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1 **The SAT protein of porcine parvovirus accelerates viral spreading through irreversible**
2 **ER stress induction**

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11 Running Head: The SATp induces irreversible ER stress

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26 **Abstract**

27 The SAT protein of porcine parvovirus (PPV) accumulates in the endoplasmic
28 reticulum (ER) and SAT deletion induces “slow spreading” phenotype. The in vitro
29 comparison of the wild type Kresse strain and its SAT⁻ knockout mutant revealed that
30 prolonged cell integrity and late viral release are responsible for the slower spreading of the
31 SAT⁻ virus. During PPV infection, regardless of the presence or absence of SATp, the
32 expression of downstream ER stress response proteins (Xbp1 and CHOP) was induced.
33 However, in the absence of SATp, significant differences were detected in the quantity and
34 the localization of CHOP, suggesting a role of SATp in the induction of irreversible ER stress
35 in infected cells. The involvement of irreversible ER stress induction in PT cell necrosis and
36 the viral egress was confirmed by treatment of infected cells by ER stress inducing chemicals
37 (MG132, DTT and Thapsigargin) that accelerated the egress and spreading both the wild type
38 and the SAT⁻ viruses. UV stress induction had no beneficial effect to PPV
39 infection underscoring the specificity of ER stress pathways in the process. However,
40 induction of CHOP and its nuclear translocation cannot alone be responsible for the biological
41 effect of SAT, since nuclear CHOP could not complement the lack of SAT in a co-expression
42 experiment.

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52 **Importance**

53 SATp is encoded in an alternative ORF of the PPV genome. Earlier we showed that
54 SATp of the attenuated PPV-NADL-2 strain accumulates in the ER and accelerates virus
55 release and spreading. Our present work revealed that “slow spreading” is a general feature of
56 the SAT⁻ PPV viruses and is the consequence of prolonged cell integrity. PPV infection
57 induced ER stress in the infected cells regardless of SATp presence, as demonstrated by the
58 morphological changes of the ER, and expression of the stress response proteins XBP1 and
59 CHOP. However, the presence of SATp made the ER stress more severe and accelerated the
60 cell death during infection as shown by the higher expression rate and the alteration of the
61 localization of CHOP. The beneficial effect of irreversible ER stress on PPV spread was
62 confirmed by the treatment of the infected cells with ER stress inducing chemicals.

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77 **Introduction**

78 Porcine parvovirus (PPV) belongs to the species *Ungulate protoparvovirus 1* in the
79 genus *Protoparvovirus*. It is the causative agent of the SMEDI syndrome (stillbirth,
80 mummification, embryonic death, infertility) in swine (1).

81 A highly pathogenic strain was isolated by Kresse with broad tissue tropism in the
82 1980s (2, 3). The Kresse strain-infected piglets showed atypical signs of PPV infection,
83 including necrotic lesions (4) on the lips, snout, tongue and foot.

84 The PPV single-stranded genome contains two major open reading frames (ORFs) and
85 one short, genus-specific ORF. The upstream major ORF encodes two non-structural proteins
86 (NS1 and NS2). The NS1 has helicase and nickase activities and it can induce apoptosis (5, 6)
87 and cell lysis (7). The NS2 protein is translated from an alternatively spliced RNA (8). The
88 downstream major ORF encodes a nested set of three C-terminally identical structural
89 proteins (VP1-3). A Ca-dependent phospholipase A2 (PLA2) enzyme located in the unique
90 N-terminal region of VP1 (9) is necessary for viral infectivity.

91 The short genus-specific ORF overlaps with the 5' end of the ORF of VP2 protein in all
92 *Protoparvovirus* genomes and it encodes a small alternatively translated protein (SATp) (10).
93 The SATp contains a single membrane-spanning α -helix and it localizes in the endoplasmic
94 reticulum-nuclear membrane network. The knockout SAT⁻ mutant of the attenuated NADL-2
95 strain of PPV shows a "slow-spreading" phenotype in cell culture. Based on *in vitro*
96 complementation studies it was presumed that the function of the SATp is to induce
97 endoplasmic reticulum (ER) stress to facilitate cell lysis (10).

98 The ER plays a key role in protein folding and maturation. The immature proteins enter
99 the ER lumen, where they are folded by molecular chaperons (e.g. Grp78, Grp 94, calnexin)
100 and protein-folding enzymes like calreticulin and protein disulphide isomerase (11). At the

101 end of the maturation process only the correctly structured proteins are transported to
102 destination sites with ER vesicles (12). Incorrectly folded proteins accumulate in the ER
103 lumen causing ER stress. Multiple transmembrane sensors of signalling pathways detect the
104 ER stress including the protein kinase R-like kinase (PERK) (13), the inositol-requiring
105 kinase/endoribonuclease 1 (IRE1) and ATF6 (14).

106 IRE1 has endoribonuclease and serine/threonine protein kinase activity and its
107 signalling pathway represents the most conserved element of the ER stress response (15, 16).
108 After activation, the IRE1 cuts out a 26 base pair length section from the mRNA of the X
109 box-binding protein 1 (Xbp1) that leads to a frame shift and a spliced Xbp1 protein (Xbp1s).
110 Xbp1s is transported to the nucleus and regulates the expression of genes contributing to
111 protein folding, glycosylation and ER membrane biogenesis (17, 18).

112 The activated PERK phosphorylates the eukaryotic translation initiation factor 2 α
113 (eIF2 α), that inhibits the translation of most mRNAs (19), thus reducing the protein load and
114 easing ER stress (20, 21). At the same time the translation rate of some ER stress-related
115 proteins – including the Activated Transcription Factor 4 (ATF4) – increases. ATF4 induces
116 the translation of genes that regulate amino acid biosynthesis and transport (22). One of the
117 ATF4-stimulated genes is the C/EBP homologous protein (CHOP) (23). Under prolonged or
118 severe ER stress CHOP irreversibly triggers programmed cell death (24, 25).

119 In the present study, we show that wild-type (wt) PPV infection induces irreversible ER
120 stress whereas the loss of SATp through mutations lessens this ER stress and leads to reduced
121 apoptosis and cell lysis in virus-infected cells. ER stress-inducing drugs can compensate for
122 the loss of SAT *in vitro*, and they accelerate the egress of the wt PPV. This process seems to
123 be ER stress specific, since UV stress doesn't induce similar effect.

124

125 **Materials and Methods**

126 ***Cells, viruses and transfection***

127 PT cell line was used in all experiments. The cells were grown in a DMEM-based
128 medium (high glucose: 4.5 g/l, PAA) supplemented with 10% serum (Fetal Bovine Serum
129 Gold, PAA), 1% penicillin-streptomycin (PAA) and 1% sodium-pyruvate solution (Lonza) in
130 the presence of 5% CO₂ at 37°C.

131 For infection and transfection, the cells were seeded in 24-well tissue culture plates
132 (1×10^5 cell/well) and they were infected or transfected with either the wt PPV Kresse or the
133 SAT⁻ mutant of PPV Kresse strain at about 50% cell confluency. We used 0.01 multiplicity of
134 infection (MOI) to monitor viral spread and 3 MOI (optimal MOI to use a minimal number of
135 viruses to target the maximal number of cells) in the experiments to investigate virus release,
136 apoptosis, viability and ER-stress. Infectious titer of the viral stocks was determined as
137 described earlier (10). Data was averaged from four independent dilutions.

138 The wt and mutant viral stocks were created by transfecting PT cells with pKresse
139 (pUC19 cloned PPV Kresse virus (26)) and pSAT⁻ plasmids, respectively. By using the
140 method described earlier, two nucleotides of pKresse (T-2842→A and T-2845→C) were
141 changed to knock out SAT and to create pSAT⁻ (10) (Figure 1).

142 Fusion constructs alone were transfected into adherent cells using the TurboFect
143 Transfection Reagent (Thermo Scientific) following the supplier's recommendations.

144 Co-transfection of the infectious clones with fusion constructs were performed in
145 suspension cell cultures as follows. 1 µg infectious clone and 1 µg fusion construct DNA were
146 mixed with 3 µl Turbofect reagent in 100 µl DMEM and incubated for 20 min at room
147 temperature. The transfection mix was then added to 1 ml freshly trypsinized, suspended PT
148 cells (1×10^5 cells/ml in DMEM with 10% FBS) and incubated on a gently rocking platform
149 for 3 h at room temperature. Cells were centrifuged at 1000g for 1 min, re-suspended in 1 ml
150 fresh medium and plated in 24-well plates.

