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2 DLK-dependent biphasic reactivation of herpes simplex virus latency established in the
3 absence of antivirals

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23 Running title: A novel in vitro model of HSV latency and reactivation

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25

26 **Abstract**

27 Understanding the molecular mechanism of herpes simplex virus type 1 (HSV-1) latent
28 infection and reactivation in neurons requires the use of model systems. However,
29 establishing a quiescent infection in cultured neurons is problematic as infectious virus
30 released from any productively infected neuron can superinfect the cultures. Here, we
31 describe a new reporter virus, HSV-1 Stayput-GFP, that is defective for cell-to-cell
32 spread and can be used to model latency and reactivation at the single neuron level.
33 Importantly, quiescent infection of neurons with Stayput-GFP can be established without
34 the use of a viral DNA replication inhibitor. The establishment of a quiescent state
35 requires a longer time frame than previous models of HSV latency using DNA
36 replication inhibitors. This results in a decreased ability of the virus to reactivate, and the
37 use of multiple reactivation triggers is required. Using this system, we demonstrate that
38 an initial Phase I wave of lytic gene expression occurs independently of histone
39 demethylase activity and viral DNA replication but is dependent on the neuronal cell
40 stress protein, DLK. Progression into the later, Phase II wave of reactivation,
41 characterized by detectable late viral protein synthesis and a requirement for histone
42 demethylase activity, is also observed with the Stayput-GFP model system. These data
43 demonstrate that the two waves of viral gene expression following HSV-1 reactivation
44 are independent of secondary infection and occur when latent infections are established
45 in the absence of a viral DNA replication inhibitor.

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48

49 **Importance**

50 Herpes simplex virus-1 (HSV-1) enters a latent infection in neurons and periodically
51 reactivates. Reactivation manifests as a variety of clinical symptoms. Studying latency
52 and reactivation *in vitro* is invaluable, allowing the molecular mechanisms behind both
53 processes to be targeted by therapeutics that reduce the clinical consequences. Here,
54 we describe a novel *in vitro* model system using a cell-to-cell spread defective HSV-1,
55 known as Stayput-GFP, which allows for the study of latency and reactivation at the
56 single neuron level. We anticipate this new model system will be an incredibly valuable
57 tool for studying the establishment and reactivation of HSV-1 latent infection *in vitro*.
58 Using this model, we find that initial reactivation events are dependent on cellular stress
59 kinase DLK, but independent of histone demethylase activity and viral DNA replication.
60 Our data therefore demonstrate the essential role of DLK in mediating a wave of lytic
61 gene expression unique to reactivation.

62

63 **Introduction**

64 Herpes simplex virus 1 (HSV-1) is a globally prevalent pathogen with the capacity to
65 infect both sensory and autonomic neurons (1-4) . Following neuronal infection, HSV-1
66 can enter a lytic replication cycle, establish a lifelong latent infection, or potentially
67 undergo a limited amount of lytic gene expression even prior to latency establishment
68 (5-13). While latency is largely asymptomatic, periodic reactivation of the virus can
69 result in cutaneous lesions, keratitis, and encephalitis. Epidemiological studies have
70 also linked HSV infection with an increased risk of developing late onset Alzheimer's
71 disease (14-22).

72

73 The regulated expression of viral lytic transcripts has been well characterized following
74 lytic infection (23-25). The HSV-1 genome enters the host cell epigenetically naked (26-
75 28) but becomes chromatinized by histones bearing transcriptionally permissive histone
76 modifications (29-38). Viral gene expression is initiated from the genome in response to
77 viral trans-activator and tegument protein VP16, which forms a complex with cellular
78 factors involved in transcriptional activation, including general transcription factors, ATP-
79 dependent chromatin remodelers, and histone modifying enzymes to promote
80 expression of immediate-early (IE) genes (32, 39-43). Synthesis of the IE proteins is
81 required for early (E) mRNA transcription. Products of the early viral genes enable viral
82 DNA replication. Viral genome synthesis is a pre-requisite for true-late (TL) mRNA
83 transcription, likely due to a shift in genome accessibility and increased binding of host
84 transcriptional machinery (44). In contrast to productive infection, during HSV-1 latency,
85 viral lytic mRNAs are largely transcriptionally repressed and promoters assemble into

86 silent heterochromatin marked by the tri-methylation of histone H3 lysine 27
87 (H3K27me3) and di/tri-methylation of histone H3 on lysine 9 (H3K9me2/3) (45-50). The
88 initiation of viral gene expression during reactivation is induced from a heterochromatin-
89 associated viral genome and occurs in the absence of viral activators like VP16. Latent
90 HSV-1 therefore relies on host factors to act on the epigenetically silent viral genome
91 and induce lytic gene expression.

92

93 The mechanisms that regulate entry into lytic gene expression to permit
94 reactivation remain elusive. Using primary neuronal models of HSV-1 latent infection,
95 reactivation has been found to progress in a two-step or bi-phasic manner. Phase I is
96 characterized as a synchronous wave of lytic viral transcripts, occurring approximately
97 20 hours post-stimulus (25, 51-54). There is evidence that this initial induction of lytic
98 gene expression is not dependent on the lytic trans-activator VP16, or viral protein
99 synthesis (25, 55). Instead, cellular factors, including the stress kinases dual leucine
100 zipper kinase (DLK) and c-Jun N terminal kinase (JNK), are required for Phase I entry
101 (52, 54). Importantly, viral gene expression occurs despite the persistence of
102 heterochromatin on viral promoters. Instead, JNK-dependent histone phosphorylation of
103 histone H3S10 results in a methyl/phospho switch, which can permit gene expression
104 even while the repressive H3K9me3 histone modification is maintained (52). The
105 second wave of viral lytic gene expression, Phase II, occurs approximately 48 hours
106 post-stimulus. Phase II reactivation is characterized by the full transcriptional viral
107 cascade, including viral DNA replication, and ultimately infectious virus production (25).

108 In contrast to Phase I, viral protein synthesis, heterochromatin removal, VP16-mediated
109 transactivation, and viral DNA replication are required for Phase II (25, 52, 54).

110

111 *In vitro* systems of HSV-1 latency are required to study the mechanisms of
112 latency establishment and reactivation that cannot be easily studied *in vivo* (56-59). *In*
113 *vitro* models more readily enable to functional studies, and immune system components
114 can be included to understand how the host immune response impacts latency and
115 reactivation (60, 61). However, there are complications involved in establishing latency
116 *in vitro*. As also observed in animal models, only a sub-population of infected neurons
117 enter a latent state whereas other neurons become lytically infected (11, 62-64). This
118 leads to superinfection of the cultures and an inability to establish a latent infection.
119 Therefore, many existing *in vitro* models have used viral DNA replication inhibitors,
120 predominantly acyclovir (ACV), to establish latency *in vitro* (52, 65-70). ACV is proposed
121 to inhibit viral DNA replication by incorporating into actively replicating viral genomes in
122 lytic cells, although there is also evidence from ACV-resistant strains that ACV can also
123 inhibit the viral DNA polymerase (71-73). There are some caveats associated with ACV
124 use as the process of DNA replication and subsequent late gene expression cannot be
125 studied in lytic neurons during latency establishment. Moreover, the fate of any
126 genomes that incorporate ACV is unknown. Therefore, new model systems are required
127 in which latency establishment can be tracked without the need for viral DNA replication
128 inhibitors. Such a system can also be used to determine whether the use of ACV in the
129 cultures alters mechanisms of lytic gene expression during reactivation. Here we
130 describe the use of a novel HSV-1 reporter virus Stayput-GFP, which provides a

131 powerful new methodology to investigate the establishment and reactivation from latent

132 infection at the single neuron level without the need for DNA replication inhibitors.

133

134 **Results**

135 Construction of a gH-null US11-GFP HSV-1.

136 To construct a recombinant HSV-1 that is deficient in cell-to-cell spread and can
137 also be used to visualize cells containing detectable viral late protein, Us11 tagged with
138 GFP (74) was inserted into an existing glycoprotein H (gH)-null virus, SCgHZ (75) (Fig.
139 1A). gH is essential for HSV-1 cell entry as it mediates fusion between the virus
140 envelope and host cell membrane (76, 77). The GFP-tagged true-late protein is a useful
141 indicator of both lytic infection and reactivation, and Us11-GFP wild-type virus has been
142 used as such in several HSV-1 latency systems (51, 54, 58, 65, 78, 79). The tagged
143 virus has previously been reported to express the full complement of HSV-1 genes, as
144 wild-type Patton strain (74).

145

146 We first verified that the resulting virus (named Stayput-GFP) was deficient in
147 cell-to-cell spread in non-neuronal and neuronal cells but otherwise undergoes gene
148 expression and replication as a wild-type virus. The genome sequence and
149 transcriptome of Stayput-GFP was validated by nanopore gDNA and direct RNA
150 sequencing, respectively (Fig. 1B-C). As determined through plaque assay, the ability to
151 produce infectious virus was perturbed in the gH-deletion strains but was rescued using
152 previously constructed gH-complementing cell line, Vero-F6 (Fig. 1D, Supplemental Fig.
153 1A). Importantly, replication in this cell line was indistinguishable from the parent
154 SCgHZ and Us11-GFP Patton strain, which we chose for comparison as this virus has
155 previously been used for latent infection studies in primary neurons.

