

1 **TITLE: CVB3 VP1 interacts with MAT1 to inhibit cell proliferation**  
2 **by interfering with Cdk-activating kinase complex activity in**  
3 **CVB3-induced acute pancreatitis**

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5 **Running title: CVB3 VP1 inhibits pancreatic cell proliferation**

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27 **Abstract**

28 Cocksackievirus B3 (CVB3) belongs to the genus *Enterovirus* of the family  
29 *Picornaviridae* and can cause acute acinar pancreatitis in adults. However, the  
30 molecular mechanisms of pathogenesis underlying CVB3-induced acute pancreatitis  
31 have remained unclear. In this study, we discovered that CVB3 capsid protein VP1  
32 inhibited pancreatic cell proliferation and exerted strong cytopathic effects on HPAC  
33 cells. Through yeast two-hybrid, co-immunoprecipitation, and confocal microscopy,  
34 we show that Menage a trois 1 (MAT1), a subunit of the Cdk-Activating Kinase  
35 (CAK) complex involved in cell proliferation and transcription, is a novel interaction  
36 protein with CVB3 VP1. Moreover, CVB3 VP1 inhibited MAT1 accumulation and  
37 localization, thus interfering with its interaction with CDK7. Furthermore, CVB3  
38 VP1 could suppress CAK complex enzymic phosphorylation activity towards RNA  
39 Pol II and CDK4/6, direct substrates of CAK. VP1 also suppresses phosphorylation of  
40 retinoblastoma protein (pRb), an indirect CAK substrate, especially at phospho-pRb  
41 Ser<sup>780</sup> and phospho-pRb Ser<sup>807/811</sup> residues, which are associated with cell  
42 proliferation. Finally, we present evidence using deletion mutants that the C-terminal  
43 domain (VP1-D8, 768-859aa) is the minimal VP1 region required for its interaction  
44 with MAT1, and furthermore, VP1-D8 alone was sufficient to arrest cells in G1/S  
45 phase as observed during CVB3 infection. Taken together, we demonstrate that CVB3  
46 VP1 can inhibit CAK complex assembly and activity through direct interaction with  
47 MAT1, to block MAT1-mediated CAK-CDK4/6-Rb signaling, and ultimately  
48 suppress cell proliferation in pancreatic cells. These findings substantially extend our  
49 basic understanding of CVB3-mediated pancreatitis, providing strong candidates for  
50 strategic therapeutic targeting.

52 **Keywords:** Coxsackievirus B3; VP1; MAT1; cell proliferation; CAK.

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71 **Introduction**

72 Cholelithiasis and alcoholism are the most common risk factors of acute pancreatitis

73 (AP). However, approximately 10% of AP occurs in patients with other miscellaneous

74 evidence such as Coxsackie Virus group B infection. Coxsackie Virus group B3  
75 (CVB3), in genus *Enterovirus* of family *Picornaviridae*, can cause a variety of human  
76 diseases, including myocarditis, pancreatitis, and can even lead to sudden infant death  
77 [1-7]. However, the mechanism by which CVB3 infection can cause pancreatitis  
78 remains unclear.

79 The crystal structure of CVB3 revealed the virus to be icosahedral, and without an  
80 envelope. CVB3 encodes four capsid proteins (VP1, VP2, VP3 and VP4), of which  
81 VP1 accumulates in the highest concentrations. Recent studies on the VP1 protein  
82 have primarily focused on vaccine development and its interactions with cell surface  
83 receptors. B-cell epitopes located on VP1 (VP1 1-15 aa, VP1 21-35 aa, and VP1  
84 229-243 aa) and T-cell epitopes on VP1 (VP1 681–700 aa, VP1 721–740 aa, and VP1  
85 771–790 aa) have been considered ideal vaccine candidates for protection against  
86 CVB3 infection[8, 9]. A VP1 protein nanoparticle vaccine and CPE30-chitosan-VP1  
87 nanoparticles were both shown to prevent CVB3 induced myocarditis. In addition,  
88 interrogation of the CVB3-DAF (human decay-accelerating factor) complex structure,  
89 determined by cryo-electron microscopy, confirmed that DAF S104 is an essential site  
90 for close contact with VP1 residue T271 during CVB3 invasion[10-13]. Our previous  
91 work also identified Golgi Matrix Protein 130 (GM130) as a direct intracellular target  
92 of CVB3 VP1, and indicated that the interaction between VP1 and GM130 could  
93 disrupt the structure of the Golgi ribbon[14]. However, few studies have reported the  
94 effects of VP1 on cell proliferation and its direct protein targets in cells.

95 Cell proliferation is the process by which cells increase in number and is mediated by

96 the core cell cycle machinery in response to various external and internal signals.

97 Typically, the cell cycle is controlled by cyclin dependent kinases (CDKs). The

98 activation of CDKs relies on the phosphorylation of essential threonine residues in the

99 T - loop by CDK-activated kinase (CAK) [15]. The CAK complex is composed of

100 MAT1, CDK7, and Cyclin H [16, 17] and is a subcomplex of TFIIH (Transcription

101 Factor II H) which is comprised of ten subunits and is essential for transcriptional

102 initiation. MAT1 is required for functional CAK assembly and induces CDK7 kinase

103 activity[18]. However, TFIIH depends on CDK7 activity to play its role in

104 transcriptional initiation[19, 20]. CDK7 has been revealed to perform dual functions,

105 contributing a central role in the enzymatic activity of the CAK complex while also

106 regulating transcription through phosphorylation of RNA polymerase II and

107 transcription factors[8, 21, 22].

108 Other CDK family proteins, such as CDK2, CDK4, and CDK6, are involved in

109 regulating cell cycle[23]. CDK2, CDK4/6, and RNA polymerase II are activated via

110 direct phosphorylation by CAK. Activated CDK2 and CDK4/6 can then

111 phosphorylate Rb (Retinoblastoma tumor suppressor protein), resulting in activation

112 of E2F and release of transcription factors necessary for G1/S phase progression[24,

113 25]. Phosphorylation of pRb at several clusters of sites could inhibit E2F binding,

114 especially at the C-terminal sites such as Ser780, Ser795 and Ser807/811[26]. The

115 activated RNA polymerase II is then able to bind DNA and start transcription of

116 S-phase genes[27-30]. The CDK-Rb-E2F pathway is undoubtedly responsible for G1

117 phase progression and transition to the S phase[25]. In this context, CDK2, CDK4/6,

118 and RNA polymerase II can be understood as direct substrates of CAK, while Rb  
119 serves as an indirect substrate. Indeed, there were a few examples of viral modulation  
120 of cyclin activity to regulate cell proliferation. Avian Reovirus p17 protein is reported  
121 to suppress cell proliferation by directly binding to CDK-associated proteins, except  
122 for the CDK1-cyclin B1 and CDK7-cyclin H complexes[31]. Furthermore, pUL21a, a  
123 Human cytomegalovirus (HCMV) protein, was found to interact with cyclin A,  
124 causing the degradation of the proteasome through binding with its cyclin-binding  
125 domain[32]. HIV Tat protein was shown to target CDK9 as part of its contribution to  
126 HIV transcription [33, 34]. However, the molecular mechanisms by which CVB3  
127 proteins directly regulate CDKs and influence cell proliferation are still unknown.

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129 In this study, we show that structural protein CVB3 VP1 can reduce HPAC cell  
130 proliferation by arresting the cell cycle at the G1/S phase. More interestingly, by  
131 interacting with MAT1, VP1 impairs the structural formation and activity of the CAK  
132 complex via mediating CAK-CDK4/6 signaling pathway, which functions as a switch  
133 for cell cycle initiation in pancreatic cells, and also reduces CDK7 expression.  
134 Moreover, we also identified that VP1-D8 (198-284aa) was the minimum domain of  
135 VP1 as function to inhibit cell cycle in the G1 phase through the deletion mutants  
136 approach. These findings describe a previously unreported phenomenon of in which  
137 the CVB3 VP1 structural protein inhibits pancreatic cell proliferation by interfering  
138 with cell cycle through impairment of CAK complex assembly and function. This  
139 work thus modifies our understanding of the role of CVB3 in causing pancreatitis

140 mechanisms and opens new avenues for targeted therapies.

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## 143 **Results**

### 144 **CVB3 capsid protein VP1 inhibits HPAC cell proliferation**

145 First, to quantitatively determine the cytotoxicity of VP1 towards HPAC cells, we  
146 observed its cytopathic effects and performed adherent cell counts. We found that  
147 transfection of VP1 resulted in a reduction in the number of adherent HPAC cells (Fig  
148 1A), we also observed that VP1 induced HPAC cell shrinking, detachment, and lysis  
149 (Supplemental Fig. 1). We then explored the effects of CVB3 VP1 on HPAC cell  
150 proliferation.

151 Analysis of CCK-8 assays confirmed that VP1 reduced the cell survival rate  
152 compared with that of the vector control group with extension of transfection time in  
153 HPAC cells (Fig. 1B). EdU assays also showed that DNA replication was  
154 significantly inhibited in HPAC cells transfected with VP1, also commensurately with  
155 increasing time of transfection (Fig. 1C). Western blot analysis revealed that the VP1  
156 expression decreased with the time course of VP1 transfection or CVB3 infection  
157 (Supplemental Fig. 2). Further, to characterize VP1 cytotoxicity towards HeLa cells,  
158 we quantified adherent, viable, and proliferating cells after exposure to VP1. We  
159 found that VP1 significantly inhibited HeLa cell proliferation, and showed the same  
160 cytotoxic effects as those observed in HPAC cultures (Supplemental Fig. 3). These  
161 results showed that the effects of VP1 alone was consistent with that of CVB3 virus



162 infection in both HPAC and HeLa cells.

163 We then targeted our investigation to the effects of CVB3 VP1 on cell cycle. To  
164 accomplish this, we first synchronized HPAC cell cultures in G1 phase, exposed them  
165 to VP1, and performed PI staining for analysis by flow cytometry. The resultant cell  
166 cycle profiles showed that in the VP1 transfection group, HPAC cells were  
167 significantly blocked at the G1 phase compared with the pBud group, while  
168 synchronized healthy cells continued their cell cycle after thymidine removal (Fig.  
169 1D). These results aligned with experiments showing that CVB3 infection blocked  
170 cell cycle in both HPAC and HeLa cells (Supplemental Fig. 4). Moreover, PI analysis  
171 confirmed that the cell cycle was unaffected by exposure to CVB3 VP2, VP3, or VP4  
172 (Supplemental Fig. 5). Taken together, these results revealed that VP1 inhibits HPAC  
173 and HeLa cell proliferation, exerts a strong cytopathic effect, and arrests cell cycle at  
174 G1/S phase.

### 175 **MAT1 was identified as a novel CVB3 VP1-binding protein**

176 To obtain deeper insight into the potential mechanisms underlying CVB3 VP1  
177 blockage of cell cycle at the G1/S phase and inhibition of cell proliferation, we  
178 screened for potential interacting proteins via yeast two-hybrid system, and  
179 subsequently identified a host cellular protein, MAT1, that acts as a VP1 binding  
180 partner (Fig. 2A). We then verified the two-hybrid binding of MAT1 to VP1, and  
181 autoradiography data confirmed that VP1 directly bound to MAT1 *in vitro* (Fig. 2B).  
182 Co-immunoprecipitation assays also confirmed the interaction in HeLa cells *in vivo*.  
183 Specifically, anti-VP1 was able to effectively precipitate MAT1, while anti-MAT1

184 also precipitated VP1, indicating that VP1 interacted with MAT1 *in vivo* (Fig. 2C). In  
185 light of these results, we then examined the cellular distribution and co-localization of  
186 VP1 and MAT1 by confocal microscopy. We observed that VP1 and MAT1 mainly  
187 shared the same spatial localization in the cytoplasm of the CVB3-infected HPAC  
188 cells (Fig. 2D) as well as in HeLa cells (Supplemental figure 6). Taken together, these  
189 results indicate that MAT1 is a binding partner for CVB3 VP1 both *in vitro* and *in*  
190 *vivo*.