151

152 ***Fusion constructs***

153 DsRed-labelled SAT and CHOP protein constructs were created in DsRed-Monomer-
154 N1 plasmids (Clontech). The SATp and CHOP sequences were amplified by the PPV-SATf
155 (GC GGTACC ATG TGG AAC AAC ACA ACC CTA), the PPV-SATr (CG GGTACC TT
156 GAT GTA TGA GTC TTG ATG CGT), the F-CHOP-DsRed (GC AAGCTT ATG GCA GCT
157 GAG TCA TTG CCT) and the R-CHOP-DsRed (GC GGATCC CG TGC TTG GTG CAG
158 ATT AAC CAT) primers, and by Phusion Hot Start DNA polymerase. To get the template for
159 CHOP, total RNA was purified with RiboZol (Amresco), and reverse transcribed with
160 Superscript III (Thermo Fisher Scientific) using the R-CHOP-DsRed primer according to the
161 manufacturer's recommendation.

162 The isolated PCR fragments and the vector were digested with KpnI or HindIII and
163 BamHI restriction enzymes and were ligated with T4 ligase (Thermo Scientific).

164

165 ***Immunofluorescence staining***

166 For immunofluorescence (IF) staining the cells were plated on coverslips. They were
167 fixed at the appropriate time with 3% formaldehyde and incubated for 30 min at room
168 temperature. After two washing steps (1.5 g bovine serum albumin dissolved in 300 ml
169 1×PBS) they were permeabilized using 1% Triton-X (Sigma-Aldrich). After 15 min the cells
170 were washed twice, 5% inactivated horse serum (diluted in PBS) was loaded into the wells
171 and the plate was incubated for 30 min at room temperature. After two washings the cells
172 were exposed to primary antibodies (3C9 (CRL-17, ATCC) mouse anti-PPV capsid-specific
173 monoclonal antibody, mouse anti-CHOP (Thermo Scientific), rabbit anti-Xbp1 (Santa Cruz
174 Biotechnology and sera of the PPV infected swine) for 60 min at room temperature. After
175 further washings the cells were incubated with their respective secondary antibodies (CF594

7

176 anti-mouse, CF488 anti-mouse, CF488a anti-rabbit or the CF 586 anti-swine antibodies
177 (Biotium)) for 60 min at room temperature in the dark. After final washings the cover glasses
178 were removed from the wells and were fixed into a slide using Fluoroshield (Sigma-Aldrich)
179 according to the manufacturer's protocol. An Axio Observer D1 inverted fluorescence
180 microscope (Zeiss) was used for visualization.

181

182 ***Fluorescent Focus growth inhibition***

183 For finding the neutralizing concentration of the 3C9 monoclonal antibody, the
184 supernatant of the 3C9 hybridoma was diluted 10× 20× 50× 100× in complete medium and 50
185 µl of the diluted solutions were mixed with 50 µl 0.01 MOI viral stock, then incubated for 1
186 hour at room temperature. After incubation 100 µl of the media containing the antibody virus
187 complexes were loaded to 50% confluent PT cells on a 96 well plate and the cells were
188 incubated at 37°C for viral growth. After 24 hours the cells were fixed and the progress of
189 infection was detected with the standard IF method described above. Twenty times diluted
190 supernatant completely blocked the PPV infection. In the next step the PT cells were infected
191 with 50 µl low MOI viral stocks (0.01) and incubated at 37°C. After 4 hours the supernatant
192 was removed, 50 µl medium with 20× diluted 3C9 was added, and the cells were incubated at
193 37°C for an additional 20 hours. After the incubation the cells were fixed and monitored for
194 viral infection with the standard IF method.

195

196 ***Apoptosis and viability experiments***

197 For the investigation of apoptosis and lysis, the cells were seeded at 50% confluency
198 ($2,5 \times 10^5$) in 24-well plates and after 3 h they were infected with viruses. Live, uninfected,
199 infected and uninfected cells were incubated with 1 µg/ml Hoechst 33342 and with 0,25 µg/ml
200 propidium iodide at different time points (18 h-88 h p.i.) for 60 min at room temperature in

201 the dark. Then they were washed with PBS, examined under a microscope, and several
202 photographs were taken from the central regions of the wells in the blue and the red channels.
203 All cells including PI positive and apoptotic cells were counted from 3-6 photographs of each
204 well and averaged. Pyknotic nuclei were identified by strong staining with Hoechst,
205 fragmentation or nuclear shrinkage to 1-4 μm (27). More than a thousand cells were counted
206 (on minimum four photos) for every time point (except when attached cell count went under
207 20% of the total) by two independent persons and data was averaged. The Mann-Whitney U
208 test was applied for the statistical analysis of the data where the null hypothesis was that the
209 two samples came from the same population.

210 Lysis was quantified based on lactate dehydrogenase enzyme (LDH) activity (28, 29) by
211 a Cytotoxicity Detection KIT (Roche) following the manufacturer's instructions. The
212 absorbance was measured in the linear range after 1:1 dilution of the supernatants with PBS
213 by an EL \times 800 ELISA plate reader (Dialab GMBH., Austria) at 490 nm. Sample absorbance
214 was calculated by subtracting the background value of the serum from the measured data.

215

216 ***Real-time quantitative PCR***

217 The supernatants of the infected cells were sampled between 0 h p.i. and 84 h p.i. The
218 viral DNAs were purified with High Pure Viral Nucleic Acid KIT (Roche) according to the
219 manufacturer's protocol. The qPCR reaction solution (25 μl) contained 18.25 μl water, 2.5 μl
220 10 \times DreamTaq Buffer (Thermo Scientific), 0.5 μl dNTP mix (2 mM each), 1 μl template
221 DNA from the supernatants, 0.5 μl DreamTaq DNA Polymerase (Thermo Scientific), 1.25 μl
222 20 \times EvaGreenTM Dye (Biotium), 0.5 μl forward primer (CTT TAG CCT TGG AGC CGT
223 GGA) and 0.5 μl reverse primer (AAC TAC CCT TAC CTC TTG CTC TT) (both 20 pM/ μl
224 concentration).

225 The thermal reaction started with a pre-denaturation step at 95°C for 5 min and
226 followed by 40 cycles (denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and
227 elongation at 72°C for 50 sec) and finished with a post-elongation step at 72°C for 5 min. The
228 specificity of the qPCR was verified with melting curve analysis.

229

230 *DNase treatment*

231 20 µl 10× DNase puffer with 25 mM MgCl₂ (Thermo Scientific) and 4 µl 50U/µl bovine
232 pancreatic DNase I (Roche) were added to 200 µl supernatant of the infected cells. After 90
233 min at room temperature the viral DNA was purified with High Pure Viral Nucleic Acid KIT
234 (Roche) according to the manufacturer's protocol.

235

236 *ER stress induction*

237 The plated and infected cells (MOI: 0.01 and 3) were incubated with 20 µM MG132 or
238 10 mM DTT (dithiothreitol) at 0-5 h, 3-8 h, 5-10 h, 7-12 h p.i. or 10 µM thapsigargin at 0-2 h,
239 2-4 h, 4-6 h, 6-8 h p.i. on 37°C. After the chemical incubation period the cells were washed
240 twice and 1 ml fresh cell culture medium was added into the wells. The supernatant was
241 harvested and the cells were fixed at 24 h p.i.

242

243 *UV stress induction*

244 The infected cells (MOI: 3) were treated with sub-lethal UV-C light (30 W light source, 50
245 cm distance) for 5 min at 0, 3, 9 and 12 h p.i. After the treatment period the cells were washed
246 twice and 1 ml fresh cell culture medium was added into the wells. The supernatant was
247 harvested at 24 h p.i. and the cells were fixed for further examinations.

248

249 **Results**

250 *Spread of the wild type and SAT⁻ mutant PPV Kresse strains*

251 Previously, we reported that the SAT⁻ mutant of the attenuated NADL-2 strain of PPV
252 has a slow spreading phenotype (10). In the present study, our first step was to investigate
253 whether the loss of SAT causes a similar effect in the virulent Kresse strain.

