

1 **Epstein-Barr Virus Induced Cytidine Metabolism Roles in Transformed B-cell Growth**
2 **and Survival**

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4 Jin-Hua Liang¹⁻⁴, Chong Wang¹, Stephanie Pei Tung Yiu^{1-3,5}, Bo Zhao¹, Rui Guo¹⁻³ and
5 Benjamin E. Gewurz^{1-3,5*}

6 ¹ Division of Infectious Disease, Department of Medicine, Brigham and Women's Hospital,
7 Harvard Medical School, Boston, MA 02115, USA

8 ² Department of Microbiology, Harvard Medical School, Boston, MA 02115

9 ³ Broad Institute of Harvard and MIT, Cambridge, MA 02142, USA

10 ⁴ Department of Hematology, the First Affiliated Hospital of Nanjing Medical University,
11 Jiangsu Province Hospital

12 ⁵ Graduate Program in Virology, Division of Medical Sciences, Harvard Medical School,
13 Boston, MA 02115

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15 *Correspondence: bgewurz@bwh.harvard.edu

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17 **Abstract:**

18 **Epstein-Barr virus (EBV) is associated with 200,000 cancers annually, including B-cell**
19 **lymphomas in immunosuppressed hosts. Hypomorphic mutations of the *de novo***
20 **pyrimidine synthesis pathway enzyme cytidine 5' triphosphate synthase 1 (CTPS1)**
21 **suppress cell mediated immunity, resulting in fulminant EBV infection and EBV+**
22 **central nervous system (CNS) lymphomas. Since CTP is a critical precursor for DNA,**
23 **RNA and phospholipid synthesis, this observation raises the question of whether the**
24 **isozyme CTPS2 or cytidine salvage pathways help meet CTP demand in EBV-infected**
25 **B-cells. Here, we found that EBV upregulated CTPS1 and CTPS2 with distinct kinetics**
26 **in newly infected B-cells. While CRISPR CTPS1 knockout caused DNA damage and**
27 **proliferation defects in lymphoblastoid cell lines (LCL), which express the EBV latency**
28 **III program observed in CNS lymphomas, double CTPS1/2 knockout caused stronger**
29 **phenotypes. EBNA2, MYC and non-canonical NF- κ B positively regulated CTPS1**
30 **expression. CTPS1 depletion impaired EBV lytic DNA synthesis, suggesting that latent**

31 **EBV may drive pathogenesis with CTPS1 deficiency. Cytidine rescued CTPS1/2**
32 **deficiency phenotypes in EBV-transformed LCL and Burkitt B-cells, highlighting**
33 **CTPS1/2 as a potential therapeutic target for EBV-driven lymphoproliferative disorders.**
34 **Collectively, our results suggest that CTPS1 and CTPS2 have partially redundant roles**
35 **in EBV-transformed B-cells and provide insights into EBV pathogenesis with CTPS1**
36 **deficiency.**

37

38 **Key words:** tumor virus, mononucleosis, chronic active EBV, gamma-herpesvirus, nucleotide
39 metabolism, pyrimidine metabolism, gamma-herpesvirus, primary immunodeficiency, B-cell
40 deficiency, lymphoproliferative disease

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43 Introduction

44 Epstein-Barr virus (EBV) causes infectious mononucleosis and persistently infects 95% of
45 adults worldwide. For most individuals, lifelong EBV colonization of the memory B-cell
46 compartment is asymptomatic. Nonetheless, EBV is associated with 200,000 human cancers
47 per year, including Burkitt lymphoma, post-transplant lymphoproliferative diseases, Hodgkin
48 lymphoma, gastric and nasopharyngeal carcinoma (1, 2). Endemic Burkitt lymphoma caused
49 by EBV remains the most common childhood cancer in sub-Saharan Africa (3-5).

50 EBV is also associated with a growing number of rare congenital immunodeficiency
51 syndromes, in which impairment of cell mediated immune responses disrupts the host/virus
52 balance (6, 7). Inborn errors of immunity associated with severe EBV disease include
53 *SH2D1A* and *XIAP* mutations, which cause the X-linked lymphoproliferative diseases 1 and 2
54 syndromes, respectively (8). Severe EBV infection is also associated with deficiency of the
55 MAGT1 transporter, GATA2, the IL-2-inducible T-cell kinase, the RAS guanyl-releasing
56 protein 1 RASGRP1, CD27, CD70 and 4-1BB (6, 7, 9-13). Chronic active EBV and EBV+
57 lymphomas occur with gain-of-function PI3 kinase catalytic subunit mutations that cause
58 T-cell senescence (14).

59 Autosomal recessive mutations of the cytidine 5' triphosphate synthetase 1 *CTPS1*
60 gene cause a primary immunodeficiency syndrome characterized by impaired T-cell
61 proliferation, low numbers of invariant and mucosal-associated T-cells and NK cells, and
62 susceptibility to encapsulated bacterial and chronic viral infections, particularly by EBV and
63 the related varicella zoster virus (15, 16). Most *CTPS1* deficient patients experienced EBV
64 disease, including severe infectious mononucleosis, chronic EBV viremia and
65 EBV-associated primary CNS lymphomas (16). Extra-hematopoietic manifestations of
66 *CTPS1* deficiency have not been reported, and hematopoietic stem cell transplantation is
67 curative (17).

68 The nucleotide cytidine is a key precursor for DNA, RNA and phospholipid biosynthesis
69 as well as protein sialylation. Cytidine triphosphate (CTP) is maintained at the lowest
70 concentration of cellular nucleotide pools and its synthesis is tightly regulated (18, 19). CTP is
71 produced through *de novo* or salvage pathways. The isozymes *CTPS1* and *CTPS2* catalyze
72 the ATP-dependent transfer of nitrogen from glutamine to uridine triphosphate (UTP), thereby

73 producing CTP and glutamate. CTPS1 and 2 share 74% amino acid sequence identity (20,
74 21). CTP can also be synthesized by salvage pathways, where uridine or cytidine are
75 imported and phosphorylated by the enzymes uridine-cytidine kinase 1 or 2 (UCK1 or UCK2).
76 Cytidine monophosphate is subsequently converted to CTP by the kinases CMPK and NDPK
77 (**Figure 1a**). CTP synthases are the rate-limiting enzymes in *de novo* CTP synthesis (22),
78 and CTPS1 is rapidly and highly upregulated in primary human T-cells upon T-cell receptor
79 stimulation (23).

80 All patients described thus far with CTPS1 deficiency harbor a homozygous missense
81 G-> C mutation at base 1692 of the *CTPS1* gene (rs145092287) that alters an intronic splice
82 acceptor site. While the mutation does not alter CTPS1 catalytic activity, it causes 80-90%
83 loss of CTPS1 protein abundance (16). The high prevalence of B-cell driven EBV-associated
84 diseases in people with CTPS1 deficiency raises the question of how EBV-driven lymphomas
85 arise at high frequency, despite pronounced defects in lymphocyte metabolism. Patients with
86 CTPS1 deficiency have normal total T and B-cell numbers, though reduced frequency of
87 memory B-cells, the site of long-term EBV persistence (16). Whether CTPS2 may have more
88 important roles in EBV-infected B-cells than in T and NK cells, and how CTPS1 deficiency
89 alters the latent versus lytic stages of the EBV lifecycle remain unknown.

90 B-cells from individuals with CTPS1 deficiency exhibit defects in proliferation (16, 23).
91 Furthermore, the *de novo* pyrimidine synthesis enzyme DHODH inhibitor leflunomide impairs
92 growth of EBV-driven lymphomas in murine models (24). Nonetheless, continuously growing
93 EBV-transformed lymphoblastoid cell lines (LCLs) can be established from B-cells of patients
94 with CTPS1 deficiency (16, 23). LCLs express the EBV latency III program also observed in
95 primary CNS lymphomas, comprised by six Epstein-Barr nuclear antigens (EBNA) and two
96 latent membrane proteins (LMP) that mimic signaling by the B-cell receptor and CD40 (25,
97 26).

98 To gain insights into EBV pathogenesis in the setting of CTPS1 deficiency, we
99 characterized roles of CTPS1, CTPS2 and cytidine salvage pathways in support of
100 EBV-transformed B-cell growth and lytic replication.

101

102 **Results**

103 **EBV upregulates CTPS1 in newly infected B cells**

104 To gain insights into EBV effects on CTPS1 and CTPS2 expression, we profiled primary
105 human B-cells at rest and at five timepoints post infection. Whereas low levels of CTPS1 or
106 CTPS2 were detected in resting peripheral blood CD19+ B-cells, EBV markedly upregulated
107 both CTPS1 and CTPS2 mRNAs. CTPS1 mRNA reached maximum levels between days 4-7,
108 the period where transforming cells undergo Burkitt-like hyper-proliferation (27, 28). By
109 contrast, CTPS2 was progressively upregulated as newly infected cells converted to
110 lymphoblastoid physiology between days 7-14 post-infection (**Figure 1b**). Similar effects
111 were evident on the protein level (**Figure 1c**), where CTPS1 exhibited comparable
112 expression patterns to Epstein-Barr nuclear antigen 2 (EBNA2) and MYC, each of which are
113 important metabolism regulators. Using our recently published RNAseq and proteomic maps
114 of EBV-driven B-cell growth transformation (28, 29), EBV was also found to upregulate nearly
115 all components of the pyrimidine *de novo* and salvage pathways at similar timepoints (**Figure**
116 **S1**).

117 CTPS1 and 2 are upregulated in primary B cells activated by combinatorial mitogenic
118 stimuli, including by CD40-ligand (CD40L) plus interleukin-4 (IL-4), but responses to
119 individual stimuli have not been studied (23). To cross-compare immune receptor and EBV
120 effects on CTPS1 and CTPS2 expression, CD19+ primary B-cells were stimulated for 24
121 hours with CD40L, the Toll-like receptor 9 agonist CpG, α IgM, the cytokine IL-4, or
122 combinations thereof (**Figure 1d-e**). Interestingly, CD40L or CpG alone were sufficient to
123 upregulate CTPS1 mRNA and protein to near maximal levels, whereas CTPS2 was
124 significantly induced on the mRNA but not protein level in response to most B-cell agonists
125 (**Figure 1d-e**). These results highlight dynamic CTPS1 and CTPS2 regulation that varies over
126 phases of EBV-mediated B-cell transformation and in response to particular receptor
127 agonists.

