

# 1 **Polyamines and eIF5A hypusination facilitate SREBP2 translation and** 2 **cholesterol synthesis to enhance enterovirus attachment and infection**

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22

## 23 **Abstract**

24 Metabolism is key to cellular processes that underlie the ability of a virus to productively infect.  
25 Polyamines are small metabolites vital for many host cell processes including proliferation,  
26 transcription, and translation. Polyamine depletion also inhibits virus infection via diverse  
27 mechanisms, including inhibiting polymerase activity and viral translation. We showed that  
28 Coxsackievirus B3 (CVB3) attachment requires polyamines; however, the mechanism was  
29 unknown. Here, we report polyamines' involvement in translation, through a process called  
30 hypusination, promotes expression of cholesterol synthesis genes by supporting SREBP2  
31 translation, the master transcriptional regulator of cholesterol synthesis genes. Measuring bulk  
32 transcription, we found polyamines support expression of cholesterol synthesis genes, regulated  
33 by SREBP2. Polyamine depletion inhibits CVB3 by depleting cellular cholesterol. Exogenous  
34 cholesterol rescues CVB3 attachment, and mutant CVB3 resistant to polyamine depletion exhibits

35 resistance to cholesterol perturbation. This study provides a novel link between polyamine and  
36 cholesterol homeostasis, a mechanism through which polyamines impact CVB3 infection.

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## 38 **Introduction**

39 Polyamines are small carbon chains with amine groups that have a positive charge at cellular pH.  
40 They play a large role within the cell and are involved in multiple cellular processes including  
41 nucleotide synthesis, DNA/RNA stability, membrane fluidity, and translation<sup>1</sup>. Ornithine, which is  
42 a derivative of arginine, is converted to the first polyamine putrescine by the rate limiting enzyme  
43 ornithine decarboxylase 1 (ODC1). Putrescine can be converted to spermidine and spermine via  
44 their respective synthases. Difluoromethylornithine (DFMO) is an FDA approved drug for  
45 trypanosomiasis and is an irreversible, competitive inhibitor of ODC1<sup>2</sup>. One key way polyamines  
46 impact cells is through translation, specifically through a process called hypusination<sup>3</sup>. Spermidine  
47 is covalently attached to eukaryotic initiation factor 5A (eIF5A) at lysine 50 by the protein  
48 deoxyhypusine synthase (DHPS) to form deoxyhypusine-eIF5A. Deoxyhypusine hydroxylase  
49 (DOHH) then adds a hydroxide in the second and final step to make hypusine-eIF5A. Hypusine-  
50 eIF5A plays a vital role in mRNA translation, ribosome function, and cell proliferation. The precise  
51 mechanisms by which hypusine-eIF5A promotes translation remain to be fully understood.  
52 However, certain amino acid motifs cause ribosomal pausing, and hypusine-eIF5A is required for  
53 the ribosome to translate through these motifs<sup>4</sup>. Poly-proline tracts have also been shown to  
54 require hypusine-eIF5A<sup>5</sup>. When the unhyposinated form of eIF5A is present, it cannot alleviate  
55 ribosomal pausing. The requirement for hypusine-eIF5A for cellular proliferation has made it an  
56 attractive target for the development of anti-cancer drugs. One such drug is the spermidine analog  
57 N1-guanyl-1,7-diaminoheptane (GC7). GC7 inhibits DHPS by directly binding to the active site  
58 and prevents spermidine from being attached to eIF5A<sup>6</sup>. Additionally, deferiprone (DEF) is an  
59 iron-chelator which has broad effects on the cell and also inhibits DOHH<sup>7</sup>.

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61 Polyamines have been found to be important for multiple RNA viruses through different  
62 mechanisms, and diverse RNA viruses are sensitive to DFMO-mediated polyamine depletion<sup>8-11</sup>.  
63 Interestingly, the enterovirus Coxsackievirus B3 (CVB3) develops mutations within its proteases  
64 as well as the capsid protein VP3 when polyamine synthesis is inhibited, suggesting a role for  
65 polyamines in protease activity and cellular attachment for enteroviruses<sup>12-14</sup>. Enteroviruses are  
66 small, non-enveloped, positive sense single-stranded RNA viruses a part of the picornavirus  
67 family that can cause a range of diseases from the mild cold to flaccid paralysis and dilated  
68 cardiomyopathy (DCM)<sup>15,16</sup>. Coxsackievirus B3 (CVB3) is a member of the enterovirus genus and

69 is well known for its ability to infect and persist in the heart and cause DCM<sup>17,18</sup>. About 50% of  
70 DCM patients have CVB3 reactive antibodies and the only cure for DCM is a heart transplant<sup>16,19</sup>.  
71 There are currently no FDA drugs approved to treat CVB3 infection. However, we previously  
72 found that inhibiting polyamines is broadly antiviral and inhibits CVB3 infection and binding<sup>12,13,20</sup>.

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74 Another key metabolite required for CVB3 infection is cholesterol. Cholesterol is important for  
75 maintaining cellular membrane integrity and fluidity as well as lipid raft formation. Removal of  
76 cholesterol from the plasma membrane blocks poliovirus entry and EV-11 entry by preventing  
77 lipid raft formation<sup>21,22</sup>. Inhibiting cholesterol homeostasis significantly inhibits CVB3 replication<sup>23</sup>.  
78 The first step in cholesterol synthesis is the conversion of Acetyl-CoA to HMG-CoA by HMGC-  
79 CoA synthase (HMGCs). HMG-CoA is then converted to mevalonic acid by the rate limiting  
80 enzyme HMG-CoA reductase (HMGCr). After 27 more reactions, the end product of cholesterol  
81 is made<sup>24</sup>. The majority of these genes, including low-density lipoprotein receptor (LDLR) is under  
82 the transcriptional control of sterol regulatory element binding protein 2 (SREBP2), which binds  
83 to sterol regulatory elements (SREs) in the promoter of target genes<sup>25</sup>. Upon depletion of  
84 cholesterol, the ER resident, multipass transmembrane protein, SREBP2 is translocated to the  
85 Golgi. Within the Golgi it gets cleaved by the proteases S1P and S2P generating the active N-  
86 terminal portion of the protein. The active SREBP2 then re-locates to the nucleus where it  
87 promotes the transcription of sterol synthesis genes<sup>26</sup>. To date, no link has been established  
88 between cholesterol synthesis and polyamines; however, mice overexpressing the polyamine  
89 catabolic enzyme spermidine-spermine acetyltransferase (SAT1) and rats treated with DFMO  
90 exhibited lower serum cholesterol levels<sup>27,28</sup>, suggesting that polyamines may facilitate cholesterol  
91 synthesis.

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93 Using a transcriptomic approach, we identified several pathways modulated by polyamines that  
94 likely impact enterovirus infection. We found that cholesterol synthesis genes were enriched in  
95 this analysis and hypothesized that polyamines may impact cholesterol synthesis and virus  
96 attachment. Here we describe a novel link between polyamines and cholesterol synthesis through  
97 the polyamine-dependent translation of SREBP2. We find that inhibition of polyamine synthesis  
98 or specific inhibition of hypusination leads to reductions in SREBP2 translation, activity, and  
99 downstream gene expression. This culminates in reduced cellular cholesterol and, in turn,  
100 reduced viral attachment and replication. These data connect previously unrelated metabolic  
101 pathways in the cell and identify cellular cholesterol depletion as an important effect of polyamines

102 on virus replication, with important implications for both virus infection and cellular metabolic  
103 status.