191

### 192 **VP1 induces CAK subunit ubiquitination to impair functional complex assembly**

193 Given our findings of VP1 binding to MAT1, we then sought to determine the  
194 effects of this interaction on MAT1 function by observing the effects on MAT1  
195 function and interactions. Confocal microscopy showed that VP1 expression  
196 increased in a time-dependent fashion with virus infection and pBud-VP1  
197 transfection. Interestingly, with increasing expression of VP1, the expression of  
198 MAT1 decreased and even disappeared in VP1-transfected and CVB3-infected HPAC  
199 cells. We found a significant decrease in MAT1 signal intensity inversely related to an  
200 increase in VP1 intensity, thereby showing that VP1 decreased the cytoplasmic  
201 accumulation and nuclear localization of MAT1 (Fig. 3A). The effect of VP1 on  
202 MAT1 can also be observed in HeLa cells using immunohistochemical fluorescence  
203 staining (Supplemental figure 7). Furthermore, we used Western blots to assess the  
204 effects of VP1 on expression of CAK complex subunits MAT1, CDK7, and Cyclin H  
205 in the nucleus and cytoplasm in cells. The results indicated that nuclear expression of

206 MAT1 and CDK7 declined with increasing time of VP1 transfection and CVB3  
207 infection. Interestingly, we observed no detectable changes in the expression levels of  
208 Cyclin H in both group (Fig. 3B). In addition, MAT1 expression decreased and CDK7  
209 / Cyclin H expression were undetectable in the cytoplasm (Supplemental Fig. 8). This  
210 result confirmed that MAT1, CDK7, and Cyclin H are all found in the cell nucleus,  
211 and that interference by VP1 attenuated the MAT1 expression in both the nucleus and  
212 cytoplasm, as well as the expression levels of nuclear CDK7.

213 To further explore the reasons why expression of CAK complex proteins MAT1  
214 and CDK7 were down-regulated by VP1, we conducted ubiquitin proteasomal  
215 degradation assays. We found that in VP1-transfected cells,  
216 anti-MAT1-immunoprecipitated MAT1 was ubiquitinated and appeared as a smear of  
217 degraded protein (Fig. 3C left). Concurrently, the MAT1 band intensity substantially  
218 increased by the addition of MG132 proteasomal inhibitor, and the intensity of MAT1  
219 ubiquitination also progressively increased with prolonged incubation with MG132  
220 (Fig. 3C right). As shown in Fig. 3D, VP1 exposure correspondingly led to CDK7  
221 degradation, as observed through ubiquitin proteasomal degradation assays (Fig. 3D).  
222 Taken together, these results strongly suggest that CAK complex assembly and  
223 function is likely impaired through the ubiquitination and proteasomal degradation of  
224 its subunits during exposure to VP1.

#### 225 **CVB3 VP1 interferes with subcellular colocalization of MAT1 and CDK7.**

226 In order to determine whether VP1 impaired MAT1 interactions with other  
227 subunits, we observed colocalization of MAT1 and CDK7 in CVB3 VP1-transfected

228 cells in G1 and S phase of the cell cycle. To this end, we first determined the optimal  
229 release time point from G1 to S phase after thymidine double blocking, thus  
230 synchronizing cells in the G1 and S phase transition. The results showed that the  
231 highest percentage of synchronized healthy cells in the G1 and S phase could be  
232 observed at 0 h and 5 h after release, respectively (Fig. 4A). We next transfected VP1  
233 and infected CVB3 (Fig. 4B), and observed through confocal microscopy that in the  
234 G1 phase, VP1 and CVB3 induced the transfer of a large proportion of  
235 nuclear-localized MAT1 to the cytoplasm, concurrent with decreased accumulation of  
236 MAT1 in both the nucleus and cytoplasm and decreased nuclear levels of CDK7 (Fig.  
237 4C). In contrast, among cells synchronized in the S phase, VP1 and CVB3 attenuated  
238 nuclear and cytoplasmic MAT1 accumulation but did not result in MAT1 export from  
239 the nucleus to the cytoplasm. Interestingly, VP1 and CVB3 induced a partial transfer  
240 of nuclear-localized CDK7 to the cytoplasm (Fig. 4D). Based on these findings, we  
241 therefore concluded that the interaction between CVB3 VP1 and MAT1 not only  
242 reduced the levels of MAT1 and CDK7, but also substantially altered the subcellular  
243 colocalization patterns of MAT1 and CDK7, likely disrupting the function of CAK  
244 complex.

#### 245 **CVB3 VP1-MAT1 interaction suppresses CAK activity via CDK7 *in vitro***

246 Since the results above suggested that the VP1-MAT1 interaction disrupted the  
247 assembly of the CAK complex, we addressed whether the interaction between VP1  
248 and MAT1 also reduced CAK activity in VP1-transfected or CVB3-infected cells.  
249 CAK indirectly phosphorylates Rb via Cyclin-dependent kinases (CDKs), and the

250 activation of CDK relies on T-loop phosphorylation by CAK[15, 35]. We therefore  
251 assessed the expression and phosphorylation levels of CDK2 or CDK4. The results  
252 showed that the protein expression and phosphorylated protein levels of CDK2 or  
253 CDK4 were progressively attenuated over the 48 h time course in pBud-VP1  
254 transfected cells (Fig. 5A).

255 Previous studies have reported that the carboxy-terminal domain (CTD) of the  
256 largest subunit of RNA Pol II is a canonical direct substrate of the CAK complex, and  
257 that the phosphorylation of RNA Pol II Ser<sup>5</sup> and Ser<sup>7</sup> residues is primarily mediated  
258 by CDK7 activity [19, 36, 37]. Therefore, we evaluated CAK activity in cells exposed  
259 to VP1 through quantification of RNA Pol II CTD Ser<sup>5</sup> phosphorylation levels. After  
260 nucleoplasm separation, we found that RNA Pol II CTD and phosphorylated RNA Pol  
261 II CTD were both down-regulated in the nucleus (Fig. 5B). In addition, we detected  
262 the phosphorylation levels of the CAK complex indirect substrate, pRb, which is the  
263 substrate of Cyclin D/CDK4 or Cyclin E/CDK2, both of which are direct substrates of  
264 the CAK complex[38]. These results showed that both pRb Ser<sup>780</sup> and pRb Ser<sup>807/811</sup>  
265 phosphorylation were down-regulated in the pBud-VP1 transfected or CVB3 infection  
266 groups. In contrast, pRb total protein levels were significantly elevated in cells  
267 exposed to VP1. However, there was no significant change in pRb Ser<sup>795</sup> site  
268 phosphorylation among any of the groups (Fig. 5C).

269 To verify whether CDK7 mediated the phosphorylation of RNA Pol II CTD and  
270 pRb in VP1-transfected cells, we silenced the CDK7 gene in HPAC cells using an  
271 shRNA vector. We also overexpressed CDK7 in HPAC cells to verify whether

272 increased CDK7 activity can rescue CAK function. The results showed that  
273 phosphorylation of RNA Pol II CTD was further decreased in cells co-transfected  
274 with the CDK7 silencing plasmid and VP1, compared with that of cells transfected  
275 with VP1 alone. In agreement with these results, phosphorylation of RNA Pol II CTD  
276 was recovered in cells co-transfected with VP1 and the CDK7 over-expression  
277 plasmid (Fig. 5D). In addition, lower phosphorylation levels of pRb Ser<sup>780</sup> and pRb  
278 Ser<sup>807/811</sup> were observed in co-transfections of VP1 and the CDK7 silencing construct  
279 compared to those transfected with VP1 alone. Similarly, pRb Ser<sup>780</sup> and pRb  
280 Ser<sup>807/811</sup> phosphorylation was apparently rescued to the high level observed in control  
281 cells after co-transfection with VP1 and CDK7 over-expression plasmid, while total  
282 pRb protein showed the opposite trend. Furthermore, we were unable to detect any  
283 changes in phosphorylation of pRb at Ser<sup>795</sup> (Fig. 5E). Collectively, these results  
284 indicate that the VP1-MAT1 interaction indeed impairs the catalytic activity of the  
285 CAK complex by interfering with phosphorylation of CDK2 or CDK4, as well as  
286 CDK7 kinase activity towards RNA Pol II and pRb phosphosites Ser<sup>780</sup> and Ser<sup>807/811</sup>.  
287

288 **CVB3 VP1 inhibits the activity of CDK4/6 and Rb phosphorylation of CDK-Rb**  
289 **signaling pathway proteins in the G1/S transition.**

290 To further investigate whether CVB3 VP1 perturbs the activity of CDK4/6 and the Rb  
291 phosphorylation of the CDK-Rb signaling pathway in the G1/S transition, we  
292 conducted a series of assays to compare the effects of the CDK4/6 inhibitor  
293 abemaciclib with that of VP1 on the inhibition of HPAC cell proliferation and

294 CDK4/6 activity. The results showed that abemaciclib and CVB3 VP1 similarly  
295 reduced cell proliferation in the pBud group, while exposure to VP1 alone led to a  
296 more powerful inhibitory effect than pBud plus abemaciclib together (Fig. 6A and B).  
297 These results were also reflected by comparable patterns of attenuation to pRb  
298 phosphorylation, with greater inhibition associated with dual treatments compared to  
299 VP1 exposure alone (Fig. 6C). Moreover, among cells treated with abemaciclib, flow  
300 cytometry showed that a greater proportion of cells also transfected with VP1 were  
301 arrested at the G1 phase compared to cells treated only with abemaciclib at both 12  
302 and 15 h after release from double thymidine (Fig. 6D). These results collectively  
303 show that VP1 indeed interrupts CDK4/6 activity and Rb phosphorylation of CDK-Rb  
304 pathway proteins, thereby leading to inhibition of cell proliferation.

305

306 **CVB3 VP1 C-terminal domain (198-284 aa) is required for interaction with**  
307 **MAT1**

308 Having established the interaction between VP1 and MAT1, we then used  
309 yeast-two hybrid to identify which regions of VP1 are required for binding with  
310 MAT1. Co-transformation of a series of VP1 truncation/deletion variants with  
311 pGADT7-MAT1 revealed that the VP1 variant carrying amino acids 198-284  
312 interacted with MAT1, while segments spanning amino acids 3-197 showed no  
313 interaction (Fig. 7A). Further, we examined the CVB3 VP1-D8 domain to determine  
314 whether it exhibited similar functions to that of full-length VP1. As shown in Fig. 7B,  
315 VP1-D8 functioned comparably to VP1 in the inhibition of cell cycle at the G1 phase,

316 and similarly attenuated the accumulation of MAT1, whereas VP1-D4 did not (Fig.  
317 7C). To strengthen the evidence for this function, we investigated whether VP1-D8  
318 was also able to affect activity towards CAK complex substrates. For this purpose, we  
319 used Western blot assays to confirm that VP1-D8 bound to MAT1 similarly to the full  
320 length VP1, and that it also reduced phosphorylation of CDK2/4, RNA Poly II CTD,  
321 and pRb, while VP1-D4 and pBud did not (Fig. 7D, E, and F). Taken together, these  
322 data collectively indicate that VP1-D8 comprises the minimum VP1 domain  
323 necessary to block the cell cycle at G1 phase, attenuate CAK complex  
324 phosphorylation activity towards its substrates via MAT1 binding, and ultimately  
325 suppress cell proliferation.