128

129 **CTPS1 is important for EBV-infected B-cell outgrowth**

130 Since LCLs from CTPS1 deficient patients retain 10-15% CTPS1 expression, this
131 residual CTPS1 activity may be necessary for EBV-transformed B-cell growth or survival.
132 Alternatively, CTPS2 redundancy may support these functions. To investigate these

133 possibilities, we first re-analyzed data from our recent human genome-wide CRISPR/Cas9
134 growth and survival screen (30). Strong selection against all four single guide RNAs (sgRNAs)
135 targeting the *CTPS1* gene was evident at screen day 21 of the LCL GM12878 and the EBV+
136 Burkitt cell line P3HR-1. By contrast, sgRNAs against *CTPS2* or the genes encoding
137 pyrimidine salvage pathway enzymes UCK1 or UCK2 were not strongly selected against,
138 suggesting that functional knockout of their targets were tolerated by these EBV-transformed
139 B-cell lines (**Figure 2a**). Next, CRISPR mutagenesis was performed with *CTPS1* targeting
140 sgRNAs to characterize acute *CTPS1* loss-of-function effects on Burkitt and LCL proliferation
141 (**Figure 2b**). Live cell numbers were measured at five timepoints following expression of
142 control versus independent *CTPS1* targeting sgRNAs and puromycin selection. *CTPS1*
143 depletion significantly impaired growth of the EBV+ Burkitt cell line Daudi, which has latency I
144 expression, and of the LCL GM12878 (**Figure 2b**). *CTPS1* editing also diminished
145 proliferation of GM11830 LCLs and Mutu I Burkitt cells (**Figure S2a-b**). A low level of
146 proliferation was nonetheless observed in *CTPS1* depleted cells, suggesting alternative
147 routes of CTP synthesis.

148 To examine effects of *CTPS1* depletion on B-cell survival, 7-AAD vital dye exclusion
149 assays were performed on LCLs and Burkitt cells 5 days post sgRNA expression and
150 puromycin selection, the timepoint at which growth curves of control and *CTPS1* depleted
151 cells diverged. Loss of *CTPS1* significantly increased 7-AAD uptake in GM12878 and Daudi
152 cells (**Figure 2c-d**) and in a second LCL and Burkitt pair (**Figure S2c-d**), indicating induction
153 of cell death. Cell cycle analysis demonstrated that *CTPS1* editing also impaired cell growth,
154 with arrest most pronounced at the G1/S stage (**Figure 2e and Figure S2e-f**). Depletion of
155 *CTPS1* induced phosphorylation of H2AX at Ser 139 (γ -H2AX), indicative of DNA damage,
156 perhaps results from imbalance in CTP nucleotide pools. PARP cleavage was also evident,
157 indicating apoptosis induction (**Figure 2g, Figure S2g**).

158

159 **Compensatory *CTPS2* role in EBV-transformed B-cell outgrowth**

160 EBV+ CNS lymphomas are often observed in patients with *CTPS1* deficiency, which are
161 estimated to have 10-20% residual *CTPS1* activity (16). Yet, the CRISPR Cancer
162 Dependency Map (DepMap) (31) found that targeting of *CTPS2* only mildly affects

163 proliferation of a broad range of cancer cells, including B-cell lymphomas (**Figure S3a**).
164 These observations raise the question of the extent to which CTPS2 can be recruited to
165 support *de novo* salvage CTP synthesis in EBV-transformed B-cells in the context of
166 hypomorphic CTPS1 activity.

167 To address this question, we next tested the effects of CTPS2 editing in LCLs and Burkitt
168 cells, alone or in the context of CTPS1 depletion. In contrast to CTPS1 depletion, CTPS2
169 targeting did not significantly impair LCL or Burkitt B-cell growth or survival (**Figure 3a-d**,
170 **Figure S3b-d**). However, concurrent CTPS1/2 targeting nearly completely abrogated LCL
171 outgrowth, to a greater extent than depletion of CTPS1 alone (**Figure 3a-d**, **Figure S3e-f**).
172 Cytidine supplementation rescued outgrowth of CTPS1 or CTPS1/2 doubly deficient
173 GM12878 LCLs or Daudi Burkitt cells (**Figure 3e-h**), suggesting that CRISPR effects were on
174 target and demonstrating that the cytidine salvage pathway was capable of restoring
175 sufficient CTP pools. Interestingly, withdrawal of exogenous cytidine at Day 7 of the
176 proliferation assay rapidly blocked outgrowth of CTPS1 deficient and CTPS1/2
177 doubly-deficient GM12878 and Daudi cells (**Figure 3i-l**), further underscoring their acquired
178 dependence on salvage CTP metabolism. CTPS1/2 depletion caused DNA damage,
179 apoptosis induction and cell death in GM12878 and Daudi, as judged by immunoblot for
180 cleaved-PARP, phospho γ H2AX signal (**Figure 3m**). 7-AAD uptake was higher following dual
181 CTSP1/2 targeting (**Figure 3n**).

182

183 **CTPS1 Transcription upregulation in EBV-transformed B-cells**

184 The T-cell receptor and ERK pathways are important for rapid CTPS1 expression in
185 activated T-cells. As pathways that control B-cell CTPS1 induction remain uncharacterized,
186 we next investigated factors important for *CTPS1* expression in EBV-transformed B-cells.
187 First, we used our and ENCODE published LCL CHIP-seq datasets to characterize
188 occupancy of EBV nuclear antigens and EBV LMP1-activated NF- κ B transcription factors
189 (32-34). This highlighted *CTPS1* promoter region occupancy by EBV transcription factor
190 EBNA-LP, by multiple NF- κ B transcription factor subunits, by c-MYC and MAX (**Figure 4a**).
191 Interestingly, EBNA2, LP, 3A, 3C and NF- κ B subunits RelB and p52 co-occupied an
192 intergenic region ~12 kilobases downstream from the *CTPS1* promoter. RNA pol II GM12878

193 Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET) analysis identifies
194 a putative long-range interaction between Pol II bound to this *CTPS1* intragenic region and
195 the *CTPS1* promoter (35).

196 To study the functional significance of the EBNA and NF- κ B-bound *CTPS1* intragenic
197 region in *CTPS1* transcription regulation, we used CRISPR-interference (CRISPR-i) (36).
198 GM12878 LCLs with endonuclease dead Cas9 fused to a KRAB transcription repressor
199 domain were transduced with lentiviruses that express control sgRNA versus one of the
200 twelve sgRNAs indicated in **Figure 4a**. Following puromycin selection, CRISPR-i effects on
201 steady state *CTPS1* mRNA abundance were quantified by real time PCR. Four of the six
202 sgRNAs targeting the putative enhancer significantly reduced *CTPS1* mRNA levels. By
203 contrast, none of the six sgRNAs targeting upstream or downstream control regions
204 diminished *CTPS1* mRNA (**Figure 4a-b**). These results suggest that the EBV latency III
205 growth program induces *CTPS1* induction through effects at the *CTPS1* promoter and at a
206 downstream interacting region.

207 EBNA2 and EBNA-LP are the first EBV proteins expressed in newly infected cells. To
208 gain insights into potential EBNA2 roles in EBV-driven *CTPS1* induction, we infected human
209 peripheral blood CD19+ B-cells, isolated by negative selection, with transforming B95.8 EBV,
210 with ultraviolet (UV) inactivated B95.8, or with non-transforming P3HR-1 EBV that lacks
211 EBNA2 and part of the EBNA-LP open reading frames. Only infection by B95.8 induced MYC,
212 a well characterized regulator of metabolism, as well as *CTPS1* by 48 hours of infection
213 (**Figure 4c**). These data suggest that EBNA2 and/or EBNA-LP, rather than an innate immune
214 response to the incoming viral particle, are required for *CTPS1* induction in newly infected
215 B-cells. We next used 2-2-3 LCLs with conditional EBNA2 expression, in which EBNA2 is
216 fused to the ligand binding domain of a modified estrogen receptor that binds to
217 4-hydroxy-tamoxifen (4HT), to study EBNA2 roles in LCL *CTPS1* expression. When grown in
218 non-permissive conditions in the absence of 4HT for 48 hours, *CTPS1* expression was
219 rapidly lost, as was expression of EBNA2 target gene MYC (**Figure 4d**). Thus, EBNA2,
220 perhaps together with its host target genes, are important for EBV-driven *CTPS1* induction.

221 To characterize putative *CTPS1* induction roles of the key host EBNA2 target MYC, we
222 used p493-6 LCLs, which carry conditional 4HT-controlled EBNA2 and tetracycline-repressed

223 *MYC* alleles (37). EBNA2 inactivation by 4HT withdrawal again caused loss of CTPS1 and
224 *MYC* expression in cells cultured with doxycycline to suppress exogenous *MYC* (**Figure 4e**).
225 Interestingly, induction of exogenous *MYC* expression by doxycycline withdrawal restored
226 CTPS1 expression in the absence of EBNA2 activity (**Figure 4e**). Similarly, *MYC* knockout in
227 GM12878 blocked CTPS1 expression at a timepoint prior to induction of cell death (**Figure**
228 **4f**), further supporting a key role for *MYC* in CTPS1 regulation and providing a potential route
229 for CTPS1 induction in Burkitt cells.