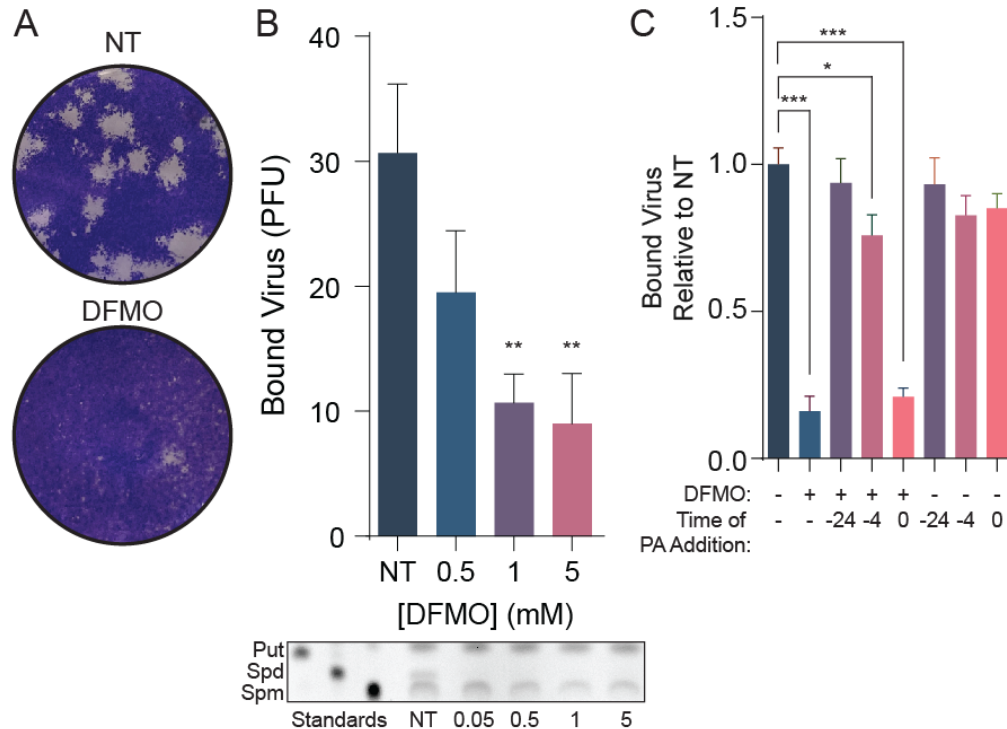
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## 105 **Results**

106 **Inhibition of polyamine synthesis inhibits CVB3 binding and is rescued with exogenous**  
107 **polyamines.** We previously found that inhibition of polyamine synthesis by the suicide inhibitor  
108 DFMO, significantly decreases CVB3 binding to cells compared to untreated cells<sup>13</sup>. To confirm  
109 this phenotype, we treated Vero cells for 4 days with increasing doses of DFMO to deplete cellular  
110 polyamines. We then added CVB3 directly to cells on ice for 5 minutes. Virus was then washed  
111 off followed by agar overlay, in media containing polyamines. Thus, in these assays, polyamines  
112 are depleted only for attachment. Plaques generated from successful attachment and entry were  
113 allowed to form and developed two days later, and bound virus was enumerated by counting  
114 these plaques (Fig. 1A). We found that DFMO significantly reduced bound virus in a dose-  
115 dependent manner (Fig 1B), in agreement with prior work and corresponding to a decrease in  
116 cellular polyamines, as measured by thin layer chromatography (Fig. 1C). To determine if this  
117 polyamine-dependent attachment phenotype relied on cellular factors, we treated cells with  
118 DFMO to deplete polyamines and subsequently replenished the polyamines (putrescine,  
119 spermidine, and spermine) in an equimolar concentration. Adding polyamines to the cells at the  
120 time of infection did not rescue viral attachment, nor did addition 4h prior to attachment. However,  
121 when polyamines were added 16h prior to infection, we observed a full rescue in CVB3  
122 attachment. These data suggest that polyamines rescue viral attachment but rely on an extended  
123 incubation period, perhaps because cellular synthesis of attachment factors required an extended  
124 time.

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**Figure 1. CVB3 requires polyamines for attachment.** (A) Representative plaques from B. (B) (Top) Quantification of plaques formed from a CVB3 binding assay with DFMO treated Vero cells. (Bottom) Thin layer chromatography of Huh7 cells treated with increasing doses of DFMO. (C) Vero cells were treated with DFMO for 96 hours then treated with 10  $\mu$ M equimolar ratio of polyamines at the indicated times before infection. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  by the Student's  $t$  test. Data from at least three independent experiments.

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**Polyamine depletion decreases expression of genes in the cholesterol synthesis pathway.**

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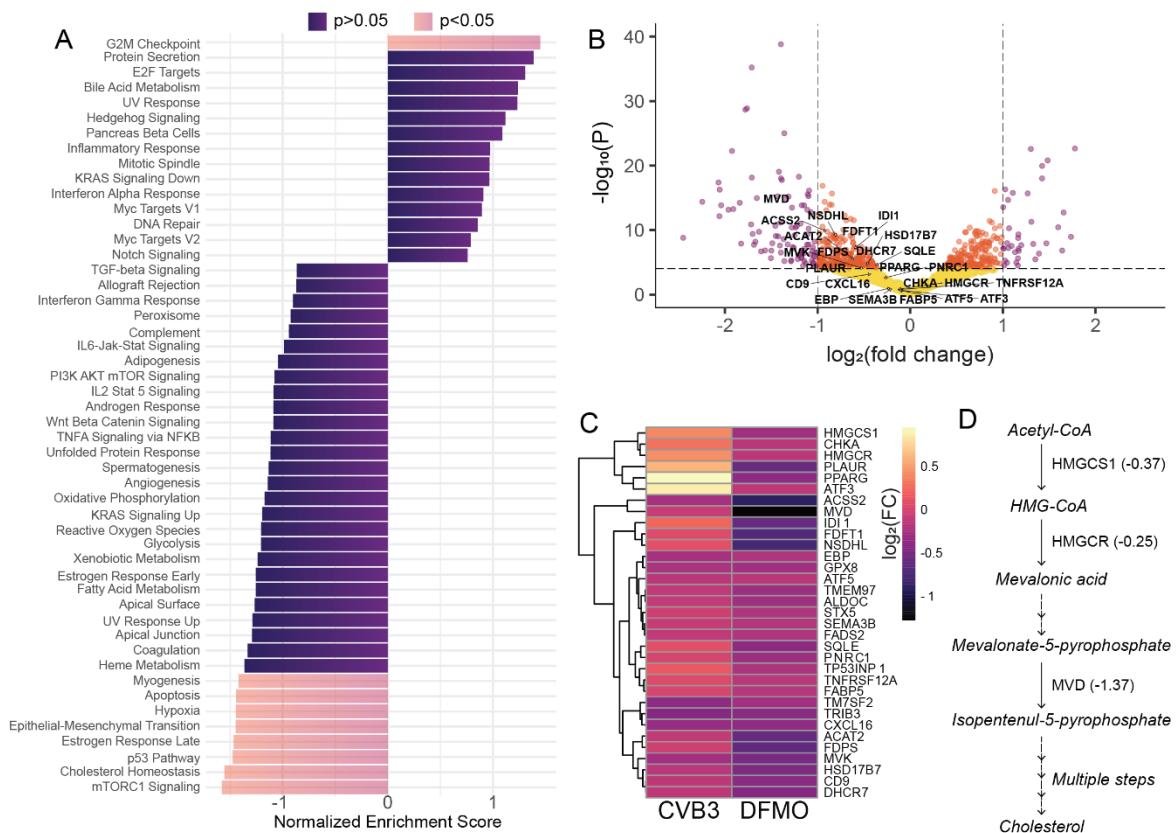
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To better understand the effects polyamine depletion has on cells and if we could identify polyamine-modulated cellular factors involved in viral attachment, we performed RNA-sequencing on untreated and polyamine-depleted cells. Huh7 cells were left untreated or depleted of polyamines with 1 mM DFMO for 96 h, at which time RNA was extracted and analyzed by Illumina paired-end reading. After the alignment of reads against human genome, a differential gene expression analysis was conducted to identify significant changes in expression. To uncover the underlying biological processes that may link these pathways, gene set enrichment analysis (GSEA) was performed. This showed multiple metabolic pathways that were significantly enriched for decreased gene expression by polyamine depletion including alcohol metabolism, cholesterol metabolism, and cellular response to a chemical (Fig 2A). Cholesterol plays a large role in