326

## 327 **Discussion**

328 In this study, we present the first report of which we are aware detailing the  
329 mechanisms of CVB3 structural protein VP1 inhibition of pancreatic cell  
330 proliferation. We discovered VP1 interacts with MAT1, leading to down-regulation of  
331 MAT1 and CDK7, and ultimately impairing CAK complex formation. Furthermore,  
332 we pinpointed the minimum functional domains of VP1 that engage in this  
333 interaction.

334 However, deregulation of cell proliferation leads to diseases characterized by either  
335 excess proliferation or cell loss, an effect that is induced by some viral infections due  
336 to exploitation of replication machinery to benefit for viral replication. Viral targeting  
337 of the cell cycle as a core regulatory component of cell proliferation has been widely  
338 studied among other viruses. For example, latent protein 3C (EBNA3C) of  
339 Epstein-Barr virus (EBV), the first reported human tumor virus, can directly bind to  
340 CDK2, and also cooperate with master transcription factor Bcl6 to regulate the  
341 expression of CDK2, thereby promoting cell proliferation [39]. In contrast, Hepatitis  
342 C virus (HCV) reduces CD 8<sup>+</sup> T cell proliferation, and leads to dysfunction in HCV  
343 chronic infection [40]. Furthermore, HCV decreases the proportion of infected cells in  
344 G1 and S phase commensurate with accumulation of G2/M cells [41]. Indeed, most  
345 viral proteins reported to participate in regulating cell proliferation are non-structural,  
346 whereas viral structural proteins more commonly contribute functions such as  
347 protection of the viral genome against inactivation by nucleases, viral attachment to  
348 host cells, providing a symmetry to virus particle structure, which can also provide

349 crucial antigenic characteristics. However, it remains largely unknown whether and  
350 how structural proteins can interact with host proteins to regulate host cell functions,  
351 such as cell cycle regulation or stress response.

352

353 In HPAC and HeLa cell models, our results indicated that the CVB3 capsid protein  
354 VP1 induced cell cycle arrest, leading to inhibition of cell proliferation and cytopathic  
355 effects. Furthermore, this process is mediated by interaction with CAK complex  
356 assembly factor, MAT1. Our findings showed that structural viral proteins can inhibit  
357 cell proliferation via cell cycle arrest. A widely accepted mechanism of G1/S phase  
358 arrest is through inactivation of cyclinD-CDK4 phosphorylation, phosphorylation of  
359 pRb, and concomitant E2F activation[42, 43]. However, our study revealed that VP1  
360 directly or indirectly inactivated phosphorylation of CDK2 and CDK4, pRb, and RNA  
361 Pol II to arrest the G1 phase, thus suggesting a new model for interference in cell  
362 proliferation.

363 We then investigated how the interaction of VP1 with MAT1 leads to the inhibition of  
364 cell cycle and found that MAT1 is expressed in the cytoplasm and nucleus in the G1  
365 phase, but primarily localized to the nucleus. Interference by CVB3 VP1 decreased  
366 MAT1 expression, destabilized CDK7, and impaired CAK complex formation in the  
367 nucleus. While in the S phase, CDK7 is expressed only in the nucleus, but readily  
368 diffused into the cytoplasm due to interference of VP1, which aligned with our results  
369 showing that CDK7 binding to MAT1 decreased upon pBud-VP1 transfection.  
370 Among several known functions of MAT1, its C-terminal domain activates CDK7

371 phosphorylation and stabilizes Cyclin H / CDK7 binding to the TFIIH core through  
372 interaction with XPB and XPD [44]. Interestingly, we observed VP1 bind to the  
373 C-terminal of MAT1 (data not shown), thus indicating that interaction between VP1  
374 and MAT1 can disrupt CAK complex assembly, and decrease phosphorylation  
375 activity by CDK7. Moreover, we found that CDK7 was degraded through the  
376 ubiquitination pathway in the presence of VP1.

377 In addition to preventing CAK complex assembly, VP1 binding to MAT1 could also  
378 impair CAK complex activity by interfering with CDK7-mediated phosphorylation.  
379 We found that phosphorylation of the direct or indirect CAK substrates CDK2,  
380 CDK4, and RNA pol CTD were all attenuated following transfection of VP1, and that  
381 inhibition of pRb phosphorylation involved its Ser<sup>780</sup> and Ser<sup>807/811</sup> sites.  
382 Overexpression or silencing of CDK7 revealed that VP1 / MAT1 interactions led to  
383 inhibition of CAK activity via CDK7 degradation. Furthermore, we found that  
384 VP1-D8 was the minimally required domain for VP1-mediated blockade of the cell  
385 cycle at G1/S phase, to successfully suppress cell proliferation and inhibit CAK  
386 complex activity.

387 We subsequently determined that CVB3 VP1 also inhibited CDK4/6 activity and Rb  
388 phosphorylation in the G1/S transition. In fact, the pocket protein Rb is among the  
389 most functionally essential proteins phosphorylated by CDK4/6 and CDK2 to regulate  
390 cell cycle[45]. Given that VP1 inhibition of CDK4/6 activity can attenuate pRb  
391 phosphorylation, thereby leading to arrest cell cycle in the G1 phase, we thus  
392 conclude that CVB3 VP1 can potentially inhibit cell proliferation through a

393 MAT1-mediated CAK-CDK4/6-Rb signaling pathway.

394 Given previous studies showing that CVB3 can cause acute pancreatitis in humans  
395 and mice, and that TNF $\alpha$  and IL6 are implicated in the development of this disease  
396 [64, 65], it is likely that cytokines are also involved in virus-induced inhibition of cell  
397 proliferation. Moreover, the expression of IL10 may suppress acute pancreatitis [66],  
398 and notably, *Sawada* found that IL-10 and its downstream STAT3 pathway regulate  
399 the proliferation of cells infected with HTLV-1 [46]. In addition, IL-6-deficient mice  
400 infected with influenza virus were found to produce high levels of TGF and enhanced  
401 the proliferation of lung fibroblasts [47]. In contrast, we found that in the G1 phase of  
402 VP1 transfection, inflammatory cytokines (TNF $\alpha$  and IL-6) were up-regulated, while  
403 anti-inflammatory cytokine (IL-10) was down-regulated (Data not shown). Taken  
404 together with our previous work showing that CVB3 infection caused pancreatitis, we  
405 thus hypothesized that the changes in the accumulation of these inflammatory factors  
406 induced by VP1 transfection in HPAC cells synergistically blocked the cell cycle in  
407 the G1 phase, potentially resulting in pancreatic inflammation. However, we cannot  
408 yet exclude the possibility of additional contributing factors, and ongoing research  
409 will clarify the full extent of host proteins participating in CVB3 induction of  
410 pancreatitis.

411

412 In this study, we provide evidence that direct interaction between CVB3 VP1 and  
413 MAT1 produces an inhibitory effect on HPAC cell proliferation by blocking the  
414 MAT1-mediated CAK-CDK4/6-Rb signaling pathway required for CAK complex

415 assembly and activity, which thereby results in cell cycle arrest during the G1 phase  
416 in pancreatic cells.

417

## 418 **Material and Methods**

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#### 420 **Cell culture, synchronization and virus**

421 The human pancreatic adenocarcinoma cell line HPAC, HeLa and 293A cells  
422 were obtained from the American Type Culture Collection (Manassas, VA, USA).  
423 Cells were grown in a 5% CO<sub>2</sub> incubator at 37°C, and maintained in Dulbecco's  
424 modified Eagle's medium (BI, Israel) and DF12 (BI, Israel) supplemented with 10%  
425 heat-inactivated 10% fetal bovine serum (BI, Israel).

426 HPAC and HeLa cells were synchronized at the G1 phase using double  
427 thymidine block treatment. Briefly, cells in six-well plates grew to 40% confluency,  
428 then thymidine was added to a 2 mM final concentration and cells were incubated for  
429 34 h (HPAC) or 19 h (HeLa). Subsequently, the medium was aspirated with  
430 thymidine and the cells were washed twice with PBS, after releasing for 10-12 h  
431 (HPAC) or 9 h (HeLa). In the second thymidine block, thymidine was added to the  
432 cells for a final concentration of 2 mM and incubated for 33 h (HPAC) or 18 h  
433 (HeLa).

434 CVB3 (Nancy strain; GI:323432) was propagated in HeLa cells and purified  
435 using a method previously described by Henke et al. [48]. Cells were infected with  
436 CVB3 (MOI of 5) throughout the study.

#### 437 **Plasmids construction**

438 To construct yeast two-hybrid plasmids, VP1 and MAT1 genes were cloned into  
439 Vector pGBKT7 (Clontech, USA). We generated pGBKT7-VP1 and pGADT7-MAT1  
440 plasmids to map the regions in VP1 that were required for their interaction in the  
441 yeast-two hybrid system. Briefly, the different truncation/deletion mutants of CVB3  
442 VP1 were cloned into pGBKT7 vector (between the Nde I & Pst I sites) in frame with  
443 the vector's GAL4 DNA Binding Domain (BD), and MAT1 was expressed in the

444 pGADT7 vector (between the EcoR I & BamH I sites) in frame with the vector's  
445 GAL4 activation domain (AD). The deletion mutants of VP1 were separately  
446 generated by PCR with pGBKT7-VP1 as the template. VP1, VP1-D2, VP1-D3,  
447 VP1-D4, VP1-D5, VP1-D6, VP1-D7, VP1-D8, VP1-D9, VP1-D10, VP1-D11,  
448 VP1-D12, VP1-D13, VP1-D14 were separately cloned into pGBKT7 vector. VP1,  
449 VP1-D4 and VP1-D8 were cloned into pBudCE4.1 (pBud) vector. CDK7 was cloned  
450 into pCAG-Flag and VP2, VP3, and VP4 were cloned into pAdtrack-Flag. All cDNAs  
451 were PCR-amplified using Phanta Max Super-Fidelity DNA Polymerase (Vazyme,  
452 China). The PCR fragments were ligated to the expression plasmid using the  
453 ClonExpress II One Step Cloning Kit (Vazyme, China). All primers in this study used  
454 for plasmid construction are listed in Supplemental Table 1.

#### 455 **Transient transfection**

456 The plasmids were transfected into HPAC and HeLa cells according to the  
457 manufacturer's instructions (Thermo Fisher Scientific, USA). Cell ( $2 \times 10^5$ ) were  
458 seeded into six-well plates, and the fusion degree of cell monolayers reached  
459 70%-80% after 24-hour incubation. Then, 400  $\mu$ l DMEM or DF12, 4  $\mu$ g plasmid and  
460 6  $\mu$ l Turbofect (Thermo Fisher Scientific, USA) were combined. The DNA-Turbofect  
461 mixture was allowed to sit for 15-20 minutes, then the cells were washed twice with  
462 PBS, and 4 ml DMEM or DF12 supplemented with 10% fetal calf serum and  
463 DNA-TurboFect mixture were added.

#### 464 **Recombinant adenovirus construction, production and use**

465 To generate the adenovirus to express *VP2*, *VP3* and *VP4* proteins, *VP2*, *VP3*  
466 and *VP4* genes from CVB3 were cloned into the pAdtrack-CMV vector. The newly  
467 constructed pAdtrack-*VP2/VP3/VP4* vectors were linearized with Pme I digestion and  
468 then cotransformed with pAdEasy-1 vector (AdEasy Adenoviral vector system;  
469 Stratagene, USA)[49] into *E. coli* BJ5183 for homologous recombination, creating  
470 *VP2/VP3/VP4* Ad vectors. Additionally, all recombinant adenoviruses were packaged

471 and propagated using 293A cells. The infection was performed as described in  
472 previous studies[50, 51]. The titer of viral stocks was assessed using real-time PCR.