254 For this purpose, first a SAT⁻ knockout mutant Kresse strain was created by eliminating
255 a potential second initiation codon of SAT (8 codons behind the initiation codon of SAT) and
256 introducing a stop codon into the SAT coding frame in the infectious clone of Kresse strain
257 without changing the protein sequence of the VP proteins (Figure 1). Then PT cells were
258 infected with the rescued SAT⁻ and wt virus at low (0.01) MOI. The differences between the
259 spreading of two virus strains were monitored by IF staining using 3C9 assembled capsid
260 sensitive primary antibody.

261 The first positive cells appeared at 12 h p.i. with both the wt and the SAT⁻ mutant virus
262 indicating similar kinetics in viral entry, decapsidation, replication, viral protein synthesis and
263 capsid assembly. The number of positive cells continuously grew until 20 h p.i. when the first
264 secondary infections could be detected in the wt virus-infected cells in the form of
265 fluorescence foci, while the first signs of re-infection were visible only at 24 h p.i. in the case
266 of the SAT⁻ mutant virus (Figure 2/A). Incubation of the low MOI virus infected cells with
267 the 3C9 neutralizing antibody abolished the appearance of large FFs (fluorescent focus) at 24
268 hours with both the wild type and the SAT⁻ viruses, proving that large FFs are indeed the
269 results of secondary viral infection. (Figure 2/B, only wild type is shown). The difference in
270 virus spreading became even more obvious as the infection progressed. At 48 h p.i., the wt
271 virus infected almost every cell, in contrast to the SAT⁻ strain. Changes in infectious titer and
272 viral copy numbers of the supernatant correlated well with the spreading pattern revealed by
273 IF staining (Figure 3/A). Thus, the loss of SATp in the pathogen Kresse strain and the
274 attenuated NADL2 strain (10) resulted in a similar phenotype.

275 High multiplicity infection indicated impaired egress for the SAT⁻ Kresse strain. The
276 copy number of the wt virus in the supernatant started increasing sharply at 20 h p.i. as a
277 consequence of mass release of the viruses and viral DNA from a large number of infected
278 cells (Figure 3/B). The copy number increase of the SAT⁻ mutant virus started later (between
279 22 h and 24 h p.i.) and the number of viral genomes in the supernatant remained constantly
280 under that of the wt virus. The biggest difference between the copy numbers was around 48 h
281 p.i. and it decreased gradually until the end of the monitoring period.

282 DNase treatment of the supernatants revealed that the majority of the qPCR-detected
283 viral copies in the supernatant of the infected cells came from DNase-sensitive replicative
284 forms and partially- or non-encapsidated genome forms released, most probably, from the
285 dying cells (Figure 3/B). The difference between the DNase-resistant copy numbers in the
286 supernatant of the two strains decreased to minimal by the end of the monitoring period (84 h
287 p.i.), indicating very similar amounts of packaged DNA and infectious virus production.
288 Titering of the wt and SAT⁻ stocks on PT cells (4.85×10^9 and 2.56×10^9) indeed revealed very
289 similar infectious particle production. This result was also congruent with our earlier findings
290 with the NADL2 strain and its SAT⁻ mutants (10).

291 We theorized that the quicker release of the wt virus must be the consequence of earlier
292 cell death and lysis induced by SATp in the host cell. To better understand the function of
293 SATp, we first studied the phenotypic effects including viability, cell lysis, and apoptosis of
294 SAT⁻ and wt viruses on infected cells.

295

296 *Cytopathogenic effects of the viral infection*

297 First LDH release as an indicator of cell lysis was quantified from high MOI-infected
298 cells. (30) (Figure 4/A). The released LDH level started to increase much faster (exponentially
299 $R^2: 0.9946$) in wt virus-infected cells from 18 h p.i. until 48 h p.i. than in SAT⁻ infected cells,

300 where the increase was logarithmic ($R^2: 0.9639$) in this interval. Measured values reached an
301 approximately twofold difference at 48 h p.i. in the supernatant of wt virus-infected cells. At
302 this time-point barely any of the wt virus-infected cells remained alive while the majority of
303 the SAT⁻-infected cells stayed adherent (Figure 4/B). Extracellular LDH activity of SAT⁻
304 infected cells started to sharply increase after 64 h and that was preceded by a rapid decrease
305 in the attached cell count. Monitoring the attached cells with propidium iodide revealed that
306 the membrane integrity of the SAT⁻-infected cells was also sustained for a longer time and in
307 more cells than that of the wt virus-infected cells (Figure 4/C) The number of pyknotic and
308 karyorrhectic nuclei remained relatively low during the course of infection of both viruses
309 (less than 14% and 8% in the case of wt and SAT⁻ viruses, respectively) (Figure 4/D).
310 Nevertheless, their rates were always higher in wt virus-infected than among SAT⁻-virus
311 infected cells, and the difference became and remained statistically significant ($p < 0.046$)
312 starting at 28 h p.i. Plasma membrane blebbing could hardly be seen in infected cells while
313 their nuclei were frequently enlarged (Figure 4/E, 5).

314 These experiments highlight that lysis, rather than apoptosis is the main form of cell
315 death during PPV infection in PT cells. They also reveal that the loss of SAT prolongs cell
316 life throughout PPV infection and it decreases the number of both lysed and necrotic cells
317 during the course of infection.

318

319 *The effect of ER stress inducers*

320 Since SATp accumulates in the ER, it seemed plausible to presume that SATp
321 facilitates early cell death and virus release through ER stress induction. To gather evidence
322 on the effect of ER stress on PPV egress, the influence of ER stress inducer drugs was
323 investigated on infected cells. PT cells were infected with wt and SAT⁻ Kresse strains at low
324 multiplicity (MOI 0.01), and the cells were treated at different time points with 10 mM DTT,

325 20 μ M MG132 or 10 μ M thapsigargin. The cells were fixed at 20 h p.i. and monitored for the
326 presence of plaque-like FFs of PPV by IF (Figure 5). In untreated cells at 20 h p.i. the SAT⁻
327 virus does not induce FFs, only individual cells are positive for the virus.

328 Treatments with all ER stress inducers moderately increased the size of FFs in wt virus-
329 infected cells, but most importantly, they also induced FFs in SAT⁻ virus-infected cells,
330 regardless of the starting time of the treatment. The MG132 treatment starting at 3 h p.i. gave
331 the biggest and highest number of FFs. Under these conditions the size of the FFs of the SAT⁻
332 virus was similar or even bigger than that of the wt virus in untreated cells (Figure 5).

333 The same treatments (10 mM DTT or 20 μ M MG132) gave similar results at high
334 multiplicity (MOI 3) infection, where the virus titer was quantified by qPCR at 24 h p.i. from
335 the culture media (Figure 6/A). Both ER stress inducers substantially increased viral egress
336 into the media. DTT treatment starting at 7 h p.i. had the strongest effect on wt virus release
337 (4.41 \times), while the 3 h p.i. MG132 treatment induced the highest titer increase in the medium
338 of cells infected with the SAT⁻ virus (71.67 \times). Unexpectedly, almost all of the tested
339 chemical treatments (with the exception of the 7 h p.i. DTT treatment) increased the SAT⁻
340 virus titer above not only the basic wt virus titer but also that of the chemically-enhanced wt
341 values.

342 Back titration of the supernatants of ER stress inducer-treated cells verified that the
343 increase of the viral copy number correlates with the increase of the number of infectious
344 particles in the supernatants (Figure 6/C).

345 To investigate the specificity of the ER stress among other stress factors to accelerate
346 viral egress, the effect of UV radiation was also examined. Short term sub-lethal UV-C light
347 treatment (5 min) did not change the viral copy numbers considerably, while it significantly
348 reduced (to \sim 2%, $p < 0.005$) the number of the infectious particles in the supernatant (Figure
349 6/B and C).

350 The results of these experiments strongly suggested that ER stress indeed facilitates the
351 release of matured particles from infected cells, and ER stress seems to be specific in this
352 regard because UV-C radiation is not able to induce a similar effect.

