et al., 2006). Images were obtained using the Opera Phoenix High Content Screening
157 System. Next, image segmentation of individual cells was performed using a custom preprocessing
158 script and a CellProfiler pipeline(Carpenter et al., 2006).

159 Once individual cells were segmented, several single-cell readouts were tested such as the average
160 intensity of the dsRed signal, total intensity of dsRed signal, median intensity of dsRed signal and
161 granularity spectrum (see Methods). The latter was chosen as the readout as it most accurately
162 reflects the presence or absence of autophagosomes within the cell, with the granules indicating
163 $LC3^{+}$ autophagosomes (**Figure 4a&b**). In this readout, the better the selected structuring element
164 size fits the signal, the higher the score. This makes it possible to detect the occurrence of larger
165 granules in the cytoplasm of the cell, upon adjustment of the structuring element size
166 hyperparameter. Thus, this is a single-cell level readout of granule formation. High granularity
167 structures correspond to the highest peak difference upon morphological opening with a structuring
168 element of a fitting radius (**Figure 4a&b**).

169 Identification of potential viral regulators of LC3 lipidation

170 For controls in this screen, we used untreated cells which were either infected (labelled as VACV) or
171 uninfected as well as cells treated with siScramble also either infected or uninfected. While
172 siScramble had no effect on LC3 granularity, VACV infection nearly doubled the granularity score
173 both in presence and absence of siScramble compared to uninfected cells (**Figure 4c**). The
174 granularity score for VACV treated with siScramble was slightly lower compared to untreated VACV
175 infected cells. While we expected to find that depletion of some VACV gene products would result in
176 reduced LC3 puncta, we also observed that depletion of some VACV early gene products led to an
177 increased granularity score. This suggests that VACV mediated LC3 lipidation is regulated in both
178 directions.

179 Applying VACV untreated as threshold resulted in 53 screen hits with an increased and 27 screen hits
180 with a decreased LC3 granularity score. Applying VACV siScramble KD as control (indicated by the
181 dotted line in **Figure 4c**) results in 25 screen hits with increased LC3 granularity and 55 with
182 decreased LC3 granularity score.

183 Statistical analysis was carried out to decide which genes to select for follow-up experiments. For
184 the genes whose gene product depletion resulted in granularity increase, we selected the four
185 highest scoring genes A44L, B1L, A29L and H3L. Additionally A28L was selected based on the
186 performance of siRNA 1. For hits showing in decreased granularity, we selected the four lowest
187 scoring genes A14L, E8R, L5R and F11L. Additionally G5L was selected also based on the
188 performance of siRNA 1.

189 VACV H3, G5, L5 and A14 may mediate VACV-induced LC3 lipidation

190 To validate the screening results and to confirm that the LC3 puncta seen in the screen correspond
191 to the LC3 lipidation results, the various candidate gene products were depleted using their
192 respective strongest performing siRNA. WB-based LC3 lipidation assays were carried out. Since cells
193 were plated at reduced confluency for siKD compared to other WB samples, the MOI was increased
194 to six to maintain the LC3 lipidation phenotype. The WB results indicated that H3 is the strongest
195 candidate for VACV-mediated negative regulation of LC3 lipidation. Depletion of H3 resulted in an
196 even stronger lipidation phenotype than siScramble with an increase of 25 % (**Figure 5a**).

197 In contrast, LC3 lipidation was significantly reduced upon siRNA mediated depletion of G5, L5 and
198 A14 protein synthesis. A14 KD resulted in a reduction of LC3 lipidation by nearly 50 % when
199 compared to VACV infected siScramble samples. Interestingly, the uninfected siScramble control
200 exhibits 60 % less LC3 lipidation than VACV siScramble. Reduction of A14 therefore nearly abrogated
201 the LC3 lipidation phenotype of VACV compared to uninfected siScramble treated cells. Other gene
202 products exposed to siRNA mediated decay resulted in significant reduction of LC3 lipidation such as
203 L5 (reduction of lipidation by 45% compared to VACV siScramble) and G5 (reduction of lipidation by
204 33 % compared to VACV siScramble) (**Figure 5b**).

205 To assure that the siRNA used in these assays could efficiently deplete the respective mRNA and
206 diminish the target protein synthesis, we used qRT-PCR to validate the KD efficiency of the siRNAs
207 (**Figure 5c**). All siRNAs showed some level of depletion of their respective target mRNAs to varying
208 degrees. An average reduction of mRNA transcripts for H3L, F11L and L5R was 30 %, while siRNAs for
209 A28L, B1R and E8R achieved a KD efficiency of 70 %. Thus, H3 depletion might have a stronger
210 impact on LC3 lipidation than suggested by **Figure 5a**, but limited siKD efficiency prevented
211 manifestation of the full phenotype in WB analysis. Likewise, the reduction of LC3 lipidation in siL5R
212 and siA14L treated samples might be more prominent if the KD efficiency was higher.

213 **Discussion:**

214 We have shown that VACV infection increased LC3 lipidation through one or more early gene
215 products. VACV causes an increase in LC3 lipidation rather than blocking the processing of pro-LC3.
216 The lipidation phenotype of LC3 manifests early during infection and increases over time resulting in
217 large amounts of lipidated LC3 at 24 hpi. We also discovered that this phenotype is caused by the
218 expression of VACV early gene(s). During the course of infection LC3 accumulates in punctae that
219 could potentially indicate fully formed autophagosomes, but might also be aggregates of LC3-II as
220 previously suggested(Moloughney et al., 2011).

221 Using a fluorescent dsRed-LC3-I-GFP cell line in an siRNA-based screen, we identified VACV early
222 gene candidates which were associated with the regulation of LC3 lipidation. The readout was based
223 on protein granularity. While the LC3 signal is specific to this screen, theoretically our approach
224 could be used to interrogate similar phenotypes such as endosomal structures or multivesicular
225 bodies. Therefore, we believe that this assay is applicable to other image-based screens and could
226 be employed in a multitude of high-content image-based screens.