147 membrane stability and fluidity which could account for the differences seen in the GSEA analysis.  
 148 In order to investigate specific cholesterol genes affected by DFMO, cholesterol genes involved  
 149 directly in cholesterol synthesis were overlaid in a Volcano plot (Fig 2B). Multiple genes were  
 150 down regulated including HMGCS1, HMGCR, MVK, and MVD. Importantly, several polyamine  
 151 metabolic genes, including SAT1 and OAZ1 exhibited reduced expression, consistent with their  
 152 role in inhibiting polyamine synthesis. We next explored how these changes caused by DFMO  
 153 related to changes caused by CVB3 infection. Again, differential gene expression analysis was  
 154 performed on CVB3-infected and mock-treated cells. Leading edge genes from the “Cholesterol  
 155 Homeostasis” category were visualized by heat map for CVB3-infected and DFMO-treated  
 156 samples (Fig 2C). Interestingly, CVB3 infection increased expression of many cholesterol  
 157 biosynthesis genes that are downregulated by DFMO, suggesting CVB3 has mechanisms to  
 158 promote this pro-viral pathway. Thus, the transcriptomic analysis of polyamine depleted and virus-  
 159 infected cells revealed that cellular metabolic processes and, specifically, cholesterol biosynthesis  
 160 (Fig 2D) may function in a pro-viral manner.  
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163 **Figure 2. Transcriptomic analysis of DFMO treated cells.** Huh7 cells were

164 treated with 1mM DFMO for 96h and subjected to RNA sequencing. Results are

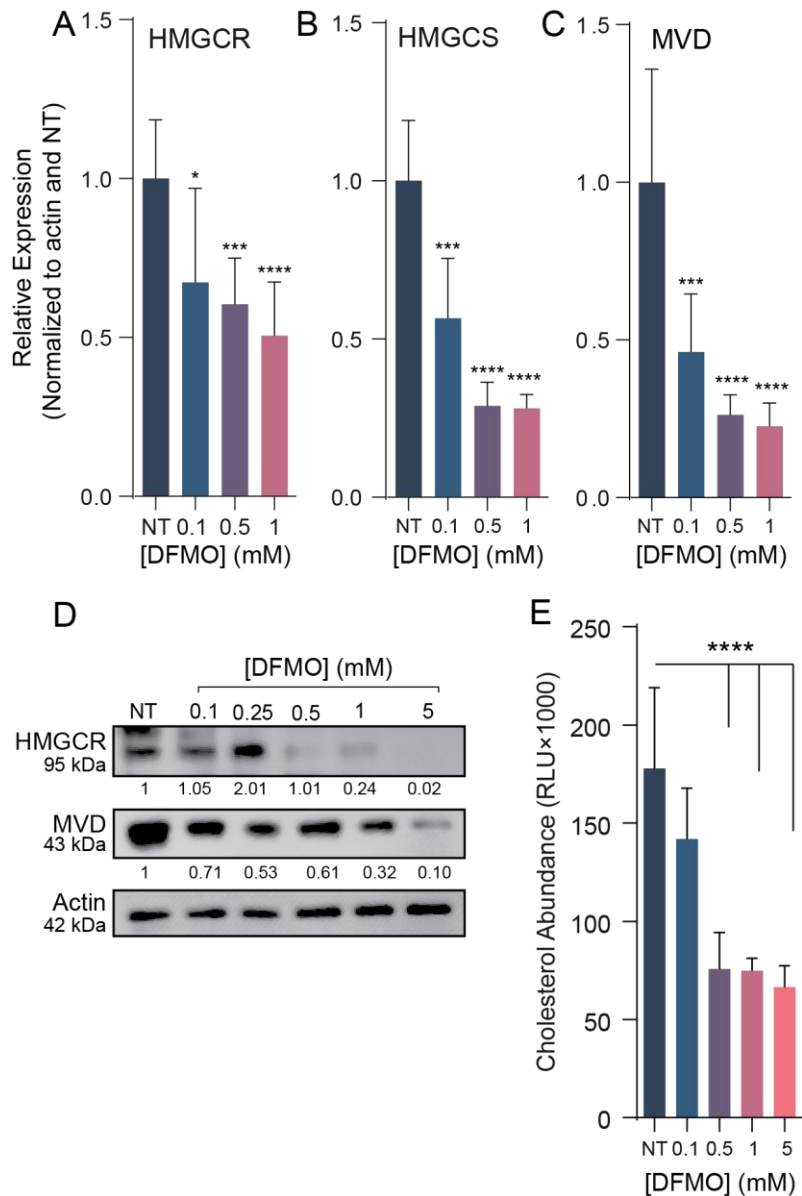


165 based on duplicates. (A) GSEA was conducted on genes differentially expressed  
166 by 1mM DFMO treated Huh7 versus untreated Huh7. The top positively and  
167 negatively enriched Hallmark pathways were plotted ( $p_{adj} < 0.05$ , purple,  $p_{adj}$   
168  $> 0.05$ , blue). (B) Volcano plot indicating  $\log_2$ FoldChange for genes from  
169 differential gene expression analysis comparing DFMO treated cells relative to  
170 untreated cells. Significant changes in gene expression are plotted in purple for  
171 genes :  $p_{adj} < 0.05$ ,  $\log_2FC > 1$ , in orange for  $p_{adj} < 0.05$ ,  $\log_2FC < 1$  and in yellow  
172 for  $p_{adj} > 0.05$ ,  $\log_2FC < 1$ . P-values were adjusted for false discovery rate using  
173 Benjamini Hochberg method. (C) Genes from the leading edge of cholesterol  
174 homeostasis pathway were subjected to hierarchical clustering for both conditions  
175 DFMO treated or CVB3 infected cells relative to untreated cells.  $\log_2$  fold change  
176 from target genes are displayed as a heat map. (D) Cholesterol synthesis pathway  
177 with the representation of down-regulated genes HMGCS1, HMGCRC and MVD.