353

354 ***Detection of ER stress***

355 To further clarify the relation of ER stress and SATp to the acceleration of viral egress,
356 the pattern of ER and ER stress markers was studied in infected cells. PT cells were infected
357 with the SAT⁻ and the wt Kresse strains (MOI: 3), and calreticulin as ER marker, as well as
358 Xbp1 and CHOP as downstream ER stress response markers were monitored by IF staining.
359 PPV infection – with or without SAT – causes condensation in the ER membranes of the
360 infected cells. Perinuclear nodes and clots could be detected implying fragmentation and
361 fusion of the tubular network as obvious signs of ER stress (31, 32, 33) (Figure 7). However,
362 no significant differences were found between the wt virus- or SAT⁻ virus-infected cells in the
363 starting time or in the pattern of the disintegration in attached cells.

364 There was also no difference between SAT⁺ and SAT⁻ viruses until 48 hours when the
365 reversible ER stress marker Xbp1 was monitored during viral infection (Figure 8/A). Xbp1
366 was mainly detected in the nuclei of infected cells with the earliest detection time of about 14
367 h p.i. The presence of the protein was temporary, its level peaked sharply at 16-18 h p.i., when
368 around 95% of the infected cells showed positivity. After that it rapidly declined: at 20 h p.i.
369 the Xbp1 could only be detected in less than 5% of the cells. Low level Xbp1 expression was
370 detected again in SAT⁻ virus infected cells at 60 h p.i. However that was most probably the
371 consequence of ER stress induced by nutrient-starvation (34) because similar level Xbp1
372 expression was also visible in non-infected cells at the same time (Figure 8/C).

373 Among the monitored markers, only the irreversible ER stress marker CHOP showed a
374 difference between wt and SAT⁻ virus infection (Figure 8/B). In the wt virus-infected cells

375 CHOP was detected first at 22 h p.i. in around 20% of the cell nuclei. Its expression plateaued
376 at 24 h p.i. with 75% positivity in the infected cells, and remained relatively unchanged until
377 36 h p.i.. After 36 hours very few attached infected cells showed positivity to CHOP.
378 Although in SAT⁻ virus-infected tissue CHOP was also detected at 22 h p.i. in around 20% of
379 the nuclei of infected cells, but instead of nuclear localization it was detected perinuclearly in
380 the cytoplasm. It also plateaued at 24 h p.i. but only with 41% positivity in the cells; this
381 value essentially remained unaffected until 36 hours in attached cells and the perinuclear
382 localization of the protein did not change either. CHOP could not be detected after 48 hours in
383 SAT⁻ virus-infected cells.

384 These experiments show that PPV infection induces ER stress regardless of the
385 presence or absence of the SATp. However, the wt virus expressing the SATp is able to
386 activate the expression of CHOP in significantly more cells than the SAT⁻ virus, and SATp
387 also influences the localization of cell death triggering CHOP in infected cells.

388 Since chemical ER stress inducers were able to compensate for the loss of SAT, their
389 effect on the induction of ER stress markers was also monitored. As expected, the chemicals
390 induced Xbp1 and CHOP expression in the treated cells (Figure 8/D). CHOP expression could
391 be detected in 100% of the treated cells at 18 h and 8 h after the beginning of MG132 and
392 DTT treatment, respectively. However, the chemical treatments always triggered the nuclear
393 localization of CHOP similarly to wt virus infection but contrary to SAT⁻ virus infection.
394 Sub-lethal UV treatment of the PT cells did not induce either Xbp1 or CHOP expression.

395 These findings gave additional support to the possible role of nuclearly localized CHOP
396 in the acceleration of cell lysis.

397

398 ***Effect of the cloned SAT protein in PT cells***

399 To further explore the role of SATp in the induction of ER stress markers, a SAT-
400 DsRed fusion protein-expressing vector was transfected into PT cells. Transfected cells were
401 fixed in the 16-48-hour post transfection period every four hours and examined for XBP1 and
402 CHOP expression by IF. Neither protein was induced in SAT-expressing cells during the
403 monitoring period (data not shown), despite the fact that in their ER similar morphological
404 alterations (condensation in the perinuclear region) could be detected as in infected cells
405 (Figure 9). During the monitoring period, the SATp always co-compartmentalized with
406 calreticulin (Figure 9). Furthermore, the SAT-DsRed protein induced apoptosis (Figure 9) in
407 the late phase of the transfection (from 30 h post transfection). After 48 h p.t., only very few
408 SAT-DsRed-expressing cells could be detected in the transfected wells while cells expressing
409 the control DsRed protein remained viable and detectable even after 90 h p. t.

410 These experiments indicate that SATp alone is toxic and its accumulation has an effect
411 on the morphology of the ER. However, without the other viral proteins SATp is not able to
412 activate either XBP1 or CHOP expression.

413

414 ***Effect of the nuclear CHOP on viral spread***

415 Since the presence of the nuclearly localized CHOP in the infected cells showed strong
416 correlation with the accelerated viral egress, we further studied the role of CHOP in the
417 process. We cloned the porcine CHOP and made a construct constitutively expressing the
418 porcine CHOP-DsRed fusion protein. Many studies reported that transiently expressed
419 mammalian CHOP from a transfected plasmid accumulates in the nucleus. Indeed, the
420 transfections of our construct alone or together with the pSAT⁻ or the pKresse infectious
421 clones resulted in the nuclear localization of the CHOP-DsRed protein (Figure 10/A). The
422 CHOP-DsRed protein proved to be functionally active because it could induce apoptosis in
423 the transfected cells as early as 18 h p.t. (Figure 10/B) and it killed almost all transfected cells

424 at 48 h p.t. (data not shown). However, when the CHOP-DsRed expressing construct was co-
425 transfected with pSAT⁻ it did not increase the size of FFs in contrast to the SAT-DsRed
426 expressing construct that readily induced large size FFs (compared to the size of FFs in cells
427 transfected with the pSAT⁻ plasmid only) (Figure 10/C). This experiment revealed that
428 nuclear CHOP alone is not able to induce such transcriptional changes in the nucleus that
429 would accelerate cell lysis and viral spread.

430

431 Discussion

432 Quantification of the medium of high and low MOI-infected cells by qPCR and titration
433 revealed that SATp induces early viral release but does not increase the final virus production
434 (Figure 3).

435 The dynamics of the viral titer increase is quite different in the medium of high and low
436 MOI infected cells. At low MOI infection orders of magnitude differences can be detected
437 between the mutant and the wild type virus during the 40-64th hours of the infection (Figure
438 3/A). The accumulating effect of the 3-4 hour delays in *first* viral release in every consecutive
439 replicative cycles, together with the sharp decrease of the dividing cells that support
440 parvoviral replication after 28 h p.i. (the cell number grows to more than 2.5 of the seeding
441 number in the first 28 hours (Figure 4/B), while in the next 60 hours the growth rate is
442 drastically reduced) can provide a plausible explanation to the phenomenon.

443 It may look surprising that at high MOI infection, when *all* of the cells are infected at
444 the same time, the difference is much smaller. However, we can only expect to see orders of
445 magnitude differences if any or both of the following premises are fulfilled: (i) one of the
446 viruses produces much more progeny than the other in the cells or (ii) at a given time point
447 orders of magnitude more cells lyse and release progeny viruses. We showed in an earlier
448 paper as well (10) that SAT⁻ and wild type viruses produce equal amounts of progeny viruses.

449 In order for the second premise to be true, magnitudes of more cells should be dying among
450 SAT⁻ than wild type virus infected cells at any given time, that is obviously not the case. The
451 biggest differences in DNase-resistant viral copy numbers (6.5×) can be seen around 48 h p.i.
452 (Figure 3/B), after 95% of the wild type infected cells detached and started to lose their
453 integrity (indicated by attached cell count at 42 h p.i.) and they lyse en mass (see jumps in
454 LDH activity at 48-64 h p.i.). At the same time around 15% of the SAT⁻ cells are detached and
455 lysed (Figure 4). The 95% versus 15% is a big difference regarding detached cell numbers,
456 however their ratio is only 6.33×, which is a pretty good match of the measured 6.5×
457 difference in viral copy number in the media, given that all the dead cells produced equal
458 amount of viruses.

