

1 **Polyamines mediate enterovirus attachment directly and indirectly through** 2 **cellular heparan sulfate synthesis.**

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16

17 **Abstract**

18 Productive viral infection begins with attachment to a susceptible cell, and viruses have evolved
19 complex mechanisms to attach to and subsequently enter cells. Prior to engagement with a
20 cellular receptor, viruses frequently interact with nonspecific attachment factors that can facilitate
21 virus-receptor interactions and viral entry. Polyamines, small positively-charged molecules
22 abundant in mammalian cells, mediate viral attachment, though the mechanism was not fully
23 understood. Using the Coxsackievirus B3 (CVB3) enterovirus model system, we show that
24 polyamines mediate viral attachment both directly and indirectly. The polyamine putrescine
25 specifically enhances viral attachment to cells depleted of polyamines. Putrescine's positive
26 charge mediates its ability to enhance viral attachment, and polyamine analogs are less efficient
27 at mediating viral attachment. In addition to this direct role of polyamines in attachment,
28 polyamines facilitate the cellular expression of heparan sulfates, negatively-charged molecules
29 found on the cell surface. In polyamine-depleted cells, heparan sulfates are depleted from the
30 surface of cells, resulting in reduced viral attachment. We find that this is due to polyamines' role
31 in the process of hypusination of eukaryotic initiation factor 5A, which facilitates cellular
32 translation. These data highlight the important role of polyamines in mediating cellular attachment,
33 as well as their function in facilitating cellular heparan sulfate synthesis.

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35 **Introduction**

36 Viral attachment to a susceptible cell is the first step in the complex process of infection. Viruses
37 have distinct mechanisms to attach to cells, evolving affinities for cellular attachment factors and

38 receptors. In the case of enteroviruses, several ubiquitous molecules serve as nonspecific
39 attachment factors, including heparan sulfates¹⁻⁶, vimentin^{7,8}, and sialic acids^{9,10}. These
40 molecules serve to enhance virus-cell interaction prior to receptor engagement. The initial
41 interaction with these nonspecific molecules serves to convert a three-dimensional search for the
42 viral receptor into a two-dimensional search, enhancing the potential for engagement with the
43 specific viral receptor. Coxsackievirus, one such enterovirus, engages with these cell surface
44 molecules prior to entry mediated by a receptor, the Coxsackievirus and adenovirus receptor
45 (CAR), to initiate infection^{4,10}. Coxsackieviruses also interact with decay accelerating factor (DAF,
46 CD55), which also mediates Coxsackievirus attachment and entry¹¹⁻¹³.

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48 A common childhood infectious agent, Coxsackievirus infection frequently resolves without the
49 need for intervention. However, CVB3 causes significant disease, including hand foot and mouth
50 disease, meningitis, encephalitis, conjunctivitis, and myocarditis¹⁴⁻¹⁶. Coxsackievirus' ability to
51 infect and persist in cardiac tissue represents not only a threat to children but also adults^{17,18}.
52 Seroprevalence is high in some areas, reaching levels as high as 50%¹⁹. Frequent outbreaks of
53 enteroviruses such as enterovirus-A71 or -D68 highlight the ability of these viruses to rapidly
54 spread and cause significant morbidity and mortality. Unfortunately no antivirals or vaccines are
55 available to treat or prevent infection.

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57 We previously showed that Coxsackievirus B3 (CVB3) attachment to cells requires polyamines²⁰,
58 small aliphatic molecules, comprised of short carbon chains and tertiary amines. Polyamines
59 function in cell cycling, translation, and nucleotide metabolism within cells²¹, and they're also
60 important for CVB3 infection²². In polyamine-depleted conditions, CVB3 replication is significantly
61 attenuated, both *in vitro* and using the *in vivo* mouse model²². By passaging CVB3 in polyamine-
62 depleted cells, we previously observed four escape mutants, three in the viral protease 2A or
63 3C^{23,24}, and one in the capsid protein VP3²⁰, suggesting a role in viral attachment and/or entry.
64 Further examination revealed a global role for polyamines in attachment of diverse enteroviruses;
65 however, the mechanism remains to be fully understood.

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67 Here, we show that CVB3 relies on polyamines to mediate viral attachment both directly and
68 indirectly. CVB3 attachment to polyamine depleted cells is diminished using several inhibitors of
69 polyamine metabolism. However, incubation of virus with polyamines enhances viral attachment
70 in a dose-dependent manner. Using a panel of natural and synthetic polyamines, we find that
71 specifically the natural polyamine putrescine enhances viral attachment, though spermidine and