156

157 To demonstrate cell-to-cell spread deficiency in neurons, we quantified GFP-
158 positive neurons over time. Infection of murine sympathetic neurons at an MOI of 0.5
159 PFU/cell resulted in detectable GFP-positive neurons, which remained constant after 24
160 hours post-infection (Fig. 1E), while the surrounding GFP-negative neurons remained
161 GFP-negative (Fig. 1F). This contrasts with the wild-type US11-GFP condition where
162 the number of lytic-infected neurons increased substantially over time. At 6 hours post-
163 infection, viral protein ICP4-positive neurons were indistinguishable between SCgHZ,
164 Stayput-GFP, or wild-type Us11-GFP infected neurons (Supplemental Fig. 1B).
165 Therefore, Stayput-GFP neuronal infection was equivalent to Us11-GFP upon initial
166 infection but failed to spread within the neuronal culture.

167

168 Reactivation of Stayput-GFP in a primary neuronal model

169 To confirm that Stayput-GFP undergoes reactivation in a manner comparable to
170 the backbone SCgHZ, we infected mouse primary sympathetic neurons in the presence
171 of viral DNA replication inhibitor ACV (52, 65-68). ACV prevents the production of
172 infectious virus, and thus superinfection of the cultures. Following infection, ACV was
173 removed, and reactivation was triggered adding a PI3-kinase inhibitor LY294002 (Fig.
174 2), which mimics loss of a branch of the NGF-signaling pathway in neurons (80) and
175 has previously been found to induce reactivation (52, 61, 65, 79). Following the addition
176 of the reactivation stimulus, viral gene expression increased uniformly between Stayput-
177 GFP, SCgHZ, and wild-type Us11-GFP at 20 hours post-stimulus (Fig. 2C). Viral gene
178 expression continued to increase from 20 to 48 hours for all three viruses, and GFP-
179 positive neurons were visible for both GFP-tagged viruses by 48 hours (Fig. 2B, 2D).

180 This indicates that even in the absence of cell-to-cell spread, reactivation progressed
181 over a 48-hour period, initiating with viral mRNA production and later detection of viral
182 late protein.

183

184 By 72 hours-post stimulus, GFP and viral gene transcription were significantly
185 up-regulated in wild-type Us11-GFP in comparison to Stayput-GFP or SCgHZ,
186 suggesting that at this time-point, the readout of reactivation for wild-type Us11-GFP is
187 confounded by cell-to-cell spread (Fig. 2B, 2E). We are therefore unable to differentiate
188 between genuine reactivation and downstream cell-to-cell spread using a wild-type
189 virus. Previous attempts to reduce cell-to-cell spread include using pooled human
190 gamma globulin or the viral DNA packaging inhibitor WAY-150138 (65, 81-83).
191 However, using Stayput-GFP offers a built-in mechanism to prevent cell-to-cell spread
192 during reactivation and permit quantification of the progression to reactivation at the
193 single neuron level.

194

195 Stayput-GFP can be used to create a quiescence model of neuronal infection in the
196 absence of viral DNA replication inhibitors

197 When wild-type virus is used for neuronal infection, superinfection of the cultures
198 occurs (Fig. 1E), and a latent infection cannot be established. By infecting with Stayput-
199 GFP, we posited that we could create a model of latency establishment without the use
200 of DNA replication inhibitors. Following the infection of neonatal sympathetic neurons at
201 an MOI of 7.5 PFU/cell, the number of GFP-positive neurons emerged by 1-day post-
202 infection, increased until 3 days post-infection, and then decreased until reaching zero

203 by 30 days post-infection (Fig. 3A). The length of time required for Us11-GFP to be lost
204 from the cultures was surprising and may result from the previously characterized
205 restricted cell death in neurons (84), or a gradual shut-off in protein synthesis in a sub-
206 population of neurons that survive even Late gene expression. Single-cell tracking
207 demonstrates that most neurons that become GFP-positive also end up staining
208 positive for cell death marker SYTOX™ Orange (Fig. 3B-C). All classes of lytic viral
209 gene expression emerged by 1-day post-infection and then decreased over the span of
210 30-days post-infection (Fig. 3D-F). In contrast to the lytic transcripts, LAT expression
211 was maintained over the infection scheme of 30-40 days and was approximately 400-
212 fold higher than lytic transcripts from 30 days onwards (Fig. 3G). This indicated that
213 LAT-positive neurons persisted over this period, likely reflecting entry into quiescence.
214 In agreement with this hypothesis, at 40 days post-infection there are approximately 200
215 viral DNA copies per cell, demonstrating that viral genomes persist (Fig. 3H). Together,
216 these data show that infection of sympathetic neurons with a cell-to-cell defective virus
217 results in a remaining population of neurons containing viral genomes and the LAT
218 transcript. Notably, this mimics events following *in vivo* infection of mice.

219

220 We were also interested to determine whether a similar quiescent infection could
221 be established in adult sensory neurons, as these are also a commonly used model of
222 HSV-1 infection *in vitro* (62). Following infection with Stayput-GFP in primary trigeminal
223 ganglia (TG) neurons, GFP also increased by 1-day post-infection and then was lost
224 over time (Supplemental Fig. 2). GFP was repeatedly lost within 15 days, a shorter
225 period than that which is observed in neonatal sympathetic neurons. Therefore, the

226 Stayput-GFP virus can be used to establish a quiescent infection of both sympathetic
227 and sensory neurons *in vitro* without the use of viral DNA replication inhibitors.

228

229 The ability of HSV-1 to undergo reactivation decreases with length of time infected

230 The presence of viral genomes and LAT transcripts suggested that HSV-1 had
231 established a quiescent infection 30 days post-infection. Therefore, we hypothesized
232 that some genomes enter latency, which is defined by an ability to reactivate in
233 response to a stimulus. We thus attempted to reactivate cultures with LY294002 (20
234 μ M). However, we were unable to detect an increase in GFP-positive neurons after the
235 addition of the trigger (Fig. 4A). We were also unable to detect a change in immediate
236 early, early, or late transcripts (data not shown). This was unexpected as LY294002 has
237 repeatedly been shown to elicit robust reactivation *in vitro* and was able to induce
238 Stayput-GFP in a model using ACV to promote latency establishment (Fig. 2).
239 Therefore, we sought to determine whether the inability to induce reactivation was due
240 to the lack of ACV or the more prolonged time between initial infection and the addition
241 of the reactivation stimulus. We infected neonatal cultures in the presence of ACV and
242 reactivated over increasing lengths of time. ACV was removed from all cultures 6 days
243 post-infection. We found that the number of GFP-positive neurons following addition of
244 LY294002 decreased as the length of time infected increased (Fig. 4B). This was not
245 likely due to a loss of viral genomes, as viral genome copy number and LAT expression
246 remained constant over this period (Fig. 3G-H) and therefore instead reflected a more
247 repressed viral genome unable to undergo reactivation upon PI3-kinase inhibition.

248

249 Neurons are known to undergo intrinsic maturation, even in culture (85, 86).
250 Therefore, the increased age of the neuron could have also impacted the ability of HSV-
251 1 to undergo reactivation. Hence, we investigated how reactivation changed with
252 increased neuronal maturation (Fig. 4C). We infected cultured neurons with Stayput-
253 GFP at an MOI of 7.5 at the postnatal (P) ages of P8, P16, and P24 and then
254 reactivated 8 days later. These postnatal ages of infection were chosen to reactivate at
255 the same ages of reactivation in Fig 4B. Importantly, we did not detect a decrease in
256 reactivation output as the age of the neuron increased. Together, these data indicate
257 that the decreased ability of Stayput-GFP to reactivate in a model that did not require
258 ACV to establish quiescence was due to the longer time frame of infection and not
259 associated with a lack of ACV in the cultures or increased age of the neuron.

260

261 Viral gene expression can be induced following long-term quiescent infection when
262 multiple triggers are combined

263 We next sought to determine whether other known stimuli of HSV-1 reactivation
264 could induce Us11-GFP expression, indicative of entry into reactivation. We attempted a
265 number of triggers including forskolin (87-90) and heat-shock/hyperthermia (91-98),
266 which are both known inducers of HSV-1 reactivation. Alone these stimuli did not induce
267 Us11-GFP expression or viral lytic mRNA induction (data not shown). However, when
268 heat-shock (43°C for 3h), in addition to forskolin (30 µM) and LY294002 (both pulsed for
269 20 hours) were combined, Us11-GFP positive neurons were detected at 48 hours post-
270 stimulus, indicating progression to reactivation (Fig. 5A). Superinfection was also used
271 as this can induce rapid and robust reactivation, likely resulting in delivery of viral

272 tegument proteins. In comparison to superinfection, the combined heat-
273 shock/forskolin/LY294002 trigger resulted in reduced entry into reactivation/Us11-GFP
274 expression, indicating that only a sub-population of neurons undergo reactivation with
275 this combined trigger, which is consistent with previous studies investigating the
276 mechanism of HSV-1 reactivation both *in vivo* and *in vitro* (25, 66, 99, 100). Importantly,
277 GFP-positive neurons at 48 hours post-stimulus co-stained with viral immediate early
278 protein ICP4, early protein ICP8, as well as late capsid protein ICP5 (Supplemental Fig
279 3). ICP8 staining appeared to form replication compartments, suggesting viral DNA
280 replication occurs at this time. ICP5 capsid staining was also detectable in GFP-positive
281 axons.