459 Interestingly, wild type virus infected cells release more DNase-sensitive viral DNA
460 than SAT⁻ virus infected cells (3/B). Since DNA is a potent immunostimulator acting through
461 several DNA-sensing receptors (35), this phenomenon may have potential therapeutic
462 significance for the development of oncolytic parvoviruses (36).

463 It was reported earlier that MVM and H1-PV, close relatives of PPV, are actively
464 transported in vesicles from the nucleus to the cell periphery and released into the culture
465 medium (37). However, we were not able to detect any assembled capsid in the cytoplasm of
466 either wt or SAT⁻ virus-infected cells at low (0.01) or even at high (3) MOI infection (Figures
467 2 & 5) until the cells started to lyse en masse (wt 40 h p.i.; SAT⁻ virus 64 h p.i. at 3 MOI) and
468 large amount of virus was released into the culture media which re-infected the remaining
469 cells. This observation suggests that vesicular transport does not play a significant role in the
470 egress of PPV, at least not in PT cells.

471 Members of the *Protoparvovirus* genus can induce either necrosis or apoptosis,
472 depending on the virus, and on the cell type (38). Parvovirus infection is also able to activate
473 early apoptotic events that do not go to completion and can lead to necrotic cell death as

474 demonstrated by H-1 infection in HeLa and P1 cells. In case of PPV it was reported that the
475 YL strain induces apoptosis in ST and PK-15 cells and in late phase of the infection (60 h p.i.)
476 the number of apoptotic cells can reach 50% (39, 40). In contrast, during Kresse strain
477 infection in PT cells the number of apoptotic cells remained low as indicated by the number
478 of pyknotic and karyorrhectic nuclei, and it never exceeded 14% regardless of the presence or
479 absence of SATp. Since subtle mutations of the PPV capsid can modify interactions with host
480 factors and change the cytopathic effect of the virus (26, 41) it is hard to pinpoint whether the
481 viral strains or the cell lines used are responsible for the observed differences.

482 It is worth mentioning that neither pyknosis nor karyorhexis are exclusive
483 characteristics of apoptosis: they are frequently detected in non-apoptotic, necrotic cells as
484 well (necrotic pyknosis). Strikingly we never detected any nuclear fragmentation inside an
485 infected cell with well-defined nuclear boundary. Apoptotic nuclei were readily produced in
486 SAT- and CHOP-expressing cells (Figure 9, 10) and they were also demonstrated in
487 parvoviral NS1-transfected cells (42). Our findings suggest that cell death in PT cells during
488 PPV infection is not characteristically apoptotic even if apoptotic pathways are involved as
489 indicated by CHOP induction. Swelling of the infected nuclei, lack of blebbing, early cell
490 membrane failure as indicated by PI uptake, rapid LDH and free viral DNA release all point
491 toward necrosis as the main form of cell death in PT cells during PPV infection.

492 ER stress inducers could mimic the effect of SATp. To minimize the chance that the
493 observed result is the consequence of unknown side effects, three differently acting chemicals
494 (DTT, MG132 and thapsigargin) that are commonly used for the activation of the unfolded
495 protein response (UPR) (43, 44, 45) have been tested. DTT reduces the disulphide bonds (46)
496 of the proteins and results in the accumulation of un- or misfolded proteins. MG132 is a
497 proteasome-specific protease inhibitor (47) that blocks the degradation of proteins, while
498 thapsigargin is a sarco-endoplasmic reticulum Ca^{2+} -ATPase inhibitor and it increases the free

499 Ca^{2+} concentration in the cytosol (48). Thapsigargin was shown to inhibit parvoviral
500 infection. Boisvert et al. (49) also showed that sustained MG132 treatment starting at the early
501 stage of the PPV NADL-2 infection blocks infection (0-20 h p.i. treatment ~100% inhibition,
502 8-20 h p.i. treatment ~50% inhibition). Similar MG132 treatment induced very similar effects
503 at PPV Kresse infection as we detected it by monitoring low MOI infection (not shown). In
504 fact all tested inducers had an inhibitory effect on viral infection when were applied at higher
505 concentration or over an extended period of time. However, they enhanced the spreading of
506 the SAT⁻ strain at the low multiplicity infection (MOI: 0.01) and two of them increased viral
507 release at high MOI (thapsigargin has not been tried) under the applied conditions. This
508 happened despite the fact that DTT and thapsigargin were visibly toxic to the cells.
509 Obviously, the concentration and the timing of the treatments fundamentally influenced the
510 outcome of the experiments, indicating a delicate balance between the opposing effects of
511 these chemicals on the infectious process. Many chemicals can be used to demonstrate a
512 negative effect on parvoviral infection. The special feature of this work is that it demonstrates
513 a positive effect on this process by using inhibitors.

514 All inducers in the applied concentration switched on XBP1 and CHOP expression that
515 led to the death of 100% of the cells in 48 h. Interestingly, switching UPR early on was not
516 only beneficially influencing viral egress as indicated by the titer of the treated high MOI-
517 infected cells (Figure 6) but, at least seemingly, it did not have a large negative effect on
518 parvoviral entry and replication, otherwise second cycle replication and the increase of FFs
519 could not have been detected at low MOI-infected cells already in the stage of UPR (Figure
520 5).

521 UV stress did not produce any enhancement on PPV release, and it did not activate
522 XBP1 and CHOP expression (Figure 8/C). Earlier it was shown that pre-treatment of cells
523 with UV light facilitates the replication of AAV (50, 51), however, it does not significantly

524 affect the replication of H-1 parvovirus, though it increases the mutation rate of the virus in a
525 dose-dependent fashion (52). We found that sub-lethal UV stress applied to the cells after
526 infection does not significantly affect PPV virus output, but – not surprisingly – considerably
527 (~50x) reduces the infectious titer of the progeny viral stocks (Figure 6/C). The probing of
528 different stress factors strongly suggested that ER stress indeed facilitates the release of
529 matured particles from infected cells, and ER stress seems to be specific in this regard,
530 because UV-C radiation is not able to induce a similar effect.

531 Our experiments with wt and SAT⁻ viruses revealed that PPV similarly to numerous
532 other viruses from different viral families (53, 54, 55, 56, 57) induces ER stress and UPR in
533 the infected cells as demonstrated by the morphological changes of the ER and expression of
534 XBP1 and CHOP (58). It is suspected that different localisation of the CHOP depends on the
535 state of the cells and the intensity of the stress effect (59). In wt virus-infected cells CHOP
536 was detected in the nuclei of the majority of the cells indicating severe ER stress, while in
537 SAT⁻ virus-infected cells CHOP was also expressed, but to a lesser extent (41% versus 76%
538 of the cells) and its localization remained perinuclear (Figure 8/B). It is generally accepted
539 that nuclear CHOP is one of the most important mediators of cell death during ER stress (25,
540 58). CHOP is a transcriptional activator of several pro-apoptotic proteins (e.g. GADD34,
541 DR5, Ero1 α) and inhibits the transcription of the anti-apoptotic Bcl-2 (60, 61). On the other
542 hand, it was also shown in mouse embryo fibroblasts that forced expression of CHOP alone or
543 together with ATF4 does not increase the expression of cell death-related genes, only
544 sensitizes to ER stress-induced cell death (62). Besides CHOP expression, increased protein
545 synthesis and oxidative stress were required to induce cell death. In our case, expressed
546 porcine CHOP was clearly detrimental to PT cells causing nuclear fragmentation and cell
547 death (Figure 10/B) in the majority of cells at 48 h post transfection. These data suggest that

548 the outcome of CHOP expression can depend on the cell type and on the actual status of the
549 cell.

550 The presence of SATp clearly influences the localization of CHOP and the outcome of
551 the ER stress response during PPV infection. It is likely that SATp increases the stress effect
552 in the infected cells, that leads to the nuclear accumulation of the CHOP and to the induction
553 of pro-apoptotic or other pathways that accelerate the death of the infected cells. The effect of
554 overexpressed SATp in transfected cells supports this theory. SATp alone is able to change
555 the morphology of the ER and can induce apoptosis (Figure 9). It is clear that the influence of
556 SATp on the activation and localization of CHOP cannot be direct, because by itself it cannot
557 induce CHOP expression (data not shown). It occurred to us that the DsRed tag can influence
558 the functional interactions of SATp, but we transfected tagless SATp expressing plasmids into
559 PT cells and were not able to detect XBP1 and CHOP expression either. The functionality of
560 SAT-DsRed is further supported by the fact that in a co-transfection experiment the protein
561 could complement the missing function of native SATp (Figure 10/C).