230 The EBV-encoded CD40 mimic LMP1 activates the NF- κ B canonical and
231 non-canonical pathways to trigger nuclear translocation of the five NF- κ B transcription factor
232 subunits. Since GM12878 ChIP-seq identified *CTPS1* locus occupancy by all NF- κ B subunits,
233 we used CRISPR to individually test their roles in LCL CTPS1 expression (**Figure 4g**).
234 Depletion of RelB, p50 and p52 each significantly reduced CTPS1 steady-state mRNA levels
235 (**Figure 4h**), implicating the non-canonical NF- κ B pathway. In support, a κ B kinase
236 (IKK)- α -selective antagonist did not visibly reduce CTPS1 protein levels at a dose where the
237 canonical pathway is selectively blocked, but impaired CTPS1 expression at a higher dose
238 that also blocks the kinase IKK β , which controls the non-canonical pathway (**Figure 4i**). By
239 contrast, ERK inhibition by an antagonist that blunts T-cell CTPS1 expression (23) failed to
240 reduce CTPS1 expression, in spite of LMP1's ability to activate ERK (**Figure 4j**). Thus,
241 distinct mechanisms that control CTPS1 expression in EBV+ B-cells versus T-cells.

242

243 **EBV+ B-cells use *de novo* and salvage UTP metabolism**

244 Uracil monophosphate (UMP), a key precursor in *de novo* CTP synthesis, can be
245 synthesized from L-glutamine and L-aspartate by the sequential enzymatic activities of CAD,
246 DHODH and UMPS. UMP can also be produced by UCK1 or UCK2 phosphorylation of
247 imported uridine (**Figure 1a**). Interestingly, UCK was originally identified as a B-cell factor that
248 associates with EBNA3A, suggesting possible roles in EBV-driven B-cell proliferation (38). To
249 gain insights into potential DHODH versus UCK1/2 roles in newly infected primary B-cells,
250 immunoblot analysis was performed on extracts from cells prior to and at five timepoints
251 post-EBV infection. UCK2 was robustly induced by day 4, whereas DHODH expression was
252 suppressed by day 10, despite each being induced on the mRNA level (**Figure 5a, Figure**

253 **S4a-b)**. By comparison, UCK1, 2 and DHODH mRNAs were upregulated by multiple primary
254 B-cell stimuli, while again UCK2 but not DHODH was upregulated on the protein level (**Figure**
255 **5b, Figure S4c)**.

256 DHODH inhibition prevents EBV lymphomas in a humanized mouse model (24). We
257 next used CRISPR to test the effects of DHODH depletion in LCLs or Burkitt cells. In media
258 containing undialyzed fetal bovine serum (FBS), which contains uridine (**Figure 1a**),
259 independent DHODH sgRNAs impaired proliferation of both LCLs and of Mutu I, but not
260 Daudi Burkitt cells (**Figure 5c-f**). To more closely examine the ability of EBV-transformed
261 B-cells to use uridine as a substrate for *de novo* CTP synthesis, control and DHODH edited
262 LCL and BL were grown with dialyzed FBS lacking uridine. In this context, DHODH depletion
263 more strongly inhibited proliferation, which was rescued by uridine add-back (**Figure 5g-j**,
264 **Figure S5a-d**). Thus, LCLs can use amino acid *de novo* or uridine salvage for CTP synthesis
265 (**Figure 1a**).

266 We next used CRISPR to test the effects of UCK1 and/or UCK2 depletion on
267 EBV-transformed B-cell proliferation. Successful CRISPR editing of UCK2 was confirmed by
268 immunoblot, whereas UCK1 editing was confirmed by T7 E1 assay, as we were unable to find
269 a suitable immunoblot antibody. Unexpectedly, depletion of UCK1, UCK2 or UCK1/2 did not
270 significantly impair GM12878 LCL or Burkitt cell proliferation (**Figure 5k-l, Figure S5e**).
271 These results suggest that LCLs and Burkitt cells can synthesize sufficient CTP *de novo*
272 when sufficient glutamine and aspartate are available, but that they are nonetheless able to
273 switch to uridine salvage when necessary.

274

275 **CTPS1 roles in EBV lytic DNA replication**

276 Knowledge remains incomplete of how EBV remodels CTP synthesis pathways in
277 support of the B-cell lytic cycle, where the viral immediate early transcription factors BZLF1
278 and BRLF1 induce early genes responsible for viral DNA replication and subsequently
279 expression of viral late genes. Lytic replication results in synthesis of hundreds to thousands
280 of EBV ~170 kilobase genomes by the viral DNA polymerase (39). To test *de novo* cytidine
281 roles in EBV lytic replication, control or *CTPS1* targeting sgRNAs were expressed in EBV+
282 Akata Burkitt cells, a model for EBV lytic replication. Following CRISPR editing, lytic

283 replication was induced by B-cell receptor cross-linking for 24 hours. Interestingly, while
284 CTPS1 depletion did not appreciably affect expression of the immediate early gene BZLF1 or
285 early gene BMRF1, production of lytic EBV genomes was significantly impaired by
286 independent CTPS1 sgRNAs (**Figure 6a**). Lytic EBV genome replication was rescued by
287 cytidine add-back, suggesting that either the *de novo* or salvage CTP pathways can support
288 the viral lytic cycle (**Figure 6b**). Similar results were obtained in P3HR-1 Burkitt cells, in which
289 lytic replication was induced by a conditional *BZLF1* allele together with sodium butyrate
290 (**Figure 6c-d**).

291

292 **Discussion**

293 The ability of B and T lymphocytes to rapidly switch from a quiescent state into rapidly
294 growing blasts upon antigen receptor and co-receptor stimulation underlies adaptive
295 immunity. CTPS1 is highly induced by T-cell stimulation through the ERK pathway and is
296 necessary for rapid T-cell proliferation and the control of acute EBV infection (23). Yet, with
297 hypomorphic CTPS1 activity, patients often exhibit severe infectious mononucleosis, chronic
298 active EBV and EBV+ CNS B-cell lymphomas. Since EBV relies on host metabolism
299 pathways to convert newly infected cells into hyperproliferating blasts and to support lytic
300 replication, these clinical observations raise the question of how individuals with CTPS1
301 deficiency exhibit elevated EBV viral loads and frequent B-cell lymphomas, despite
302 hypoactivity of a key CTP generation enzyme, yet high CTP demands of B-cell growth (16,
303 23). Further adding to this question, classic studies indicate that CTP synthase activity is
304 frequently upregulated in cancer (40).

305 We therefore tested the effects of CTPS1, CTPS2 and CTP salvage pathways to
306 support EBV-infected B-cell growth, survival and EBV lytic replication. EBV-mediated B-cell
307 growth transformation proceeds through a genetically encoded process that proceeds
308 through Burkitt-like hyperproliferation and lymphoblastoid phases, whose physiology
309 resembles the Burkitt and LCL cell lines used in this study (28, 40). LCLs are an established
310 tissue culture model for immunoblastic lymphomas of immunosuppressed hosts. The results
311 presented here suggest that while CTPS1 activity is important for EBV-transformed B-cell
312 proliferation, cells can also utilize CTPS2 activity and cytidine salvage metabolism to support

313 CTP synthesis for DNA, RNA phosphatidylcholine and phosphatidylserine needs. While
314 knowledge of cytidine concentrations in tonsil and lymphoid tissues remains limited, robust
315 deoxycytidine kinase activity is evident in human tonsil tissue (41).

316 We speculate that in patients with CTPS1 deficiency, CTPS2 compensation is insufficient
317 for cell-mediated control of EBV infection, but is nonetheless able to support EBV+ B-cell
318 growth, together with hypomorphic CTPS1. Given our findings that CTPS2 supports
319 proliferation of EBV-transformed CTPS1 deficient B-cells, we speculate that CTPS2 plays
320 important roles in the CNS lymphomas observed clinically. Consequently, CTPS1/2 may
321 serve as a therapeutic synthetic lethal target in this setting, for instance if cytidine
322 replacement fails to restore anti-tumor immunity. While our quantitative proteomic analyses
323 identified that CTPS1 is approximately four times more abundant than CTPS2 in primary
324 human B-cells undergoing EBV-mediated growth transformation (28), EBV-infected
325 lymphomas may compensate for CTPS1 deficiency by increasing CTPS2 levels and/or
326 activity. It would be of interest to test this hypothesis through RNAseq and enzymatic profiling
327 of CNS lymphoma samples.

328 CTPS1 and CTPS2 are each highly regulated at the post-translational level, including
329 by assembly into filaments called cytoophidium that regulate catalytic activity (42-44).
330 Whether cytoophidium form in EBV-infected B-cells generally or with CTPS1 deficiency is
331 unknown. Furthermore, cryo-EM studies recently identified a novel filament-based
332 mechanism of CTPS2 regulation that stabilizes nucleotide levels (45). Filaments containing
333 CTPS1/2 and IMPDH, the rate limiting enzyme in *de novo* GTP synthesis have also been
334 described (46), and may play important roles in EBV-infected B-cell metabolism.

335 CTP salvage pathways may also play important roles in support of pathogenic B-cell
336 roles in CTPS1 deficient patients. Cytidine is imported into the CNS by nucleoside
337 transporters located at the blood-brain barrier (47). That CTPS1/2 deficient LCL growth could
338 be restored by cytidine supplementation highlights the potentially important role of the CTP
339 salvage pathway in B-cells with the viral latency III program observed in CNS lymphoma.
340 Interestingly, since oral intake may alter CNS pyrimidine levels (47), dietary cytidine and
341 uridine restriction could potentially have antineoplastic effects in CTPS1 deficient patients
342 with CNS lymphoma.

343 Patients with CTPS1 deficiency often have elevated EBV genome copy number, which
344 may result from latent or lytic states with impaired T-cell surveillance (48). Viral lytic gene
345 expression has been associated with EBV lymphomagenesis (49-51). Interestingly, the
346 DHODH chemical inhibitor teriflunomide blocks EBV lytic gene expression and DNA
347 replication (24). Here, we found that CTPS1 knockout instead reduces EBV lytic DNA
348 replication, without impairing immediate early and early gene expression. Consistent with a
349 potential role in supplying CTP for lytic genome synthesis, cytidine supplementation rescued
350 EBV lytic DNA replication, newly identifying their ability to utilize cytidine salvage metabolism.
351 It will be of interest to define relative CTPS1 and CTPS2 roles in epithelial cell EBV lytic
352 replication.