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179 **Polyamine depletion decreases cholesterol gene expression, protein levels, and**  
180 **intracellular cholesterol.** To test polyamines' effect on the transcription of cholesterol synthesis  
181 genes and confirm the RNA-seq data, cells were treated for four days with increasing  
182 concentrations of DFMO. RNA was then extracted and RT-qPCR was performed using optimized  
183 and specific primers. HMGCRC, HMGCS, and MVD showed moderate but significant reductions in  
184 expression (Fig. 3A-C), aligning with the RNA-sequencing data. To determine if this reduction in  
185 transcription affected protein synthesis, we examined total cellular levels of HMGCRC and MVD by  
186 western blot. Both proteins showed reduced levels with polyamine depletion (Fig. 3D). Finally, to  
187 determine if reduction of transcription and translation of cholesterol synthesis proteins affected  
188 intracellular cholesterol, cells were treated with DFMO for 96h, and the total amount of cellular  
189 cholesterol was measured via a luciferase-based cholesterol assay. We found that total cellular  
190 cholesterol was significantly reduced with DFMO treatment, consistent with a decrease in  
191 expression of cholesterol synthesis genes (Fig. 3E). Thus, cellular cholesterol synthesis relies on  
192 polyamines through the expression of cholesterol synthetic proteins.

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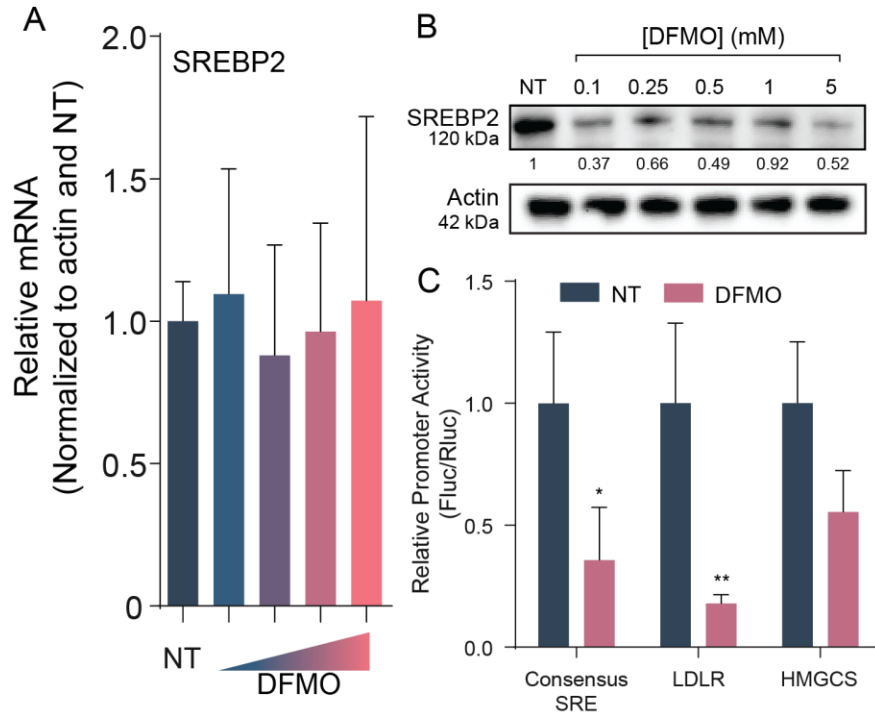
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**Figure 3. Depletion of polyamines decreases cholesterol synthesis gene expression, translation, and intracellular abundance.** (A-C) Normalized qPCR of cholesterol synthesis genes relative to actin expression in Huh7 cells treated with DFMO. (D) Western blot of Huh7 cells treated with increasing doses of DFMO. Actin was used as a loading control. (E) Intracellular cholesterol abundance in DFMO treated Huh7 cells. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$  by the Student's *t* test. Data from at least three independent experiments.

**Polyamine-dependent SREBP2 translation but not transcription facilitates transcriptional activity at cholesterol gene promoters.** Having observed that an array of cholesterol synthetic



205 enzymes were reduced in transcription and translation by polyamine depletion, we considered  
206 that polyamines may affect a master regulator of their expression, rather than on each gene  
207 individually. We hypothesized that polyamines were affecting the transcription factor sterol  
208 regulatory binding protein 2 (SREBP2), one such regulator. SREBP2 is a multipass  
209 transmembrane protein found within the ER. When cholesterol levels are low within the cell,  
210 SREBP2 relocates to the Golgi where it is cleaved by S1P and S2P. The N-terminus of SREBP2  
211 then relocates to the nucleus where it binds to sterol regulatory elements (SRE) to increase  
212 transcription of cholesterol synthesis genes. To test the impact polyamines have on SREBP2,  
213 Huh7 cells were treated with increasing doses of DFMO followed by qPCR (Fig. 4A). Unlike the  
214 cholesterol synthetic genes, we observed no significant change in SREBP2 transcripts. However,  
215 examining SREBP2 protein levels by western blot revealed that polyamine depletion caused a  
216 reduction of SREBP2 protein (Fig. 4B). To determine if this reduction of SREBP2 translation was  
217 sufficient to impact the expression of cholesterol synthesis genes, we measured the activity of  
218 SREBP2 binding to its promoter, the SRE. We transfected cells with or without polyamines with  
219 a construct encoding firefly luciferase driven by distinct cellular SREs. We used the HMGCS SRE,  
220 the LDLR SRE, and a generalized SRE created with the SRE consensus sequence. Additionally,  
221 we transfected renilla luciferase to control for effects of polyamine depletion on luciferase  
222 translation. When we measured SRE activity, we noted a significant reduction in activity in  
223 polyamine depleted cells for all SREs tested, suggesting that polyamine depletion impacts SRE  
224 promoter activity, likely due to a reduction in SREBP2 translation. Thus, polyamines facilitate  
225 translation and activity but not transcription of SREBP2.  
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**Figure 4. SREBP2 translation is dependent on polyamines.** (A) Normalized qPCR of SREBP2 with increasing doses of DFMO (0.1, 0.5, 1, 5mM) relative to actin expression in Huh7 cells. (B) Western blot of SREBP2 in DFMO treated Huh7 cells. Actin was used as a loading control. (C) Huh7 cells were treated with 1mM DFMO for 96h followed by transfection with the promoter luciferase constructs and siCheck. Results are normalized to NT and are relative to renilla activity. \* $p < 0.05$  and \*\* $p < 0.01$  by two-way ANOVA. Data from at least three independent experiments.