282

283 We went on to investigate whether viral mRNA expression was induced prior to
284 the detection of Us11-GFP positive neurons. Using the triple stimulus, we detected an
285 increase in viral lytic transcripts as early as 10 hours post-stimulus, which continued to
286 increase, peaking at 48 hours post-stimulus (Fig. 5B-D, Supplemental Fig. 4). Detection
287 of immediate-early and early transcripts was slightly more robust than late transcripts,
288 although all late transcripts were significantly induced by 20 hours post-stimulus (Fig.
289 5D, Supplemental Fig. 4B-E).

290

291 We also confirmed that the triple combinatorial stimulus elicits robust GFP re-
292 emergence in adult TGs (Fig. 5E). Similar to what we observed in the sympathetic
293 neurons, Us11-GFP positive neurons were detected by 48 hours post-stimulus.
294 Intriguingly, superinfection only induced GFP expression to equivalent levels as the

295 triple stimuli, which may be reflective of the repressive nature of sub-population of
296 mature sensory neurons to lytic replication and reactivation (62, 63). Together, these
297 data indicate that *in vitro* models of latency and reactivation can be established in
298 sympathetic and sensory neurons in the absence of ACV and reactivation can be
299 induced using a combination of triggers. In both models, a wave of lytic mRNA
300 expression was detected prior to the appearance of Us11-GFP positive neurons (Fig. 5
301 F-H, Supplemental Figure 5).

302

303 Neurons infected with Stayput-GFP undergo a DLK-dependent Phase I of reactivation

304 Phase I reactivation has largely been investigated using *in vitro* models in which
305 ACV has been used to promote latency establishment. In addition, Phase I has been
306 found to occur with the single triggers of forskolin or LY294002 (25, 52, 54). The
307 requirement of multiple triggers for reactivation suggested that multiple cell-signaling
308 pathways converged to have a synergistic effect and induce reactivation in this more
309 repressive model. Therefore, we were interested to determine whether the
310 characteristics of Phase I reactivation occurred in the model of quiescent infection
311 established in the absence of ACV and using the more robust trigger to induce
312 reactivation. Potential Phase I viral transcription was investigated at 12.5 hours post-
313 stimulus by RT-qPCR and Phase II was investigated when Us11-GFP positive neurons
314 could be detected (48 hours post-stimulus). A characteristic of Phase I expression is the
315 requirement of the stress kinase dual leucine zipper kinase (DLK) (52, 54). Therefore,
316 using the DLK-specific inhibitor GNE3511 4 μ M (101), we investigated the effect of DLK
317 on reactivation in our system. We found up-regulation of immediate early/early

318 transcripts 12.5 hours post-stimulus was eliminated with the addition of the DLK inhibitor
319 (Fig. 6A-C). Further, full reactivation, demonstrated by peak GFP expression at 48
320 hours, was also reduced to baseline levels upon the addition of the DLK inhibitor (Fig.
321 6D). Therefore, using our new model of HSV-1 latency our data demonstrate that the
322 initiation of viral lytic gene expression is dependent on DLK activity.

323

324 In addition to the dependence on DLK activity, a further characteristic of Phase I
325 gene expression is the induction of viral mRNA transcripts independently of the activity
326 of histone de-methylase enzymes required for full reactivation. Therefore, we also
327 triggered reactivation in the presence of de-methylase inhibitors. GSK-J4 is known to
328 specifically inhibit the H3K27 histone demethylases UTX and JMJD3 (102) and has
329 previously been found to inhibit HSV-1 reactivation but not Phase I gene expression
330 (52, 54). OG-LOO2 is an LSD1 specific inhibitor. LSD1 has previously been shown to
331 be involved in removal of H3K9me2 from HSV-1 genomes and its activity is required for
332 full reactivation but not Phase I gene expression (41, 52, 54). Full reactivation was
333 reduced in the presence of OG-LOO2 or GSK-J4 as demonstrated by GFP-positive
334 neurons at 48 hours (Fig. 7C). However, the initial expression of lytic transcripts at 12.5
335 hours post-stimulation was not inhibited by either OG-LOO2 or GSK-J4 (Fig. 7A-B).
336 Therefore, the initiation of gene expression in a manner that is independent of histone
337 demethylase activity occurs when reactivation is induced by a triple stimulus and in a
338 primary neuronal model in which latency was established without ACV.

339

340 During Phase I, there is no detectable replication of viral genomes even though
341 true late gene expression occurs (52, 54). In addition, using ACV models to establish
342 latency, late gene expression has previously been found to occur to equivalent levels
343 when viral DNA replication is inhibited during reactivation. Although we did observe late
344 gene expression during the initial period of lytic gene induction (12.5-20 hours), the
345 increase was less robust than IE and E genes and appeared slightly delayed, not
346 reaching significance for all analyzed late transcripts until 24 hours post-stimulus (Fig.
347 5D, Supplemental Fig. 3). Therefore, we investigated whether this late gene induction
348 was dependent on viral DNA replication by reactivating in the presence of ACV. The
349 addition of ACV inhibited entry in full reactivation, demonstrated by Us11-GFP positive
350 neurons at 48 hours post-stimulus, indicating that ACV was capable of blocking robust
351 late gene expression at this late time-point (Fig. 8D). However, the addition of ACV did
352 not inhibit the induction of lytic transcripts at 22 hours post-stimulus. Importantly, we
353 included multiple true Late genes in this analysis and all were induced to equivalent
354 levels in the presence and absence of ACV (Fig. 8A-C, Supplemental Fig 6). Therefore,
355 the initial expression of late genes following a reactivation stimulus is independent of
356 viral DNA replication. In summary, using a model system in which a quiescent infection
357 is established without the need for ACV, all the previous characteristics of Phase I gene
358 expression (dependence on DLK and independence of histone demethylase activity and
359 viral DNA replication) were still observed.

360

361

362 **Discussion**

363 We envision multiple uses for the Stayput-GFP virus model developed here for
364 investigating HSV-1 neuronal infection *in vitro*. The Stayput-GFP virus is advantageous
365 in models that otherwise use DNA replication inhibitors to promote latency
366 establishment because it allows for the separation of initial viral gene expression/protein
367 synthesis events and readouts from events that result from cell-to-cell spread. In
368 addition, even in systems where ACV is used, there can be low levels of lytic replication
369 or spontaneous reactivation after removal of ACV from cultures. The use of Stayput-
370 GFP helps limit the confounding effects of spontaneous reactivation events by inhibiting
371 subsequent cell-to-cell spread, while at the same time identifying neurons that escape
372 quiescence. Further, the GFP tag serves as an imaging indicator in real time of when *de*
373 *novo* lytic infection is resolved and latency is considered established. We are also able
374 to track viral DNA replication and downstream late viral transcription and protein
375 synthesis during the latency establishment process *in vitro*. The fate of lytic neurons,
376 whether they undergo cell death or turn off gene expression programs and enter the
377 latency pool, can also be investigated by tracking GFP and cell death at a single-cell
378 level.

379

380 There are limitations to our system. Although there is some discrepancy in what
381 defines reactivation (103), it is ultimately defined by the production of infectious virus.
382 Due to the nature of the gH-deletion virus, *de novo* virus is by design non-infectious and
383 we are unable to demonstrate reactivation in its strictest definition. That said, we can

384 readily demonstrate the re-emergence of all classes of viral gene transcripts, synthesis
385 of viral capsid protein and replication compartment formation.

386

387 An intriguing finding in our study is that reactivation output decreases as length of
388 infection increases. A potential explanation is that the viral genome becomes
389 increasingly chromatinized over time, leading to a more repressive phenotype. In
390 support of this hypothesis, the association of the facultative heterochromatin mark
391 H3K27me3 with the HSV-1 genome increases dramatically between 10- and 15-days
392 post-infection *in vivo* (46). The kinetics of H3K27me3 deposition remain to be
393 investigated *in vitro*, but if they are mirrored this could suggest that active
394 chromatinization and reinforcement of silencing continues even after initial shut-down of
395 viral gene expression. In the cellular context, H3K27me3 is linked with the recruitment
396 of canonical polycomb repressor complex 1 (cPRC1), which may reinforce silencing
397 through long-range chromosomal interactions or 3D compaction (50, 104, 105). It is
398 therefore possible that even following H3K27me3 formation on the genome, there are
399 additional layers of protein recruitment that build up over time. In addition, it is also
400 possible that the accumulation of viral non-coding RNAs expressed in latency could
401 impact cellular pathways resulting in decreased signaling to the viral genome for
402 reactivation. The use of the Stayput-GFP model system will permit these different
403 avenues to be explored.