353 CTP levels increase in EBV-transforming B-cells, though to lower magnitude than other
354 deoxynucleotide triphosphates (52). Feedback and allosteric regulation renders CTPS1
355 activity sensitive to the levels of all four nucleotides (22, 53). EBNA2 and MYC are important
356 regulators of EBV-driven one-carbon metabolism and purine nucleotide synthesis in newly
357 infected cells (28). Thus, their roles in CTPS1 induction likely coordinates pools of all four
358 deoxynucleotides to avoid imbalances and DNA damage. Indeed, CRISPR knockout of
359 CTPS1 resulted in γ -H2AX phosphorylation. While NF- κ B is not required for initial
360 EBV-driven B-cell growth (54), it is essential for LCL growth and survival, where we found a
361 non-canonical NF- κ B role in CTPS1 expression. Our findings also suggest that hyper-active
362 MYC activity is also sufficient to drive CTPS1 expression and likely supports CTPS1
363 expression in Burkitt cells. By contrast, ERK and SRC have obligatory roles in robust CTPS1
364 upregulation upon T-cell activation that are critical for T-cell expansion (**Figure 7**). Notably,
365 MYC has key roles in *de novo* pyrimidine nucleotide synthesis (55).

366 In summary, these studies highlight that multiple CTP synthesis pathways are operative
367 in EBV-infected B-cells, and that each can play important roles in EBV transformed B-cell
368 growth and lytic DNA replication. Collectively, these results highlight CTP synthesis pathways
369 as important therapeutic targets in EBV-driven lymphoproliferative disorders.

370

371 **Methods**

372 **CRISPR/Cas9 gene editing.** CRISPR editing was performed as previously described (56).

373 Briefly, Broad Institute pXPR-011 pXPR-515 control sgRNA, Avana or Brunello library
374 sgRNAs, as listed in **Supplementary Table 1**, were cloned into lentiGuide-Puro (Addgene
375 Cat#52963) or pLenti SpBsmBI sgRNA Hygro (Addgene Cat# 62205). sgRNA expressing
376 lentiviruses were produced by 293T transfection and used to transduce LCLs or Burkitt cells
377 with stably expressed Cas9.

378

379 **Growth Curve Analysis.** Cells were normalized to the same starting concentration. Cell
380 numbers were then quantified, using the Cell Titer Glo assays at the indicated times.

381 **Recombinant EBV.** EBV B95-8 virus was produced from B95-8 cells with conditional BZLF1
382 expression. The P3HR-1 EBV strain was produced from P3HR-1 cells with conditional BZLF1
383 and BRFL1 expression. UV irradiation of B95-8 virus supernatants was performed at a
384 cumulative intensity of 3J per square centimeter on ice, to prevent heat-induced virus
385 degradation. The quantitation of equal infection of B95-8, UV B 95-8 and P3HR1 was
386 performed as previously described (57).

387

388 **Statistical Analysis.** Data are presented as means \pm standard errors of the means. Data
389 were analyzed using analysis of variance (ANOVA) with Sidak's multiple-comparison test or
390 two-tailed paired Student's *t* test with Prism7 software. For all statistical tests, a *P* cutoff value
391 of <0.05 was used to indicate significance.

392

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397

398 **Compliance with ethical standards**

399 Conflict of interest BG receives research funding from Abbvie
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401

402 **Author contributions**

403 J.L. performed the experiments. C.W, R.G., S.Y., B.Z. provided technological assistance. R.G.
404 performed bioinformatic analysis. J.L, R.G. and B.E.G. prepared the manuscript. B.E.G
405 supervised the study.

406

407 **Disclosure of Conflicts of Interest**

408 The authors declare no competing interests

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593

594 **Figure Legends**

595 **Figure 1. Upregulation of *de novo* CTP synthesis enzymes CTPS1 and CTPS2 in**
596 **primary human B-cells stimulated by EBV or B-cell agonists.**

597 **(A)** Schematic diagram of *de novo* and salvage CTP synthesis pathways. The *de novo*
598 pathway synthesizes CTP from L-glutamine and L-aspartate or uridine substrates, whereas
599 the salvage pathway uses cytidine for CTP synthesis.

600 **(B)** Quantitative PCR (qPCR) analysis of CTPS1 and CTPS2 mRNA levels in primary human
601 CD19+ peripheral blood B-cells infected by EBV B95.8 strain for the indicated days.

602 **(C)** Immunoblot analysis of CTPS1, CTPS2, MYC, EBNA2 and load control DDX1 levels in
603 whole cell lysate (WCL) from primary human CD19+ peripheral blood B-cells infected by the
604 EBV B95.8 strain for the indicated days.

605 **(D)** qPCR analysis CTPS1 and CTPS2 mRNA levels in primary human CD19+ B-cells
606 stimulated for 24 hours by Mega-CD40 ligand (CD40L, 50ng/mL), the toll-receptor 9 agonist
607 CpG (1 μ M), \square IgM crosslinking (1 μ g/mL), interleukin 4 (IL-4, 20ng/mL) or combinations
608 thereof, as indicated.

609 **(E)** Immunoblot analysis of CTPS1, CTPS2 and DDX1 abundances in WCL of primary human
610 CD19+ B-cells stimulated for 24 hours by CD40 ligand Meda-CD40L (50ng/mL), CpG (1 μ M),
611 \square IgM (1 μ g/mL), IL- 4 (20ng/mL), or combinations thereof, as indicated.

612 For d and d, mean and SEM values from n=3 replicates are shown. *, $p<0.05$; **, $p<0.01$; ***,
613 $p<0.001$; ****, $p<0.0001$; ns, non-significant using one way Anova with multiple comparisons.

614 For c and d, representative blots from n=3 replicates are shown.

615

616 **Figure 2. CTPS1 roles in EBV transformed B-cell growth and survival.**

617 **(A)** Distribution of Avana human genome-wide CRISPR screen sgRNA Log2 fold-change
618 values at Day 21 versus input in Cas9+ P3HR-1 Burkitt lymphoma (BL) left or GM12878 LCL
619 (right). Values for CTPS1, CTPS2, UCK1 or UCK2 targeting sgRNAs (red lines) are overlaid
620 on gray gradients depicting all Avana sgRNA library values (30). Average Day 21 vs input
621 values from four screen biological replicates are shown.

622 **(B)** Immunoblot analysis (top) and growth curve analysis (bottom) of Cas9+ GM12878 LCLs
623 or Daudi Burkitt lymphoma (BL) expressing non-targeting control (C) or independent sgRNAs
624 against *CTPS1*. WCL were obtained at day 7 post sgRNA expression. Growth curves were
625 begun at 96 hours post-sgRNA expression and 48 hours post-puromycin selection.

626 **(C)** Flow cytometry (FACS) analysis of vital dye 7-AAD uptake in GM12878 LCL at Day 7
627 post-expression of control or sgRNAs targeting *CTPS1*. Shown at right are median + SEM
628 values from n=3 replicates.

629 **(D)** FACS analysis of 7-AAD uptake in Daudi BL at Day 7 post-expression of control or
630 *CTPS1* targeting sgRNAs. Shown at right are median + SEM values from n=3 replicates.

631 **(E)** Propidium iodide cell cycle analysis of GM12878 LCL (left) or Daudi BL (right) expressing
632 control or *CTPS1* sgRNAs at Day 7.

633 **(F)** Immunoblot (top) and growth curve analysis (bottom) of GM12878 or Daudi expressing
634 control or independent sgRNAs against *CTPS2*. WCL were obtained at day 5 post sgRNA
635 expression. Growth curves were begun at 96 hours post-sgRNA expression and 48 hours
636 post-puromycin selection.

637 **(G)** Immunoblot analysis of WCL from GM12878 or Daudi, 7 days post expression of control
638 or *CTPS1* sgRNAs.

639 For B, C, D, E and F, mean and SEM values from n=3 replicates are shown. *, $p < 0.05$; **, $p < 0.01$;
640 ***, $p < 0.001$; ****, $p < 0.0001$; ns, non-significant using one way Anova with multiple
641 comparisons. For B, F and G, representative blots or n=3 replicates are shown.

642

643 **Figure 3. Partially redundant CTPS1/2 roles in EBV-transformed B cell growth and**
644 **survival.**

645 **(A)** Immunoblot of WCL from GM12878 LCL on Day 7 post expression of the indicated control,
646 CTPS1 and/or CTPS2 sgRNAs.

647 **(B)** Growth curve analysis of GM12878 LCL with control, CTPS1 and/or CTPS2 sgRNAs, as
648 indicated.

649 **(C)** Immunoblot of WCL from Daudi BL Day 7 post expression of the indicated control, CTPS1
650 and/or CTPS2 sgRNAs.

651 **(D)** Growth curve analysis of Daudi BL with control, CTPS1, and/or CTPS2 sgRNAs, as
652 indicated.

653 **(E-H)** Growth curve analysis of GM12878 LCL (E-F) or Daudi BL (G-H) that express control,
654 CTPS1 or CTPS1 and CTPS2 sgRNAs as indicated, with 200 μ M cytidine supplementation.

655 **(I-L)** Growth curve analysis of GM12878 (I), GM11830 (J), Daudi (K) or Mutu I (L) with CTPS1
656 sgRNA expression, cultured in the presence of 200 μ M cytidine from days 1-7 or throughout.

657 **(M)** Immunoblot analysis of WCL from GM12878 (left) or Daudi (right) at Day 7 post expression
658 of the indicated sgRNAs.

659 **(N)** Mean + SEM values of FACS 7-AAD values from GM12878 (left) or Daudi (right)
660 expressing the indicated sgRNAs for 7 days from n=3 replicates.

661 Blots in A, C, and M are representative of n=3 replicates. Growth curves were begun at 96
662 hours post-sgRNA expression and 48 hours post-puromycin selection. Growth curves show
663 mean + SEM fold change live cell numbers from n = 3 replicates. *, $p < 0.05$; **, $p < 0.01$; ***,
664 $p < 0.001$; ****, $p < 0.0001$; ns, non-significant using one way Anova with multiple comparisons.