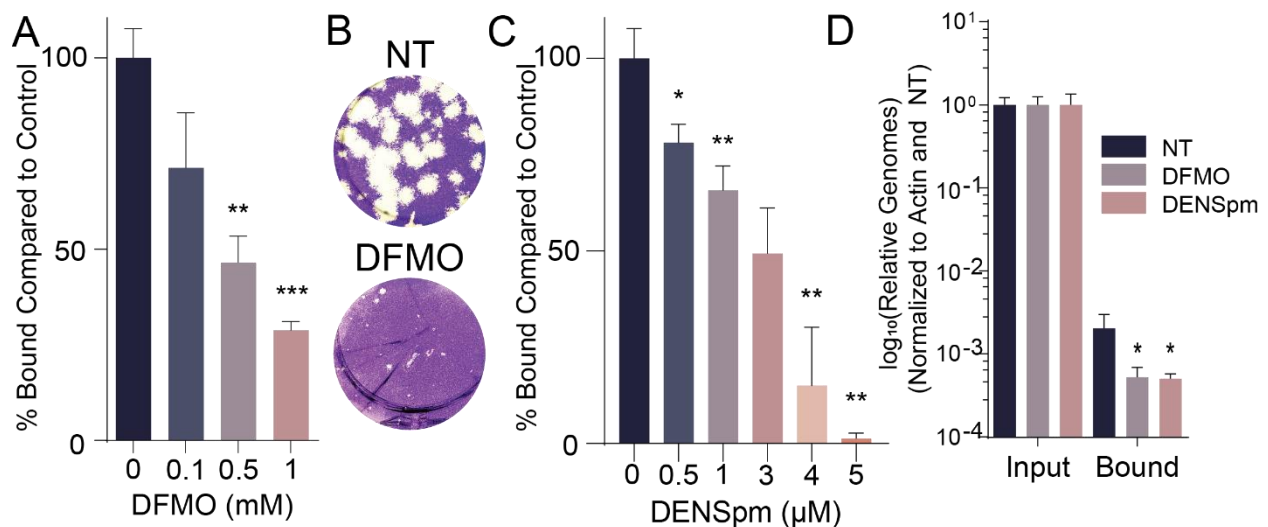
72 spermine also function to a slightly reduced degree. We find that the viral escape mutant CVB3
73 VP3^{Q234R}, which is resistant to polyamine depletion, does not require polyamines, nor do
74 polyamines enhance attachment. However, CVB3 with a negative or neutral charge at this amino
75 acid do rely on putrescine. Finally, we find that cellular factors also contribute to viral attachment.
76 Heparan sulfate, a nonspecific attachment factor, is reduced in cell surface abundance in
77 polyamine-depleted cells, which likely depends on polyamines for the translation of a heparan
78 sulfate synthetic enzyme. Together, these results demonstrate roles for polyamines in directly
79 and indirectly mediating CVB3 attachment, highlighting the importance of these molecules in
80 CVB3 infection.

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82 Results

83 *Polyamines facilitate CVB3 attachment.* We previously demonstrated that polyamines facilitate
84 viral attachment²⁰, though the mechanism was not clear. To recapitulate these results, we
85 performed a viral attachment assay by applying virus to cells on ice for a five-minute period before
86 washing away unbound virus. Immediately after washing, cells were overlaid with agarose-
87 containing media to limit virus spread. To this attachment assay, we added increasing doses of
88 DFMO to deplete polyamines, and we observed a significant reduction in attached virus with
89 increasing DFMO concentrations (Figure 1A, representative plaques shown in Figure 1B).
90 Importantly, in this assay, polyamines are replenished in the agarose-containing media, so
91 polyamine depletion is limited strictly to the binding phase of infection.

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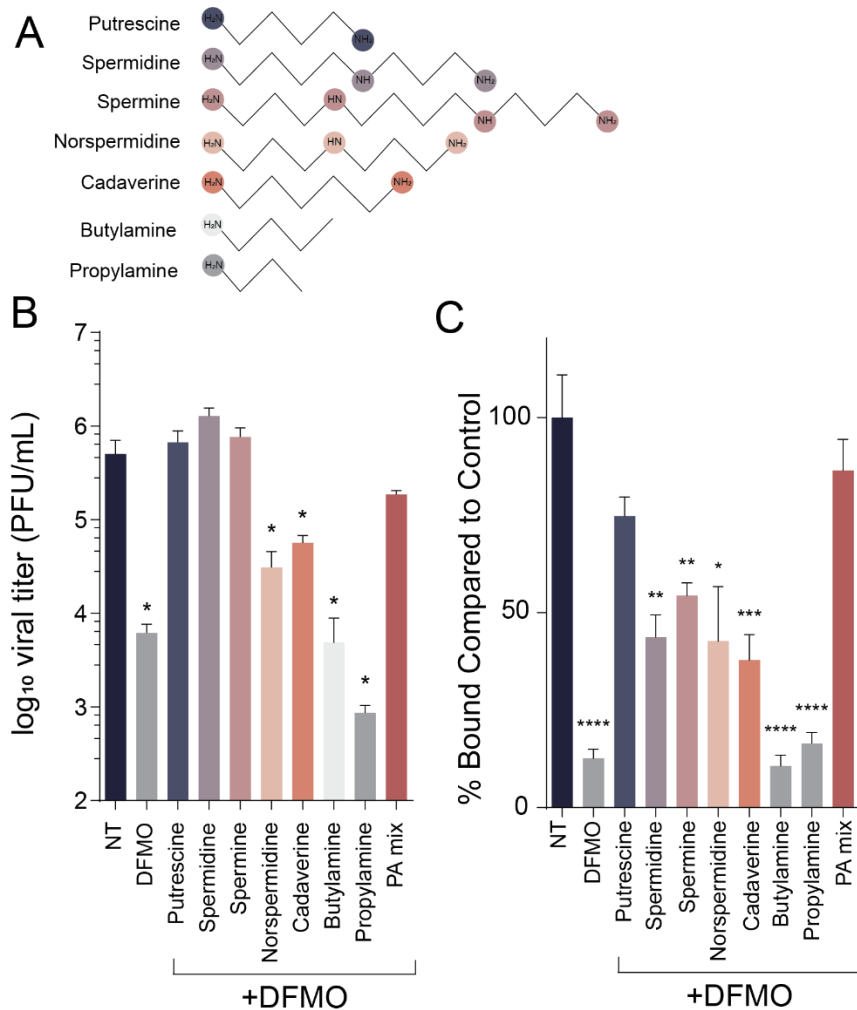
94 **Figure 1. Polyamines facilitate CVB3 attachment.** (A) Vero cells were treated
95 with increasing doses of DFMO for four days prior to measuring viral attachment

96 by plaque assay. Bound virus was quantified and compared to untreated
97 conditions. (B) Representative image of assay as performed in (A). (C) Vero cells
98 were treated with increasing doses of DENSpm 16h prior to performing a binding
99 assay as in (A). (D) Cells were treated with DFMO or DENSpm, infected with
100 CVB3, and bound virus quantified by qRT-PCR after washing away unbound virus.
101 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Student's T test ($N \geq 3$).