### 473 **Cell proliferation assay**

474 The effect of cell proliferation was analyzed using a CCK-8 assay. HPAC and  
475 HeLa cells were seeded in 96-well plates at the density of 3,000 cells into each well,  
476 and after nearly 24 hours of incubation, the two cells were transfected with pBud or  
477 pBud-VP1 plasmids at different times. A total of 10  $\mu$ l CCK-8 solution (Vazyme,  
478 China) was added each well in the plate and incubated for 2-4 hours in a 5% CO<sub>2</sub>  
479 incubator at 37°C, and the absorbance was recorded at 450 nm in a microplate reader  
480 (SpectraMAX® Paradigm®).

### 481 **EdU incorporation assay**

482 The capacity of DNA replication was determined using a  
483 5-Ethynyl-2'-deoxyuridine (EdU) assay according to the manufacturer's protocol  
484 (RiboBio, China). Cells in a logarithmic state were seeded in a 96-well culture plate at  
485 the density of 5,000 cells per well and incubated for 24 h, then transfected with  
486 plasmids pBud and pBud-VP1 for 0, 24, 48, 72 h. Next, cells were treated with 50  $\mu$ M  
487 EdU for 4 h (HPAC) or 2 h (HeLa), then fixed with 4% polyformaldehyde for 30 min,  
488 and finally incubated with 2 mg/ml Glycine for 5 min. After treating with 0.5% Triton  
489 X-100 for 10 min, cells were stained with Apollo 567 for 30 min, and then cell nuclei  
490 were incubated with Hoechst33342 for 30 min. All samples were measured using a  
491 fluorescence microscope (Olympus IX17).

### 492 **Flow cytometry**

493 HPAC cells ( $2 \times 10^5$ ) were seeded in each well of a six-well plate, then treated  
494 with plasmid (pBud and pBud-VP1) transfection and CVB3 infection within the  
495 treatment of double-thymidine. Cells were then released and collected at indicated  
496 time, the number of collected cells ranged from  $1-5 \times 10^6$ . The collected cells were



497 washed with cold PBS, fixed using 75% ethanol and stored overnight at 4°C. Before  
498 detection, the fixed cells were washed with cold PBS, bathed with RNase A at 37°C,  
499 and the cell adhesives were filtered with 400 mesh screen, then stained with  
500 propidium iodide (PI) for analysis, and kept away from light for 30 minutes. Flow  
501 cytometry analysis was used to determine cell percentages at different stages of the  
502 cell cycle (BD FACSCVerse).

### 503 **Antibodies**

504 Proteins were detected using the following primary antibodies: anti-enterovirus  
505 VP1 clone 5-D8/1 antibody purchased from Dako (Denmark); Mouse anti-Ub, Mouse  
506 anti-MAT1, Mouse anti-CDK7, and Mouse anti-Cyclin H antibodies purchased from  
507 Santa Cruz (USA); Mouse monoclonal to RNA polymerase II CTD and Rabbit  
508 monoclonal to RNA polymerase II CTD (phosphor S5) antibodies purchased from  
509 Abcam (USA); Mouse anti  $\beta$ -actin purchased from Proteintech; Rabbit monoclonal  
510 CDK2 and CDK4, Rabbit monoclonal phosphor-CDK2 and CDK4, Mouse  
511 monoclonal pRb, Rabbit monoclonal pRb-phospho Ser780, pRb-phospho Ser795 and  
512 pRb-phospho Ser807/811 antibodies purchased from Cell Signaling (USA); Rabbit  
513 polyclonal Lamin B1, Mouse monoclonal c-Myc, Mouse monoclonal HA-Tag and  
514 Mouse Monoclonal Flag antibodies purchased from Sigma (USA); Goat anti-mouse  
515 IgG horseradish peroxidase-conjugated secondary antibody and goat anti-rabbit IgG  
516 horseradish peroxidase-conjugated secondary antibody purchased from Thermo Fisher  
517 Scientific (USA).

### 518 **Western blot**

519 The total protein was washed with PBS and lysed with lysis buffer (150 mM  
520 NaCl, 20 mM Tris HCl, 0.1% NP-40, pH 7.4). The protein supernatant was collected  
521 after centrifugation. The same amount of protein was injected into a 10% SDS-PAGE  
522 gel for separation and transferred to a polyvinylidene fluoride (PVDF) membrane  
523 (Millipore, Germany). Cell membranes were blocked at room temperature for 2 hours

524 with 5% skim milk and incubated with primary antibodies overnight at 4°C, then  
525 incubated with secondary antibodies for 1-2 hours at room temperature. The protein  
526 was detected with an enhanced Chemiluminescence Kit (Thermo Fisher Scientific,  
527 USA).

### 528 **Co-immunoprecipitation**

529 The VP1 interactome was captured from the cells with Mouse anti-enterovirus  
530 VP1. Briefly, CVB3-infected cells at 3 hpi were lysed with lysis buffer (150 mM  
531 NaCl, 20 mM Tris HCl, 0.1% NP-40, pH 7.4) and nuclei were removed by a 10 min  
532 low-speed centrifugation step. Precleared cell lysates were incubated with or without  
533 monoclonal antibodies against MAT1 (1:500) or irrelevant IgG for 1 h, and for an  
534 additional hour with 20 µl Protein A/G Agarose beads (Thermo Fisher Scientific,  
535 USA) by gentle rotation at 4 °C. The beads were washed three times in lysis buffer  
536 with protease inhibitors, and two times in a wash buffer (100 mM Tris pH 7.4) with  
537 500 mM LiCl before suspending in 5× SDS sample buffer. The supernatant and crude  
538 extracts were analyzed using 10% SDS/PAGE, transferred to PVDF membranes  
539 (Millipore, Germany) and detected with specific antibodies against VP1 (1:1,000).

### 540 **Ubiquitin proteasomal degradation assay**

541 The ubiquitin proteasomal degradation was induced by HPAC cells with 10 µm  
542 MG132. Briefly, HPAC cells were seeded in six-well culture plates for 24 h and  
543 transfected with pBud-VP1 for 40 h, and then incubated with MG132 at a 10 µm final  
544 concentration for 8h. Cells were lysed with lysis buffer and supplemented with  
545 protease inhibitor PMSF, the cell lysate was centrifuged, and the supernatant was  
546 collected. Before incubation with 20 µl Protein A/G Agarose beads and specific  
547 antibodies, antibody against MAT1 or CDK7 and Protein A/G Agarose beads were  
548 additionally combined for 4 h, then incubated with protein supernatant overnight at  
549 4°C. Subsequently, the protein supernatant was washed three times with ice-cold lysis  
550 buffer and eluted in 5× SDS sample buffer. Western blot was performed for anti-Ub

551 and anti- $\beta$  actin antibodies with protein samples.

## 552 **Indirect immunofluorescence labelling and confocal microscopy**

553 To visualize the co-localization of VP1 and MAT1 in HPAC cells and the  
554 changes of their expression, the CVB3-infected HPAC cells and pBud  
555 VP1-transfected HPAC cells were incubated with fluorescein conjugated goat  
556 anti-rabbit IgG (1:200) and rhodamine-conjugated goat anti-mouse IgG (1:200) at  
557 room temperature for 1.5 h with primary antibodies. The primary antibodies used  
558 were rabbit anti-MAT1 (1:50), Mouse anti-enterovirus VP1 clone 5-D8/1 (1:50) and  
559 Mouse anti-CDK7 (1:50). The cells were rinsed extensively with PBS, and DAPI was  
560 used for counterstaining. The stained slides were analyzed with a FLUOVIEW  
561 FV1000 confocal laser scanning microscope (Olympus) with Olympus FV1000  
562 software.

## 563 **Yeast two-hybrid screen and assay**

564 Yeast two-hybrid screen and assay were performed as previously described[14].  
565 In order to determine which region of CVB3 VP1 interacts with MAT1, various  
566 truncated deletion mutants of VP1 were cloned into pGBKT7 bait vectors with the  
567 Matchmaker System. The yeast strain AH109 was co-transformed with  
568 pGADT7-MAT1 prey vector and bait vectors for expression of different truncated  
569 coding sequences of VP1 using lithium acetate. Transformants positive for prey-bait  
570 interaction were grown on selection plates lacking tryptophan, leucine, adenine and  
571 histidine but containing X- $\alpha$ -Gal.

## 572 **Protein-protein binding assay *in vitro***

573 An *in vitro* protein-protein binding assay was performed as previously  
574 described[14]. pGBKT7-VP1 bait vector and pGADT7-MAT1 prey vector were used  
575 as templates to transcribe and translate *in vitro*, then labeled with <sup>35</sup>S-Methionine  
576 (Amersham Pharmacia Biotech) *in vitro* transcription-translation system (Promega),

577 respectively, to obtain <sup>35</sup>S-labeled fusion proteins HA-MAT1 and c-Myc-VP1. The  
578 <sup>35</sup>S-Methionine-labeled HA-MAT1 and c-Myc-VP1 were incubated at room  
579 temperature, then incubated with antibody against c-Myc (Clontech) in lysis buffer,  
580 subsequently mixed with protein A/G plus-agarose (Thermo Fisher, USA) and  
581 incubated for 3 h at 4°C. The beads were washed three times with lysis buffer. The  
582 radioactive antibody–protein complexes were eluted, and subjected to SDS/PAGE and  
583 then autoradiography.

584

### 585 **Statistical analysis**

586 All statistical analyses were performed using GraphPad Prism 6.0 and SPSS  
587 software. The raw data are expressed as means ± standard error of the mean (s.e.m.)  
588 of at least three independent repeats. Unpaired two-tailed Student's *t*-tests were  
589 applied to analyze the differences for all comparisons in SPSS version 17.0. Band  
590 density on western blot was evaluated and quantified using Image J software.

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597 **Author contributions**

598 Lingbing Zeng and Xiaotian Huang conceived and designed the experiments;  
599 Hongxia Zhang, Lingbing Zeng, Qiong Liu, Guilin Jin, Jieyu Zhang and Zengbin Li  
600 performed the experiments. Hongxia Zhang and Qiong Liu analyzed the data;  
601 Hongxia Zhang, Lingbing Zeng and Xiaotian Huang wrote the manuscript.

602 **Conflicts of interest**

603 The authors declare that they do not have any conflicts of interest.

604

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724 **Figure legends**

725 **Figure 1. CVB3 VP1 affects cell proliferation and blocks cell cycle at the G1/S**  
726 **phase. (A)** The numbers of adherent HPAC cells in different groups were counted  
727 using Image Pro Plus (IPP) software. **(B)** HPAC cells were transfected by pBud-VP1  
728 and pBud plasmids, and the cell proliferation was evaluated using the CCK-8 assay.  
729 **(C)** The activity of DNA replication was examined using EdU incorporation in HPAC  
730 cells. **(D)** G1/S phase arrest induced by VP1 transfection. HPAC cells were treated  
731 with 36-hour plasmid transfection (pBud and pBud-VP1) within the treatment of  
732 double-thymidine, and collected at 0, 6 and 15 hpi separately, then cells were  
733 analyzed by flow cytometry. Values are shown as the mean  $\pm$  SEM of three  
734 independent experiments. (\* $P$ <0.05, \*\* $P$ <0.01).