562 However, no matter which way SATp stimulates the expression of CHOP, it seems that
563 the induction of this protein and its nuclear localization cannot alone be responsible for the
564 biological effect of SATp, since nuclear CHOP could not complement the lack of SATp in a
565 co-expression experiment (Figure 10/C). Since severe ER stress emulates the effect of SATp,
566 one of the most likely mechanism of action of SATp is that in the ER it influences protein
567 interactions, which make the UPR response more severe. This process may lead to the
568 activation of the PERK-eIF2 α -ATF4-CHOP pathway where one or more of the proteins
569 upstream of CHOP also induce alternative pathways. These may supplement the effect of
570 CHOP leading to early cell death. In an alternative scenario, SATp may induce other ER
571 stress response pathway/s beside UPR (e.g. ER overload response), and this effect alone or
572 synergistically with the CHOP pathway cause early cell death.

573

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578

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764

765 **Legends**

766 Figure 1. The DNA and amino acid sequences of the wild type (A) and the SAT⁻ (B)
767 mutant Kresse strain (based on the U44978.1 sequence). The 2842th and 2845th nucleotides
768 were changed (T→A and T→C). These modifications did not change the amino acid

769 sequence of the VP1, however they led to a STOP codon in the SAT ORF and to the
770 substitution of the nearest methionine.

771

772 Figure 2. Spread of the wild type and the SAT⁻ mutant viruses in PT cells at a low
773 multiplicity (MOI: 0.01) infection. Infected cells (red) were visualized with the assembled
774 capsid specific 3C9 primary and CF594 labelled secondary antibody. (A) Cells were fixed in
775 the indicated time points and their nuclei were labelled with Hoechst 33342. (B) 3C9 antibody
776 was added to Kresse infected cells to monitor the inhibition of the secondary infections. The
777 beginning of the treatments is indicated on the pictures. The cells were fixed at 24 h p.i..

778

779 Figure 3. The change of the viral copy numbers during infection in the medium of PT
780 cells. (A) Copy numbers and infectious titer (indicated by triangles and columns respectively)
781 during low multiplicity (MOI: 0.01) infection. (B) Total copy numbers and copy numbers
782 measured after DNase treatment (represented by triangles and columns respectively) at high
783 multiplicity infection (MOI: 3). Error bars indicates standard deviation.

784

785

786 Figure 4. The different forms of the cells death during the PPV Kresse infection in
787 porcine testis cells. The error bars represent one standard deviation. (A) LDH activity in the
788 supernatant of the infected and control cells as indicator of the total cell death. Maximum
789 absorption value at 18 h p.i. (lysed control uninfected cells) are indicated by x. (B) The
790 attached cell count as indicator of the viability of cells. Number of uninfected cells at 24 h
791 represents 100%. (C) Rate of the attached propidium-iodide positive cells. (wt values were
792 calculated until 48 h p.i). (D) The rate of the attached pyknotic and karyorrhectic cells,

793 calculated by Hoechst staining. (wt values were calculated until 48 h p.i). (E) Swelling of
794 nuclei at at 22 h p.i.

795

796 Figure 5. Spreading of the PPV wild type and the SAT⁻ strains after ER stress inducer
797 treatments at low MOI (0.01) infection. Infected PT cells were fixed at 20 h p.i. Monoclonal
798 3C9 anti-capsid antibody was used for the detection of the infected cells (red). Cell nuclei
799 (blue) were visualized by Hoechst staining. Concentration of the chemicals and the duration
800 of treatments (hours post infection) are indicated.

801

802 Figure 6. Changes of viral copy numbers in the medium at high multiplicity infection
803 (MOI: 3) after stress inducer treatments. All supernatants was harvested at 24 h p.i. (A) Cells
804 were infected with wild type and SAT⁻ strains and treated with ER stress inducers (MG132 20
805 μ M and DTT 10 mM) during different time periods. (B) Cells were infected with wild type
806 and SAT⁻ strains and treated with UV-C light (sub-lethal) at different time points for a 5-
807 minute time period. (C). Infectious titer of the supernatants of differently treated infected
808 cells.

809

810 Figure 7. Morphological changes of the ER in PT cells during PPV infection. To
811 visualize the ER and the viral particles anti-calreticulin antibody (red) and anti-capsid (green)
812 anti-bodies were used, respectively.

813

814 Figure 8. Detection of ER stress protein markers during PPV infection. Cell nuclei
815 (blue) were visualized by Hoechst staining. (A) Activation of Xbp1 reversible ER stress
816 marker after wt and SAT⁻ strain infection. Xbp1 (green) and viral capsid (red) were labelled
817 with anti-Xbp1 and anti-capsid antibodies, respectively at 18 h p.i. (B) Activation of CHOP

818 irreversible ER stress marker after wt and SAT⁻ strain infection. CHOP (green) and viral
819 capsid (red) were labelled with anti-CHOP and anti-capsid antibodies, respectively at 24 h p.i.
820 (C) Activation of Xbp1 in SAT⁻ virus infected and non-infected cells at 60 h p.i. (D)
821 Induction of the Xbp1 and CHOP after 20 μ M MG132 treatment and sub-lethal UV treatment
822 at 12 h.

823

824 Figure 9. Morphological changes of the ER in the SAT-DsRed fusion protein expressing
825 PT cells. Cells were transfected with SAT-DsRed and as a control with DsRed (red)
826 expressing plasmids. The calreticulin was labelled by anti-calreticulin monoclonal antibody
827 (green) and the cell nuclei (blue) were visualized by Hoechst staining. The cells were fixed in
828 different time points after transfection. Apoptotic nuclei are pointed out with arrows.

829

830 Figure 10. Localization of the porcine CHOP and its effect on the spreading of the SAT⁻
831 virus strain. Viral capsid is labelled with anti-capsid antibodies (green) while nuclei (blue) are
832 visualized by Hoechst staining. (A) Nuclear localization of the CHOP-DsRed fusion protein
833 (red) in infected and non-infected cells. CHOP-DsRed expressing plasmids were transfected
834 with wild type and pSAT⁻ infectious clones or alone into PT cells and fixed at 24 h post
835 transfection. (B) Cytopathic effect of the CHOP-DsRed at 18 h post transfection. Arrows
836 indicate fragmented nuclei. (C) Spreading of the SAT⁻ strain after co-transfection of the
837 pSAT⁻ infectious clone with CHOP-DsRed and SAT-DsRed expressing plasmids. As positive
838 control, wild-type clone was transfected with DsRed expressing plasmid. Cells were fixed 96
839 h post transfection, blue (nucleus) and green (virus positive cells) channels were merged.