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103 While DFMO treatment depletes polyamines by inhibiting ODC1, polyamines can also be depleted
104 by the drug diethylnorspermidine (DENSpm), which activates polyamine catabolism through the
105 enzyme spermidine-spermine acetyltransferase. To determine if DENSpm similarly restricted
106 virus attachment, we treated cells with increasing doses of DENSpm and measured virus
107 attachment as with DFMO. Again, we observed a dose-dependent reduction in virus attachment
108 (Figure 1C, representative plaques in Figure 1D), suggesting that polyamine depletion, and not
109 DFMO or DENSpm themselves, reduces viral attachment. To confirm these results with a more
110 specific attachment assay, we performed the assay as previously, treating with both DFMO and
111 DENSpm, but immediately after virus attachment and washing of excess virus, we collected cells
112 and bound virus in Trizol, purified RNA, reverse transcribed, and measured viral genomes via
113 qRT-PCR, normalizing to cellular actin. Again, we observed a significant reduction in virus
114 attachment in polyamine depleted cells, both for DFMO and DENSpm treatment (Figure 1E),
115 again implicating polyamines in viral attachment.

116
117 *Exogenous polyamines rescue virus replication and attachment in DFMO-treated cells.* Cells
118 acquire polyamines either through synthesis or via dedicated transporters on the cellular surface.
119 We previously demonstrated that applying exogenous polyamines to cells rescue virus replication,
120 including for CVB3. To determine if specific polyamines enhance virus replication, possibly
121 indicating specificity in polyamine-virus interactions, we added individual polyamines to DFMO-
122 treated cells and measured viral titers after a 24-h infection. When we treated cells with any of
123 the biogenic polyamines (putrescine, spermidine, spermine, or a mix of the three), we observed
124 a full rescue in virus replication (Figure 2A). Interestingly, when we applied cadaverine,
125 norspermidine, or propylamine, we observed a modest rescue of virus titers. These data suggest
126 that CVB3 replication relies on the three polyamines synthesized by eukaryotic cells.

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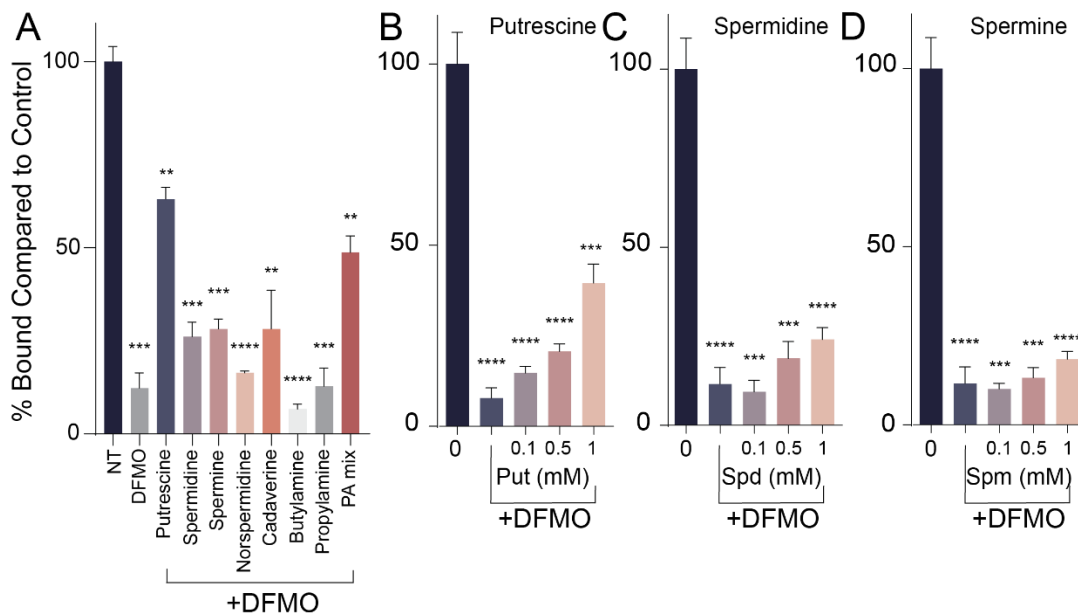
Figure 2. Exogenous polyamines rescue CVB3 replication and attachment in DFMO-treated cells. Vero cells were treated with DFMO for four days prior to supplementation with exogenous polyamines as shown in (A). Cells were subsequently infected and (B) viral titers were measured 24 hpi. (C) Viral attachment was measured in cells treated as in (A). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Student's T test ($N \geq 2$).

To more precisely determine which polyamine, if any in particular, enhanced virus attachment, we performed an attachment assay with the individual polyamines, adding the polyamines to the viral inoculum prior to applying to the cells. When we did this, we observed that curiously only putrescine rescued virus attachment (Figure 2B, representative plaques shown in Figure 2C). Additionally, when we mixed the three eukaryotic polyamines (including putrescine), we observed a full rescue for attachment. These data suggest that specifically putrescine enhances CVB3 attachment.