665

666 **Figure 4. CTPS1 Transcription Regulation in EBV-infected B-cells.**

667 **(A)** Chromatin immunoprecipitation (ChIP)-sequencing (ChIP-seq) tracks for the indicated
668 transcription factors or histone 3 lysine 27 acetyl (H3K27Ac) at the LCL *CTPS1* locus. Y-axis
669 ranges are indicated for each track. Also shown below is LCL RNA Pol II ChIA-PET signal
670 indicating a long-range DNA interaction between the *CTPS1* promoter region and an intronic
671 site. Schematic diagram of the *CTPS1* locus shown below, with exons indicated by rectangles.
672 CRISPR-i sgRNA targeting sites are shown.

673 **(B)** Normalized CTPS1 mRNA levels in dCAS9-KRAB+ GM12878 expressing the indicated
674 sgRNAs for CRISPR-i analysis for 5 days. CTPS1 mRNA level in cells with non-targeting
675 control sgRNA was set to 1.

676 **(C)** Immunoblot of WCL from primary human B-cells that were either mock-infected or
677 infected with equal amounts of the non-transforming P3HR-1, UV-irradiated B95-8 or B95-8
678 EBV strain for four days.

679 **(D)** Immunoblot analysis of samples prepared from 2-2-3 EBNA2-HT LCLs grown in the
680 absence (EBNA2 non-permissive) or presence (EBNA2 permissive) of 4HT (1 μ M) for 48
681 hours.

682 **(E)** Immunoblot analysis of WCL from conditional P493-6 LCLs treated with 1 μ g/ml
683 doxycycline (DOX) to suppress exogenous *MYC* allele expression and/or with 1 μ M 4HT to
684 induce EBNA2 activity for 48 hours, as indicated.

685 **(F)** Immunoblot analysis of WCL from GM12878 LCL expressing control or
686 independent *MYC*-targeting sgRNAs for 5 days, as indicated.

687 **(G)** Immunoblot analysis of WCL from GM12878 LCL expressing the indicated sgRNA for 6
688 Days.

689 **(H)** qPCR analysis of CTPS1 and CTPS2 mRNA levels in GM12878 LCL expressing the
690 indicated sgRNAs for 7 days. Levels in cells with sgControl were set to 1.

691 **(I)** Immunoblot of WCL from GM12878 or GM11830 LCLs treated with the indicated
692 concentrations of the IKK antagonist Calbiochem VIII for 48 hours. 1 μ M selectively blocks
693 the canonical pathway IKK α kinase.

694 **(J)** Immunoblot of WCL from GM12878 or GM11830 LCLs treated with the ERK inhibitor
695 PD0325901 at the indicated concentrations for 48 hours.

696 Blots in C, D, E, F, G, I and J are representative of n = 3 experiments. SEM + Mean values
697 are shown in B and H from n=3 replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ using one
698 way Anova with multiple comparisons.

699

700 **Figure 5. De novo pyrimidine synthesis enzyme DHODH and uridine salvage roles in**
701 **EBV-transformed B-cell growth.**

702 **(A)** Immunoblot analysis of UCK2, DHODH and load-control DDX1 levels in whole cell lysate

703 (WCL) from human CD19+ peripheral blood B-cells infected by the EBV B95.8 strain for the
704 indicated days.

705 **(B)** Immunoblot analysis of UCK1, DHODH and DDX1 abundances in primary human CD19+
706 B-cells stimulated for 24 hours by CD40L (50ng/mL), CpG (1 μ M), \square IgM (1 μ g/mL), IL-4
707 (20ng/mL), or combinations thereof, as indicated.

708 **(C-F)** Immunoblot analysis (top) and growth curve analysis (bottom) of GM12878 (C),
709 GM11830 (D), Mutu I (E) and Daudi (F) expressing non-targeting control or independent
710 sgRNAs against *DHODH* in media containing 10% undialyzed fetal bovine serum (FBS).
711 WCL were obtained at day 7 post sgRNA expression.

712 **(G-J)** Growth curve analysis of GM12878 (G), GM11830 (H), Mutu I (I) and Daudi (J)
713 expressing control or independent sgRNAs against DHODH. Cells were grown in media
714 containing dialyzed FBS and supplemented with 200 \square M uridine where indicated.

715 **(K)** Growth curve analysis (left) and immunoblot of WCL or T7 endonuclease I (T7E1) assays
716 of CRISPR editing of the *UCK1* locus (right) from GM12878 expressing the indicated
717 sgRNAs.

718 **(L)** Growth curve analysis (left) and immunoblot of WCL or T7E1 assays of CRISPR editing of
719 the *UCK1* locus (right) from Daudi expressing the indicated sgRNAs.

720 Blots in A-F, K and L are representative of n = 3 experiments. SEM + Mean values are shown
721 in C-L from n=3 replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, non-significant using one
722 way Anova with multiple comparisons.

723

724 **Figure 6. Key CTPS1 role in EBV lytic DNA replication**

725 **(A)** Immunoblot analysis of WCE from EBV+ Akata BL expressing the indicated sgRNAs for
726 96 hours and mock-induced or induced for lytic replication by \square IgG (10 \square g/ml) cross-linking
727 for 24 hours. Blots for immediate early BZLF1 and early BMRF1 lytic antigens, CTPS1 and
728 GAPDH are shown. Shown below are EBV genome copy numbers determined by qPCR
729 analysis. Values for mock-induced cells with sgControl were set to 1. Relative intracellular
730 EBV DNA copy numbers were determined by qPCR using primers to the *BALF5* gene and
731 were normalized for input cell number using primers to *GAPDH*.

732 (B) Immunoblots and qPCR analysis of EBV genome copy number as in panel A, from cells
733 grown in the presence of 200 μ M cytidine rescue.

734 (C) Immunoblot analysis of WCE from Cas9+ P3HR-1 Burkitt cells expressing a conditional
735 4-HT regulated BZLF1 allele and the indicated sgRNAs, mock-induced or induced for EBV
736 lytic reactivation by addition of 400nM 4HT and 500 μ M sodium butyrate for 24 hours.
737 sgRNAs were expressed for 6 days. Blots for EBV-encoded wildtype BZLF1 and BMRF1 as
738 well as for CTPS1 and GADPH are shown. Shown below are qPCR analysis of EBV genome
739 copy number, as in A.

740 (D) Immunoblots and qPCR analysis of EBV genome copy number as in panel C from cells
741 grown in the presence of 200 μ M cytidine rescue.

742 Blots in A-D are representative of n=3 replicates. Mean + SEM values from n=3 of biologically
743 independent replicates are shown in A-D. ****, $p<0.0001$; *** $p<0.001$; NS, non-significant
744 using one way Anova with multiple comparisons.

745

746 **Figure 7. Schematic model of CTPS1 roles in T vs EBV-infected B-cells.**

747 T-cell receptor stimulation induces CTPS1 expression, at least in part through the ERK and
748 SRC pathways. By contrast, EBV induces CTPS1 in LCLs through EBNA2, MYC and
749 LMP1-activated non-canonical NF- κ B pathway, whereas hyper-expressed MYC has key
750 roles in Burkitt CTPS1 induction. CTPS1 and CTPS2 activity together supply CTP pools for
751 EBV-transformed B-cells to prevent nucleotide imbalance, DNA damage, cell cycle arrest and
752 apoptosis. CTPS1 depletion also impairs EBV lytic DNA replication.

753

754 **Supplementary Figure Legends**

755 **Figure S1. EBV-induced *de novo* pyrimidine synthesis pathway expression.**

756 (A-F) CAD (A), DHODH (B), UMPS (C) CMPK1 and CMPK2 (D), CTPS1 and CTPS2 (E),
757 NME1 and NME2 encoded NDPK (F) relative protein abundances (top) and mRNA
758 abundances (bottom) over the first 28 days of peripheral blood CD19+ B-cell infection by EBV
759 B95.8 at a multiplicity of infection of 0.1. Mean + SEM values from n=4
760 tandem-mass-tag-based proteomic and n=3 RNAseq replicates were used for these analyses
761 and were obtained from recently published datasets (28, 29). *, $p<0.05$; **, $p<0.01$; ***,

762 $p < 0.001$; ****, $p < 0.0001$; ns, non-significant, using one-way Anova with multiple comparisons.

763

764 **Figure S2. CTPS1 roles in EBV-transformed B-cell growth and survival.**

765 **(A-B)** Immunoblot analysis (top) and growth curve analysis (bottom) of GM11830 LCLs (A)
766 and Mutu I BLs (B) expressing control or independent sgRNAs against CTPS1. WCL were
767 obtained at day 7 post sgRNA expression.

768 **(C)** FACS analysis of 7-AAD uptake in GM11830 LCL (left) or Mutu I BL (right) expressing
769 control or CTPS1 sgRNAs for 7 days.

770 **(D)** Median + SEM values from $n=3$ replicates, as in C.

771 **(E)** Propidium iodide cell cycle analysis of GM12878 LCL, GM11830, Daudi BL or Mutu I BL
772 expressing control or CTPS1 sgRNAs at Day 9 post sgRNA expression.

773 **(F)** Median + SEM values from $n=3$ replicates, as in E. Percentages of cells in G2/M, S,
774 G0/G1 or with $<2n$ DNA content indicative of cell death are shown.

775 **(G)** Immunoblot analysis of WCL from GM11830 (left) or Mutu I cells (right) expressing control
776 or CTPS1 sgRNAs for 7 days.

777 Blots in A and G are representative of $n = 3$ experiments. SEM + Mean values are shown in A,
778 D and F from $n=3$ replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns,
779 non-significant using one-way Anova with multiple comparisons.

780

781 **Figure S3. Analysis of CTPS2 roles in EBV-transformed B-cell growth.**

782 **(A)** DepMap CRISPR screen dependency scores (31) for sgRNAs targeting CTPS1 (left) and
783 CTPS2 (right) across cell lines from the indicated cancer cells of origin. Each oval
784 represents the DepMap screen value for a cell line from the tissue indicated at right.