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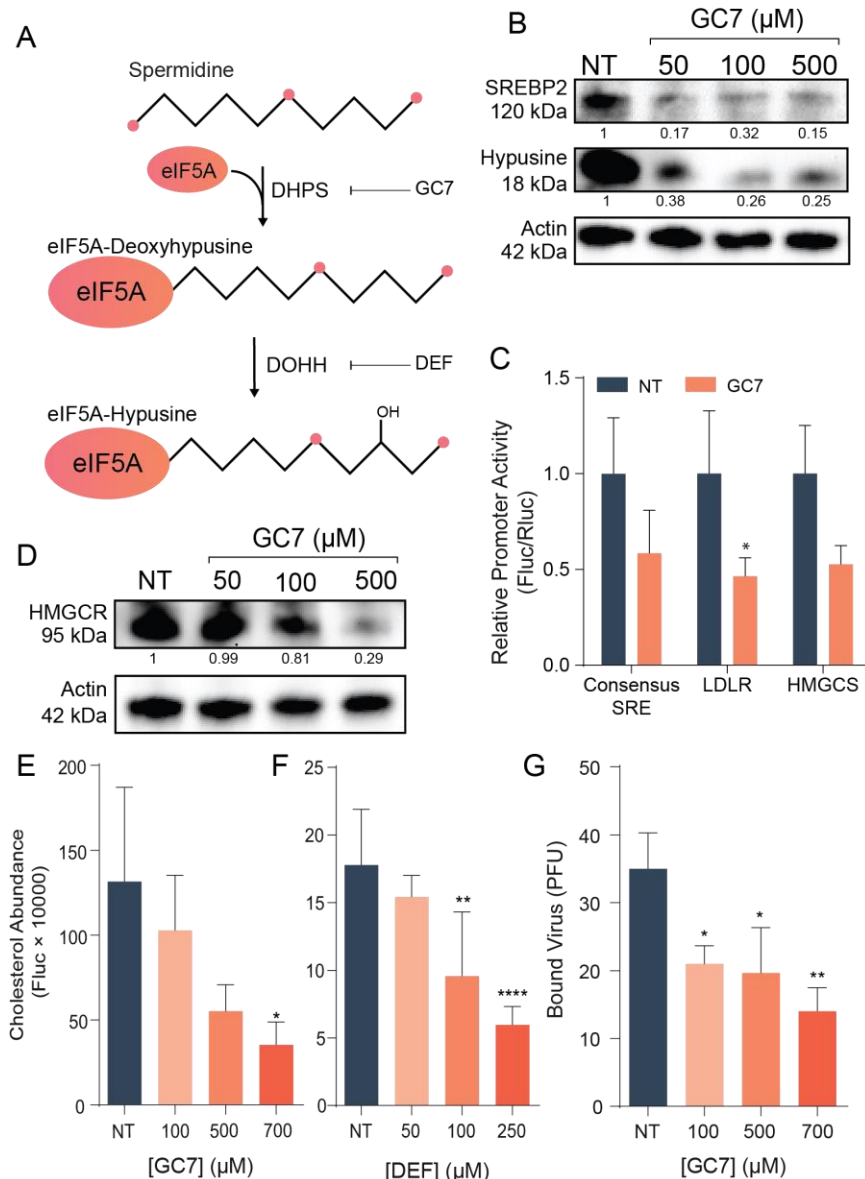
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**Polyamine-dependent eIF5A hypusination supports SREBP2 translation and cholesterol synthesis.** A well-described mechanism by which polyamines support cellular translation is through the post-translational modification of eIF5A, in which spermidine is conjugated and hydroxylated, forming a unique amino acid called hypusine (Fig. 5A). While the precise mechanism(s) by which hypusinated eIF5A facilitates translation remain incompletely understood, it is known that a subset of cellular proteins rely on this enzyme for efficient translation. To determine if SREBP2 is included in this subset, we treated cells with inhibitors of the hypusination pathway. Inhibition of the deoxyhypusine synthase (DHPS) inhibitor GC7 resulted in a dose-dependent reduction in SREBP2 protein levels, concomitant with a reduction in cellular hypusinated eIF5A (Fig 5B). In concurrence with this, treatment with GC7 resulted in significant reduction of SRE promoter activity (Fig. 5C). Additionally, we observed a reduction in HMGCR

248 protein levels with increasing GC7 or deferiprone treatment (Fig. 5D). To confirm that these  
249 changes in SREBP2 and cholesterol synthesis gene expression affected cellular cholesterol, we  
250 again measured total cellular cholesterol in cells treated with increasing doses of GC7 (Fig. 5E)  
251 or the deoxyhypusine hydroxylase (DOHH) inhibitor deferiprone (DEF) (Fig. 5F). Similar to our  
252 results with DFMO, we observed a significant, dose-dependent reduction in cellular cholesterol  
253 when hypusination was inhibited, suggesting that polyamines facilitate cholesterol synthesis  
254 through hypusination. Finally, to confirm that hypusinated eIF5A contributes to viral attachment,  
255 as we see with DFMO, we treated cells with increasing doses of GC7, performed an attachment  
256 assay, and counted attached viruses (Fig. 5G). We observed a dose-dependent decrease of viral  
257 attachment with GC7 treatment, suggesting hypusinated eIF5A facilitates viral attachment.  
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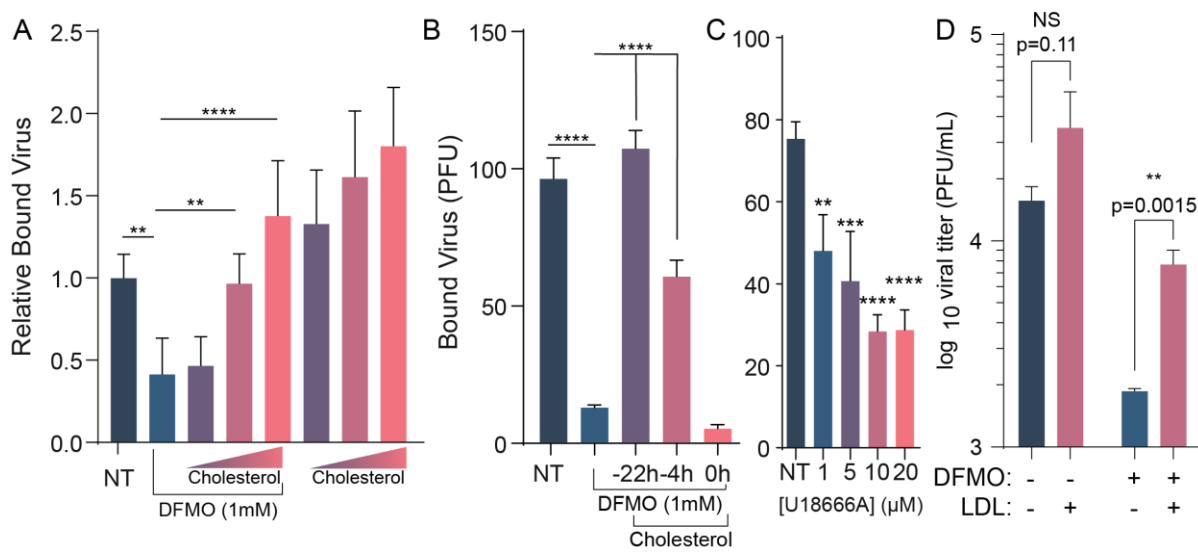
**Figure 5. Hypusination supports cholesterol synthesis.** (A) Pathway of eIF5A hypusination and its inhibition. (B) Western blot of Huh7 cells treated with increasing doses of GC7 probed for SREBP2 and hypusine-eIF5a. Actin was used as a loading control. (C) Huh7 cells treated with 500  $\mu\text{M}$  GC7 for 24h followed by transfection with the promoter luciferase constructs and siCheck as a transfection control. Results were normalized to NT and are relative to renilla activity from siCheck. Two-way ANOVA was used to analyze statistical significance. (D) Western blot of Huh7 cells treated with increasing doses of GC7 and probed for HMGCR and actin was used as the loading control. (E-F) Intracellular cholesterol abundance of GC7 (E) and DEF (F) treated Huh7 cells. Y-axis is Fluc value

270 x10000. (G) Number of CVB3 plaque forming units bound to GC7 treated Vero  
271 cells. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$  by the Student's  $t$  test.  
272 Data from at least three independent experiments.