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144 *Putrescine enhances CVB3 binding.* Having observed that specifically putrescine enhances virus
145 attachment, we next investigated whether incubating polyamines directly with virus prior to
146 attachment mediated this rescue. To this end, we incubated CVB3 with a panel of polyamines
147 prior to attachment to cells on ice for five minutes, in our standard attachment assay. We observed
148 that a mixture of the biogenic polyamines (“PA Mix”) enhanced attachment, but only putrescine
149 enhanced attachment, while none of the other polyamines were functional (Figure 3A). To
150 determine if this was concentration dependent, we incubated CVB3 with increasing doses of
151 putrescine as before. When we did this, we observed a dose-dependent rescue of virus
152 attachment in DFMO-treated cells. In a similar vein, we incubated CVB3 with increasing doses of
153 spermidine and spermine, and we observed a modest rescue of virus attachment (Figure 3C, D).
154 Together, these data suggest that specifically putrescine mediates viral attachment when directly
155 incubated with CVB3.

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158 **Figure 3. The biogenic polyamines facilitate CVB3 attachment in a dose-**

159 **dependent manner.** (A) Vero cells were treated with 1 mM DFMO for four days

160 prior to infection with CVB3 that was incubated with 5 mM of the indicated

161 polyamines. Viral attachment assays were subsequently performed. (B-D) cells

162 were treated as in (A) but virus was incubated with increasing doses of (B)

163 putrescine, (C) spermidine, and (D) spermine. Unattached virus was washed and

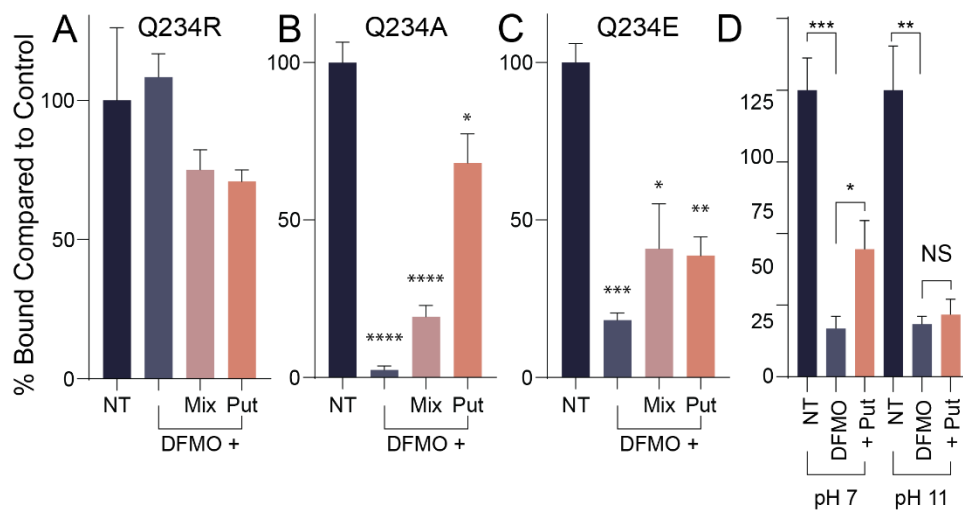
164 attached virus revealed and quantified after plaque development. *p<0.05,

165 **p<0.01, ***p<0.001, ****p<0.0001 by Student's T test (N≥3).

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167 *VP3^{Q234R} mediates attachment independently of polyamines.* We previously identified a CVB3
168 mutant that attached to cells independently of polyamines. The mutant in VP3 at glutamine 234
169 altered the negatively-charged glutamine to a positively-charged arginine (hereafter referred to
170 as VP3^{Q234R}). We reasoned that polyamine depletion limits positively-charged polyamines within
171 the cell and that the virus responds to the polyamine depletion through the incorporation of this
172 positively-charged amino acid. Interestingly, we observed this phenotype in other mutants
173 resistant to polyamine depletion, both for CVB3 and CHIKV. To determine if this mutant attached
174 to cells independently of polyamines, we incubated CVB3 VP3^{Q234R} with increasing doses of
175 polyamines and performed an attachment assay. When we did this, we observed that the amount
176 of attached virus did not change with putrescine concentration (Figure 4A), suggesting that this
177 mutant does not rely on polyamines, putatively because of this positively-charged amino acid. In
178 contrast, when we performed these assays with VP3^{Q234A} or VP3^{Q234E} mutants, we observed
179 continued dependence on putrescine for attachment (Figure 4B, C).

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Figure 4. Positively-charged putrescine or Q234R mutation of VP3 mediates

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attachment. (A) Vero cells were treated with 1 mM DFMO for four days prior to

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attachment assay with CVB3 incubated with or without 5 mM putrescine at pH 7 or

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pH11. Attached virus was revealed and quantified after plaque formation. (B) Cells

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were treated as in (A) and subsequently infected with infectious clone-derived

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VP3^{Q234R}, VP3^{Q234A}, or VP3^{Q234E} CVB3. Attachment was measured as in (A). (D)

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Attachment assay was performed as in (A) but inoculum was incubated at pH 7 or

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pH 11 during incubation and attachment. NS not significant, *p<0.05, **p<0.01,

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****p<0.0001 by Student's T test (N≥2).