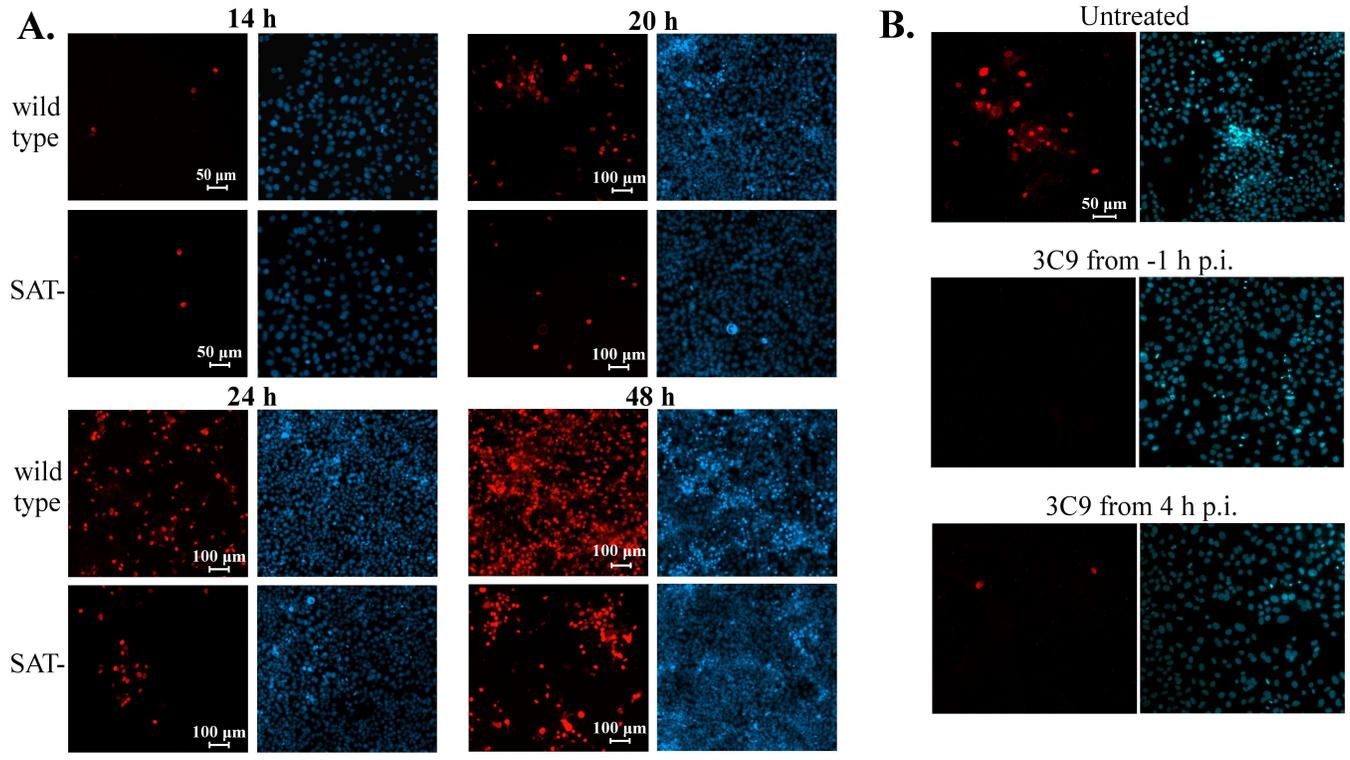
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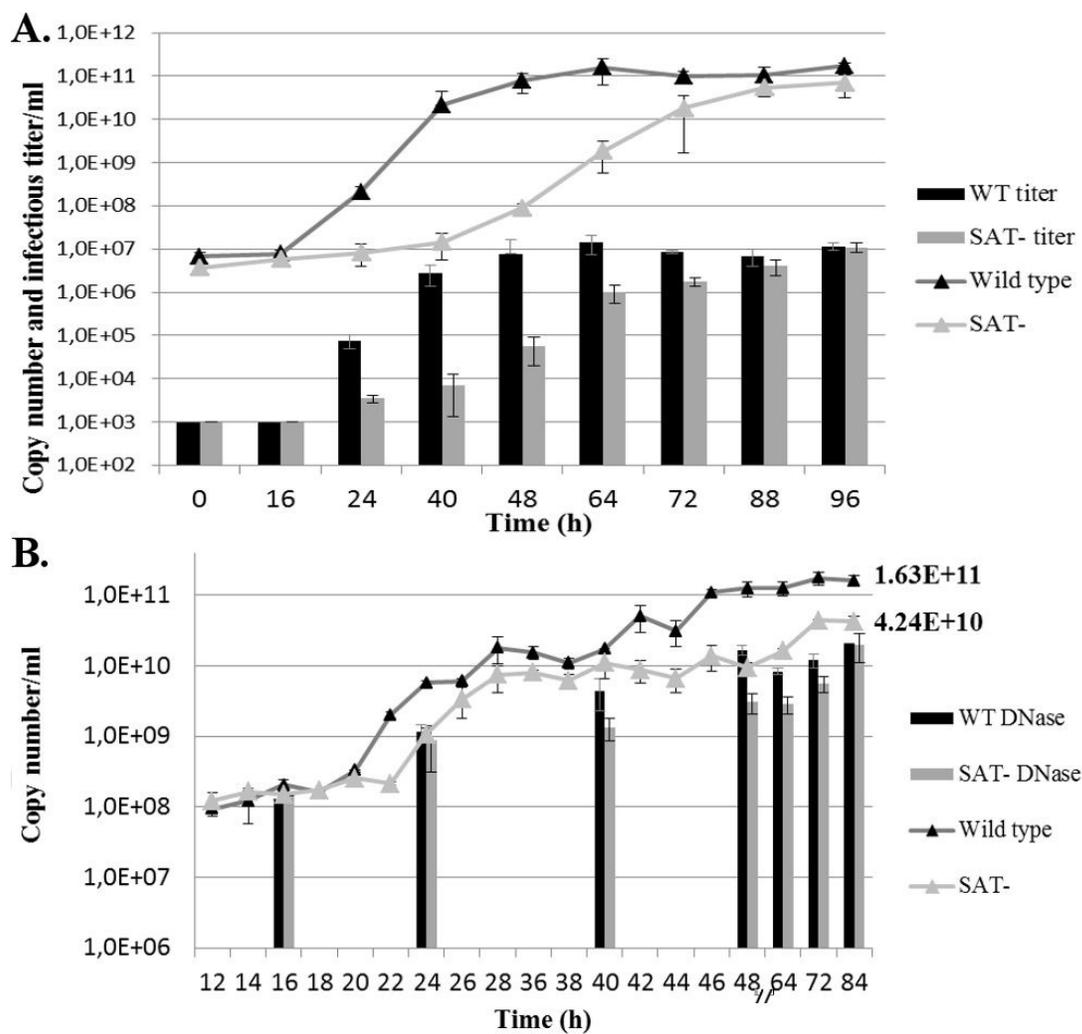
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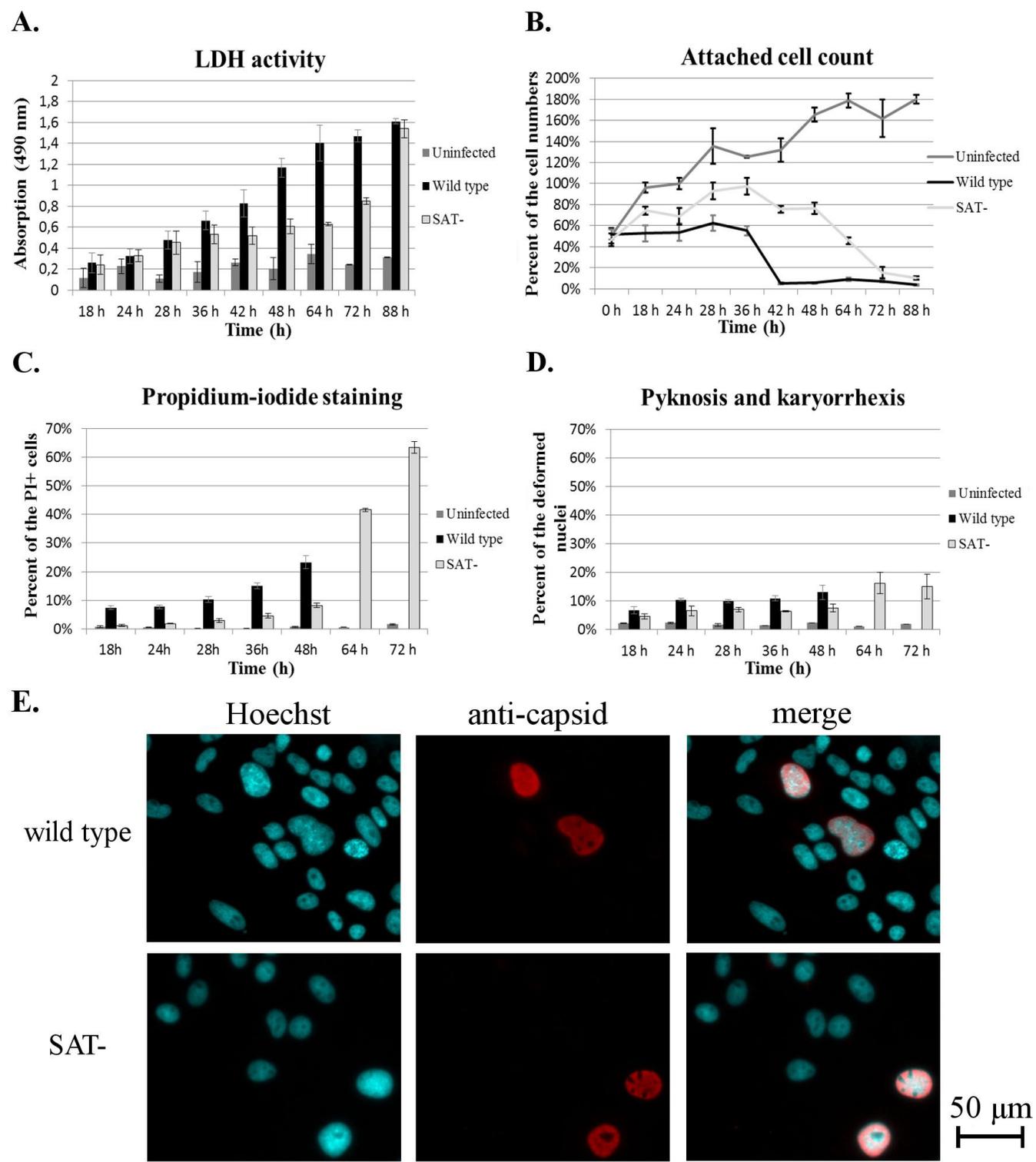
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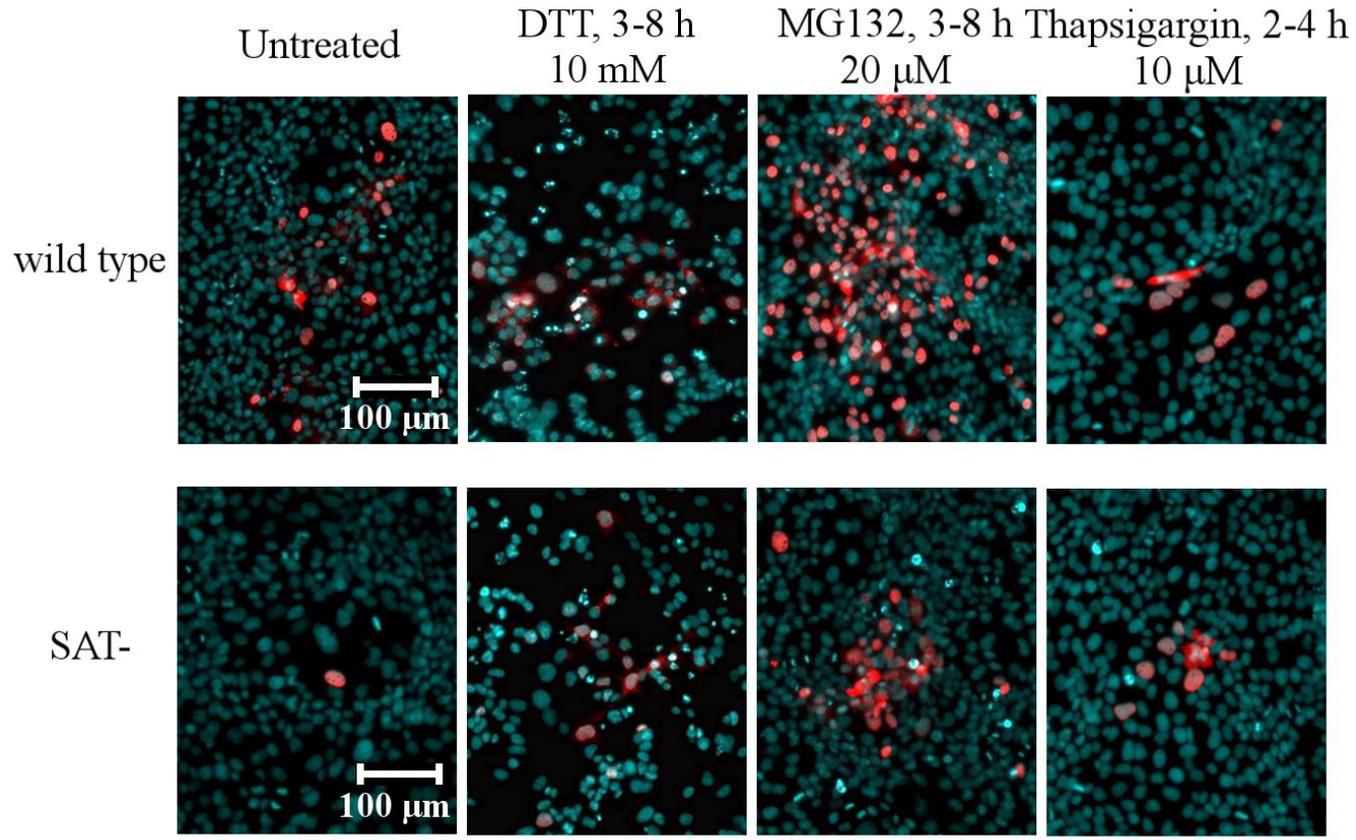
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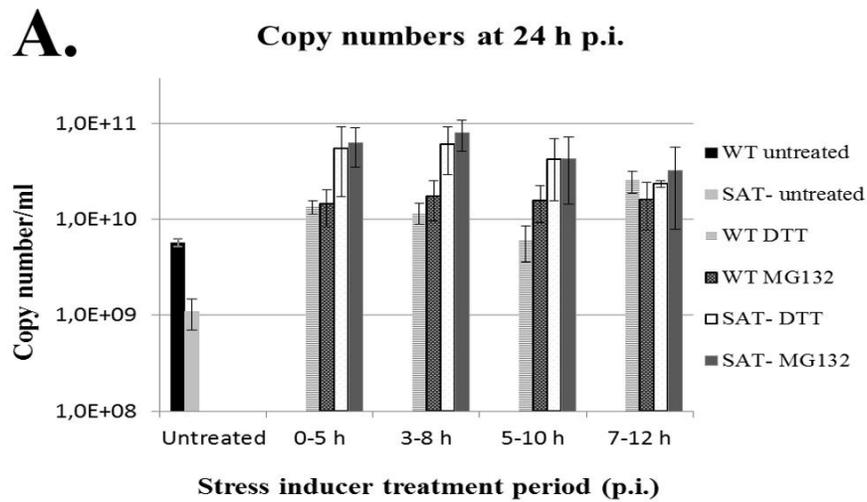
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+2 Frame (SAT)M W N N T T L **STOP T** Q A L N C L Q Q E