404

405 Our model system recapitulates the hallmarks of reactivation Phase I, which has
406 previously been explored in *in vitro* systems using a DNA replication inhibitor. These

407 data are interesting considering the discrepancies in conclusions drawn about
408 reactivation between *in vitro* and *in vivo* modeling. There is evidence *ex vivo* for a
409 Phase I as all classes of viral gene are expressed in a disordered, non-cascade fashion
410 when a combination of explant and nerve-growth factor deprivation are used (106).
411 However, in other models of reactivation *ex vivo*, there is evidence that Phase-I-like
412 gene expression may not occur, especially from studies investigating the requirement
413 for histone demethylase inhibitors. These latter experiments used explant (axotomy) to
414 induce reactivation and found that the earliest induction of lytic gene expression is
415 dependent on lysine 9 demethylase activity (40, 41). In a recent study from our lab, we
416 have found that Phase I reactivation can occur *ex vivo* when axotomy is combined with
417 PI3-kinase inhibition, although with more rapid kinetics than those observed here (107).
418 These discrepancies between may result from the different trigger used to induce
419 reactivation or currently unknown effects of latency established *in vivo*. Importantly, here
420 we have demonstrated that any potential differences between *in vivo* and *in vitro*
421 observations on the mechanisms of reactivation do not result from the use of ACV to
422 establish a quiescent infection. Further work using the Stayput-GFP model system will
423 help elucidate how changes in the host immune response, neuronal subtype, and
424 stimulus used can potentially alter the mechanisms of viral gene expression during
425 reactivation.

426

427 Phase I, in addition to occurring synchronously and independently of histone
428 demethylases, also occurs in the absence of viral protein synthesis. In an *in vitro* model
429 employing ACV during latency establishment and stimulus LY294002 during

430 reactivation, it is demonstrated that initial viral transcription occurs before the
431 appearance of viral late protein synthesis and, specifically, independently of viral
432 transactivator VP16 (25, 51). Therefore, cellular host factors must be responsible for
433 instigating the initial reactivation process. Evidence from an *in vitro* model system has
434 demonstrated these events are in fact navigated by cellular proteins JNK and DLK (52).
435 Interestingly, host cell proteins may also be implicated in restricting the full reactivation
436 process, including Gadd45b which appears to antagonize the HSV-1 late expression
437 program to prevent full reactivation (51). Interestingly, Gadd45b mRNA is increased in
438 response to LY294002 only in infected neurons, suggesting perhaps that a viral factor
439 may be mediating the gatekeeping from Phase I to Phase II reactivation.

440

441 In our system, Phase I gene expression was dependent on the neuronal
442 regulator of JNK activity, DLK, highlighting the central role of DLK in HSV-1 reactivation.
443 DLK is a cell protein implicated in neuronal stress signaling upstream of cellular protein
444 JNK (108). It has previously been found to be essential for HSV-1 reactivation following
445 PI3-kinase inhibition (52), as well as neuronal hyper-excitability through forskolin (54).
446 However, it has not, until now, been shown to be central to reactivation mediated by
447 heat shock. Although heat shock has been used as a trigger for HSV-1 reactivation (91-
448 98), the downstream molecular events following this stimulus are not well elucidated.
449 Multiple studies have demonstrated that heat shock during reactivation leads to the up-
450 regulation of heat shock proteins, although none of them knowingly relate to DLK.
451 Following hyperthermia-induced reactivation *in vivo*, heat shock protein HSP60 and
452 HSP40 have been demonstrated to be up-regulated (98). Components of the heat

453 shock response pathway have also been demonstrated to be up-regulated by
454 LY294002 treatment in an *in vitro* system (51), including HSP70. In fact, in this same
455 system, treatment with cultures of heat shock factor 1 (HSF-1) activator compound
456 causes robust reactivation. Outside of the virological context, heat shock protein
457 chaperone HSP90 has been shown to bind and maintain DLK stability *in vivo* and it is
458 specifically required for DLK function following axon injury signaling (109). It is a
459 possibility that heat shock in our system is enhancing the function of DLK. Therefore,
460 multiple signals may converge on DLK, which is then able to activate JNK and protein
461 histone phosphorylation and to promote lytic gene expression from the heterochromatin-
462 associated viral genome for reactivation to occur. Indeed, synergy has been
463 demonstrated to enhance DLK activity in neurons (110). This central role for DLK is
464 especially important as it is largely a neuron-specific protein that regulates the response
465 to multiple forms of stress (111) and is therefore a potential target for novel therapeutics
466 that would prevent HSV-1 gene expression and ultimately reactivation.

467

468 **Methods**

469 Primary neuronal cultures

470 Sympathetic neurons from the superior cervical ganglia (SCG) of post-natal day 0–2
471 (P0-P2) CD1 Mice (Charles River Laboratories) were dissected as previously described
472 (52). Sensory neurons from the trigeminal ganglia (TG) or adult (P21-24) CD1 Mice
473 (Charles River Laboratories) were dissected as previously described (112). Rodent
474 handling and husbandry were carried out under animal protocols approved by the
475 Animal Care and Use Committee of the University of Virginia (UVA). Ganglia were

476 briefly kept in Leibovitz's L-15 media with 2.05 mM L-Glutamine before dissociation in
477 Collagenase Type IV (1 mg/mL) followed by Trypsin (2.5 mg/mL) for 20 min each at 37
478 \square C. Dissociated ganglia were triturated, and approximately 5,000 neurons per well were
479 plated onto rat tail collagen in a 24-well plate. Sympathetic neurons were maintained in
480 CM1 (Neurobasal Medium supplemented with PRIME-XV IS21 Neuronal Supplement
481 (Irvine Scientific), 50 ng/mL Mouse NGF 2.5S, 2 mM L-Glutamine, and Primocin).
482 Aphidicolin (3.3 mg/mL) was added to the CM1 for the first 5 days post-dissection to
483 select against proliferating cells. Sensory neurons were maintained in the same media
484 supplemented with GDNF (50 ng/ml; Peprotech 450-44), and more Aphidicolin (6.6
485 mg/mL) was used for the first 5 days as more non-neuronal cells tend to be dissected
486 with this neuron type.

487
488 Establishment and reactivation of latent HSV-1 infection in primary neurons
489 Neonatal SCGs were infected at postnatal days 6-8 with either Us11-GFP, SCgHZ, or
490 Stayput-GFP at an MOI of 7.5 PFU/cell assuming 5,000 cells/well in DPBS +CaCl₂
491 +MgCl₂ supplemented with 1% Fetal Bovine Serum, 4.5 g/L glucose, and either with ou
492 without 10 mM Acyclovir (ACV) for 3 hr at 37 \square C. Post-infection, inoculum was
493 replaced with CM1 containing with or without 50 mM. For infections with ACV, the ACV
494 was washed out 5-6 days post-infection. Reactivation was carried out with LY294002 20
495 μ M, forskolin 60 μ M (pulsed for 20 hours), and heat shock (3 hours at 43 \square C) in
496 BrainPhys (Stem Cell Technologies) supplemented with 2 mM L-Glutamine, 10% Fetal
497 Bovine Serum, Mouse NGF 2.5S (50 ng/mL) and Primocin. Reactivation was quantified

498 by counting number of GFP-positive neurons or performing Reverse Transcription

499 Quantitative PCR (RT-qPCR) for HSV-1 lytic transcripts.

500

501

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509

510 **Data Availability**

511 All nanopore sequencing datasets associated with this study are available via the

512 European Nucleotide Archive under the accession PRJEB51869.

513

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846

847

848 **Figure legends**

849 FIG 1: Stayput-GFP replicates as wild-type but is unable to spread.

850 (A) Schematic overview of HSV-1 strain SC16, the gH-deletion mutant SCgHZ, and
851 Stayput-GFP. The gH deletion / LacZ insertion and Us11-GFP insertion sites are shown
852 by blue and green triangles, respectively. (B-C) Coverage plots derived from (B)
853 nanopore gDNA sequencing and (C) nanopore direct RNA sequencing of SCgHZ and
854 Stayput-GFP. Sequence read data were aligned against the SC16 reference genome
855 and demonstrate a drop in coverage at the gH locus. (D) Vero-F6 cells were infected
856 with Stayput-GFP, SCgHZ, or Us11-GFP at an MOI of 5. Infectious virus was collected
857 over time and titrated on Vero-F6 cells (n=3 biological replicates). (E-F) Neonatal
858 sympathetic neurons were infected at an MOI of 0.5 PFU/cell with Stayput-GFP or
859 Us11-GFP in the absence of DNA replication inhibitors. Us11-GFP-positive neurons
860 were counted over time (n=3 biological replicates). Shapiro-Wilk normality test.
861 Unpaired student's t test between Us11-GFP and Stayput-GFP. ***p<0.0001. The
862 means and SEMs are shown.