735

736 **Figure 2. CVB3 VP1 may directly interact with MAT1. (A)** Plasmids  
737 pGBKT7-VP1 and pGADT7-MAT1 were co-transformed with yeast strain AH109,  
738 and the positive result selected on high-stringency medium (SD/-Ade/-His/-Leu/-Trp).  
739 Interaction is reflected by blue color, while white colonies suggest no interaction. **(B)**  
740 the VP1-MAT1 interaction identified by transcription-translation system *in vitro*.  
741 Plasmids HA-MAT1 and c-Myc-VP1 were labeled with  $^{35}\text{S}$ -Methionine, and  
742 immunoprecipitated with antibody against c-Myc; the binding proteins with  
743  $^{35}\text{S}$ -Methionine were analyzed by 10% SDS-PAGE and autoradiography. **(C)**  
744 Co-immunoprecipitation detected the interaction of VP1 and MAT1 in CVB3 infected  
745 cells. At 3 hours post infection, the cells lysates of mock and CVB3 infected cells  
746 were incubated with or without monoclonal antibody against MAT1 or irrelevant IgG  
747 affinity gel, and analyzed by western blotting with the specific antibodies after  
748 immunoprecipitation. **(D)** MAT1 and VP1 intracellular localization in CVB3 infected  
749 HPAC cells. Representative confocal immunofluorescence microscopic images of  
750 MAT1 and VP1 stained with rabbit anti-MAT1 (green) and mouse anti-VP1  
751 antibodies (red), respectively, the MAT1 and VP1 images were also merged; the  
752 nuclei are labeled with DAPI. Scale bar = 10  $\mu\text{m}$ . Values are shown as the mean  $\pm$

753 SEM of three independent experiments. (\* $P$ <0.05, \*\* $P$ <0.01).

754

755 **Figure 3. CVB3 VP1 impairs the assembly of a functional CAK complex. (A)**

756 Confocal microscopy analysis of the abundance of VP1 and MAT1 in HPAC cells,

757 Cells were detected with monoclonal antibodies to MAT1 (Alexa-488) and polyclonal

758 anti-VP1 (fluorescein; red), and counterstained with DAPI to show the nucleus. The

759 MAT1 and VP1 images were merged. Scale bar: 10  $\mu$ m. **(B)** Immunoblot analysis of

760 the nuclear-localized accumulation of MAT1, CDK7, Cyclin H in CVB3 infected,

761 VP1 and pBud transfected HPAC cells by specific antibodies. **(C)** CVB3 VP1 induces

762 ubiquitination-proteolysis of MAT1. The cell lysates of pBud-VP1 and pBud

763 transfected cells were incubated with monoclonal antibody against MAT1. The

764 generated blot was then analyzed by immunoblotting with anti-ubiquitin antibody. **(D)**

765 CVB3 VP1 induces ubiquitination-proteolysis of CDK7. Values are shown as the

766 mean  $\pm$  SEM of three independent experiments. (\* $P$ <0.05, \*\* $P$ <0.01).

767

768 **Figure 4. CVB3 VP1 alters the subcellular colocalization of MAT1 and CDK7 in**

769 **G1/S phase. (A)** Probe showing the time points when the cells were mainly at G1 and

770 S phases by flow cytometry. The cells were synchronized using double thymidine

771 block treatment, and released every hour. **(B)** Patterns of infection and transfection

772 time points. The cells are synchronized at G1 (46 hrs) and S phase (51 hrs) by

773 thymidine double blockade. **(C and D)** The alteration in spatial localization of MAT1

774 and CDK7 by thymidine double blockade at G1 and S phases, respectively. The cells

775 were transfected with pBud-VP1 and infected with CVB3, and then imaged using

776 confocal microscopy. Confocal image shows MAT1 (green), CDK7 (red), DAPI (blue)

777 and merged views. Scale bar: 10  $\mu$ m. The integrated optical density (IOD) was used

778 to represent the fluorescence using Image Pro Plus (IPP). Values are shown as the

779 mean  $\pm$  SEM of three independent experiments. (\* $P$ <0.05, \*\* $P$ <0.01)

780

781 **Figure 5. CVB3 VP1 could suppress CAK activity *in vivo*. (A)** Time-course

782 analysis of the accumulation of phosphorylated/nonphosphorylated CDK2 and CDK4

783 by indicated antibodies. **(B)** The expression level and phosphorylation of RNA Pol II  
784 CTD were detected by antibodies against RNA Pol II CTD and phospho-RNA Pol II  
785 CTD. CVB3 infection was the positive control. **(C)** Immunoblot analysis of the  
786 accumulation of phosphorylated/nonphosphorylated pRb with phospho-pRb Ser<sup>780</sup>,  
787 <sup>795, 807/811</sup> antibodies. CVB3 infection was the positive control. **(D)** The abundance of  
788 RNA Pol II CTD, phospho-RNA Pol II CTD and CDK7 were analyzed by Western  
789 blot. **(H)** Western blotting analysis of the expression of  
790 phosphorylated/non-phosphorylated pRb and CDK7 with phospho-pRb Ser<sup>780, 795,</sup>  
791 <sup>807/811</sup> and CDK7 antibodies.

792

793 **Figure 6. CVB3 VP1 inhibited the activity of CDK4/6 and Rb phosphorylation of**  
794 **the CDK-Rb signaling pathway in the G1/S transition.** Abemaciclib (5 mg/ml  
795 stock solution in DMSO) was diluted to a final concentration of 10  $\mu$ M in culture  
796 medium to treat cells. **(A)** Abemaciclib (Abema) further inhibits cell proliferation  
797 with VP1. An EdU assay was used to analyze the cell proliferation of Mock and  
798 pBud-VP1 transfected HPAC cells with or without abemaciclib treatment. **(B)**  
799 Abemaciclib further inhibits cell viability with VP1. A CCK-8 assay was used to  
800 analyze the cell viability of Mock and pBud-VP1 transfected HPAC cells with or  
801 without abemaciclib treatment. **(C)** Abemaciclib further inhibits phosphorylated pRb  
802 with VP1. Western blotting analysis of the expression of  
803 phosphorylated/nonphosphorylated pRb with phospho-pRb Ser<sup>780, 807/811</sup> antibodies.  
804 **(D)** CDK4/6 inhibitor further inhibits the cell cycle based on pBud-VP1. HPAC cells  
805 were treated with pBud and pBud-VP1 transfection within the treatment of the  
806 double-thymidine and abemaciclib, then released and separately collected at 12 and  
807 15 hpi; the right figure is flow cytometric results. Values are shown as the mean  $\pm$   
808 SEM of three independent experiments. (\* $P$ <0.05, \*\* $P$ <0.01)

809

810 **Figure 7. Mapping the minimum domain of VP1 interaction with MAT1.** **(A)**  
811 Construction of a series of VP1 truncation/deletion mutants. The dark blue box is the  
812 full-length sequence encoded by VP1, the gray boxes are the VP1 deletion mutants

813 that cannot interact with MAT1, the light blue boxes are the VP1 mutants that were  
814 detected to interact with MAT1, the D8 (red box) was the minimum domain of VP1  
815 for the interaction (768-859 aa) in the VP1 C-terminus. **(B)** The interaction between  
816 VP1-D8 and MAT1 arrested cell cycle at the G1/S phase. pBud, pBud-VP1,  
817 pBud-VP1-D4 and pBud-VP1-D8 transfected cells for 48 h, and the cells were  
818 harvested and analyzed by flow cytometry. **(C)** VP1-D8 transfection down-regulated  
819 the expression of MAT1. Immunoblot analysis of the abundance of MAT1 in pBud,  
820 VP1, VP1-D4 and VP1-D8 transfected cells. **(D)** VP1-D8 transfection down-regulated  
821 the phosphorylation of CDK2 and CDK4. Western blot analysis of the accumulation  
822 of phosphorylated/nonphosphorylated CDK2 and CDK4 in different groups. **(E)** The  
823 accumulation of Pol II CTD, phospho-RNA Pol II CTD and  $\beta$ -actin were analyzed by  
824 Western blot. **(F)** Western blotting analysis of the expression of  
825 phosphorylated/nonphosphorylated pRb with specific antibodies.  
826 as in (D) using indicated antibodies. Values are shown as the mean  $\pm$  SEM of three  
827 independent experiments. (\* $P$ <0.05, \*\* $P$ <0.01).

828

829 **Figure 8. A schematic diagram representing how CVB3 VP1 inhibits cell**  
830 **proliferation via impairing the CAK complex through its interaction with MAT1.**

831 As CVB3 invades HPAC and Hela cells, CVB3 VP1 interacts competitively with  
832 MAT1, leading to the degradation of CDK7, thus the assembly of the CAK complex  
833 is inhibited, and the activity of CAK is simultaneously inhibited. CAK affects the  
834 activity of cyclin-dependent kinases CDK2 and CDK4, and then inhibits the  
835 phosphorylation level of pocket Rb protein, inducing the persistent binding of Rb and  
836 E2F protein, and finally inhibits the expression of downstream transcription factors by  
837 E2F and cell cycle arrest occurs in the G1 phase. CAK also affects the  
838 phosphorylation of its substrate RNA Pol II CTD Ser<sup>5</sup>, which eventually leads to the  
839 inability of RNA Pol II to participate in cell transcription and cell cycle arrest at the  
840 G1 phase.

841

842 **Supplemental Figure 1. CVB3 VP1 results in a specific cytopathic effect to**  
843 **HPAC cells.** Cell adherence was observed at 48 h after transfection or infection  
844 through the comparative analysis of bright-field images. Scale bar = 100  $\mu$ m.

845

846 **Supplemental Figure 2. The expression levels of VP1 increased in a**  
847 **time-dependent.** Western blot analysis of VP1 expression in Mock, pBud, pBud-VP1  
848 and CVB3 groups.

849

850 **Supplemental Figure 3. CVB3 VP1 inhibits cell proliferation in HeLa cells. (A)**  
851 HeLa cells were transfected with pBud-VP1 and pBud plasmid, and the cell  
852 proliferations were separately evaluated using a CCK-8 assay. **(B)** The activity of  
853 DNA replication was examined using EdU incorporation in HeLa cells. **(C)** The  
854 number of adherent HeLa cells in different groups was counted using Image Pro Plus  
855 (IPP) software. Mean  $\pm$  range of values for the counts of cell adherence in replicate  
856 experiments.

857

858 **Supplemental Figure 4. CVB3 infection induces G1/S phase accumulation of**  
859 **HPAC and HeLa cells.** After HPAC and HeLa cells were treated with thymidine  
860 again, HPAC **(A)** and HeLa **(B)** cells were mock infected or infected with CVB3 at a  
861 MOI of 5. These cells were released from the thymidine block and collected  
862 according to the indicated release time (0, 6 and 9 or 12 hrs); the cells were analyzed  
863 by flow cytometry. The percentage of cells in each phase of the cell cycle is showed  
864 as mean  $\pm$  SEM of three independent experiments. (\* $P$  < 0.05, \*\* $P$  < 0.01).

865

866 **Supplemental Figure 5. Other structural proteins of CVB3 cannot arrest the cell**  
867 **cycle at the G1/S phase.** The structural proteins of CVB3 VP2, VP3 and VP4  
868 infected double-thymidine synchronized cells, with GFP as a control. These cells  
869 were then released and collected according to the indicated release time (0, 3, 6, or 9  
870 hrs). The percentage of cells in each phase of the cell cycle is shown as mean  $\pm$  SEM  
871 of three independent experiments. (\* $P$  < 0.05, \*\* $P$  < 0.01).

872

873 **Supplemental Figure 6. MAT1 and VP1 intracellular localization in CVB3**  
874 **infected HeLa cells.** Representative confocal immunofluorescence microscopic  
875 images of MAT1 and VP1 stained with rabbit anti-MAT1 (green) and mouse  
876 anti-VP1 antibodies (red); the nuclei are labeled with DAPI. Scale bar = 10  $\mu\text{m}$ .

877

878 **Supplemental Figure 7. Confocal microscopy analysis of the abundance of VP1**  
879 **and MAT1 in HeLa cells.** Cells were detected with monoclonal antibodies to MAT1  
880 (Alexa-488) and polyclonal anti-VP1 (fluorescein; red), and counterstained with  
881 DAPI to show the nucleus. The MAT1 and VP1 images were merged. Scale bar: 10  
882  $\mu\text{m}$ .

883

884 **Supplemental Figure 8.** Immunoblot analysis of the cytoplasmic-localized  
885 accumulation of MAT1, CDK7, Cyclin H in CVB3 infected, pBud-VP1 and pBud  
886 transfected cells by specific antibodies.