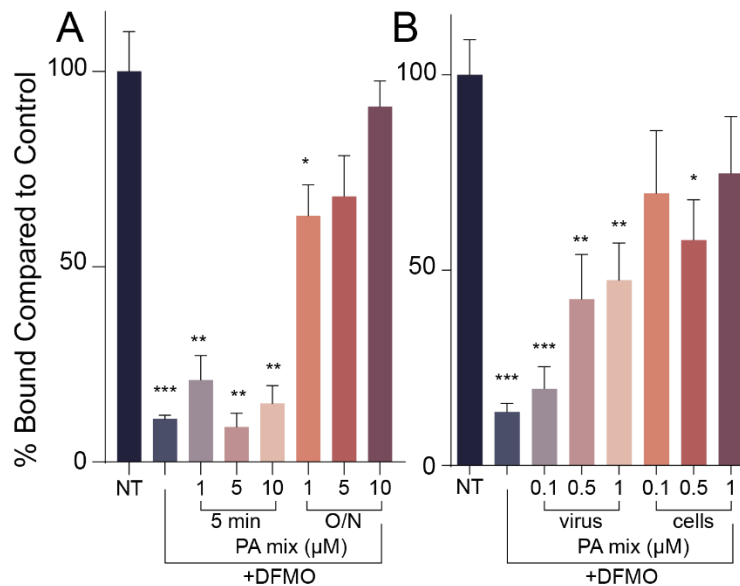
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192 *Putrescine's positive charge mediates viral attachment.* At physiological pH, polyamines are
193 positively charged. We considered that the positive charge on putrescine likely mediates viral
194 attachment and that neutralizing this charge would abrogate viral attachment. To test this, we
195 incubated virus and putrescine together as previously, but we increased the pH to 11 to reduce
196 the amount of positive charge on putrescine. We also incubated virus without putrescine at pH 11
197 to control for any effect of this change in pH on virus attachment itself. When we applied this virus
198 to cells, we observed that in virus incubated with putrescine at pH 7, the molecule mediated
199 attachment. However, incubation of CVB3 with putrescine at pH 11 eliminated the ability of the
200 polyamine to mediate attachment (Figure 4D), suggesting that the positive charge on putrescine
201 is required for it to facilitate CVB3 attachment.

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203 *Cellular factors contribute to polyamine-mediated attachment.* When we incubate virus with
204 polyamines, we observe a rescue in virus attachment, specifically with putrescine and in a dose-
205 dependent manner. We wished to investigate whether incubation of cells with polyamines directly
206 might also rescue virus attachment, and to this end, we applied polyamine-containing media to
207 DFMO-treated cells, removed this media, and then applied CVB3 for five minutes on ice, washing
208 away unbound virus and adding agarose-containing overlay medium. Despite adding polyamines
209 to the inoculum, we observed no rescue in virus attachment, suggesting that polyamines
210 (including putrescine) may not mediate viral attachment when applied to cells. However, we
211 previously demonstrated that adding polyamines to the cellular media 16h prior to viral attachment
212 rescued virus replication. We recapitulated these results, which suggest that polyamine rescue of
213 viral attachment requires a prolonged incubation time, perhaps suggesting that cellular processes
214 dependent on polyamines need to be modulated to mediate viral attachment.

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Figure 5. Cellular factors contribute to polyamine-mediated attachment. Vero cells were treated with 1 mM DFMO for four days prior to measuring CVB3 attachment. (A) Cells were incubated with exogenous polyamines as a mixture of spermidine, spermine, and spermine for either 5 min or overnight (O/N). (B) cells were treated as in (A) but either virus or cells were treated with a mixture of polyamines immediately prior to attachment assay. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Student's T test (N≥3).

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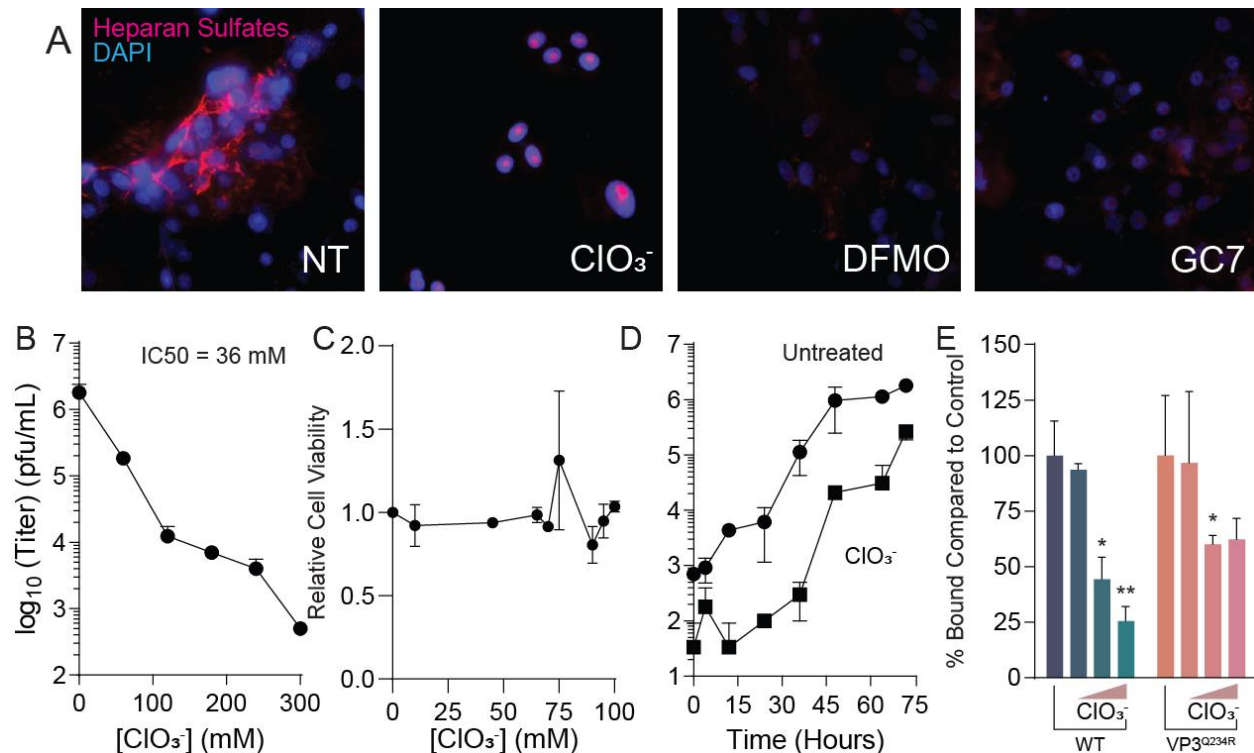
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Polyamines facilitate heparan sulfate cell-surface presentation. Our prior work showed that diverse viruses rely on polyamines for attachment, including Zika virus, human rhinovirus, and Rift Valley fever virus²⁰. Given the diversity of viruses exhibiting this phenotype, we considered that perhaps polyamines facilitate the expression of a common attachment factor for each of these viruses. With this consideration, we investigated whether heparan sulfates, negatively-charged cell surface molecules involved in virus attachment, were impacted by polyamine depletion. We measured cell surface expression of heparan sulfates via immunofluorescence (Figure 6A), using an antibody specific to heparan sulfates. To these cells, we also added sodium chlorate, a sulfation inhibitor, as well as DFMO to deplete polyamines. We observed significant cell surface staining in untreated cells, but in DFMO-treated cells, we found that heparan sulfates were significantly reduced, to a level similar to sodium chlorate treatment.