227 We confirmed that siRNA mediated depletion of specific mRNAs derived from some early VACV
228 candidate genes changed the VACV LC3 lipidation phenotype. Further analysis will be necessary to
229 study these candidate gene products and their potential interaction with LC3 in more detail.

230 We are proposing three hypotheses regarding VACV and LC3 lipidation (**Figure 5d**): 1) LC3 lipidation
231 could be aberrant as suggested by Moloughney *et al*. This could be an autophagy evasion strategy by
232 the virus. As cargo is recruited into the forming autophagosome via autophagy receptors which bind
233 to LC3, aberrant LC3 lipidation would impede this process, leaving forming autophagosomes
234 'empty'. 2) Perhaps lipidated LC3 contributes to the formation of new virions. It was originally
235 though that VACV membranes may utilize autophagy machinery for membrane formation(Zhang et
236 al., 2006). While Beclin1, ATG5 and ATG7 have been ruled out by Zang et al., a role for lipidated LC3
237 has not been addressed. 3) LC3 lipidation is a side effect of autophagy activation during infection.
238 Upon sensing of invading VACV virions the cell may initiate autophagosomal membrane formation to
239 combat infection. However, VACV-induced depletion of autophagy receptor proteins(Krause et al.,
240 2024) results in excess amounts of autophagosomal membranes.

241 While several siRNAs were initially found to cause an increase in LC3 puncta formation, follow up
242 experiments revealed only one siRNA that significantly upregulated LC3 lipidation upon infection.
243 This siRNA targeted the production of viral protein H3, which is a heparin sulphate binding protein
244 on the surface of IMVs. Some publications indicate that heparin prevents autophagy activation in
245 mesangial cells(Wang et al., 2014) and diabetes(Wang et al., 2015). In these studies, diabetic rats

246 were treated with heparin over an extended period which led to a reduction in LC3 levels and
247 number of autophagosomes. It is possible that H3 interactions with heparin during infection may
248 increase the effect the polysaccharide has on LC3, thus acting as a negative regulator of VACV-
249 induced LC3 lipidation.

250 Amongst the candidate gene products to cause increased LC3 lipidation, confirmed through WB
251 analysis, were G5, L5, and A14. A14 is essential for viral membrane biogenesis and a major
252 component of the mature virion (MV) membrane. Together with A17 it forms viral membrane
253 crescents which progress into the immature virion (IV) membrane. A14 and A17 also form a lattice
254 that is stabilized by disulphide bonds which serves as an anchor within the viral membrane for other
255 proteins important for virion structure and morphogenesis(Betakova et al., 1999; Mercer and
256 Traktman, 2003; Rodríguez et al., 1998). G5 is thought to be a putative endonuclease required for
257 double-strand break repair, homologous recombination, and production of full-length viral genomic
258 DNA(da Fonseca et al., 2004; Moussatche and Condit, 2015; Senkevich et al., 2009). L5 is another
259 viral membrane protein and part of the entry fusion complex in VACV. Virions lacking L5 can bind to
260 cells, but not enter the cytoplasm(Laliberte et al., 2011; Townsley et al., 2005). E8 was another gene
261 selected for WB based validation. While E8 siKD did not lead to a significant decrease in VACV
262 mediated LC3 lipidation by WB, the characteristics of this protein still make it an interesting target
263 for follow-up experiments. E8 is an ER-localized membrane protein and ER stress is known to induce
264 autophagy(Kawakami et al., 2009) and to serve as a source of LC3 precursors(Ge et al., 2015).

265 Furthermore, studies on VACV suggest that soon after initial viral DNA synthesis, the replication sites
266 become entirely enwrapped by membranes of the ER which is essential for efficient VACV DNA
267 replication(Tolonen et al., 2001). Thus, initial VACV replication seemingly takes place near sites of
268 autophagosomal origin, further suggesting an option for disabling the autophagy pathway for
269 successful replication. We have previously shown that VACV utilizes ESCRT-mediated multi-vesicular
270 bodies during virion wrapping on intracellular enveloped viruses (IEVs)(Huttunen et al., 2021). The
271 formation of aberrant autophagosomal structures may be an additional source for wrapping
272 membranes for IEVs. Of the five proteins selected based on the screening results, three are
273 membrane proteins. This could be an indication for an involvement of LC3 in the formation of viral
274 membranes. Zhang *et al.*, showed that VACV membrane formation occurs independently of ATG5
275 and Beclin1(Zhang et al., 2006). LC3 lipidation however also takes place in ATG5 and ATG7 deficient
276 cells during VACV infection(Moloughney et al., 2011). Moloughney *et al.*, suggested that the virus
277 encodes a viral protein that compensates for the lack of these proteins which are required for the E3
278 ligase activity of the ATG12-ATG5-ATG16L complex that leads to conjugation of LC3 to PE on the

279 autophagosomal membrane. A recent pre-print suggested that VACV protein A52 prevents the
280 formation of autolysosomes by binding SNAP29, a protein that facilitates the fusion of
281 autophagosomes with lysosomes, which would provide an additional layer of autophagy pathway
282 modulation by VACV for autophagosomal escape (Niu et al., 2024).

283 In conjunction with previous literature, the findings described in this study suggest a closer interplay
284 of VACV with the host autophagy machinery than previously assumed. Deciphering the involvement
285 of these viral proteins in abrogating autophagy will be of interest for future research.

286 **Methods:**

287 Cell culture & Viruses

288 HeLa cells (WT as well as a HeLa based fluorescent cell line) were maintained in Dulbecco's Modified
289 Eagle Medium (DMEM, Life Technologies) supplemented with 10 % Foetal Bovine Serum (FBS, Life
290 Technologies), 1 mM sodium pyruvate (Thermo Fischer Scientific), 100 µM non-essential amino
291 acids, 2 mM L-alanyl-L- glutamine dipeptide (GlutaMAX, ThermoFisher) and 1% Penicillin-
292 Streptomycin (Life Technologies) under at 37°C and 5 % CO_2. Cell lines were maintained in either T75
293 or T175 flasks and passaged two to three times per week using Phosphate-buffered Saline (PBS) and
294 Trypsin/EDTA (2.5g Trypsin/litre, 0.2g EDTA/litre). The following viruses were used: Wild-type
295 Western Reserve strain (VACV WR WT) and VACV WR encoding an endogenous C-terminal mCherry
296 tagged A4 core protein (WR mCherry-A4)(Mercer and Helenius, 2008; Schmidt et al., 2013).

