Cell Reports, Volume *29*

Supplemental Information

Membrane-Associated Enteroviruses Undergo Intercellular Transmission as Pools of Sibling Viral Genomes

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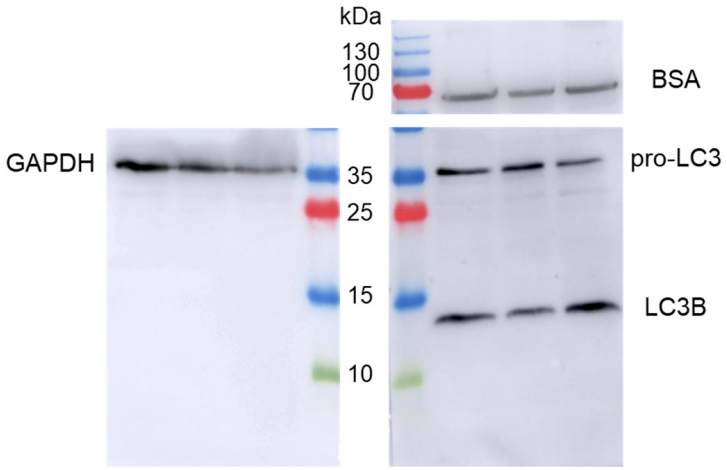

Figure S1. Western blotting of the P_3 fraction, related to Figure 1. Cells were inoculated at 10 PFU/cell and the P_3 fraction was prepared from culture media harvested at 12 hpi. GAPDH was used as a positive control for structures containing cytoplasmic content, and should be present in vesicles. In contrast, BSA should not be present inside vesicles or other structures derived from the cytoplasm. In control assays in which we performed the same preparation protocol (P_3 fraction and Western blot) from uninfected cells lysed with ddH_20, the BSA band was not present (not shown). This suggests that our P_3 fractions carried over some BSA from the medium. P_3 fractions were positive for LC3B, a typical autophagosome marker. However, the immature form of the protein (pro-LC3) was also detected. Each lane represents a technical replicate. To help visualization and since GAPDH and pro-LC3 proteins are similar in size, the regions of interest of the membrane were cut prior to the primary antibody incubation.