863

864 FIG 2: Stayput-GFP in a latency and reactivation model using ACV to promote latency
865 establishment

866 (A) The latency and reactivation model scheme. Neonatal sympathetic neurons were
867 infected with Stayput-GFP, parent virus SCgHZ, or wild-type Us11-GFP at an MOI of
868 7.5 PFU/cell in the presence of ACV (50 μ M). 6 days later, ACV was removed, and 2
869 days later, cultures were reactivated with LY294002 (20 μ M). The numbers of GFP
870 positive neurons in a single well (containing approximately 5,000 neurons) for Stayput-

871 GFP and wild-type Us11-GFP were counted over time (B). Viral gene expression also
872 was quantified by RT-qPCR for immediate early (*ICP27*), early (*ICP8*), and late (*gC*)
873 genes at 20 hours (C), 48 hours (D), and 72 hours (E) post-stimulus. Relative
874 expression to un-reactivated samples and cellular control (mGAPDH). n=6 biological
875 replicates from 3 litters. Normality determined by Kolmogorov-Smirnov test (B-E). Mann-
876 Whitney (B) or Kruskal-Wallis with comparison of means (C-E). *p<0.05, **p<0.01,
877 ***p<0.001. The means and SEMs are represented. Individual biological replicates are
878 indicating in C-E.

879

880 FIG 3: Stayput-GFP can be used to create a quiescence model in the absence of viral
881 DNA replication inhibitors in neonatal sympathetic neurons

882 Neonatal sympathetic neurons were infected with Stayput-GFP at an MOI of 7.5
883 PFU/cell and the numbers of Us11-GFP-positive neurons were quantified. n=9
884 biological replicates from 3 litters (A). SYTOXTM Orange-positive neurons were also
885 quantified over time (n=3) (B). Following infection, the same field of view was imaged to
886 track GFP and SYTOXTM Orange (250 μ m scale bar for FOV, 25 μ m scale bar for zoom)
887 over time (C). Lytic (D-F) and latent (G) viral transcripts (n=6) were quantified up to 40
888 days post-infection. Viral DNA load (n=6) (H) was also quantified up to 40 days post-
889 infection. Individual biological replicates along with the means and SEMs are shown.

890

891 FIG 4: Reactivation decreases with length of time infected

892 Sympathetic neurons were infected with Stayput-GFP at an MOI of 7.5 PFU/cell and
893 were treated with LY294002 when GFP-positive neurons were no longer detected

894 (approximately 30 days post-infection). GFP-positive neurons were quantified over time;
895 peak GFP (48 hours post-stimulus) is represented (A). Neonatal SCGs were infected at
896 age postnatal day 8 (P8) with Stayput-GFP in the presence of ACV. ACV was removed
897 6 days post-infection and reactivation was triggered at the indicated times post-infection
898 (B). Neonatal SCGs were infected as above after different lengths of time *in vitro*,
899 representing indicated postnatal ages, and reactivated 8 days post-infection with
900 LY294002 (C). n=12 biological replicates from 3 litters. Normality determined by
901 Kolmogorov-Smirnov test. Unpaired student's t-test (B) or Mann-Whitney (A, C) based
902 on normality of data. **p<0.01, ***p<0.001, ****p<0.0001. Individual biological replicates
903 along with the means and SEMs are shown.

904

905 FIG 5: Viral gene expression can be restarted following latency establishment

906 Neonatal sympathetic neurons or adult sensory neurons were infected at an MOI of 7.5
907 PFU/cell with Stayput-GFP in the absence of viral DNA replication inhibitors. Following
908 the loss of GFP, signaling quiescence of the culture, wells were reactivated with a
909 variety of triggers, including combinations of LY-294002 (20 μ M), forskolin (60 μ M), and
910 heat shock (43°C for 3 hours), as well as a superinfection with untagged F strain at an
911 MOI of 10 PFU/cell. GFP was quantified over time, and the peak GFP, at 48 hours post-
912 stimulus, is depicted. N=12 biological replicates (A, E). Immediate early (B, F), early (C,
913 G) and late (D, H) viral transcripts were investigated over time following the stimulus.
914 Neonatal sympathetic neurons n=9 biological replicates from 3 litters. Mann-Whitney
915 against 0 hours (A-D). Adult sensory neurons n=9 biological replicates (E) or n=6
916 replicates (F, G, H). Normality determined by Kolmogorov-Smirnov test. Mann-Whitney

917 against 0 hours. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Individual biological
918 replicates along with the means and SEMs are shown.

919

920 **FIG 6: Reactivation is dependent on DLK**

921 Cultures were infected with Stayput-GFP at an MOI of 7.5 PFU/cell in the absence of
922 ACV. Following loss of GFP, cultures were reactivated with a combination of LY294002,
923 forskolin, and heat shock in the presence of DLK inhibitor GNE-3511 (4 μ M). Immediate
924 early (*ICP27*) and early (*ICP8/UL30*) viral genes (A-C) were investigated at 12.5 hours-
925 post stimulus, and GFP was counted over time (D). Peak GFP, consistently around 48
926 hours post-stimulus, is presented. n=9 biological replicates from 3 litters. Normality
927 determined by Kolmogorov-Smirnov. Mann-Whitney test. (*p<0.05, **p<0.01,
928 ***p<0.001, ****p<0.0001). The mean and SEM are shown.

929

930 **Figure 7: The early phase of lytic gene expression following a reactivation stimulus is**
931 **independent of demethylase activity.**

932 Cultures were infected with Stayput-GFP at an MOI of 7.5 PFU/cell. Following loss of
933 GFP, cultures were reactivated with a combination of LY-294002, forskolin, and heat
934 shock in the presence of H3K27 demethylase inhibitor GSK-J4 (2 μ M) or H3K9
935 demethylase inhibitor OG-L002 (20 μ M). Immediate early (*ICP27*) and early
936 (*ICP8/UL30*) viral genes (A-C) were investigated at 12.5 hours-post stimulus and GFP
937 was counted over time (D). Peak GFP is presented. n=3 biological replicates from 3
938 litters. Normality determined by Kolmogorov-Smirnov. Mann-Whitney. *p<0.05,
939 **p<0.01, ***p<0.001, ****p<0.0001. The mean and SEM are shown.

940

941 Figure 8: Differential dependence on viral DNA replication between Phase I and II

942 reactivation.

943 Cultures were infected with Stayput-GFP at an MOI of 7.5 PFU/cell in the absence of

944 ACV. Following loss of GFP, cultures were reactivated with a combination of LY-

945 294002, forskolin, and heat shock in the presence of ACV (50 μ M). Late (*VP16, gC,*

946 *UL10*) genes (A-C) were investigated at 22 hours-post stimuli. GFP was counted over

947 time and peak GFP is presented (D). n=12 biological replicates, normality determined

948 by Kolmogorov-Smirnov. Mann-Whitney. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