297 Reagents & Antibodies

298 Cytosine arabinoside (AraC) and cycloheximide (CHX) were obtained from Sigma-Aldrich, while
299 MG132 was purchased from MERCK Millipore. The drugs were used at 10 µM, 50 µM and 25 µM
300 respectively. DMSO (Sigma-Aldrich) was used to dissolve drugs and as negative control in all drug
301 related assays. Bafilomycin A1 from Streptomyces griseus (Sigma Aldrich) was used at 10 nM. Torin1
302 (MERCK Millipore) was used at 250 nM, 3-Methyladenine (3-MA) (Sigma Aldrich) was used at 5 mM.
303 The following antibodies were used: Anti-LC3b (CST #3868S) used in IF at 1:200 and in WB at 1:1000;
304 Anti-Tubulin (CST #9701S) used in WB at 1:5000. Hoechst Trihydrochloride Trihydrate 33342
305 (Invitrogen #H3570) was used for DNA staining in Immunofluorescence (IF) at 1:10,000. Secondary
306 antibodies were purchased from Invitrogen and LI-COR.

307 Imaging

308 For confocal imaging cells were seeded on 13 mm glass coverslips (VWR) at 60,000 cells per coverslip
309 the day prior to infection. Infection was carried out with WR mCh-A4 or WT virus at MOI 1 in DMEM,
310 and incubated for 1 hr at 37°C. The media was removed and replaced with full medium and
311 incubated for the desired time. Cells were fixed with 4 % FA-PBS for 15 mins. Cells were
312 permeabilised with ice cold MeOH at -20°C for 20 min, blocked with 3 % bovine serum albumin (BSA,
313 from Sigma Aldrich) in PBS for 1 hr, and stained with 30 µl primary antibody diluted in 3 % BSA-PBS
314 for at least 1 hr at RT or overnight at 4°C. The coverslips were washed three times for 5 mins in 3 %
315 BSA-PBS before secondary antibody and Hoechst staining for 1 hr at RT in 30 µl 3 % BSA- PBS and
316 mounted on glass mounting slides. Samples were imaged using a 63 x oil immersion objective (ACS
317 APO) on a Leica TCS 2012 model SPE confocal microscope. High content imaging was carried out as
318 follows: Prior to image analysis cells were fixed with 4 % FA. Antibody and Hoechst staining was
319 carried out in a 40 µl volume on a shaker using dilutions as stated above. The Opera Phenix High
320 Content Screening System was used at 40 x magnification with an air objective using 405 nm, 488
321 nm, 594 nm or 647 nm lasers and at least 15 images taken per well. CellProfiler was used to detect
322 individual cells, based on Hoechst stained nuclei.

323 Infection time kinetics

324 For western blot or fractionation samples, HeLa cells were seeded in either 60 mm or 35 mm dishes
325 for confluency at infection. Infection was carried out in DMEM without FBS with VACV WR, or any
326 tagged virus, at MOI 3. Virus was incubated with the cells for 1 hr at 37°C, before aspirating and
327 replacing with full medium. Cells were either left untreated or treated with the indicated
328 compounds from the start of infection, and incubated at standard conditions. Samples were
329 harvested at their respective time points, by removing media and washing cells in PBS. Cells were
330 harvested in lysis buffer as indicated below.

331 PLD band shift assay

332 To determine the difference between lipidated LC3 and pro-LC3 on a western blot, a
333 phospholipase D (PLD) band shift assay was carried out following the previously published
334 methodology(Agrotis et al., 2019). The reaction was stopped by the addition of 3 x blue loading
335 dye (CST #56036S) containing DTT and immediately boiled at 95°C for 5 mins.

336 Western blot analysis

337 Cells were washed with cold PBS and subsequently scraped into 50 - 200 µl (depending on dish size)
338 lysis buffer containing protease inhibitor (#5872, NEB). The sample was then left on ice for at least
339 20 mins and subsequently spun down at 20,000 x *g* for 10 mins at 4°C. The supernatant was either
340 frozen and stored at -20°C or directly supplemented with 3 x blue loading dye containing DTT. Prior
341 to western blot analysis the samples were subject to 5 min incubation at 95°C. All protein samples
342 were loaded into 12 % Bis-Tris polyacrylamide gels and ran with MES SDS buffer (both Thermo Fisher
343 Scientific) at 60 Volt (V). Transfers were carried out onto 0.2 µm nitrocellulose membrane (Life
344 Technologies) using the semi-dry transfer system (both Biorad) at 10 V for 1 hr 15 mins, with transfer
345 buffer containing methanol (NuPAGE buffer, Thermo Fisher Scientific). Primary antibodies were
346 applied in 5 % BSA TBS-T over night at 4°C, then was washed three times in TBS-T. Membranes were
347 incubated with LiCor secondary antibodies for 1 hr at RT. Imaging of membranes was carried out
348 with using a LiCor Odyssey. Western blot quantifications were done using ImageJ (Version 2.0.0)
349 with tubulin used as a loading control where applicable.