887

888



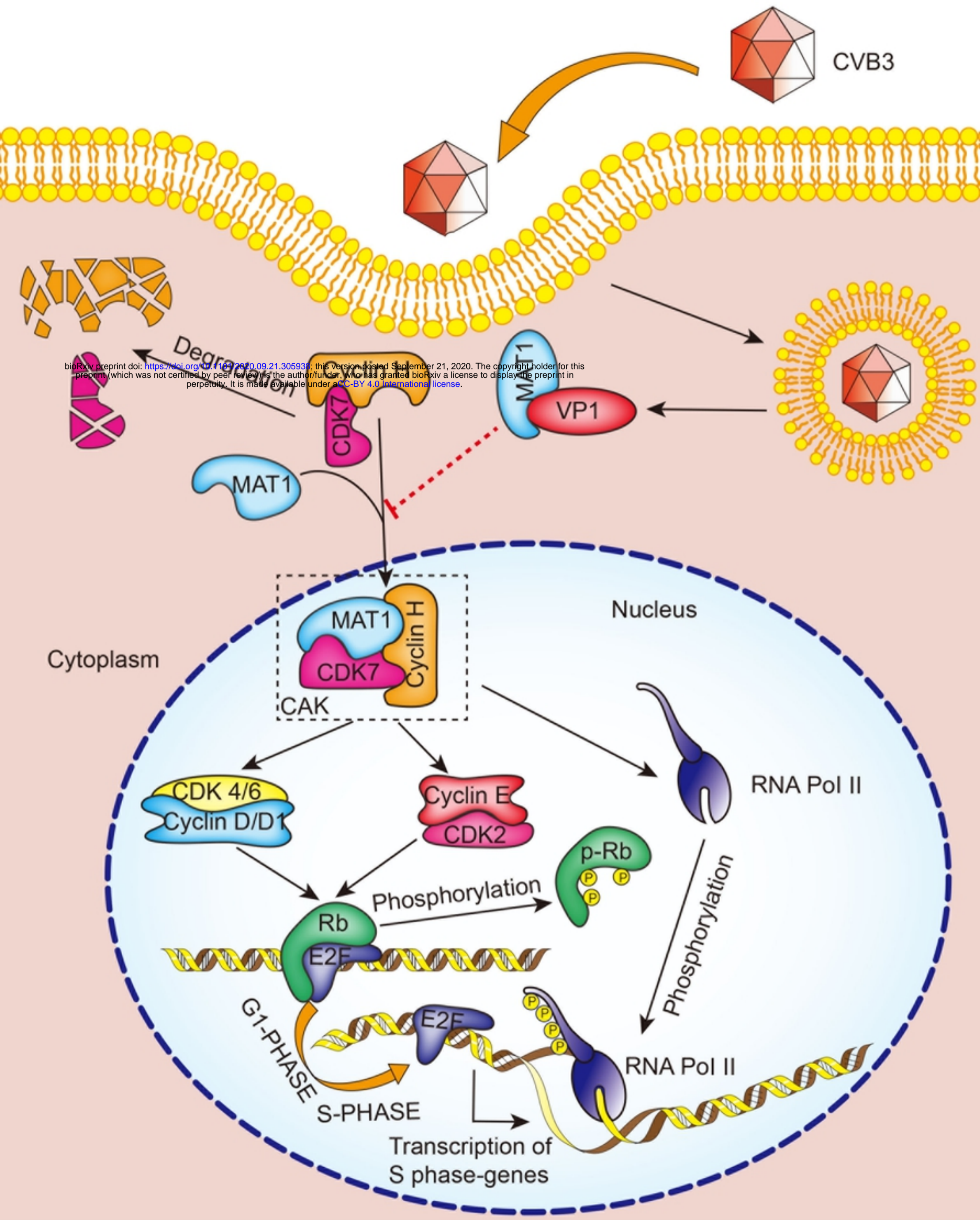


Figure 8



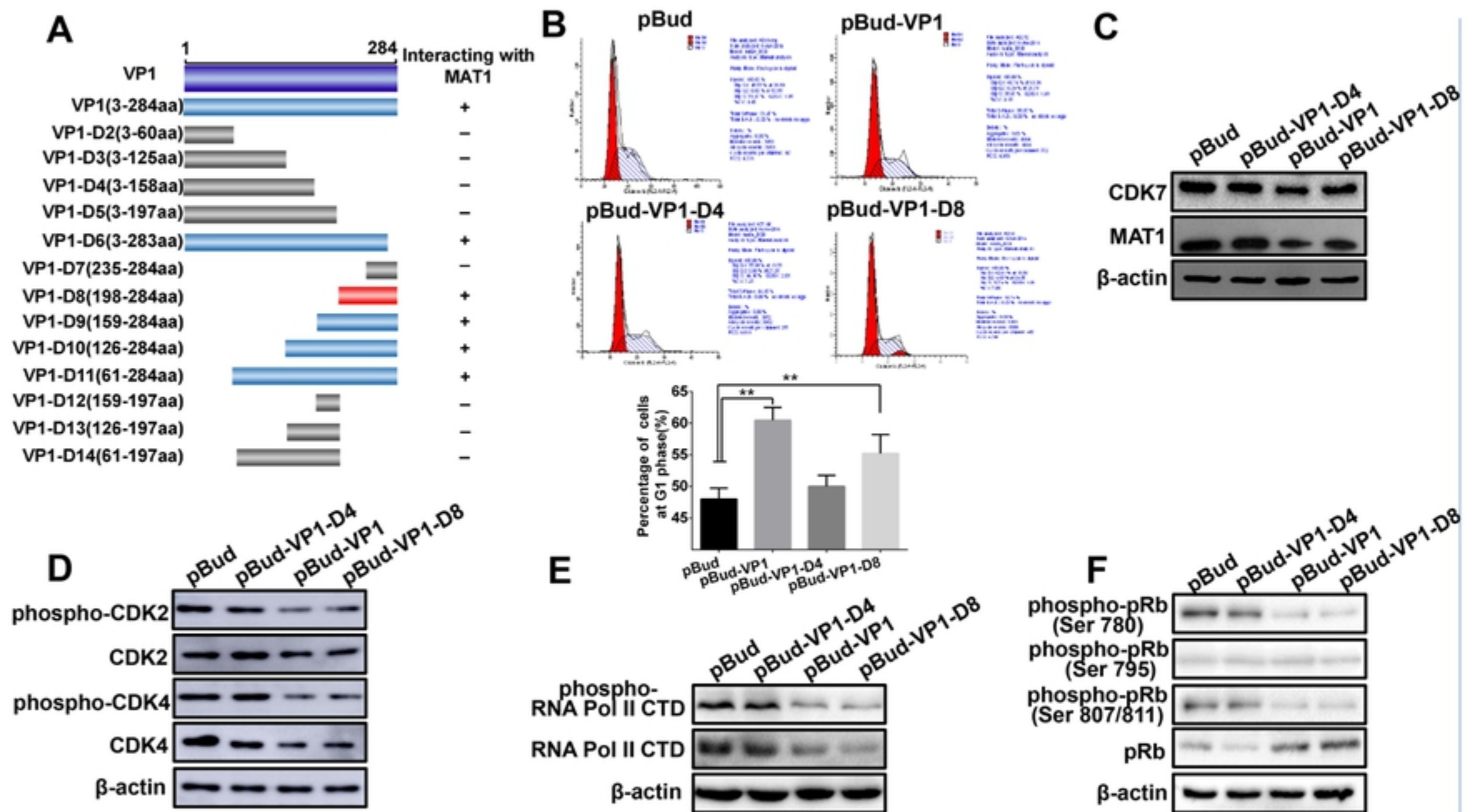


Figure 7



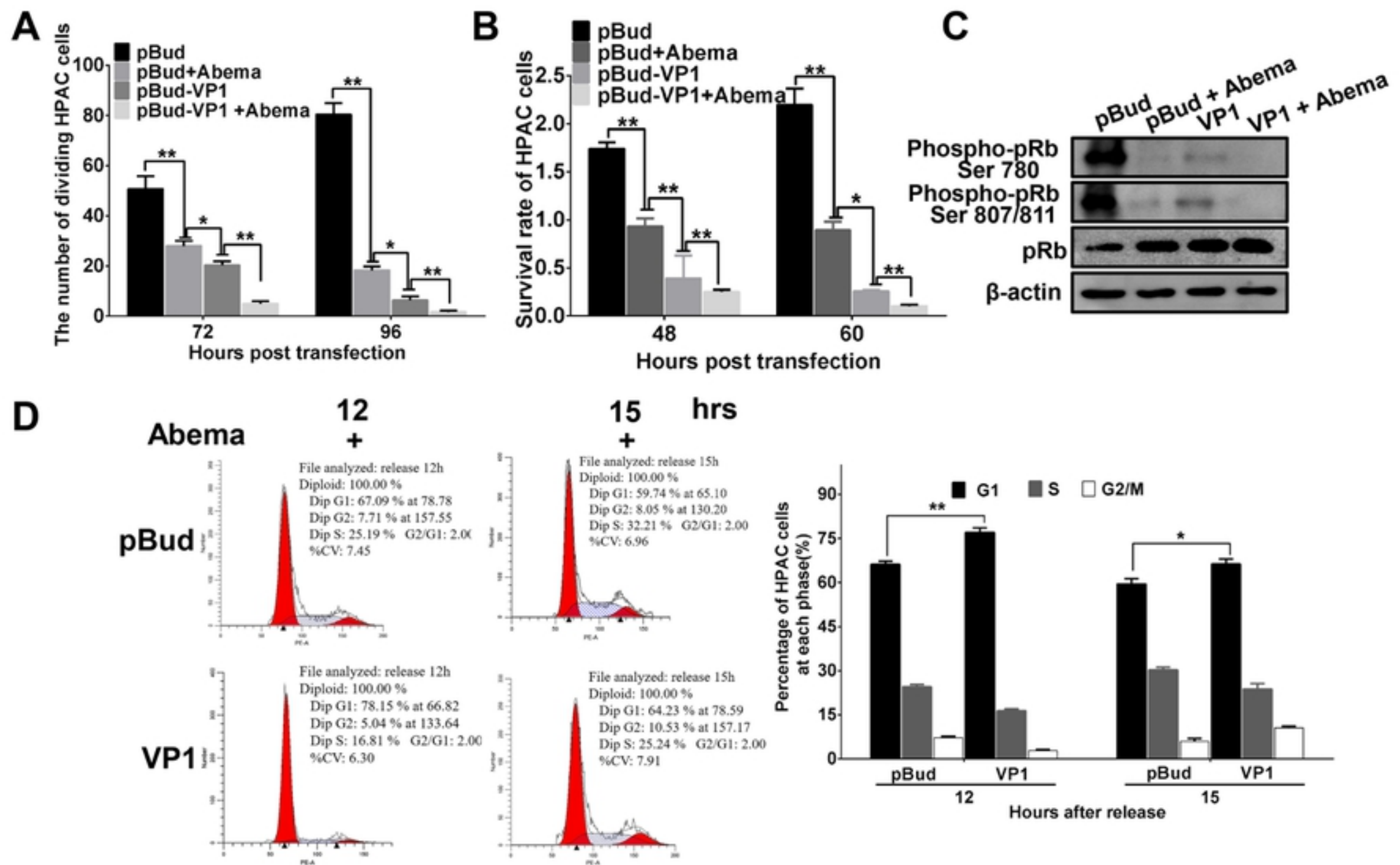


Figure 6

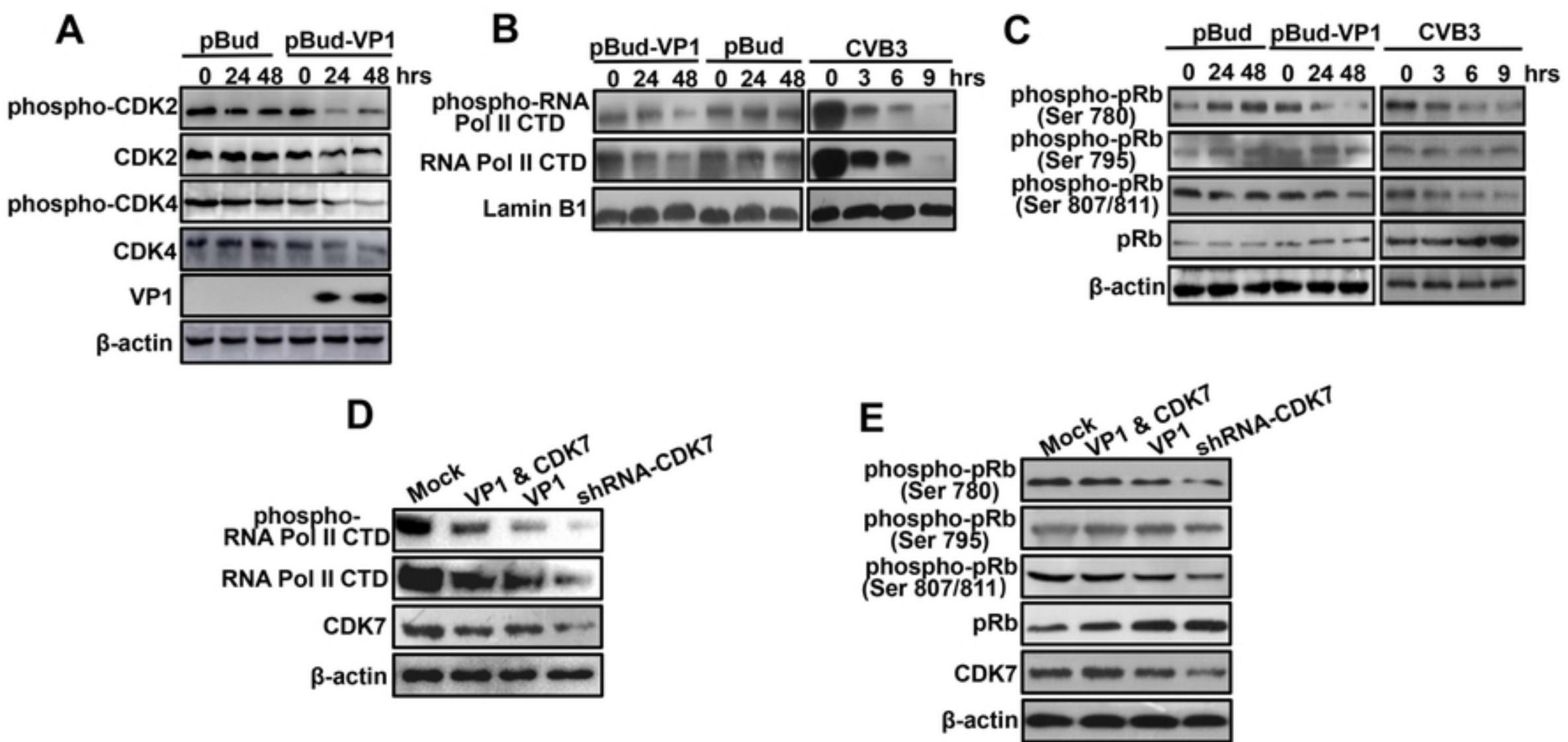