To begin understanding how polyamines affect heparan sulfate synthesis and presentation, we measured expression of a variety of heparan sulfate synthetic genes, involved both in the

239 synthesis of the core protein, polymerization of carbohydrate moieties, and sulfation of those
240 carbohydrates. In all scenarios, we observed no significant change in gene expression in DFMO-
241 treated cells, suggesting that perhaps DFMO does not regulate heparan sulfate synthesis at the
242 level of gene expression. However, polyamines are involved in translation through the unique
243 modification of eIF5A called hypusination, in which spermidine is conjugated to eIF5A and
244 carboxylated. To determine if hypusination contributes to heparan sulfate synthesis, we imaged
245 heparan sulfates by immunofluorescence using a specific inhibitor of hypusination called GC7.
246 GC7 inhibits the first step in hypusination (conjugation of spermidine to eIF5A by deoxyhypusine
247 synthase). Interestingly, we observed that heparan sulfates were significantly reduced in GC7-
248 treated cells, suggesting that hypusination contributes to heparan sulfate synthesis and/or
249 modification.
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252 **Figure 6. Polyamines facilitate cellular heparan sulfate synthesis.** (A) Vero
253 were treated with 100 mM chlorate (ClO₃⁻), 1 mM DFMO, or 500 μM GC7 and
254 subsequently stained for heparan sulfates and visualized by immunofluorescence.
255 (B) Vero cells were treated with increasing doses of chlorate for four days prior to
256 infection with CVB3 at MOI 0.1. Viral titers were determined at 48 hpi. (C) Cells
257 were treated as in (A) and cellular viability measured. (D) Vero cells were treated
258 with 100 mM chlorate and infected with CVB3 at MOI 0.1. Viral titers were

259 determined at the times indicated. (E) Veros were treated with increasing doses of
260 chlorate and WT and VP3^{Q234R} mutant CVB3 attachment was measured. *p<0.05,
261 **p<0.01 by Student's T test (N≥3).

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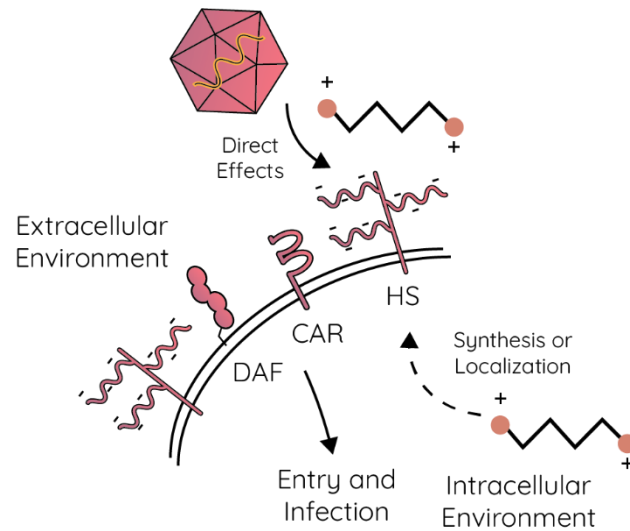
263 The Nancy strain of CVB3 has been reported to rely on heparan sulfates for attachment, but to
264 confirm that heparan sulfates are important for virus attachment and infection, we first measured
265 virus replication in sodium chlorate-treated cells. When treating cells with increasing doses of
266 sodium chlorate, we observed a dose-dependent decrease in CVB3 titers (Figure 6B), with
267 modest impacts on cellular viability (Figure 6C). This was true over several rounds of virus
268 replication, as viral titers were reduced with sodium chlorate treatment over a timecourse (Figure
269 6D). Similarly, we found that sodium chlorate reduced virus attachment in a dose-dependent
270 manner (Figure 6E). We used our VP3^{Q234R} mutant in these attachment assays and observed a
271 modest rescue in viral attachment in these cells, suggesting that a portion of this mutant's
272 resistance may originate from its ability to bind cells depleted of heparan sulfates or that the CVB3
273 VP3^{Q234R} mutant has overall enhanced cellular attachment. In sum, these data highlight the role
274 of polyamines and hypusination in heparan sulfate synthesis.