350 qRT-PCR

351 Quantitative real-time PCR (qRT-PCR) was carried out to determine knock-down efficiency of early
352 VACV genes that were top hits in the LC3 lipidation screen. siRNA treated 33 mm dishes of HeLa cells
353 were infected with WT VACV at MOI 6 for 1 hr. Unbound virus was removed and replaced with full
354 medium. Cells were harvested at 24 hpi. RNA extraction, cDNA transcription and qRT-PCR were
355 carried out according to a recently published protocol(Huttunen and Mercer, 2019). Viral mRNA
356 threshold cycle (C_T) values were calculated and expression relative to and glyceraldehyde-3-
357 phosphate dehydrogenase (GAPDH) housekeeping gene determined. The qRT-PCR primers used are:

Primer name	**Sequence (5'-3')**
E8R FWD Set 2	AAG GCT GGC GAC ATT TAG AG
E8R REV Set 2	GTG TGG GCG ATT ACC TTT CT
G5R FWD Set 3	GTA CAG ACC AGG TGG CTT AAA
G5R REV Set 3	CGG TAC ATT CAG CCT CGT T
L4R FWD Set 4	GAA CCT TAG CCG TTC AGA TGT A
L4R REV Set 4	TCT AAG GAG CAG GGA ACA ATT T
H3L FWD Set 4	GGA TAC TCA CGT GAA GGT CTT G
H3L REV Set 4	GAT TAT TCC AGT TGG GCT TGT TG
A28L FWD Set 5	ATG AGG CAA GGC GAG AAG
A28L REV Set 5	CAG TGG TAC GAC CTG ATA GAT G
A29L FWD Set 5	TCT ACC AGG AAG AAC TAG CAA ATC
A29L REV Set 5	CAA ACA GCA CAA CCA GTG TC
A44L FWD Set 5	TCG GGC AGA GGA AGT TCT AT
A44L REV Set 5	GCA CGA GGA TTT CTC AGA AGG
B1R FWD Set 3	GTT TCC GGA TCC TCT GAG TTT
B1R REV Set 3	GGA GAG TGT TGG AAT GAG TGT
F11L FWD Set 1	GAG GCT TCA TGC TGG GAT AC

F11L REV Set 1	GTA CCA CAG TCC TTG GAT TAC C
A14L FWD Set 2	GTT AGA CCA CAG CAG AGA AGT C
A14L REV Set 2	GGC ACG AGG GCT TTA ATA CT
GAPDH fw (RT-PCR, Ctrl)	AAG GTC GGA GTC AAC GGA TTT GGT
GAPDH rv (RT-PCR, Ctrl)	ACA AAG TGG TCG TTG AGG GCA ATG

358 siRNA-transfection

359 Lipofectamine RNAiMax (Thermo Fisher Scientific) was diluted 1 in 125 in pure DMEM for 5 minutes
360 at RT. siRNA dilutions and media containing Lipofectamine were then incubated together for a
361 further 1 hr at RT. The AllStar Hs cell death control (Quiagen) was used as a control for transfection
362 efficiency, and AllStar negative (Quiagen) as an off-target, scrambled siRNA control. For western blot
363 analysis and confocal imaging, the siRNA-lipid complex mixture was then plated into a 30 mm dish.
364 300 000 HeLa cells were seeded on top of the mixture in 600 µl DMEM containing FBS.

365 For high content imaging of the siRNA LC3 screen a recently published protocol for high content
366 screening of VACV early genes(Caroline Martin et al., n.d.) was used. The primers used were
367 previously published(Kilcher et al., 2014).

368 Computational Analysis

369 The granularity spectrum measurement(Luc Vincent, 2000) used here is obtained through a
370 morphological opening operation(Luc Vincent, 1992) with disc structuring element of varying radius
371 to the cytoplasmic LC3 puncta observed in the cytoplasm of individual cells. The disc radius used for
372 the readout of all siRNA-treated cells was determined by comparing uninfected cells with infected
373 cells that had not been treated with siRNA.

374 Computational analysis of the image-based data has been performed using a combination of open-
375 source software and custom developed code. Image analysis was performed on a Desktop PC
376 equipped with Intel Core i7-8700K CPU at 3.7 GHz and 32 GB of RAM as well as GeForce 1080 Ti
377 GPU. Image analysis for the LC3 lipidation screen was performed using a custom pipeline for the
378 CellProfiler software(Carpenter et al., 2006) to detect LC3 granularity. Prior to granularity
379 assessment the images were processed for computational analysis: Images of cell nuclei were
380 corrected for optical shading and then convolved using a median filter. Next, the sum of all channel
381 images was produced to obtain a whole cytoplasm signal. Subsequently, processed nuclei and total
382 images allowed for detection of cell nuclei and cells as primary and secondary objects, respectively.

383 Based on the detected cells, LC3 signal granularity was measured for each single cell. To do so, a
384 morphological opening operation(Luc Vincent, 1992) with a disc structuring element of varying
385 radius was applied to the cytoplasmic LC3 puncta (594 nm channel) selected by the cytoplasm mask
386 of individual cells. The disc radius used for the readout of all siRNA-treated cells was determined by
387 comparing uninfected cells with infected cells that had not been treated with siRNA. The readout
388 size was selected based on comparisons of LC3 granularity between infected and uninfected HeLa
389 cells. The better the fit for the defined structuring element size, the higher the granularity score. To
390 account for variability of the granule size, the sum of all scores per cell was used as readout for LC3
391 granularity on a single-cell level. CellProfiler outputs produced for each cell were then processed
392 using a custom KNIME-based workflow, normalizing and grouping the obtained measurements.

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404 request.

405

406 **Figure Legends:**

407

408 **Figure 1: VACV infection causes an increase in LC3b puncta formation and in LC3-II.** A)
409 HeLa cells were infected with VACV for the time indicated and stained for LC3b or treated
410 with 3-MA, Bafilomycin and Torin1 at standard concentrations for 4 hrs. Representative
411 images of three biological replicates are shown. Scale bars represent 10µM. B) HeLa cells
412 were infected with VACV or incubated with BafilomycinA1 and Torin1 (B+T) or 3-MA for 8