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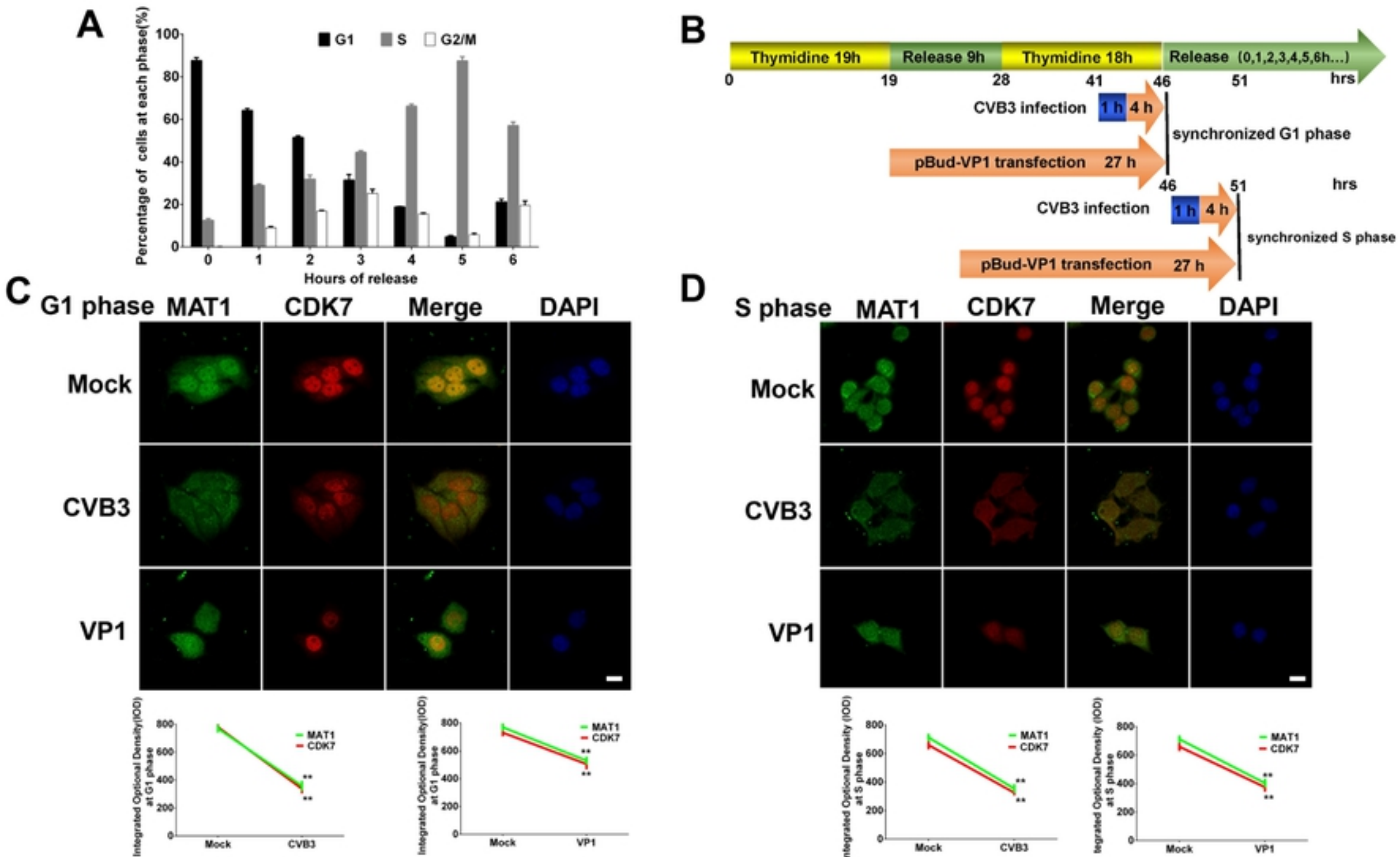


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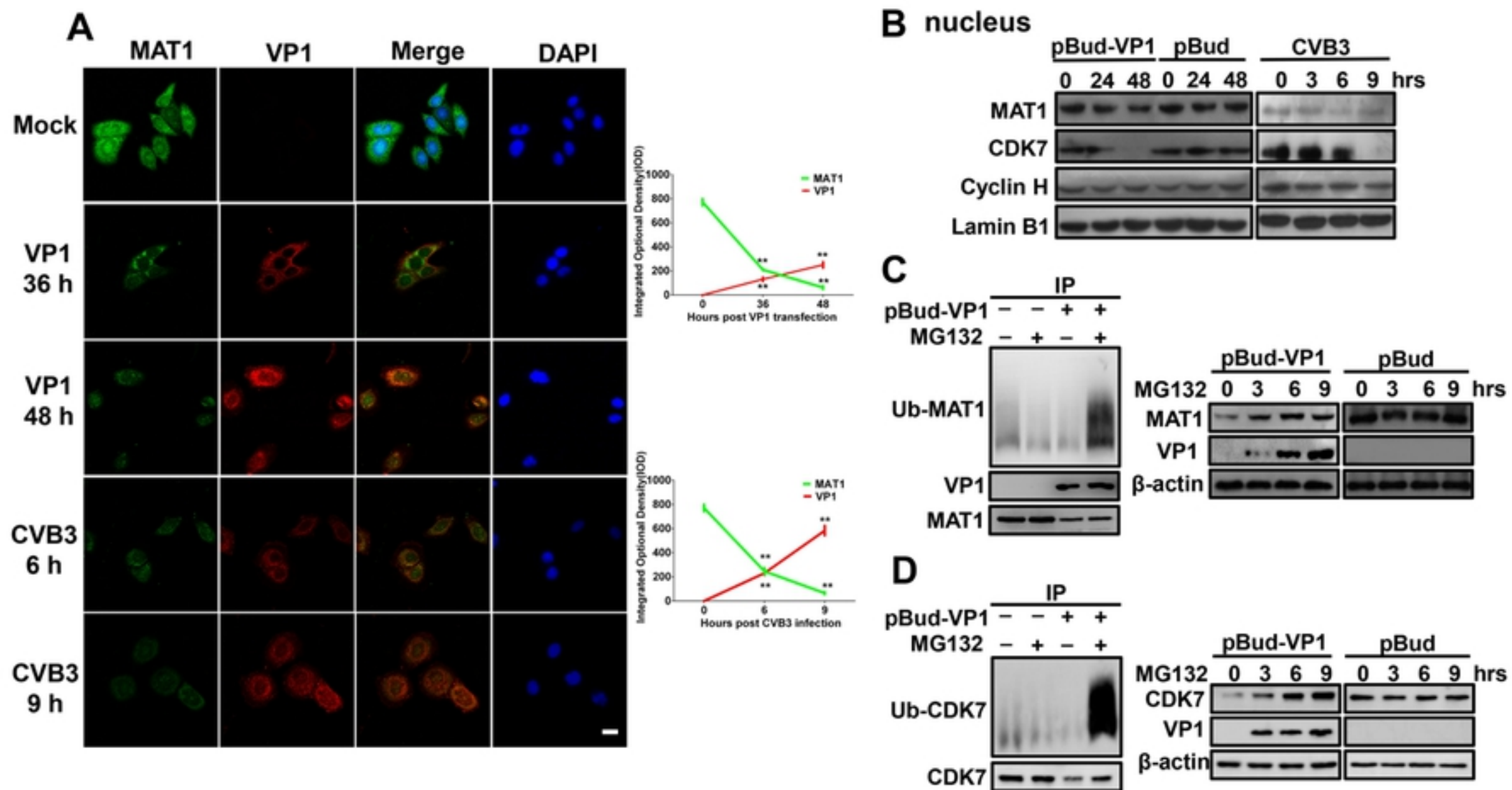