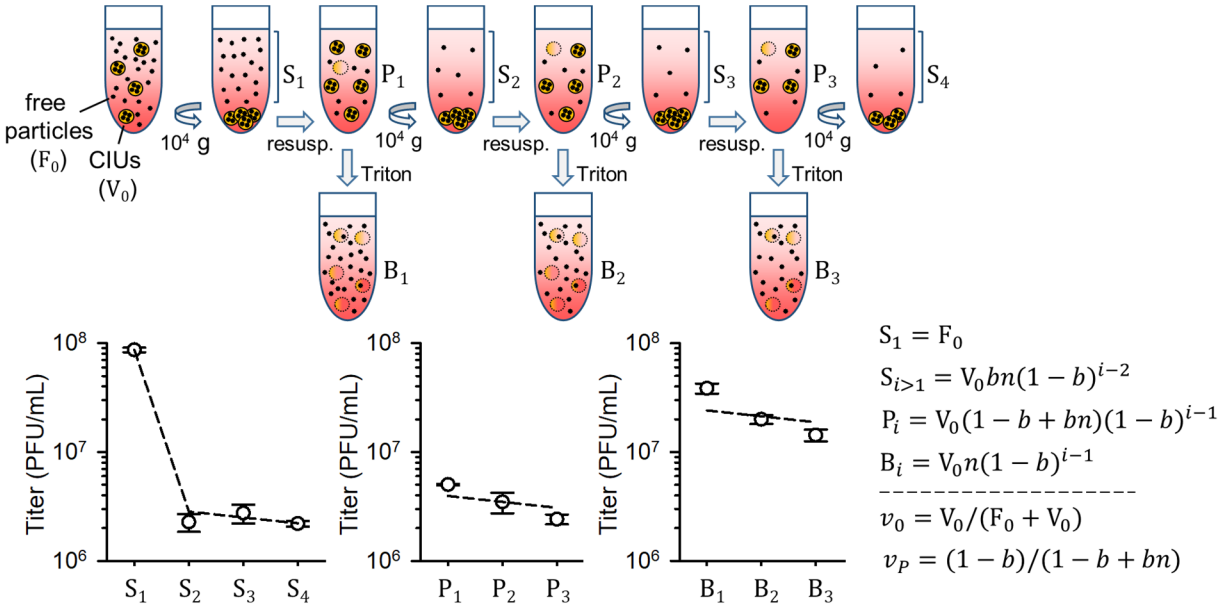

Figure S2. Model for quantifying contamination of pellet fractions with free viral particles, related to Figures 1 and 2. The protocol for preparing high-weight infectious units by slow-speed centrifugation is shown on top. The initial titer (PFU/mL) of free particles is F_0, whereas the initial titer corresponding to membrane-associated CIUs is V_0 (each CIU produces 1 PFU). The initial proportion of collective to total infectious units is thus $v_0 = V_0/(F_0 + V_0)$. The titer of the first supernatant fraction (S_1) equals the initial titer of free particles (F_0). The first resuspended pellet fraction (P_1) contains $V_0(1-b)$ CIUs, where b is the probability that CIUs are broken during resuspension. The P_1 fraction also contains $V_0 bn$ free infectious particles, where n is the number of infectious particles released per broken CIU. Hence, the titer of the first resuspended pellet is $P_1 = V_0(1-b+bn)$. Following detergent treatment, we assume that all CIUs are disrupted ($b = 1$) and, thus, the titer becomes $B_1 = V_0 n$. The second supernatant fraction (S_2) contains the free particles released during resuspension of the first pellet, that is, free particles present in P_1. Hence, $S_2 = V_0 bn$. The expression for the second pellet (P_2) is the same as for P_1, multiplied by the probability of no CIU breakage during the first resuspension. Hence $P_2 = V_0(1-b+bn)(1-b)$. The titer of the B_2 fraction is thus $B_2 = V_0 n(1-b)$. Hence, for the i-th centrifugation/resuspension round, $P_i = V_0(1-b+bn)(1-b)^{i-1}$, and $B_i = V_0 n(1-b)^{i-1}$. The i-th supernatant fraction (S_i) contains the free particles released from CIUs during resuspension of the previous ($i-1$) pellet, that is, free particles present in P_{i-1}. Hence, $S_i = V_0 bn(1-b)^{i-2}$. In the graphs below are shown the experimental titers for the first 10 fractions (S_1 to S_4, P_1 to P_3, and B_1 to B_3). This was done in three independent experiments. Dashed lines in each graph show the best fit of the model to data by non-linear least-squares regression. This allowed us to infer the four

parameters of the model: V_0, F_0, b, and n. This inference was done independently for each of the three experimental replicates, allowing us to obtain the mean and error (SEM) of each parameter. In resuspended pellets, the estimated proportion of PFUs corresponding to CIUs is $v_P = (1-b)/(1-b+bn)$, that is unbroken CIUs divided by total PFUs. The model thus allowed us to estimate the size of CIUs (n) and to quantify contamination of pellet fractions with free viral particles resulting from CIU breakage. For simplicity, the model assumed that after centrifugation all free particles remained in the supernatant and all CIUs sedimented. Also, CIU breakage during centrifugation was not considered. Notice, though, that breakage during centrifugation should not lead to contamination of resuspended pellets with free viral particles and, hence, should not be a source of concern for the purpose of this study.