413 hours. Right: Quantification of four biological repeats of experiment shown. Statistical
414 analysis: Unpaired t-test with * P<0.05. Error bars represent SEM
415
416 **Figure 2: VACV infection causes LC3 lipidation, not pro-cleavage block through expression**
417 **of an early viral gene.** A) PLD assay carried out for VACV WR WT infection at 8 hpi with
418 BafilomycinA1 & Torin1 ctrl with quantification of n=3 biological replicates below. B) PLD
419 assay carried out for VACV WR WT infection at 24 hpi with BafilomycinA1 & Torin1 ctrl with
420 quantification of n=3 biological replicates below. C) HeLa cells were infected with VACV WR
421 WT at MOI30 for the time indicated in presence of 50 µM CHX, 25 µM MG132, 10 µM AraC
422 or DMSO with quantification of n=3 biological replicates on the right. Statistical analysis:
423 Unpaired t-test with * P<0.05 and **P<0.01. Error bars represent SEM.
424
425 **Figure 3: A cell line expressing a dsRed-LC3-I-GFP construct is used as readout for LC3**
426 **lipidation.**
427 A) Schematic representation of dsRed-LC3-I-GFP. B) Representative confocal images of the
428 cell line expressing the dsRed-LC3-I-GFP construct shown in A. HeLa cells were treated with
429 3-MA, Bafilomycin and/or Torin1 at standard concentrations for 4hrs or infected with VACV.
430 Scale bar indicates 15 µM.
431
432 **Figure 4: An early VACV gene siRNA screen identifies several candidates for VACV**
433 **mediated increase and decrease of LC3 puncta formation.** A&B) Principle approach to
434 granularity measurement. A Top: An example of a high-granularity cell. B Top: An example
435 of a low-granularity cell. Left-to-right channel merge, LC3 channel and morphologically
436 processed LC3 micrograph using ray morphology open with a 10px circle as a structuring
437 element. The yellow dashed line corresponds to the position the linear profile was
438 measured. A,B bottom: Line profile intensity measurement of unprocessed (raw) and
439 processed micrographs of high and low granularity, highlighting the principle approach of
440 granularity quantification. C) Cells were treated with siRNA for 16 hrs and infected with WR
441 WT at MOI 10. Red puncta of lipidated LC3 were detected and used for granularity readout.
442 Figure shows pooled results for all three siRNAs per gene with each dot representing an n=3
443 for one specific siRNA. Error bars indicate SEM. Scale bar indicates 10µm. Statistical analysis:
444 Unpaired t-test with * P≤0.05, **P≤0.01 and ***P≤0.001

445

446 **Figure 5: Hit Validation of LC3 screen.** A) Validation of hits for LC3 lipidation decrease,
447 sample western blot (left) and quantification of three western blots (right). B) Validation of
448 hits for LC3 lipidation increase, sample western blot (left) and quantification of three
449 western blots (right). C) qRT-PCR validation of KD efficiency n=3 independent repeats in
450 comparison to gene expression in siScr samples. Statistical analysis of n=3 repeats for A and
451 B: Unpaired T-test with * $P<0.05$. All controls significant with at least *** $P<0.0001$.
452 Absence of significance indication in target genes means non-significant. Error bars
453 represent SEM. **D Three different models of LC3 lipidation:** 1) A viral protein causes ATG12–
454 ATG3 conjugation. The ATG12–ATG3 complex results in aberrant LC3 lipidation dislocated
455 from phagophore leading to failure in autophagosome formation. 2) Lipidated LC3
456 contributes to the formation of new virions. 3) LC3 lipidation is a side effect of autophagy
457 activation during infection due to depletion of autophagy receptor proteins.

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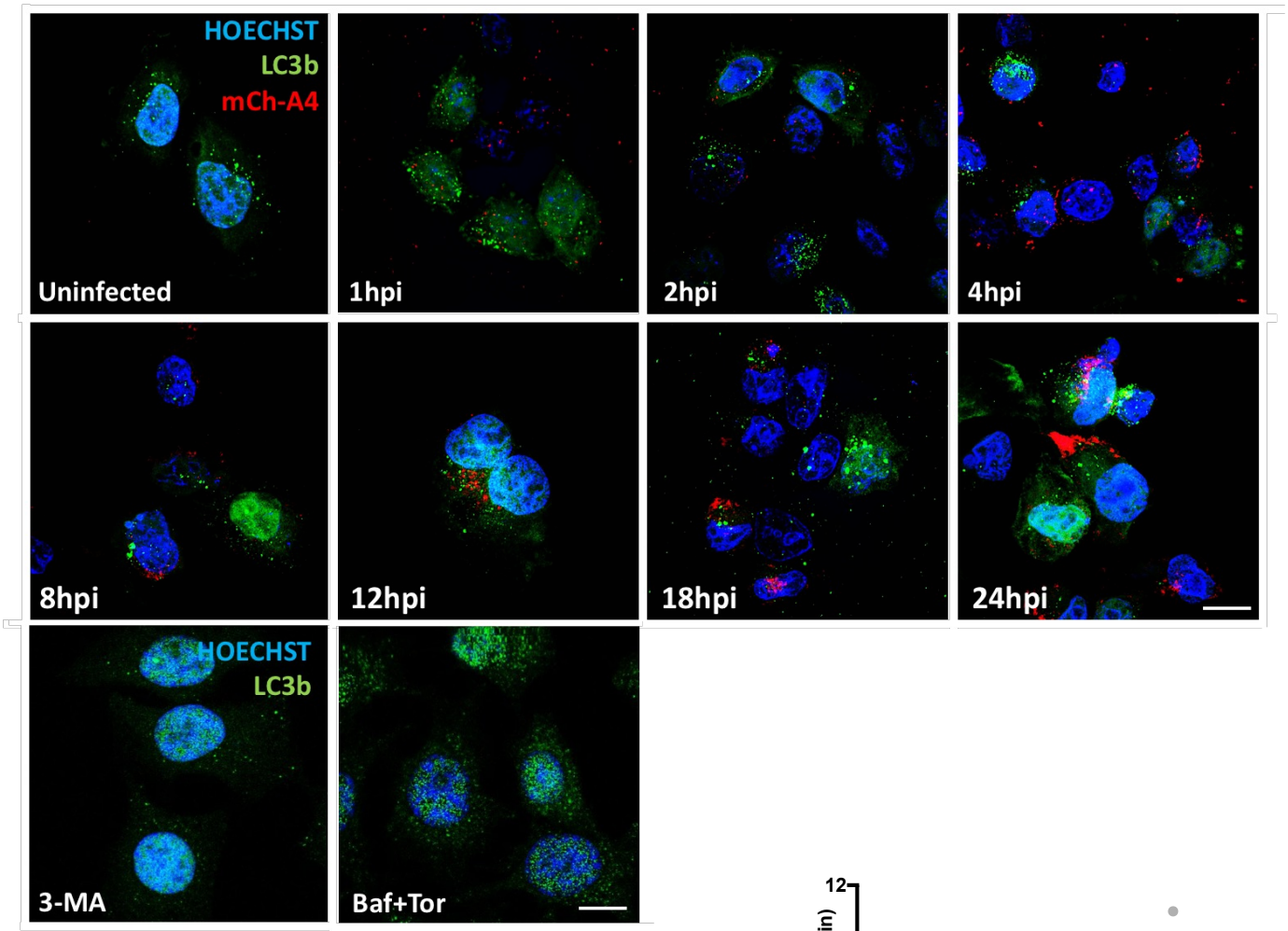
HOECHST
LC3b
mCh-A4
Uninfected
1hpi
2hpi
4hpi
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12hpi
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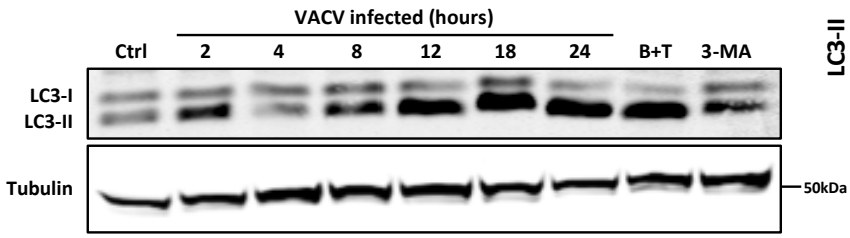
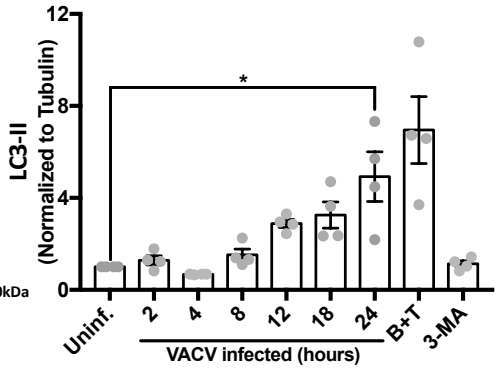
24hpi
HOECHST
LC3b
3-MA
Baf+Tor