Figure 3



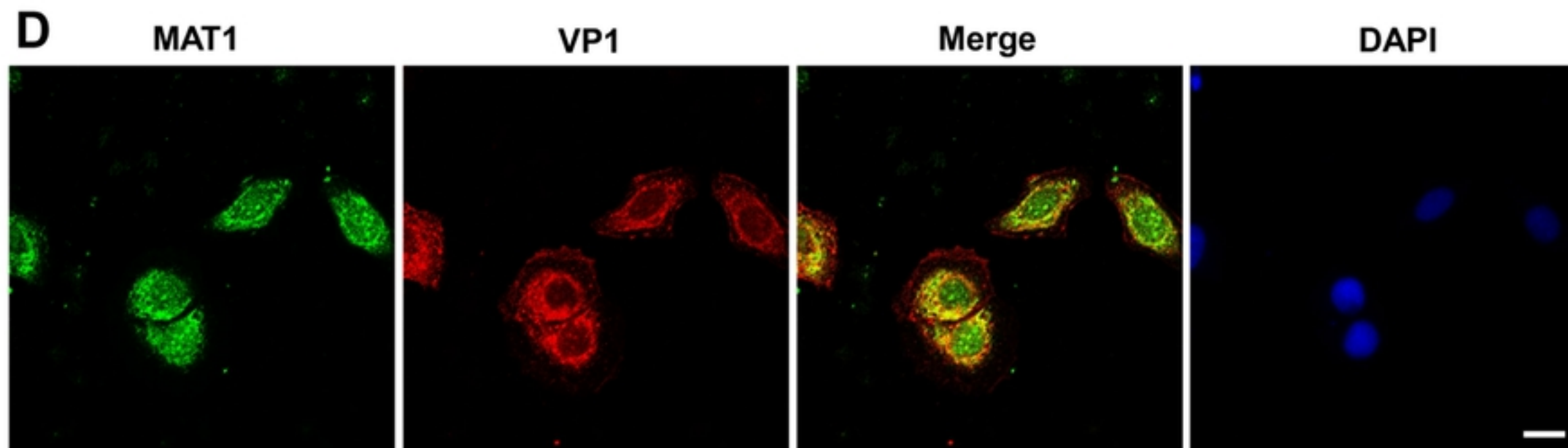
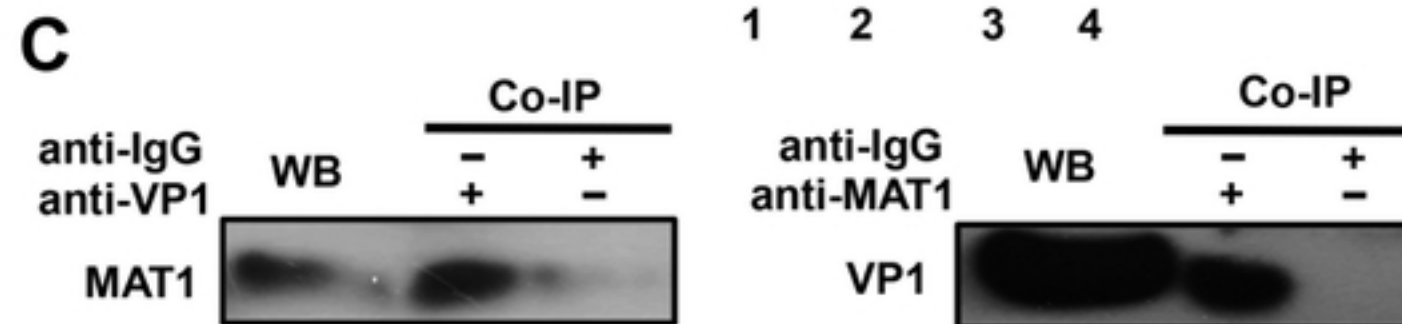
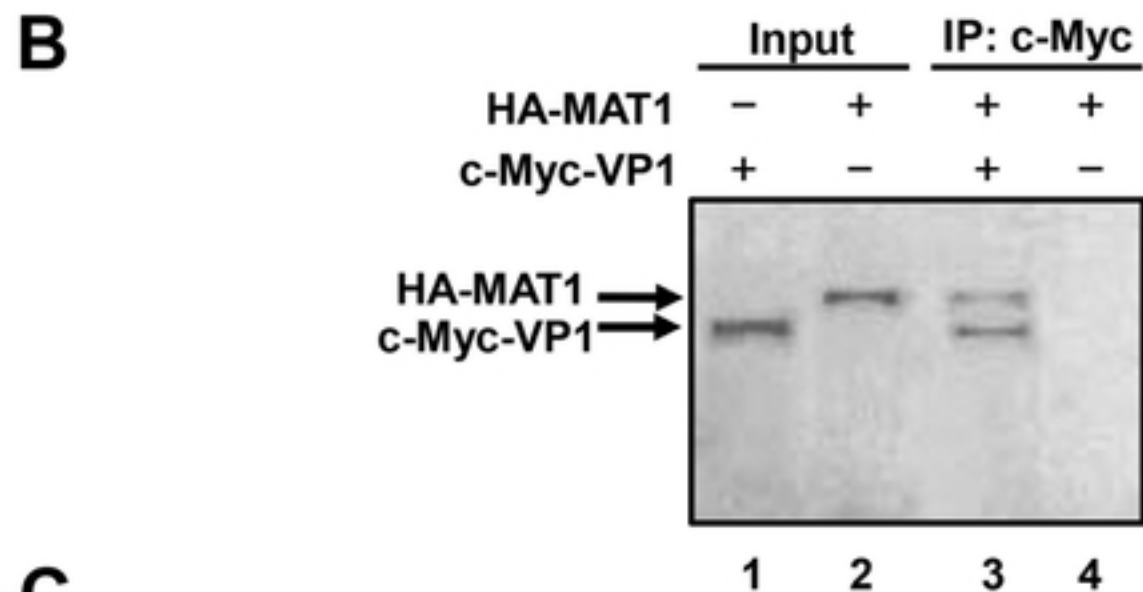
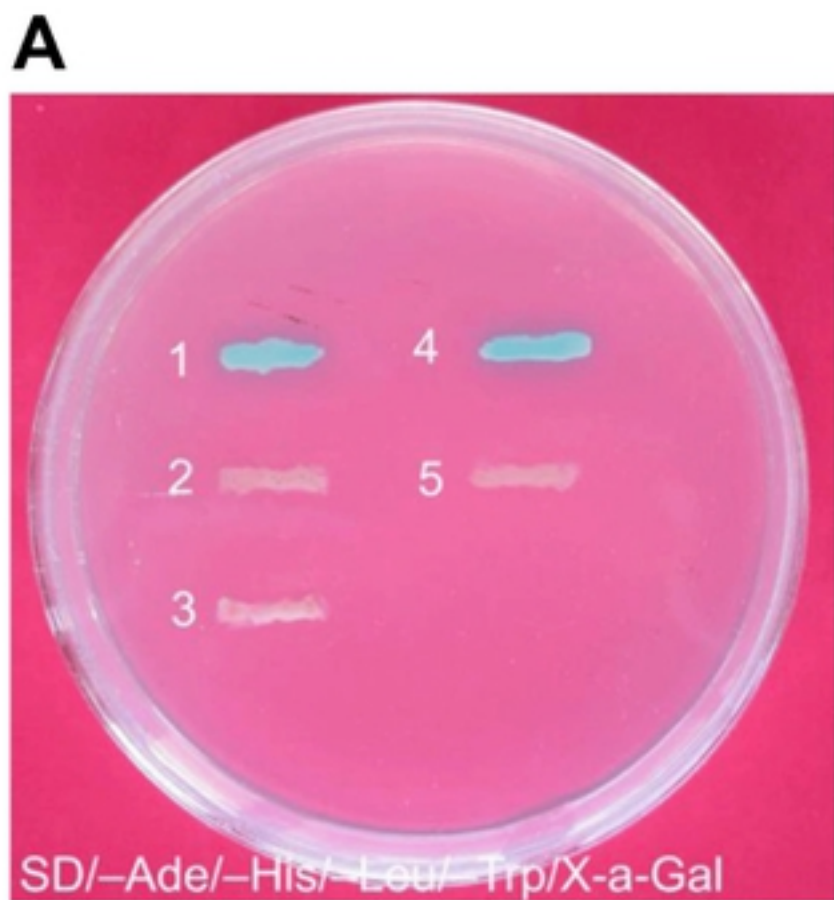


Figure 2

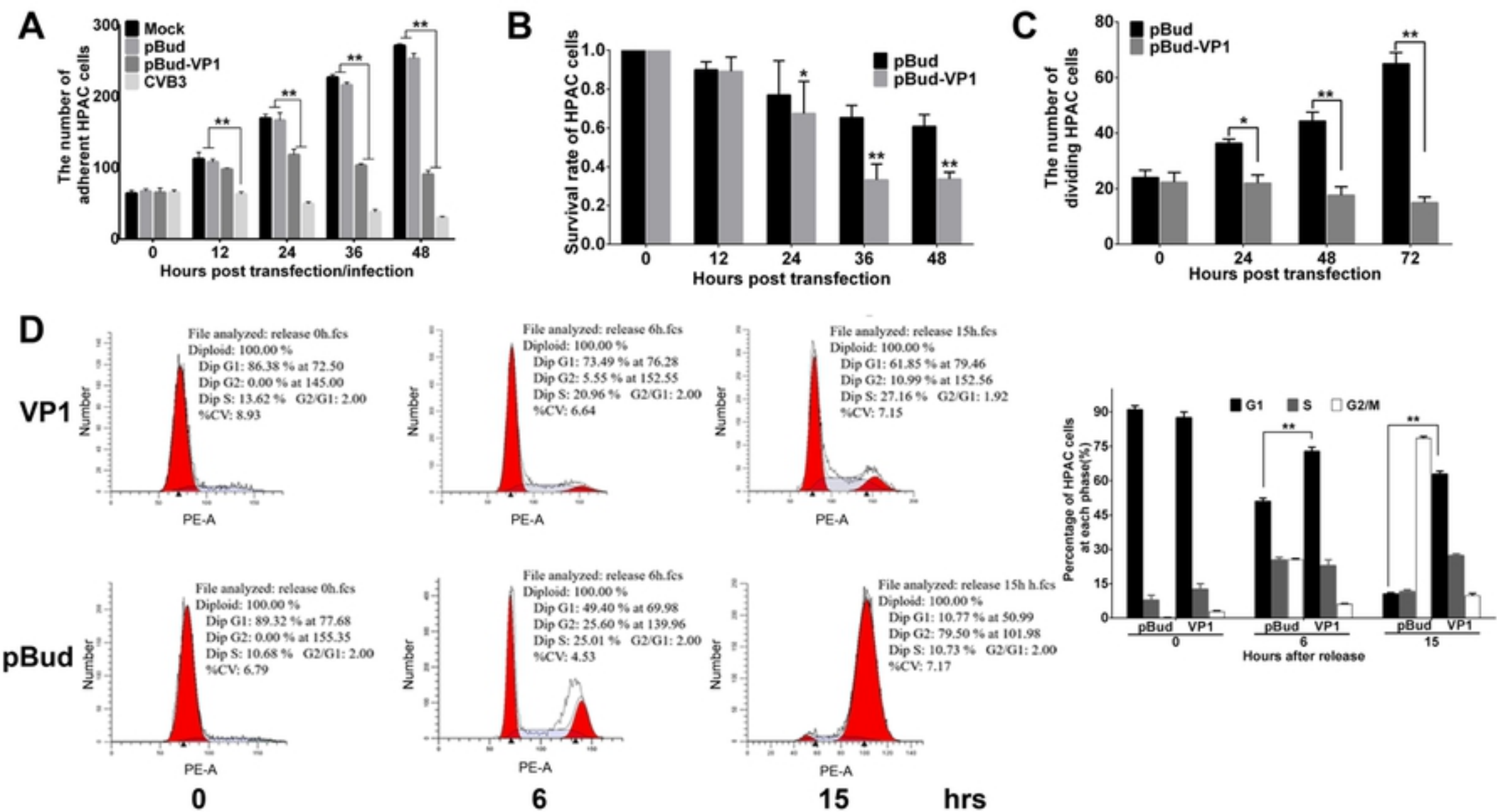


Figure 1