949 The mean and SEM are shown.

950

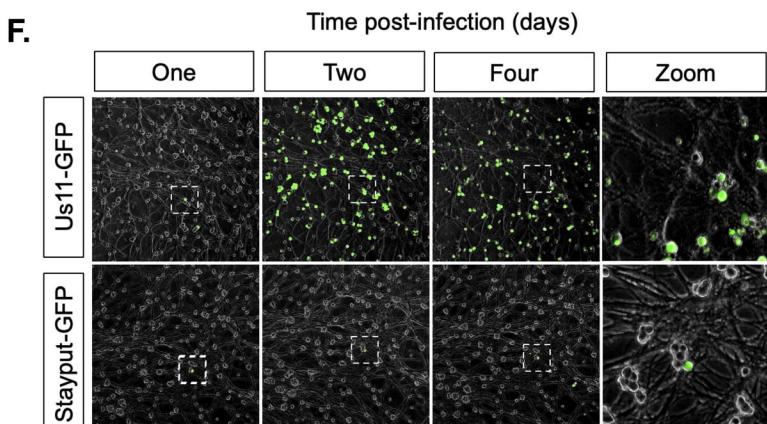
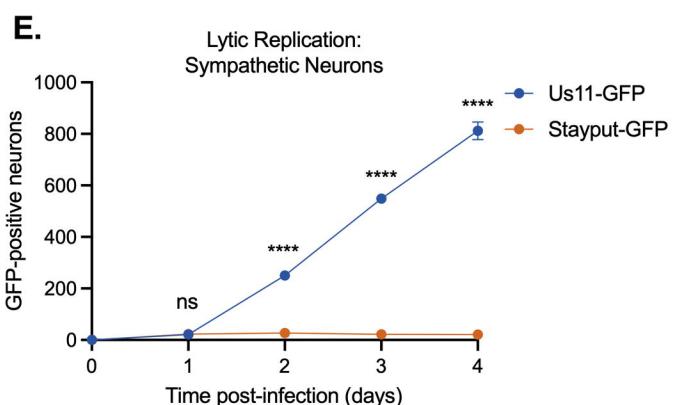
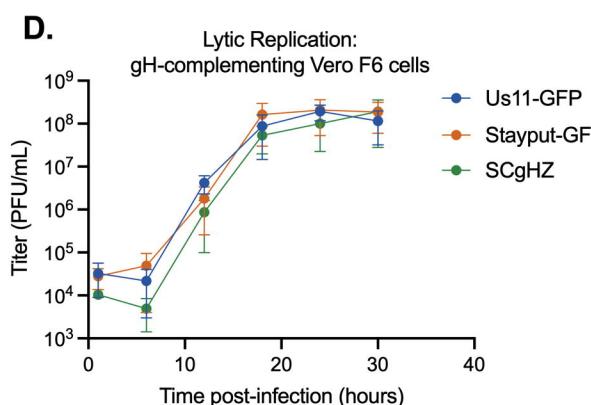
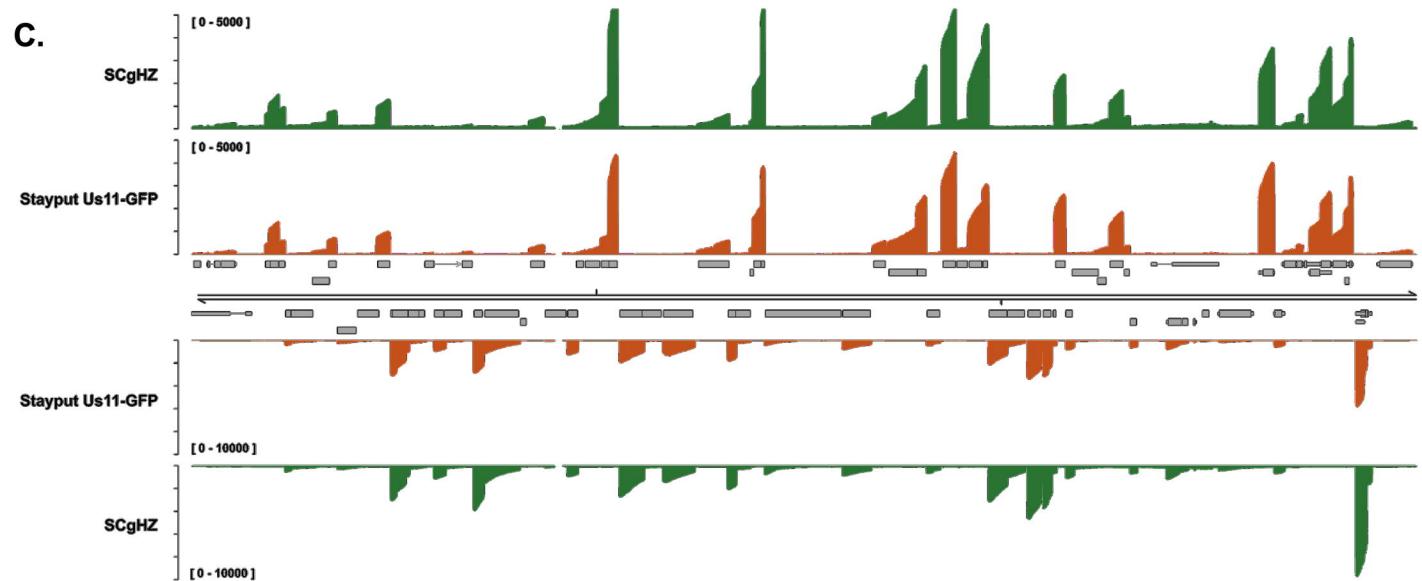
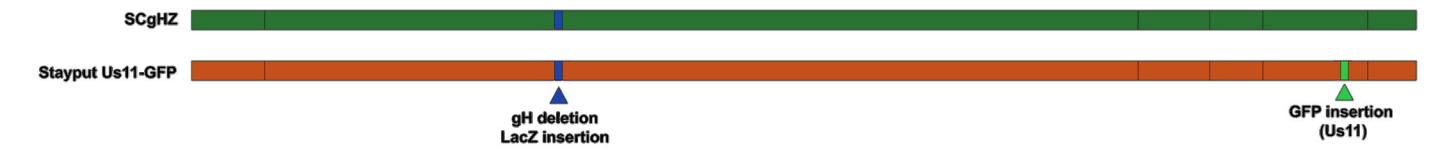
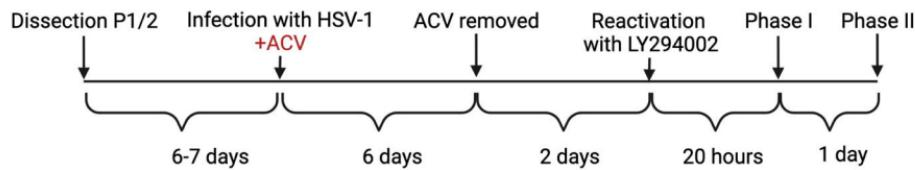
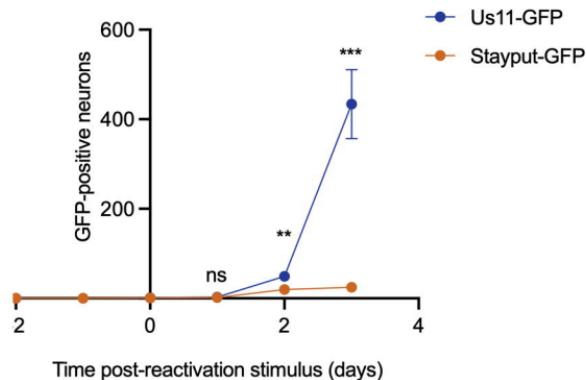
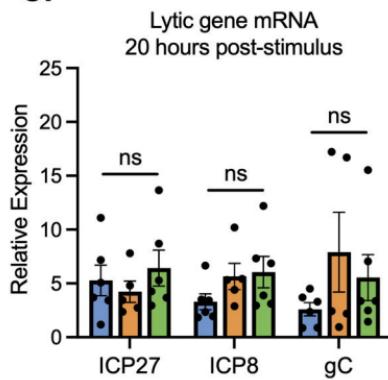
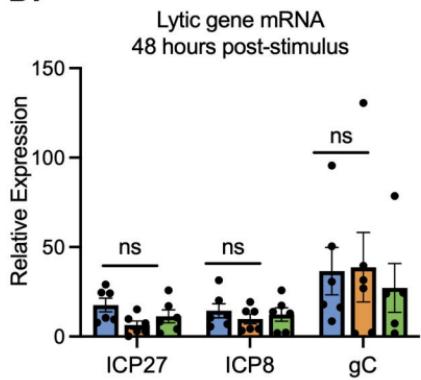
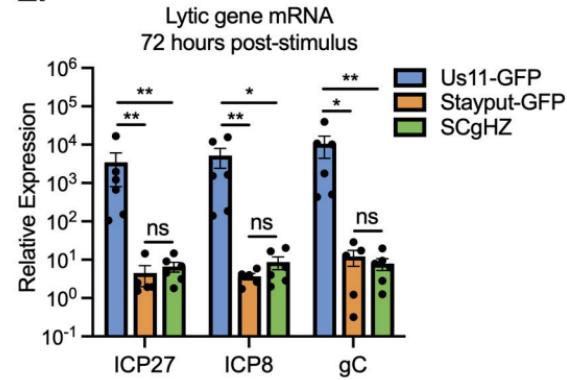