275

276 Discussion

277 Our prior work with CVB3 showed that polyamines facilitate at least one step during viral
278 attachment and entry, as depletion of polyamines limits the amount of virus associated with
279 susceptible cells²⁰. This phenotype is reverse with exogenous polyamines, though it was unclear
280 whether polyamines directly or indirectly facilitate attachment. Our current work demonstrates that
281 polyamines function both at the level of directly enhancing virus-cell association and by facilitating
282 the synthesis of cellular heparan sulfates, a common attachment factor for several viruses^{1,5}.
283 Thus, these results likely hold true for other viruses that rely on heparan sulfates for attachment,
284 as we observed previously.

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Figure 7. Working model. Polyamines facilitate CVB3 attachment and entry by directly mediating cellular attachment through the positive charge of the polyamines. Polyamines also support synthesis and localization of heparan sulfates, a nonspecific attachment factor, to facilitate CVB3 attachment.

We find that specifically putrescine, the “simplest” of the polyamines, facilitates direct attachment of virus to cells. Precisely why putrescine, rather than the other polyamines with more charges and longer carbon chains, specifically enhances attachment is unclear, though one could hypothesize that this polyamine specifically associates with the charge landscape of the enterovirus virion. The capsid proteins exhibit canyons and valleys that mediate interaction with cellular molecules, including heparan sulfates and the cellular receptors. Whether polyamines and specifically putrescine bind to a specific portion of the CVB3 capsid is unclear. Structural analysis of the viral capsid proteins has never cocrystallized a polyamine with a structural protein; however, these proteins are often highly purified and may lose polyamine association upon purification.

Passaging CVB3 in polyamine-depleted cells, treated with DFMO, we observed a mutation in VP3 that confers resistance through enhanced cellular attachment²⁰. The VP3^{Q234R} mutation was previously described to enhance association with the CVB3 receptor CAR (Coxsackie and adenovirus receptor)^{25,26}, which we hypothesized was overcoming a deficit in cells treated with DFMO. It is tantalizing to consider that polyamines, specifically putrescine, may facilitate VP3’s association with CAR, and that upon polyamine depletion, CVB3 adapts by conferring a positive charge to this amino acid, negating the requirement for putrescine. Current work is addressing this model.

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311 The connection between polyamines and other metabolic pathways has been established by our²⁷
312 and others' research^{28,29}. Polyamines have previously been shown to associate with heparan
313 sulfates and that cells can take up polyamines from the extracellular environment via heparan
314 sulfates³⁰⁻³³. We find that heparan sulfate synthesis and surface expression is facilitated by
315 polyamines, as treatment of cells with DFMO diminishes their presence on cells. When we
316 investigated whether the expression of heparan sulfate synthesis or modification genes, we found
317 no significant changes in expression (not shown); however, the regulation of heparan sulfate
318 synthesis could be at the level of translation, through hypusination of eIF5A³⁴. Whether
319 polyamines facilitate the expression and presentation of other viral attachment factors on the
320 surfaces of cells remains incompletely understood, but future work will need to characterize cell
321 surface molecules impacted by polyamines and how this impacts virus attachment.

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323 Several molecules like polybrene or protamine sulfates enhance viral attachment to cells and are
324 frequently used to enhance transduction efficiency³⁵⁻³⁷. Interestingly, the structure of polybrene is
325 highly similar to polyamines, comprised of carbon chains with quaternary amine groups, which
326 confers a repeating positive charge. Polybrene has been shown to enhance attachment of viruses
327 to cells in the absence of viral receptors³⁵. Additionally, polybrene may act by aggregating viruses,
328 enhancing their potential to productively infect³⁸, or by neutralizing the negative cell surface
329 charge (potentially through negatively-charged heparan sulfates)³⁹ to enhance virus association.
330 Thus, polyamines and putrescine in particular may be functioning similarly. In our assays, we
331 used high levels of polyamines, in the millimolar range^{40,41}; however, cellular levels of polyamines
332 can be in this range. As a pathogen transmitted by the fecal-oral route, CVB3 also likely
333 encounters high polyamine levels within the intestinal tract, where both bacterial and mammalian
334 cells produce polyamines⁴²⁻⁴⁵. While our work does not address the *in vivo* physiological
335 relevance of polyamines during natural infection with CVB3, it highlights a novel role for
336 polyamines in both directly and indirectly facilitating viral attachment.

337

338 **Acknowledgments**

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340 assistance with microscopy.

341

342 **Materials and Methods**

343 **Cell culture.** Cells were maintained at 37°C in 5% CO₂, in Dulbecco's modified Eagle's medium
344 (DMEM; Life Technologies) with bovine serum and penicillin-streptomycin. Vero cells were
345 obtained through Biodefense and Emerging Infections (BEI) Research Resources, NIAID, NIH
346 (NR-10385), and were supplemented with 10% new-born calf serum (NBCS; Thermo Fisher).

347
348 **Drug treatments.** Difluoromethylornithine (DFMO; TargetMol) and N¹,N¹¹-Diethylnorspermine
349 (DENSp_m; Santa Cruz Biotechnology, Santa Cruz, CA, USA), GC7 and sodium chlorate (Sigma-
350 Aldrich, St. Louis, MO) were diluted to 100× solution (100mM, 10mM, 50 mM, and 10 M,
351 respectively) in sterile water. For DFMO treatments, cells were trypsinized (Zymo Research) and
352 reseeded with fresh medium supplemented with 2% NBCS. Cells were treated with 1 mM DFMO
353 unless otherwise indicated. Cells were incubated with DFMO for 96 h, DENSp_m for 16 h, GC7
354 for 16 h, or sodium chlorate for 96 h to allow for depletion of polyamines or heparan sulfates.
355 Experiments involving polyamine rescues were performed using 10 μM polyamines (Sigma-
356 Aldrich) unless otherwise indicated and added to either the cell supernatant or viral inoculum, as
357 indicated. The polyamine mix is a 1:1:1 equimolar solution of putrescine, spermidine, and
358 spermine.