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274 **Exogenous cholesterol rescues CVB3 attachment to polyamine depleted cells and**  
275 **replication.** Viruses require diverse cellular factors to mediate attachment and entry, including  
276 cholesterol. To determine if cholesterol is a key polyamine-modulated molecule in this process,  
277 we attempted to rescue viral attachment and replication by adding cholesterol to cells  
278 exogenously. Cells depleted of polyamines were treated with increasing doses of cholesterol  
279 overnight, followed by washing away excess cholesterol and performing a binding assay as  
280 before. When plaques were revealed, we found that cholesterol significantly rescued CVB3  
281 binding in a dose dependent manner (Fig. 6A). To test if this rescue was due to a direct interaction  
282 of CVB3 and cholesterol or if cholesterol had to be incorporated into cellular membranes,  
283 cholesterol was added to cells at 22 h, 4 h, and 0 h before CVB3 binding. Only cholesterol added  
284 22 h and 4 h before binding was able to significantly rescue CVB3 binding to polyamine depleted  
285 cells (Fig. 6B), suggesting that viral attachment requires cellular cholesterol incorporation. Finally,  
286 to determine if cholesterol could rescue virus replication in polyamine-depleted cells, we  
287 measured viral titers in DFMO- and cholesterol-treated cells. DFMO significantly reduced viral  
288 titers, and treatment with exogenous cholesterol significantly increased these titers, though not to  
289 untreated levels, highlighting that polyamines affect cholesterol to support viral replication but also  
290 that polyamines play multiple roles in infection.

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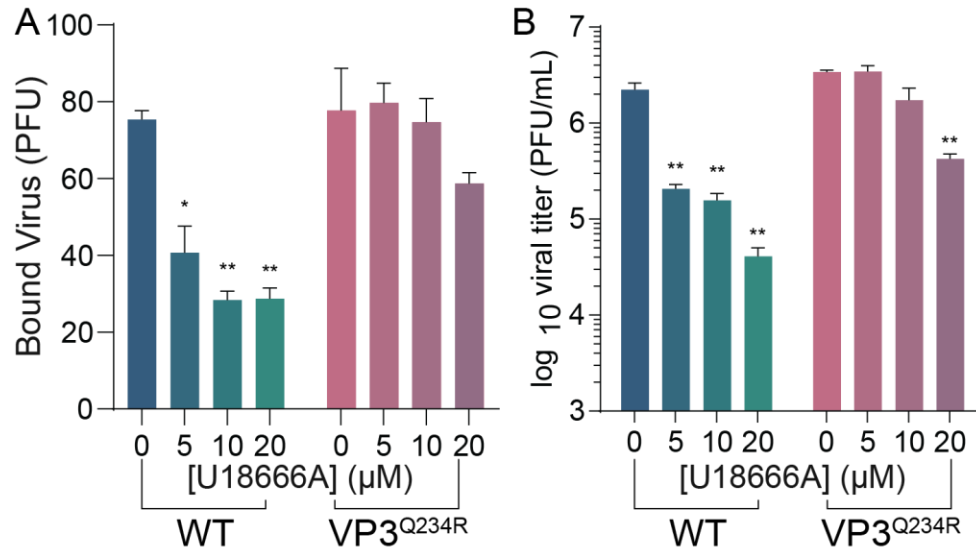


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293 **Figure 6. Addition of cholesterol rescues CVB3 attachment and replication**  
294 **in polyamine depleted cells.** (A) Binding assay of Vero cells were left untreated  
295 or treated with DFMO for 96 h. After 72 h, cells were treated with 100, 250, or 500  
296  $\mu\text{g/mL}$  for 16-24h. (B) Binding assay of DFMO treated Vero cells treated with 500  
297  $\mu\text{g/mL}$  cholesterol for the indicated times. (C) Binding assay of Vero cells treated  
298 with increasing concentrations of U1866A. (D) Huh7 cells were left untreated or  
299 treated with DFMO for 96 h. After 72 h, the cells were treated with 100  $\mu\text{g/mL}$  LDL  
300 for 24 h followed by infection with CVB3 at an MOI of 0.1. Virus was collected after  
301 24 h and the PFU/mL was quantified via titration. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p <$   
302 0.001, and \*\*\*\* $p < 0.0001$  by the Student's  $t$  test. Data from at least three  
303 independent experiments.

304  
305 We previously described a viral mutant that exhibits enhanced viral attachment in polyamine  
306 depleted cells via mutation of VP3 at position 234. This mutant was found when we passaged  
307 virus in DFMO-treated cells, suggesting that CVB3 harboring this mutation may be resistant to  
308 polyamine depletion by overcoming a block in attachment. To determine if this block was  
309 cholesterol, we considered viral attachment in cells where cholesterol transport is impaired. The  
310 inhibitor U18666a, an NPC1 inhibitor, impacts cellular transport of cholesterol and reduces  
311 plasma membrane levels. We found that treatment of cells with U18666a reduced CVB3  
312 replication in a dose-dependent manner, as well as attachment. We next considered that CVB3  
313 VP3<sup>Q234R</sup> may be resistant to this inhibitor. When we performed an attachment assay on U18666a  
314 treated cells using CVB3 VP3<sup>Q234R</sup>, we observed a modest reduction in viral attachment compared  
315 to WT CVB3, suggesting that the mutant may overcome polyamine depletion via bypassing the  
316 need for cholesterol. Additionally, we measured viral titers in U18666a-treated cells infected with  
317 CVB3 VP3<sup>Q234R</sup> and observed titers significantly higher than WT CVB3. Together, these data  
318 suggest that polyamines support cholesterol synthesis to promote viral attachment and that CVB3  
319 is able to overcome polyamine depletion by mutation of VP3, which bypasses the need for  
320 cholesterol in viral attachment.

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## Discussion

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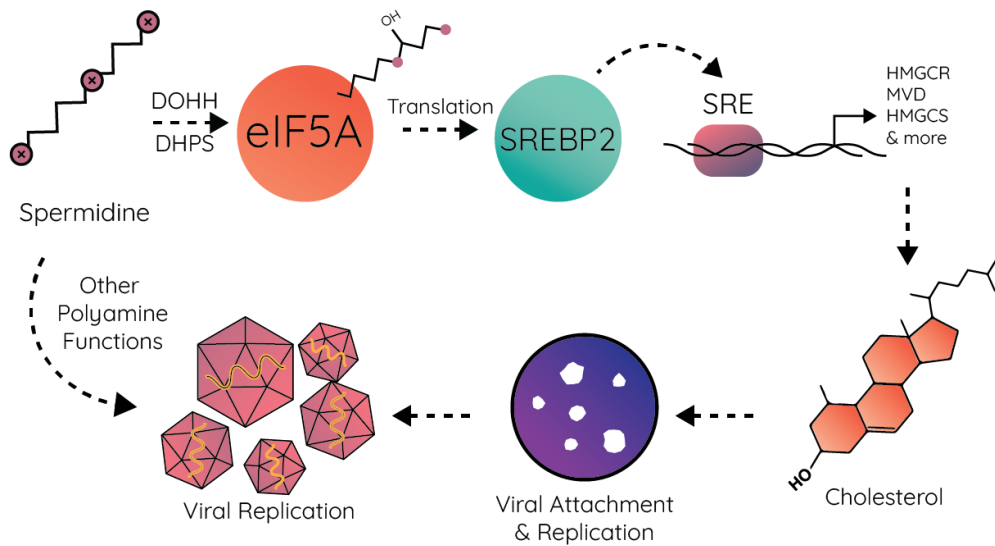
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**Figure 7. CVB3 VP3<sup>Q234R</sup> mutant is resistant to reduction of cytoplasmic cholesterol.** (A) Huh7 cells were treated with increasing doses of U18666A for 16h prior to binding WT and VP3<sup>Q234R</sup> mutant CVB3. Bound virus was enumerated by counting plaques indicative of attached virus. (B) Cells were treated as in (A) and infected at MOI 0.1 with WT and VP3<sup>Q234R</sup> mutant CVB3. Viral titers were determined at 24 hpi. \*p<0.05, \*\*p<0.01 via Student's *t* test from three independent experiments.