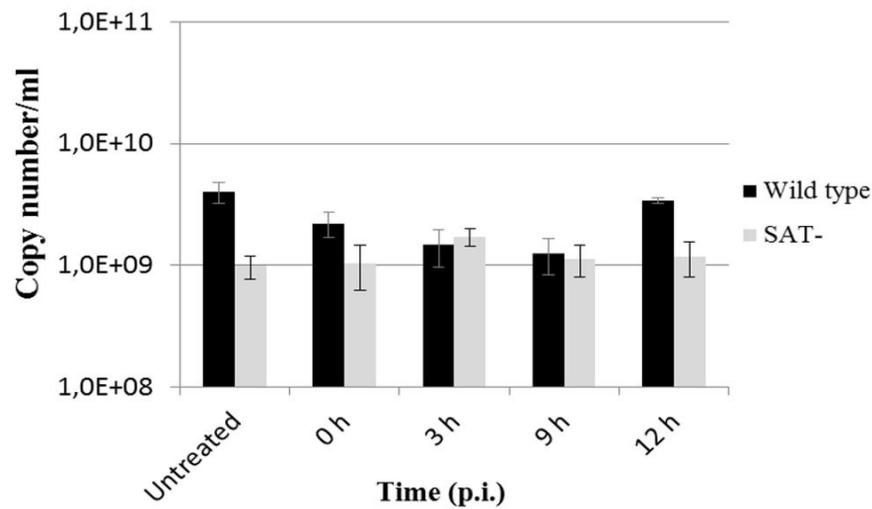








B. Copy numbers after the UV treatment at 24 h p.i.



C. Viral titer after the treatments at 24 h p.i.