Figure 1

A.**B.****C.****D.****E.****Figure 2**

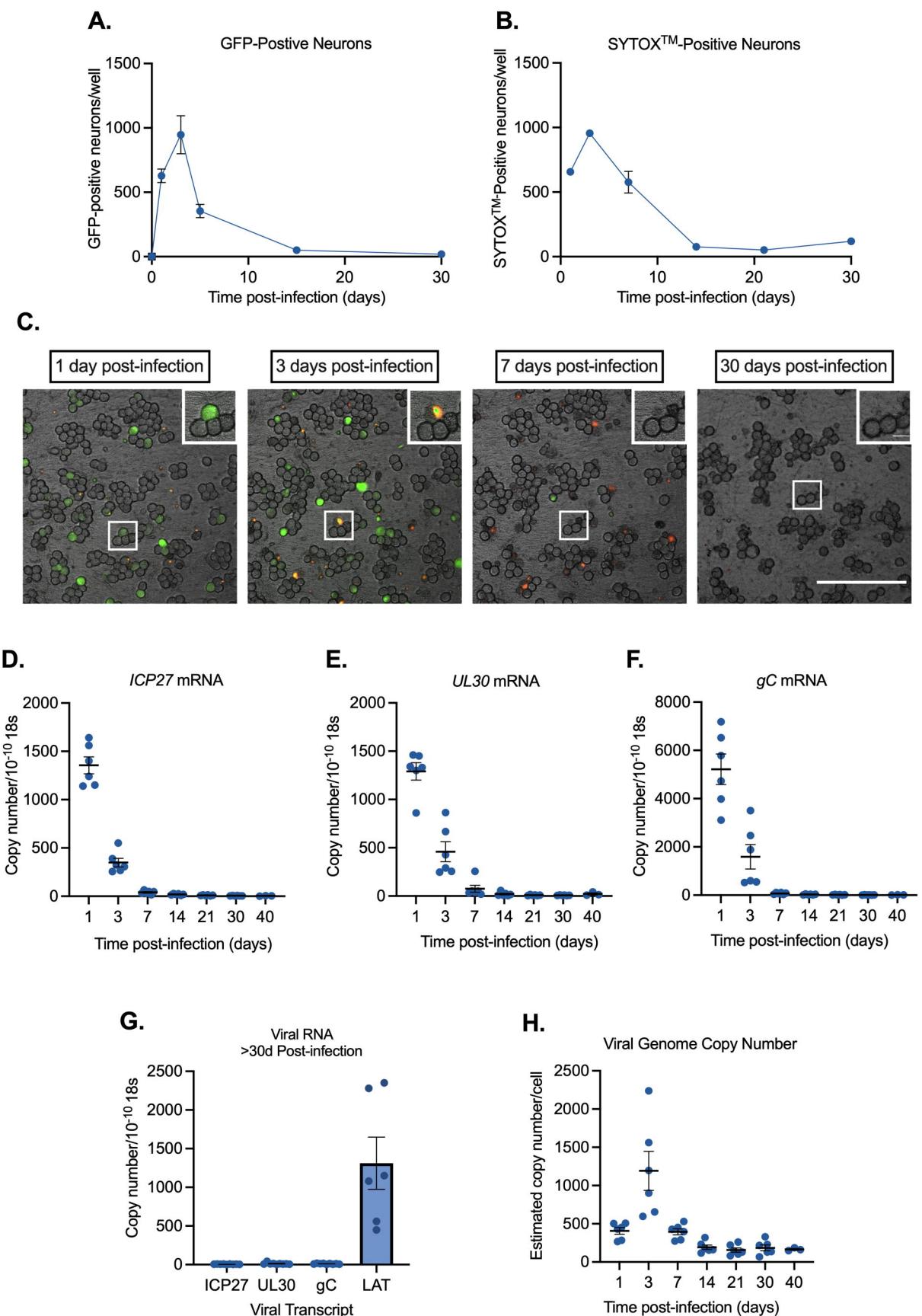


Figure 3

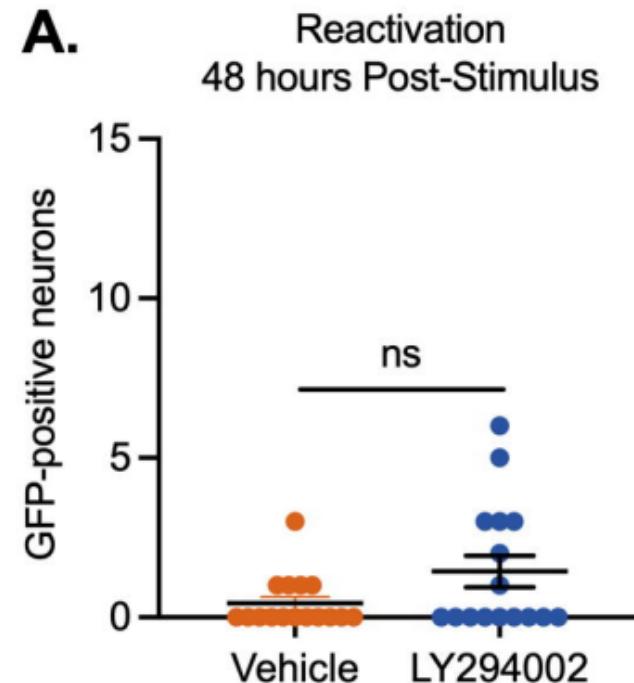
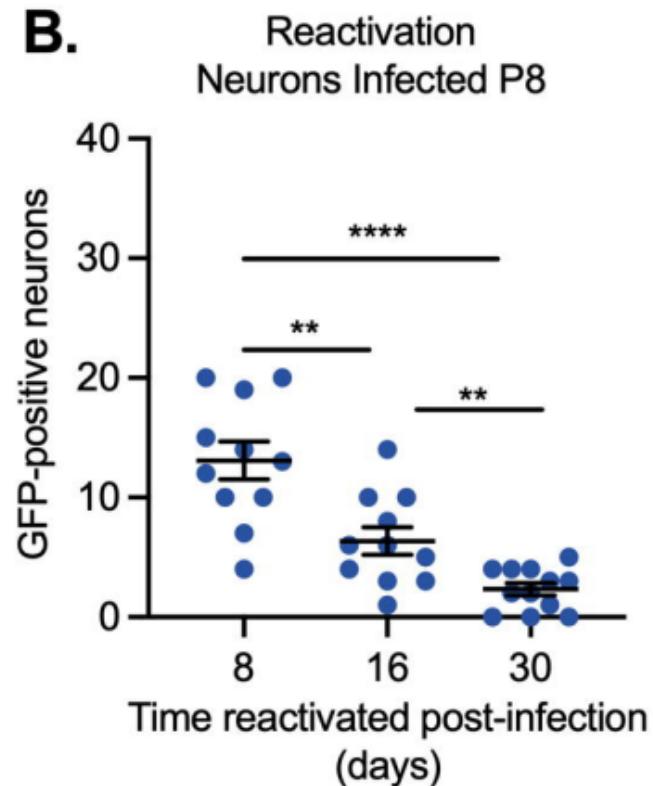
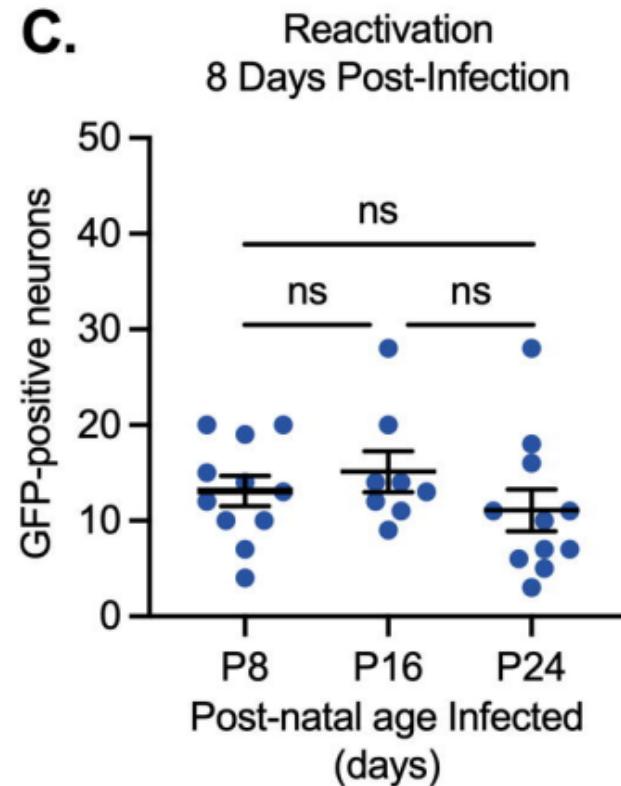
A.**B.****C.**

Figure 4

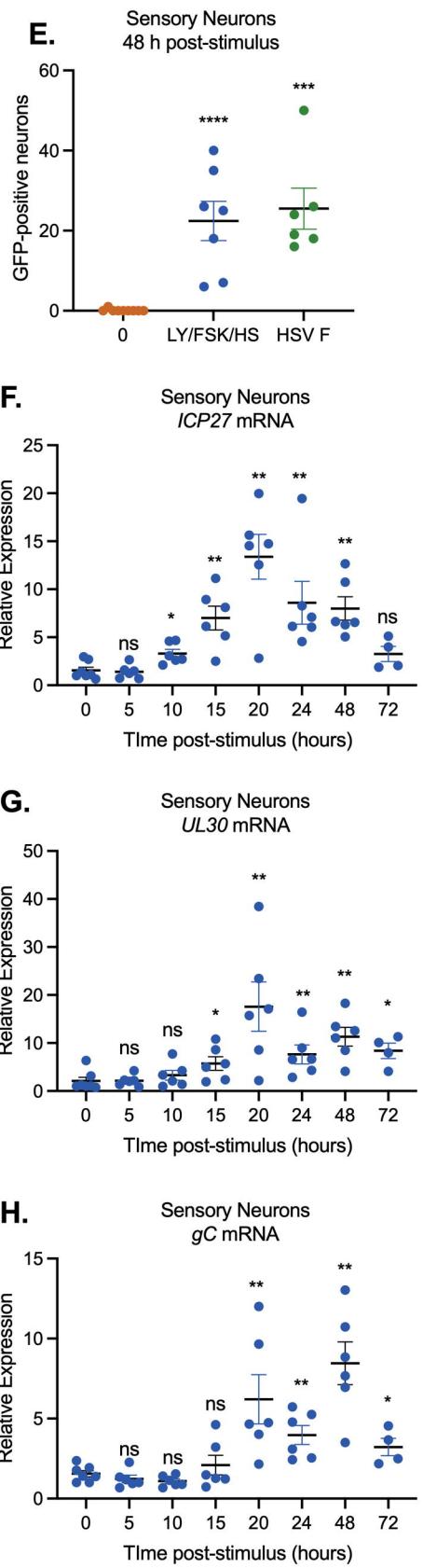
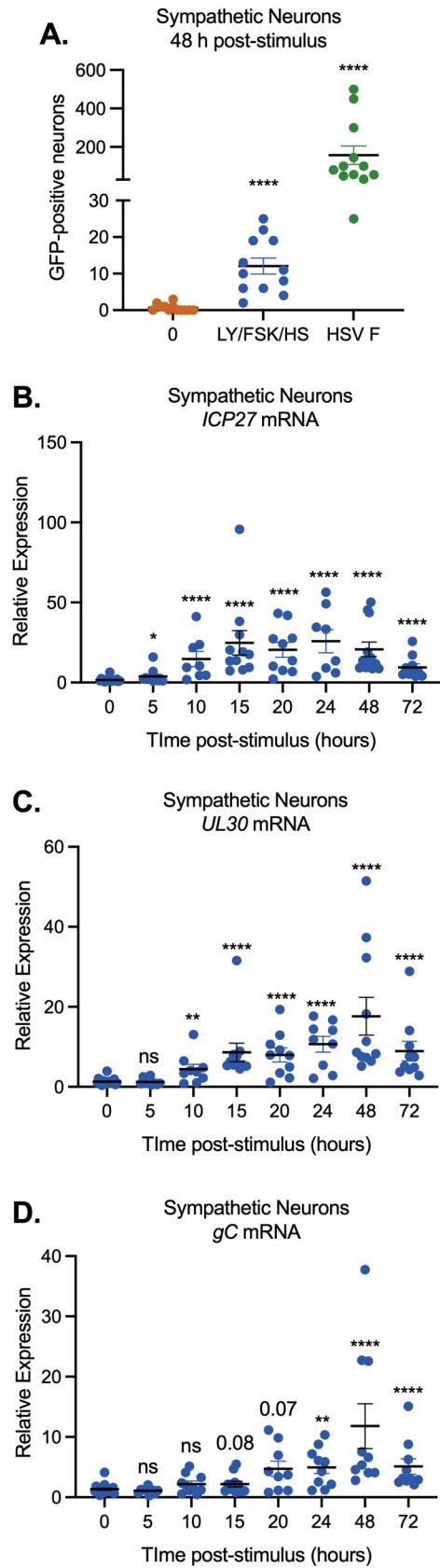