B


VACV infected (hours)
Ctrl
2
4
8
12
18
24
B+T
3-MA
LC3-I
LC3-II
Tubulin
50kDa


LC3-II
(Normalized to Tubulin)
12
8
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Uninf.
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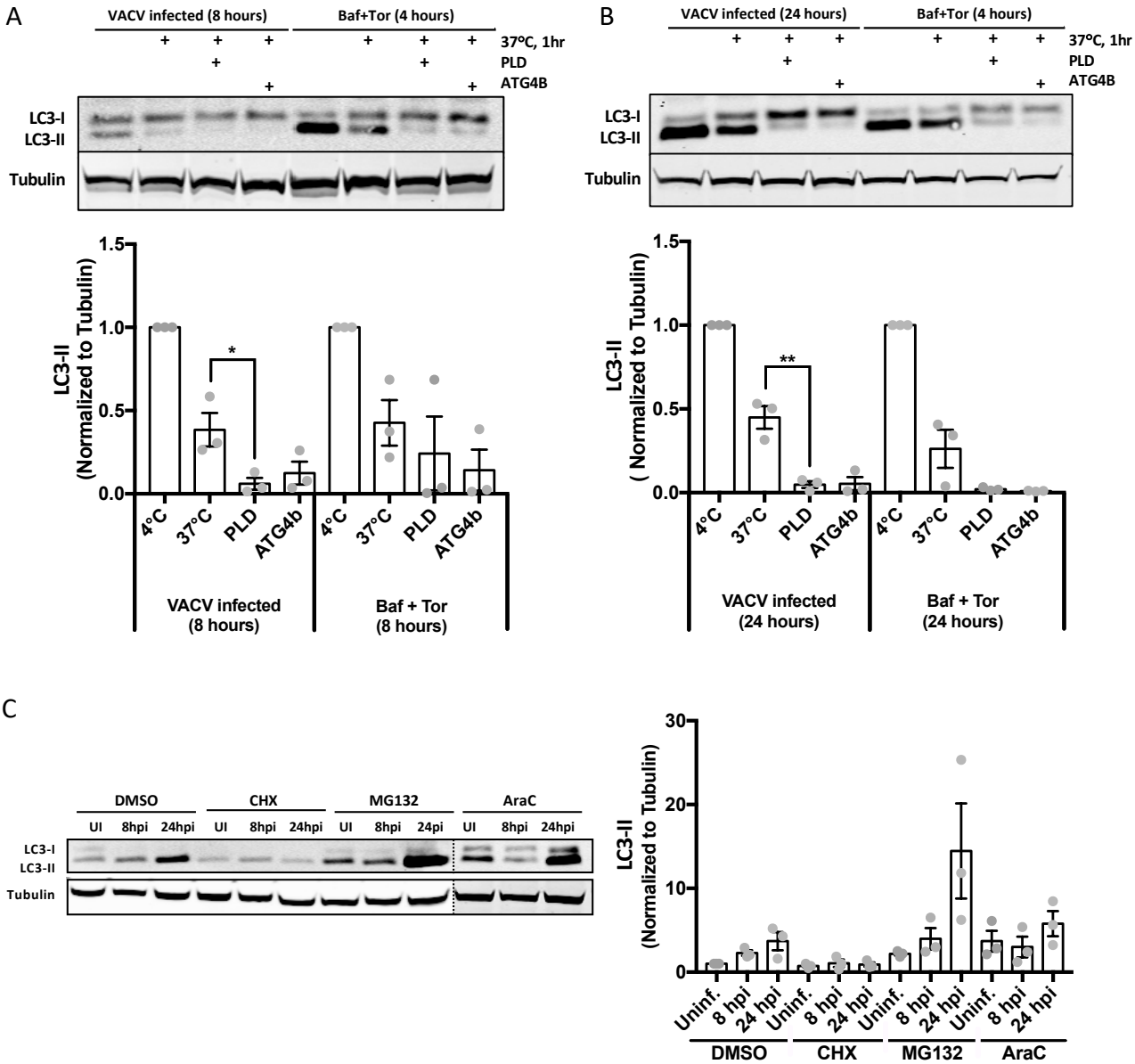
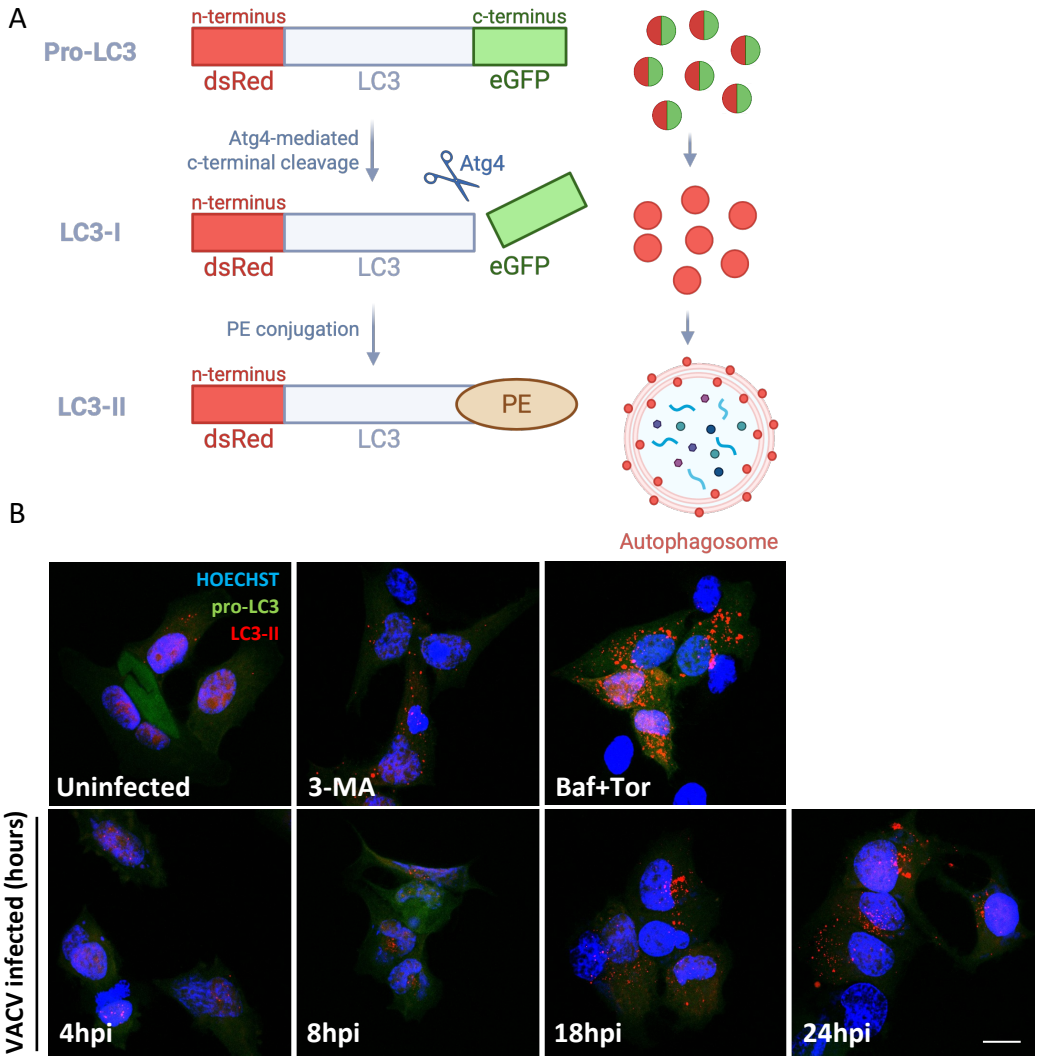
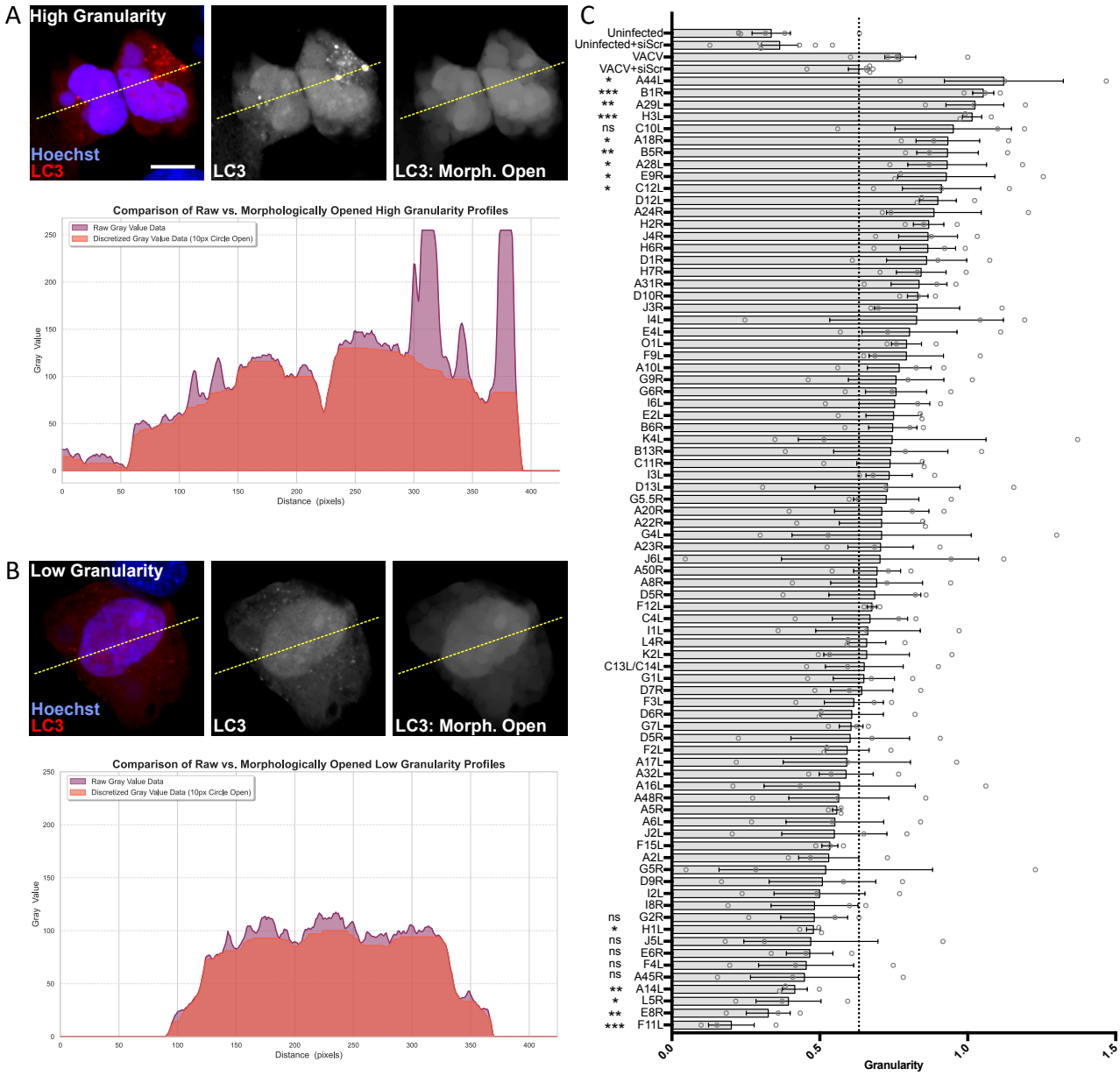
8
12
18
24
B+T
3-MA
VACV infected (hours)