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**Figure 8. Working model.** Polyamines facilitate hypusination of eIF5A, which promotes SREBP2 translation. SREBP2 binds to SREs on the promoters of cholesterol synthesis genes, leading to their expression and cholesterol synthesis. Cellular cholesterol synthesis enhances CVB3 attachment and, subsequently viral replication.

The process of hypusination has been well studied and defined, though the precise mechanisms by which hypusination of eIF5A supports translation remains to be fully elucidated. Hypusine-eIF5A is required for cellular proliferation and translation of “hard-to-read” sequences, such as poly-proline motifs. However, the specific genes that require hypusine-eIF5A are not well known. Here we have found that SREBP2 requires hypusine-eIF5A to be translated. While HMGCR does not have any poly-prolines, it does have a 3 amino acid motif, GGT, at position 807, that has been shown to be require hypusine-eIF5A<sup>4</sup>. SREBP2 on the other hand has multiple poly-proline repeats as well as other 3 amino acid motifs shown to require hypusine-eIF5A. Furthermore, a study found hypusine-eIF5A is involved in cotranslation translocation of proteins into the ER, where SREBP2 is found<sup>29</sup>. Our findings demonstrate another level of regulation of the cholesterol synthesis pathway, translational regulation of SREBP2. Since polyamines are also vital for cellular proliferation, a lack of polyamines will prevent crucial proliferation genes from being translated.

The roles of polyamines in viral infection are diverse and appear to be distinct for different viral families. In the case of enteroviruses, like CVB3, prior work showed that polyamines facilitate cellular attachment and protease activity. However, the mechanism(s) by which polyamines promote these activities was unclear. Cholesterol is a key molecule in enterovirus attachment,

368 and its association with lipid rafts has been demonstrated to facilitate CVB3 engagement with its  
369 receptor (Coxsackie- and adenovirus receptor, CAR). For many viruses, cholesterol and lipids  
370 promote not only entry, but also viral replication, either through the formation of viral replication  
371 compartments on specific cellular membranes or through the hydrolysis of lipids to release energy  
372 for replication. While polyamines have previously been described to facilitate viral genome  
373 replication for both chikungunya virus and Ebolavirus, it remains to be determined if this  
374 phenotype could be through the synthesis of cellular cholesterol. Prior work highlighted effects of  
375 polyamines on viral proteins, such as the polymerase, but our work highlights an indirect effect  
376 on virus infection, specifically through cholesterol synthesis.

377

378 Although we show that SREBP2 relies on polyamines for translation through hypusinated eIF5A,  
379 regulation of SREBP2 and its activity is complex. Polyamines play a wide variety of roles within  
380 the cell, one of which is stabilizing DNA and promoting transcription factor engagement. Another  
381 potential area of involvement for polyamines within the cholesterol pathway is the stabilization of  
382 SREBP2 binding of SRE. It has previously been shown that polyamines maintain the estrogen  
383 receptor elements (ERE) in the correct motif to allow for estrogen receptor (ER) to bind, and a  
384 lack of polyamines decreased the ability of ER to bind to EREs. Polyamines also play a role in  
385 protease function. Our lab previously demonstrated that both of CVB3's proteases develop  
386 mutations in response to polyamine depletion, making them resistant to the lack of polyamines,  
387 suggesting a role for polyamines in protease activity. SREBP2 processing requires two proteases,  
388 S1P and S2P, for its maturation. It is unclear whether SREBP2 cleavage or S1P/S2P protease  
389 activity relies on polyamines. It is unlikely that hypusine-eIF5A is affecting another protein  
390 upstream of SREBP2 as its expression levels do not significantly change with DFMO treatment.

391

392 eIF5A is the only enzyme within the eukaryotic cell to be hypusinated, and it supports the  
393 translation of diverse cellular proteins. Prior work showed that Ebolavirus relies on hypusination  
394 specifically for the translation of VP35, a viral transactivator that facilitates viral gene expression.  
395 Other work showed that inhibitors of hypusination reduce translation of retroviruses, including  
396 HIV-1. The direct roles of eIF5A hypusination in viral protein synthesis remain to be fully explored  
397 for many viruses, including CVB3. However, hypusinated eIF5A's roles in cellular translation also  
398 affect viral replication, as seen here. Thus, hypusinated eIF5A is an important drug target due to  
399 its potential direct and indirect effects on virus replication. Additional exploration of mechanisms  
400 connecting hypusinated eIF5A to viral and cellular factors involved in infection will further  
401 illuminate how this molecule and polyamines support the replication of diverse viruses.

402

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408

## 409 **Materials and methods**

### 410 **Cell Culture and Virus Enumeration**

411 Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) with  
412 bovine serum (FBS; Thermo-Fischer) and penicillin-streptomycin at 37°C and 5% CO<sub>2</sub>. Huh7 cells  
413 were supplemented with 10% fetal bovine serum (FBS; Thermo-Fischer). Vero cells were  
414 obtained through BEI Resources. Vero cells were supplemented with 10% new-born calf serum  
415 (NCBS; Thermo-Fischer). CVB3 (Nancy strain) was derived from the first passage of virus in Vero  
416 cells after rescue from an infectious clone. Viral stocks were maintained at -80°C. Viral titers were  
417 enumerated as previously described<sup>13</sup>.

418

### 419 **Drug Treatments**

420 Difluoromethylornithine (DFMO; TargetMol) was diluted to a 100mM solution in sterile water. For  
421 DFMO treatments, cells were trypsinized (Zymo Research) and reseeded with fresh medium  
422 supplemented with 2% FBS (Huh7) or 2% NCBS (Vero). Cells were treated with 1mM DFMO  
423 unless otherwise indicated. Cells were incubated with DFMO for 96h to allow for depletion of  
424 polyamines. GC7 was diluted to a 100mM solution in sterile water. Freshly seeded cells were  
425 treated with GC7 along with 500µM aminoguanidine for 16-24h. Cholesterol was diluted to 10  
426 mg/mL in ethanol then added to adherent cells for 16-24h.

427

### 428 **RNA Purification and cDNA Synthesis**

429 Media were cleared from cells, and Trizol reagent (Zymo Research) was added directly. Lysate  
430 was then collected, and RNA was purified through a Zymo RNA extraction kit. Purified RNA was  
431 subsequently used for cDNA synthesis using High Capacity cDNA Reverse Transcription Kits  
432 (Thermo-Fischer), according to the manufacturer's protocol, with 10–100 ng of RNA and random  
433 hexamer primers.