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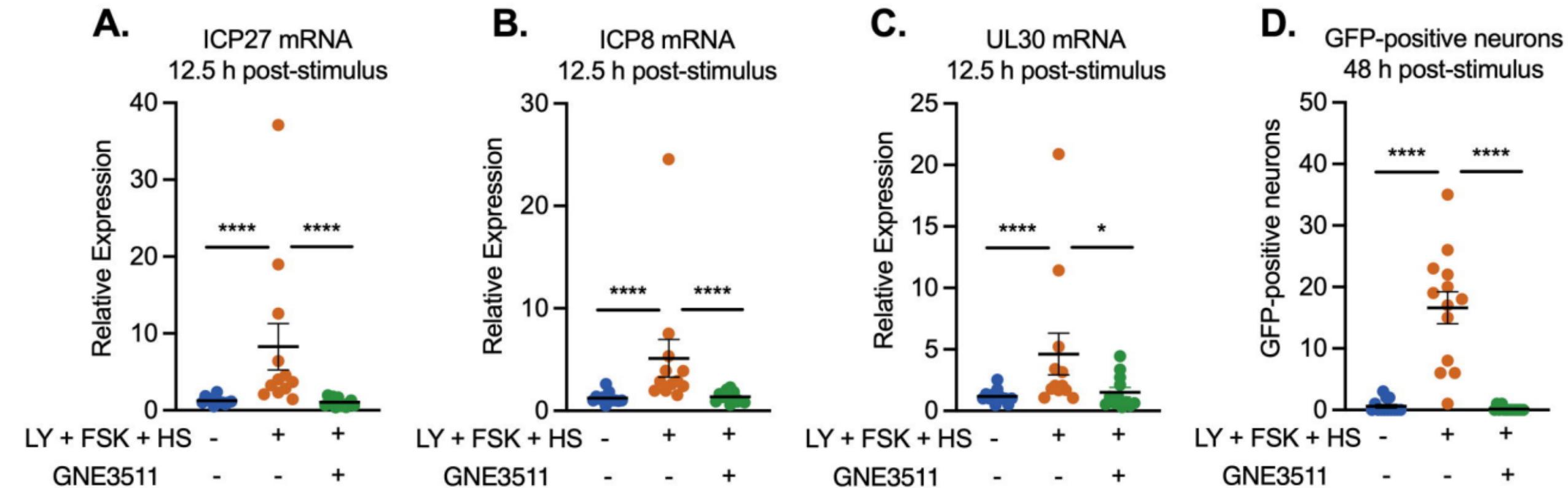


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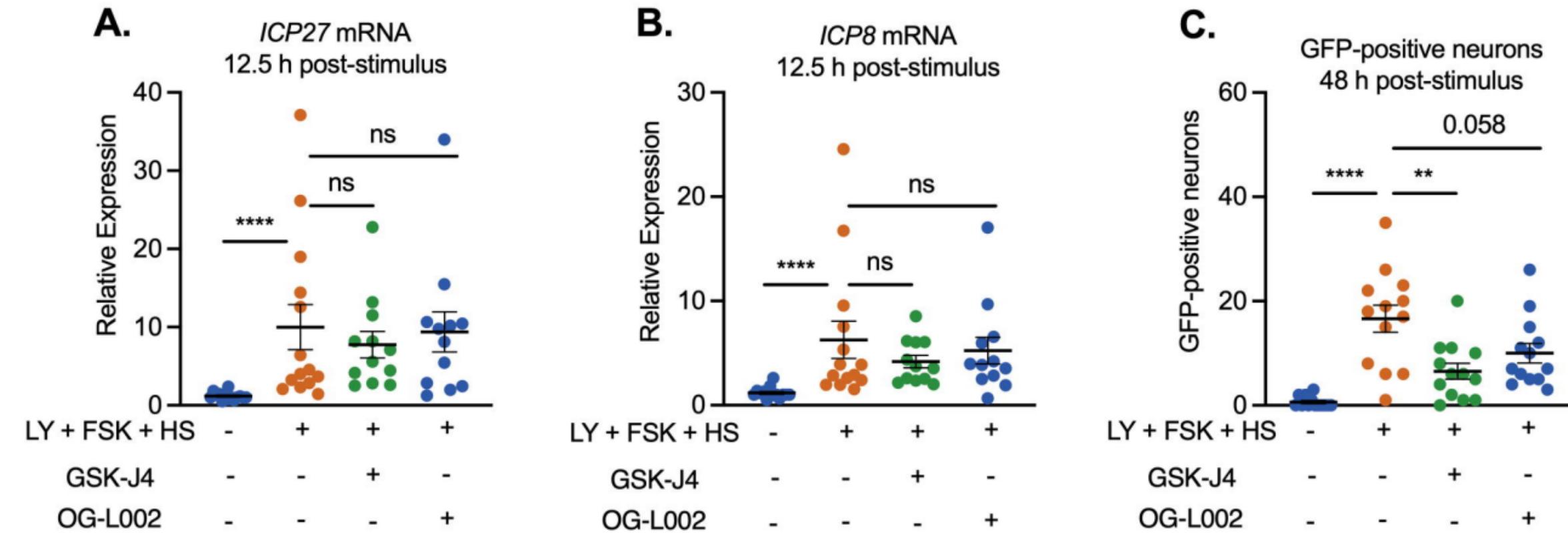


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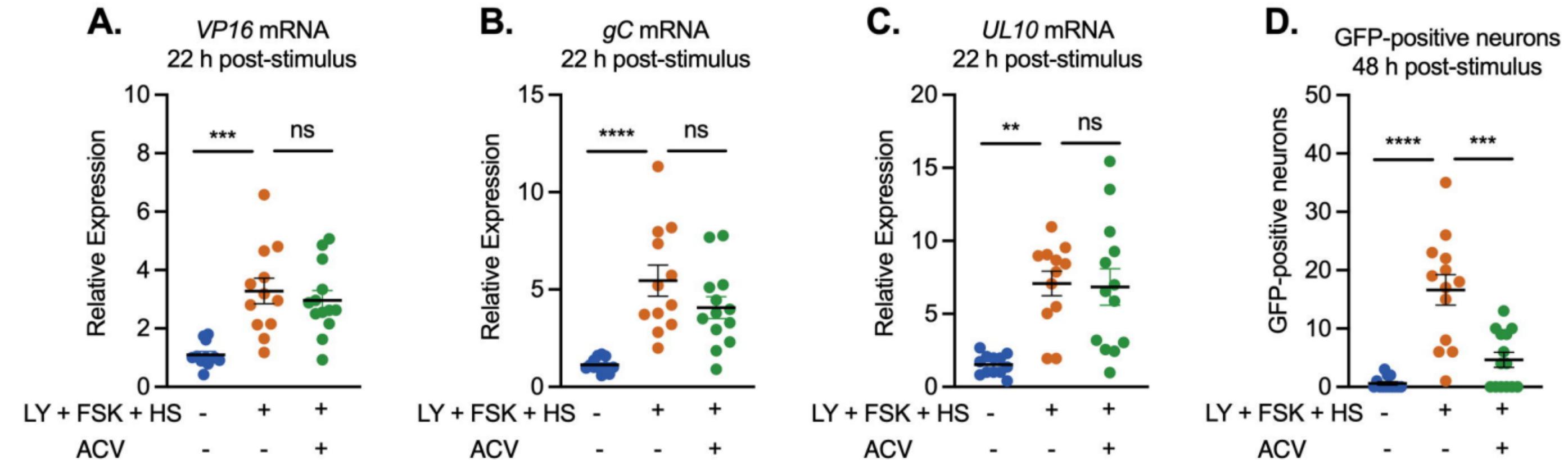


Figure 8