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360 **Infection and enumeration of viral titers.** CVB3 (Nancy strain) was derived from the first
361 passage of virus in Vero cells after rescue from an infectious clone. VP3^{234R}, VP3^{234E}, and VP3^{234A}
362 mutants were generated as previously described²⁰ using the following primers: VP3^{234A}, 5'-CCT-
363 TTC-ATT-TCG-GCC-AAC-TTT-TTC-C-3' (F) and 5'-CCC-TGG-AAA-AAG-TTG-GCC-TGC-GAA-
364 ATG-3' (R); VP3^{234E}, 5'-CCT-TTC-ATT-TCG-CAG-GAA-AAC-TTT-TTC-C-3' (F) and 5'-CCC-
365 TGG-AAA-AAG-TTT-TCC-TGC-GAA-ATG-3' (R). For all infections, DFMO, DENSp_m, sodium
366 chlorate, and GC7 were maintained throughout infection as designated. Viral stocks were
367 maintained at -80°C. For infection, virus was diluted in serum-free DMEM for a multiplicity of
368 infection (MOI) of 0.01 on Vero cells, unless otherwise indicated. Viral inoculum was added to the
369 cells and supernatants were collected at specified time points. To quantify viral titers via plaque
370 assay, dilutions of cell supernatant were prepared in serum-free DMEM and used to inoculate
371 confluent monolayers of Vero cells for 10 to 15 min at 37°C. Cells were overlain with 0.8% agarose
372 in DMEM containing 2% NBCS. CVB3, VP3^{234A}, VP3^{234E} samples were incubated for 2 days and
373 the VP3^{234R} mutant for 3 days at 37°C. Cells were fixed with 4% formalin and revealed with crystal
374 violet solution (10% crystal violet; Sigma-Aldrich). Plaques were enumerated and used to back
375 calculate the number of PFU per ml of collected volume.

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377 **Plaque formation attachment assay.** Vero cells were seeded in 6-well plates and grown to
378 confluence in DMEM with 2% NBCS. The cells were treated for 96 h with 1 mM DFMO. For
379 polyamine rescue experiments, cells were treated overnight before the infection with 10 μ M or
380 incubated with the viral inoculum for 5 min at room temperature with .1, .5, 1, or 5mM of
381 polyamines (Millipore Sigma) unless otherwise indicated. After the 96-h DFMO treatment, the
382 cells were placed on ice and the medium was aspirated from the cells and replaced with .5 ml
383 serum-free medium containing either 1,000 or 2,000 PFU. The infected cells were incubated on
384 ice for a specified amount of time. After the specified time, the cells were washed 3x with PBS
385 and then overlaid with 0.8% agarose containing DMEM with 2% NBCS. The plates were incubated
386 at 37°C for plaques to develop. CVB3, VP3^{234A}, and VP3^{234E} were incubated for 2 days; the
387 VP3^{234R} mutant for 4 days. The cells were fixed with 4% formalin, and the plaques were visualized
388 with crystal violet staining.

389
390 **qPCR based attachment assay.** Vero cells were seeded at 1.5×10^5 cells per well in 12-well
391 plates in DMEM with 2% NBCS. The cells were treated for 96 h with 1mM DFMO or 16 h with
392 DENSp_m. After 96 h or 16 h, the media were aspirated from the cells and replaced with 100 μ L
393 of serum free media containing virus. The infected cells were incubated for 10 min at room
394 temperature or on ice. The cells were then washed 1x with PBS, and then, 200 μ L of Trizol was
395 added to the cells. The RNA was extracted with the Zymo RNA extraction kit, converted to cDNA,
396 and quantified by real-time PCR with SYBR Green (DotScientific) using the one-step protocol
397 QuantStudio 3 (ThermoFisher Scientific). Relative genomes were calculated using the Δ CT
398 method, normalized to the β -actin qRT-PCR control, and calculated as the fraction of the
399 unwashed samples. Primer sequences are as follows: CVB3, 5'-AGG-GCG-AGA-TCA-ATC-ACA-
400 TTA-G-3' (F) and 5'-CTC-TGC-TGT-TGC-CTC-ACT-ATC-3' (R); β -actin, 5'-CAC-TCT-TCC-
401 AGCCTT-CCT-TC-3' (F) and 5'-GTA-CAG-GTC-TTT-GCGGAT-GT-3' (R). Primers were verified
402 for linearity using 8-fold serial diluted cDNA and checked for specificity via melt curve analysis.

403
404 **Immunofluorescence imaging.** Cells grown on coverslips were either treated with 1 mM DFMO,
405 100mM sodium chlorate, 10 μ M DENSp_m, 500 μ M GC7 or untreated. Cells were fixed with 4%
406 formalin for 15 min, washed with PBS, permeabilized, and blocked with 0.2% Triton X-100 and
407 2% BSA in PBS (blocking solution) for 30 min at room temperature (RT). Cells were sequentially
408 incubated as follows: primary mouse anti-10E4 (Amsbio, 370255-S) with blocking for 2 h at room
409 temperature. Cells were subsequently washed then incubated with secondary goat anti-mouse
410 antibodies (1:500 with blocking, 30 min, RT). Mounting media with DAPI was used to visualize

411 nuclei. Samples were imaged with a Zeiss Axio Observer 7 with Lumencor Spectra X LED light
412 system and a Hamamatsu Flash 4 camera using appropriate filters using Zen Blue software with
413 a 40x objective.

414

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