434

### 435 **RNA Sequencing**

436 RNA was purified and prepared as described from Huh7 cells treated for 96h with DFMO or  
437 infected for 24h with CVB3. Libraries were prepared by the University of Chicago Genomics  
438 Facility and analyzed by Illumina NovaSeq 6000. Read quality was evaluated using FastQC  
439 (v0.11.5). Adapters were trimmed in parallel to a quality trimming (bbduk,  
440 [sourceforge.net/projects/bbmap/](https://sourceforge.net/projects/bbmap/)). All remaining sequences were mapped against the human  
441 reference genome build 38 with STAR (v2.5.2b)<sup>30</sup>. HTseq (v0.6.1) was used to count all reads for  
442 each gene and set up a read count table<sup>31</sup>. Differential gene expression analyses were performed  
443 using the DESeq2 Bioconductor package (v1.30.1)<sup>32</sup>. The default “ashr” shrinkage (v2.2-47)<sup>33</sup> set  
444 up was used for our analysis. Gene set enrichment analysis (GSEA) was performed with the fgsea  
445 Bioconductor package<sup>34</sup>, using Hallmark gene sets downloaded from the Molecular Signatures  
446 Database<sup>35</sup>.

447

#### 448 **Plaque Formation Attachment Assay**

449 Vero cells were seeded in 6-well plates and grown to 100% confluence in DMEM with 2% NCBS  
450 and treated for 96h with the indicated concentrations of DFMO. After 96 h of DFMO treatment,  
451 cells were placed on ice and the media aspirated from the cells. 500 uL of serum free media  
452 containing 1000 PFU CVB3 was added to cells on ice for 5 min. Cells were washed 3x with PBS  
453 and then overlaid with 0.8% agarose containing DMEM with 2% NCBS. The plates were incubated  
454 at 37°C for 2 days for plaques to develop. The cells were fixed with 4% formalin, and the plaques  
455 were visualized with crystal violet staining. For the cholesterol rescue, cells were washed 3x with  
456 PBS before infecting with CVB3.

457

#### 458 **qPCR Gene Expression Assay**

459 Huh7 cells were seeded at  $4 \times 10^4$  cells per well in 24-well plates in DMEM with 2% FBS. Cells  
460 were treated with varying concentrations of DFMO for 96 h. After 96 h, the media was aspirated  
461 off cells, washed 1x with PBS, and then, 200 uL of Trizol was added to the cells. The RNA was  
462 extracted with the Zymo RNA extraction kit, converted to cDNA, and quantified by real-time PCR  
463 with SYBR Green (DotScientific) using the one-step protocol QuantStudio 3 (ThermoFisher  
464 Scientific). Relative expression was calculated using the  $\Delta\Delta C^T$  method, normalized to the  $\beta$ -actin  
465 qRT-PCR control, and calculated as the fraction of the untreated samples. Primers were verified  
466 for linearity using 8-fold serial diluted cDNA and checked for specificity via melt curve analysis.  
467 The primer sequences are as follows: HMGCRCR, (F) 5'-GAG ACA GGG ATA AAC CGA GAA AG-  
468 3' and (R): 5'-GGA GGA GTT ACC AAC CAC AAA-3'; HMGCS, (F): 5'-CCT GCC AAG AAA GTA  
469 CCA AGA-3' and (R): 5'-GTC TTG CAC CTC ACA GAG TAT C-3'; MVD (F): 5'-TGG TTC TGC

470 CCA TCA ACT C-3' and (R): 5'-GGT GAA GTC CTT GCT GAT GA-3'; SREBP2 (F): 5'-CTG TAG  
471 CGT CTT GAT TCT CTC C-3' and (R): 5'-CCT GGC TGT CCT GTG TAA TAA-3'.

472

### 473 **Western Blot**

474 Samples were collected with Bolt LDS Buffer and Bolt Reducing Agent (Invitrogen, Waltham, MA,  
475 USA) and run on polyacrylamide gels. Gels were transferred using the iBlot 2 Gel Transfer Device  
476 (Invitrogen). Membranes were blocked with 5% BSA in TBST then probed with primary antibodies  
477 for HMGCR (Ms mAb, 1:000, abcam), MVD (1:1000, Santa Cruz), SREBP2 (Gt pAb, 1:1000, R&D  
478 Systems), Hypusine (Rb pAb, 1:2000, EMD Millipore) or  $\beta$ -actin (Ms mAb, 1:1000, proteintech)  
479 overnight at 4°C. Membranes were then washed 3x in TBST followed by 1h incubation of in  
480 secondary antibody (Gt $\alpha$ Ms/Gt $\alpha$ Rb/Donkey $\alpha$ Gt HRP, 1:15000, Jackson Labs). After 3 additional  
481 washes in TBST, membranes were treated with SuperSignal West Pico PLUS Chemiluminescent  
482 Substrate (ThermoFisher Scientific) and visualized on Fluorchem E imager (Protein Simple, San  
483 Jose, CA, USA). Quantification of western blots were done by using ImageJ and normalizing to  
484 NT and relative to actin density.

485

### 486 **Intracellular Cholesterol Abundance Assay**

487 Huh7 cells were plated at a density of 5000 cells/well in a 96 well plate in DMEM with 2% FBS.  
488 Cells were treated with DFMO for 96h or after 72h, treated with DEF or GC7 for 24h. The following  
489 day, the media was removed from cells followed by a PBS wash. To measure total intracellular  
490 cholesterol abundance, we used the Cholesterol/Cholesterol Ester-Glo Assay<sup>TM</sup> (Promega) in  
491 accordance to manufacturer's protocol.

492

### 493 **SRE promoter luciferase**

494 Complimentary primers were made containing SRE consensus sequence were ordered flanked  
495 by Sfil cut site overhangs (FWD: 5'-CGGCC ATCACCCAC GGCCTCGG-3'; REV 3'-  
496 GCCGCCGG TAGTGGGGTG CCGGA-5'). Primers were phosphorylated and annealed at 37°C  
497 for 30 minutes then 95°C for 5 minutes and were allowed to cool to 25°C. pGL4.10 (Promega)  
498 was digested with Sfil in Fast Digest buffer for 15 min at 50°C. The cut plasmid was ran through  
499 DNA clean up kit. The annealed primers were then ligated into the cut plasmid using T4 ligase  
500 followed by transformation into chemical competent E. Coli. Colonies were picked and grown up  
501 followed by sequencing to confirm the SRE sequence was present.

502

### 503 **Promoter Luciferase Assay**

504 Huh7 cells were plated in a 96 well plate with 2% FBS DMEM then treated with 1 mM DFMO for  
505 96 h or after 96 h, treated with 500 uM GC7. Cells were transfected with SRE-pGL4.10, 5'  
506 HMGCS-Fluc (addgene #60444), or pLDLR-Luc (addgene #14940) after cells had been plated  
507 for 96 h. All cells were transfected with the renilla control plasmid siCheck (Promega). 100 ng of  
508 plasmid were transfected with LipoD293 according to manufacture's protocol. 24 h after  
509 transfection, media was removed followed by one wash with PBS. Cells were then lysed with  
510 gentle lysis buffer for 15 min.

511

## 512 **Statistical analysis**

513 Prism 6 (GraphPad) was used to generate graphs and perform statistical analysis. For all  
514 analyses, a two-tailed Student's *t* test was used to compare groups, unless otherwise noted.

515

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