785 Negative values indicates selection against the sgRNAs target the gene over a 21 day
786 growth and survival screen. Values < -1 (red vertical line) indicates cell line dependency on
787 the targeted gene for growth or survival.

788 **(B-C)** Immunoblot analysis (top) and growth curve analysis (bottom) of GM11830 LCL (B) or
789 Mutu I BL (C) expressing control or independent CTPS2 sgRNAs for 7 days.

790 **(D)** FACS analysis of 7-AAD uptake in GM12878 (left) or Daudi cells (right) expressing control
791 or CTPS2 sgRNAs for 7 days.

792 **(E)** Median + SEM values from n=3 replicates, as in D, of 7-AAD uptake FACS analysis in
793 GM12878, GM11830, Daudi or Mutu I cells.

794 **(F)** Mean + SEM values of FACS propidium iodide cell cycle analysis of GM12878 (left) or
795 Daudi (right) expressing control, CTPS2, CTPS1 or CTPS1/2 sgRNAs for 7 days.

796 Significantly differences from sgControl values are indicated.

797 Blots in B and C are representative of n = 3 experiments. SEM + Mean values are shown in

798 B-F are from n=3 replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns,

799 non-significant using one-way Anova with multiple comparisons.

800

801 **Figure S4. Profiling of EBV and immune receptor stimulation effects on UCK1, UCK2**
802 **and DHODH expression in primary B-cells.**

803 **(A)** qPCR analysis of UCK1 and UCK2 mRNA levels in human peripheral blood CD19+

804 B-cells at the indicated day post infection by B95.8 EBV at MOI=0.1. Day 0 values were set to

805 1.

806 **(B)** UCK1, UCK2 and DHODH relative protein abundances from published analyses of

807 tandem-mass-tag-based proteomic analysis at rest and at nine time points after EBV B95.8

808 infection of human peripheral blood CD19+ B-cells at a multiplicity of infection of 0.1 (28).

809 **(C)** qPCR analysis UCK1, UCK2 or DHODH mRNA levels in human peripheral blood CD19+

810 B-cells stimulated for 24 hours by Mega-CD40L (50ng/mL), CpG (1 μ M), \square IgM crosslinking

811 (1 μ g/mL), IL-4 (20ng/mL) or combinations thereof, as indicated.

812 SEM + Mean values are shown in A-C from n=3 replicates. *, $p < 0.05$; **, $p < 0.01$; ***,

813 $p < 0.001$; ****, $p < 0.0001$; ns, non-significant using one-way Anova with multiple comparisons.

814

815 **Figure S5. Analysis of DHODH, UCK1/2 and uridine salvage metabolism roles in**
816 **EBV-transformed B-cell growth.**

817 **(A-B)** Growth curve analysis of GM11830 (A) or Mutu I (B) expressing control or DHODH

818 sgRNAs and puromycin selected for 48 hours, then grown in media containing

819 uridine-depleted 10% dialyzed fetal bovine serum, in the absence or presence of 200 \square M

820 uridine supplementation. Immunoblot analysis of WCL from cells with the indicated sgRNAs

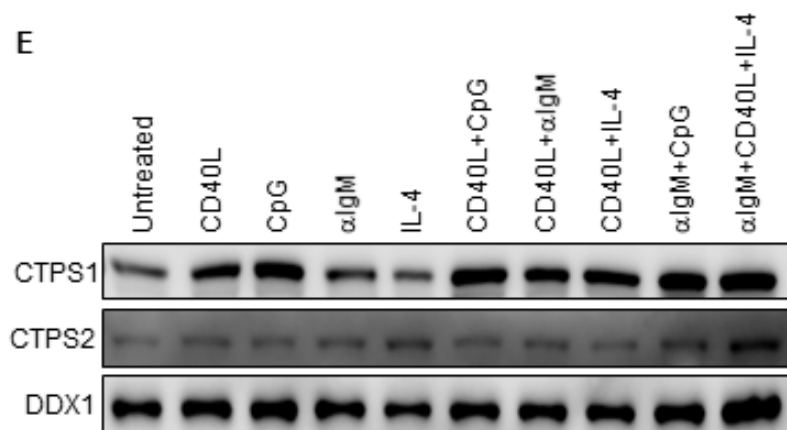
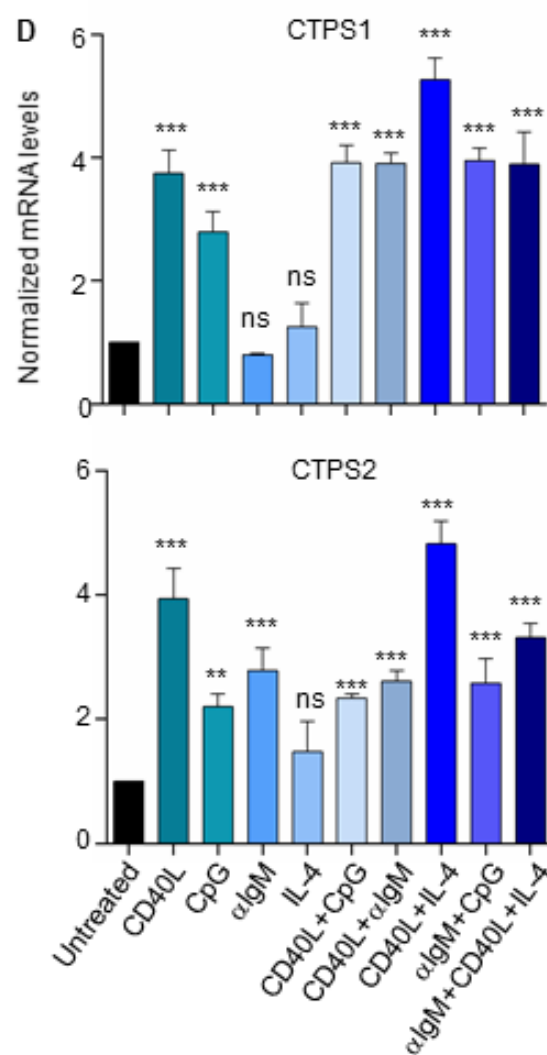
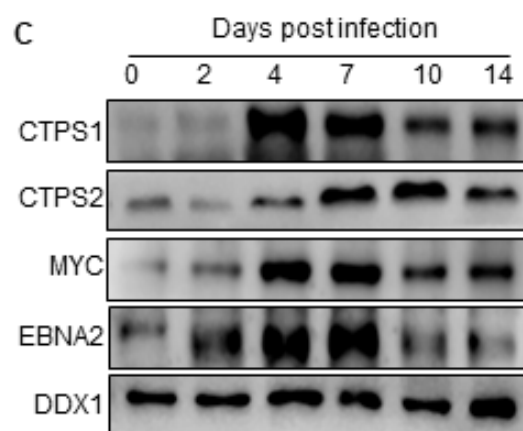
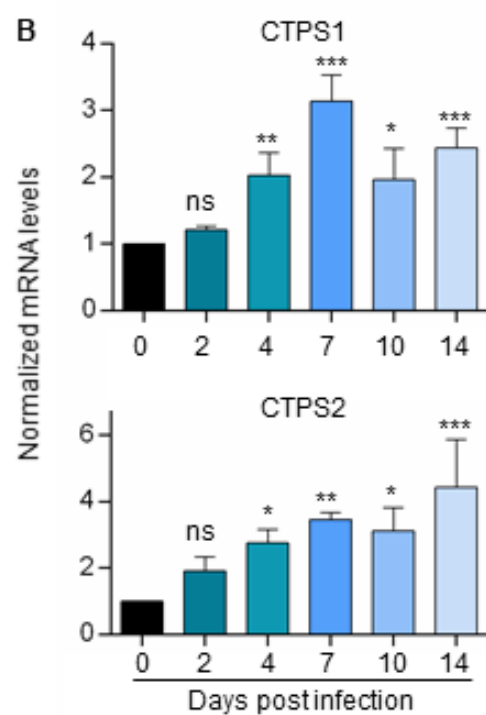
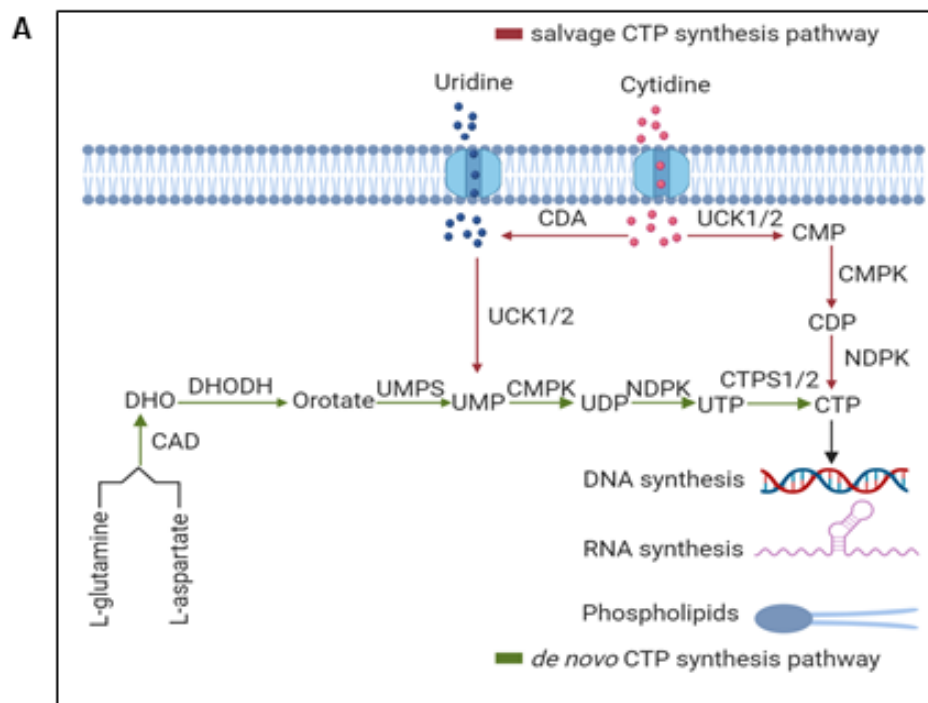
821 are shown to the right.