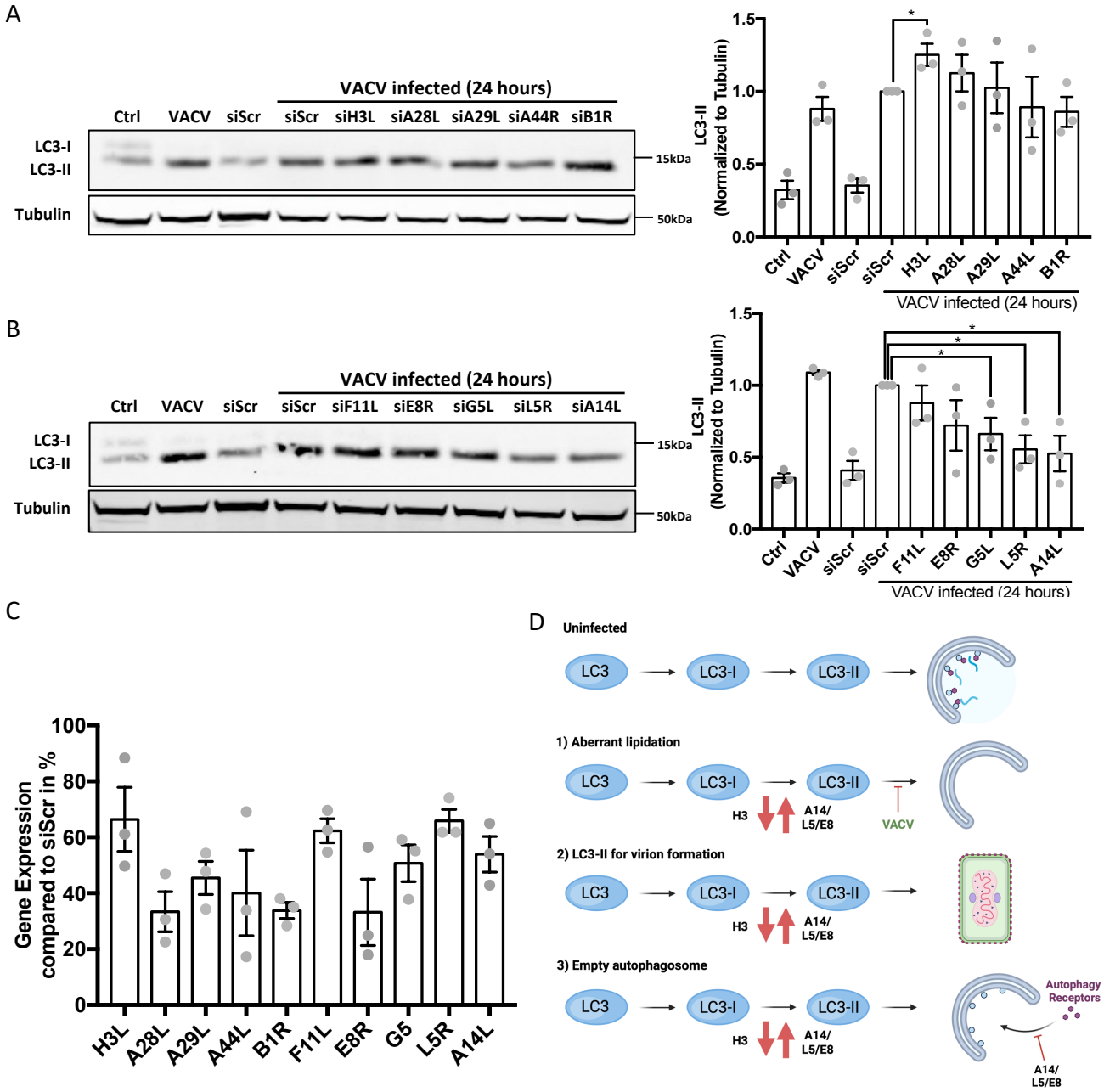
Krause et al., A granularity screening approach identifies candidate genes for VACV induced LC3 lipidation



Krause et al., A granularity screening approach identifies candidate genes for VACV induced LC3 lipidation



Krause et al., A granularity screening approach identifies candidate genes for VACV induced LC3 lipidation



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