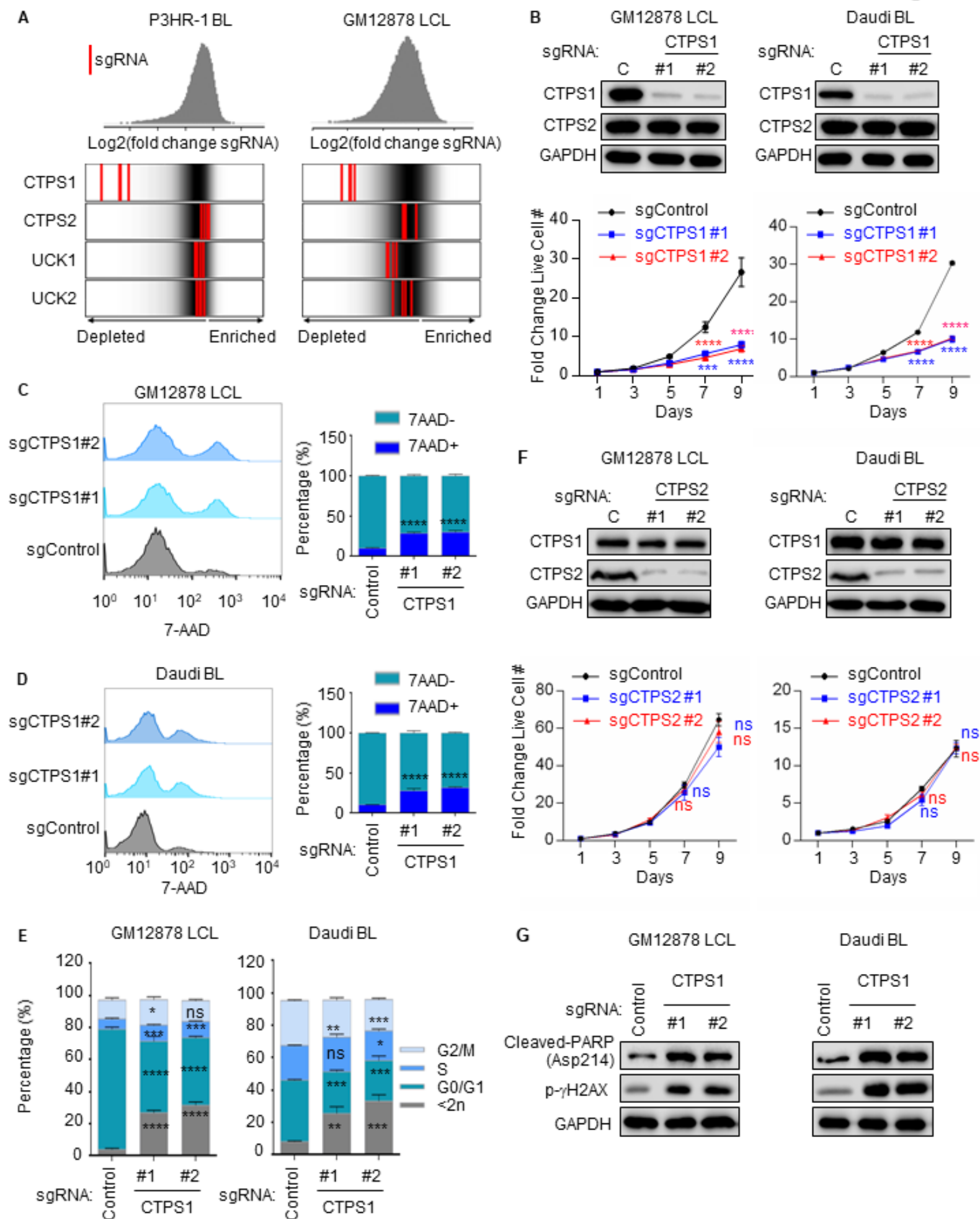
822 **(C)** FACS analysis of 7-AAD abundances in GM12878, GM11830, Mutu I or Daudi expressing
823 the indicated sgRNAs for 7 days and grown in media containing uridine-depleted, dialyzed
824 FBS in the absence or presence of 200 μ M uridine supplementation.

825 **(D)** Mean + SEM values of FACS analysis of n=3 replicates, as in C.

826 **(E-F)** Growth curve analysis (left) and immunoblot of WCL for UCK2 and GAPDH load control
827 or *UCK1* locus T7E1 assay results (right) of GM11830 (E) or Mutu cells (F) expressing the
828 indicated sgRNAs.

829 Blots in A, B, E and F are representative of n = 3 experiments. SEM + Mean values are
830 shown in A, B, D, E and F from n=3 replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns,
831 non-significant using one-way Anova with multiple comparisons.





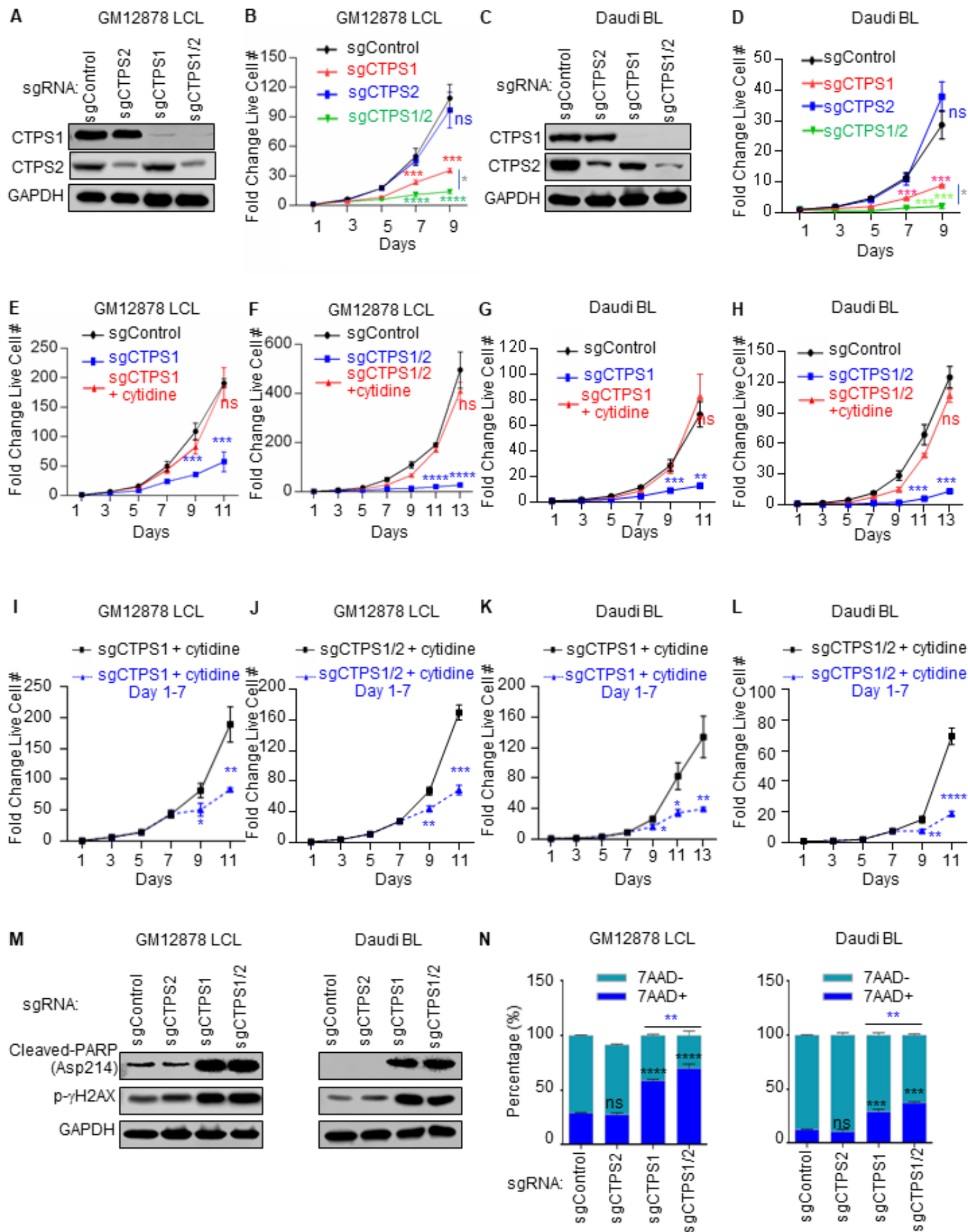
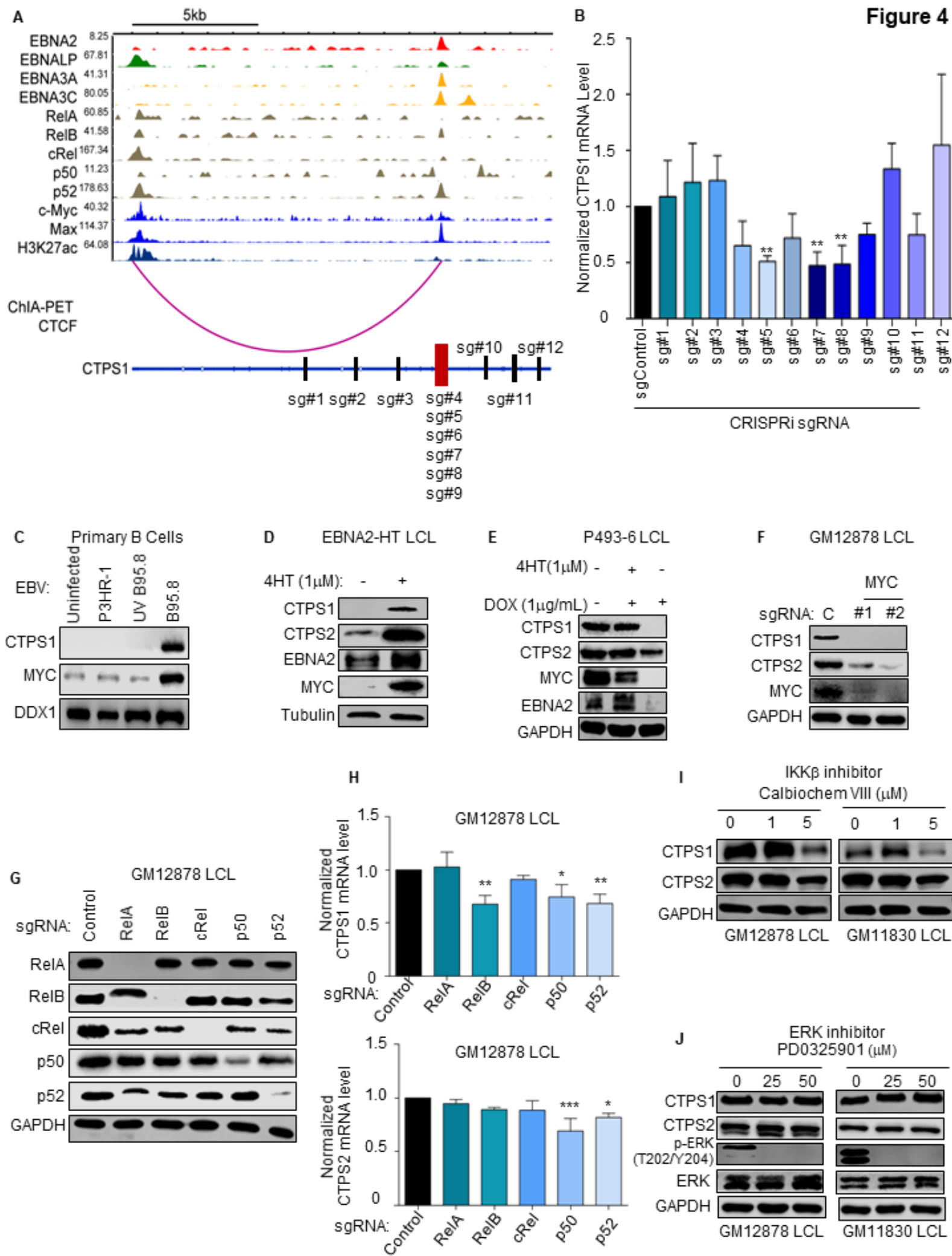
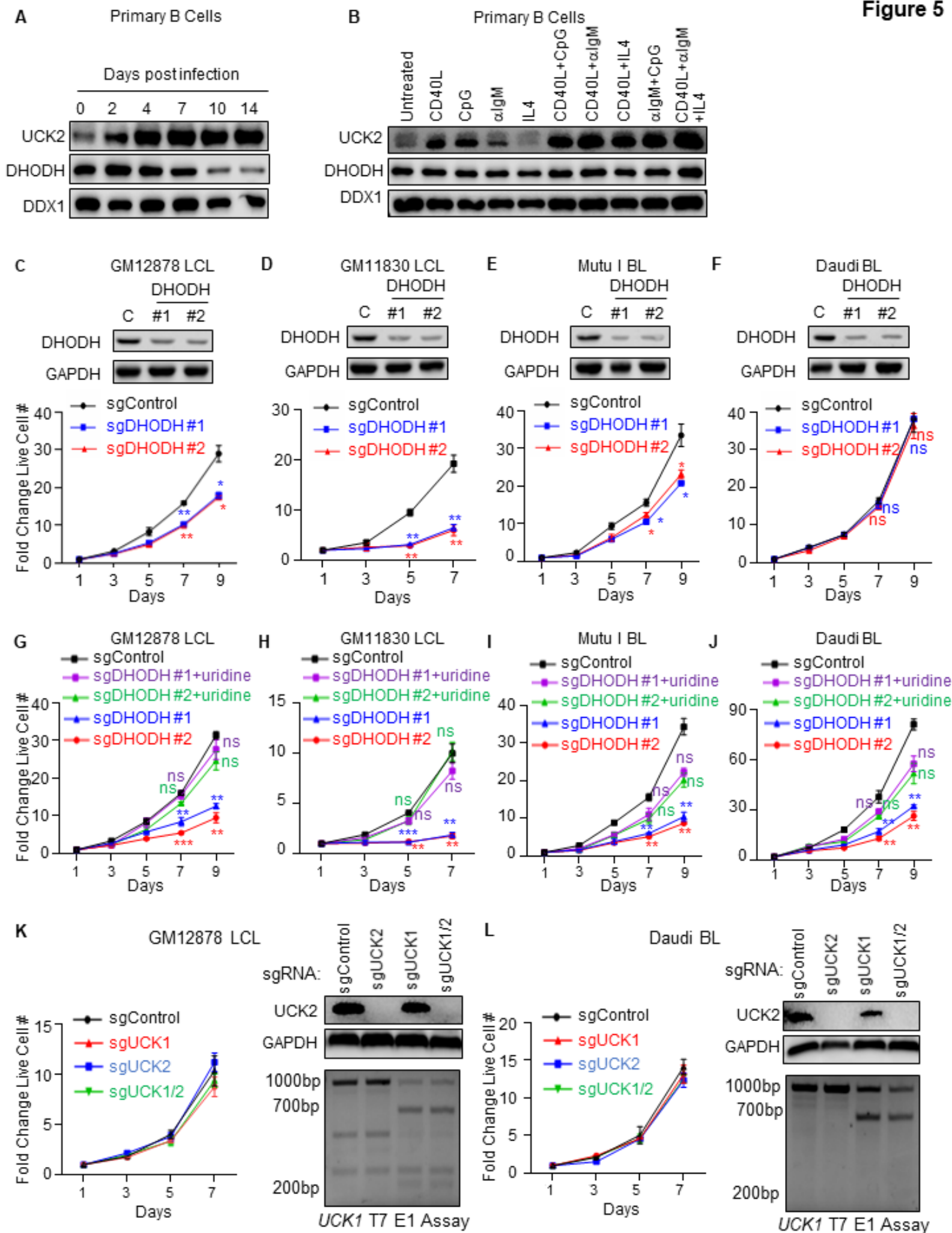


Figure 4




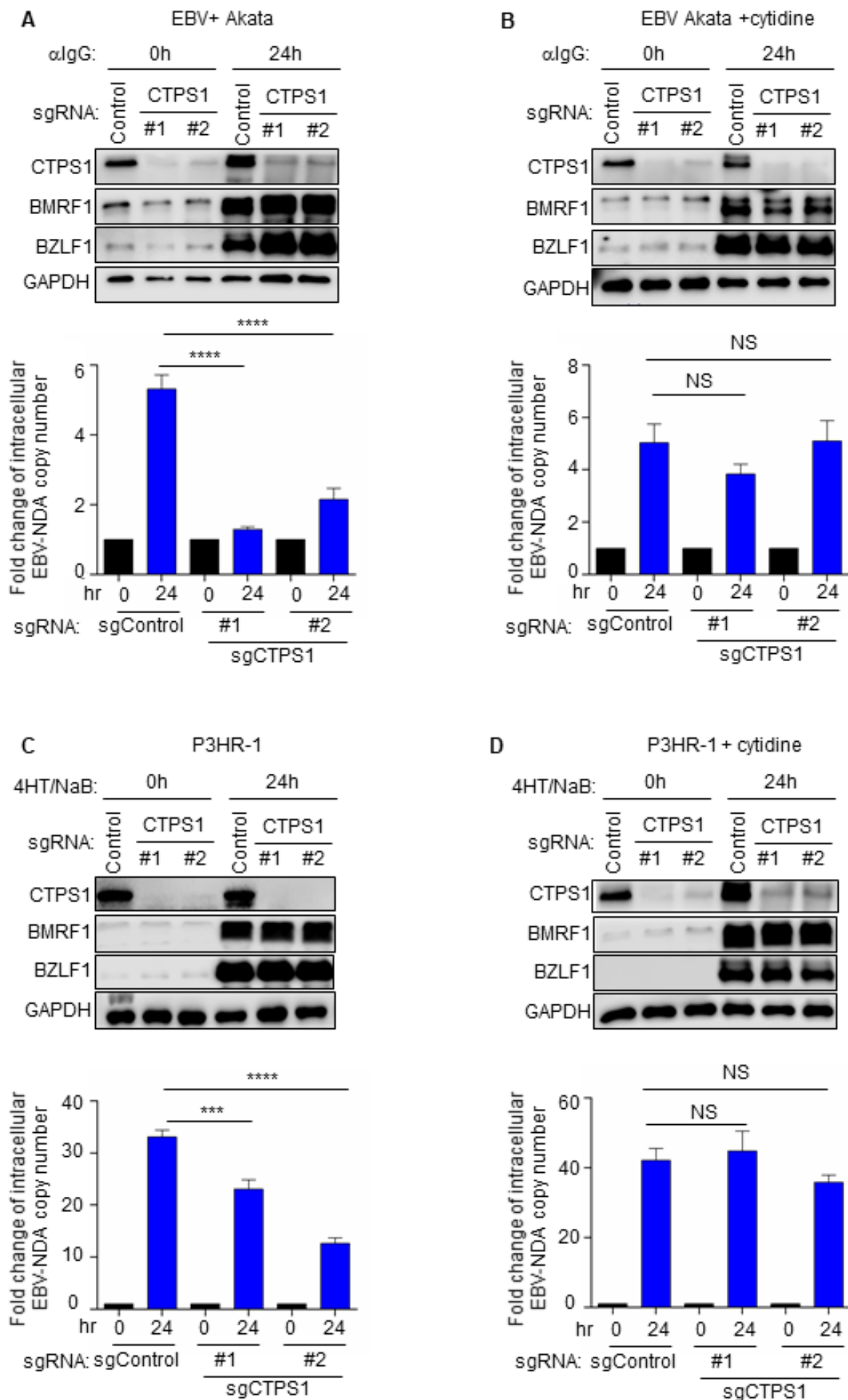


Figure 7