$Q_0 = e^{-D}$

No fluorescence

$Q_1 = De^{-D}$

$(1-v_P)g$	$(1-v_P)(1-g)$	$v_P g(1-m)$	$v_P(1-g)(1-m)$	$v_P m$

$(1-v_P)$ $v_P(1-m)$ v_P

$Q_2 = \frac{1}{2}D^2e^{-D}$

	$(1-v_P)^2 g^2$	$(1-v_P)^2$ $g(1-g)$	$v_P g^2$ $(1-m)(1-v_P)$	$v_P(1-g)(1-m)$ $(1-v_P)g$	$v_P m(1-v_P)g$
	$(1-v_P)^2$ $g(1-g)$	$(1-v_P)^2(1-g)^2$	$v_P g(1-m)$ $(1-v_P)(1-g)$	$v_P(1-m)$ $(1-v_P)(1-g)^2$	$v_P m$ $(1-v_P)(1-g)$
	$(1-v_P)g^2$ $v_P(1-m)$	$(1-v_P)(1-g)$ $v_P g(1-m)$	$v_P{}^2 g^2(1-m)^2$	$v_P{}^2(1-m)^2$ $g(1-g)$	$v_P{}^2 gm(1-m)$
	$(1-v_P)g$ $v_P(1-g)(1-m)$	$(1-v_P)(1-g)^2$ $v_P(1-m)$	$v_P{}^2(1-m)^2$ $g(1-g)$	$v_P{}^2(1-g)^2$ $(1-m)^2$	$v_P{}^2(1-g)$ $m(1-m)$
	$(1-v_P)gv_P m$	$(1-v_P)(1-g)$ $v_P m$	$v_P{}^2 gm(1-m)$	$v_P{}^2(1-g)$ $m(1-m)$	$v_P{}^2 m^2$

Figure S3. Estimation of the proportion of polymorphic CIUs in the presence of contaminant free viral particles, related to Figure 2. The P_3 fraction was used for infecting cells at ca. D = 0.1 PFU/cell. Flow cytometry was used for counting non-fluorescent, GFP-positive, mCherry-positive, and doubly fluorescent cells at 7 hpi. The proportion of cells receiving zero (Q_0), one (Q_1), or two (Q_2) PFUs (CIUs or free particles) was assumed to follow a Poisson distribution. Cells receiving more PFUs were neglected. Based on this, the actual D was calculated from the observed proportion of non-fluorescent cells, $Q_0 = e^{-D}$. This allowed us to infer Q_1 and Q_2. The probability of receiving a CVB3-EGFP free particle, a CVB3-mCherry free particle, a CVB3-EGFP CIU, a CVB3-mCherry CIU, or a polymorphic CIU containing both virus variants among cells receiving one PFU is shown in the first table. Grid colors indicate the corresponding phenotype of the cell (green: GFP positive; red: mCherry positive; orange: doubly fluorescent). For cells receiving two PFUs, the probability of each possible combination is shown in the second table. These probabilities depend on the proportion of CVB3-GFP to total virus (g), the proportion of collective to total infectious units in the inoculum (v_P), and the proportion of polymorphic to total CIUs (m). Parameter g was directly estimated as the ratio of green to (green + red) fluorescent cells (g = 0.464), whereas v_P was determined as detailed in Fig. S2 (v_P = 0.284). Parameter m was numerically calculated from the indicated values of g and v_P and the observed proportions of EGFP-positive, mCherry-positive, and doubly fluorescent cells.

Table S1. Flow cytometry counts of cultures inoculated with P_3 fractions harvested from cells coinfected with CVB3-EGFP and CVB-mCherry at 12 hpi, related to Figure 2.

		Observed count (%)			**Expected count[c] (%)**
Category	N	G	R	D	E(D)
EGFP	–	+	–	+	+
mCherry	–	–	+	+	+
Assay 1	90,598 (91.2%)	3507 (3.5%)	4192 (4.2%)	1081 (1.1%)	243 (0.24%)
Assay 2	90,104 (90.8%)	3648 (3.7%)	4205 (4.2%)	1268 (1.3%)	271 (0.27%)
Assay 3	90,241 (91.0%)	3678 (3.7%)	4092 (4.1%)	1144 (1.2%)	255 (0.26%)
Average[a,b]	90314 (91.0%)	3611 (3.6%)	4163 (4.2%)	1164 (1.2%)	256 (0.25%)

[a]On average 9.0 ± 0.1 % of the cells were fluorescent, calculated as (G+R+D)/(N+G+R+D).

[b]On average, among fluorescent cells, 13.0 ± 0.5 % were doubly fluorescent, calculated as D/(G+R+D).

[c]Calculated as E(D)=(G+D)×(R+D)/(N+G+R+D). The average observed count was 4.5-fold higher than the expected count.

Table S2. Flow cytometry counts of cultures inoculated with P_3 fractions harvested from cells coinfected with CVB3-EGFP and CVB-mCherry at 8 hpi, related to Figure 2.

		Observed count (%)			**Expected count[c] (%)**
Category	N	G	R	D	E(D)
EGFP	–	+	–	+	+
mCherry	–	–	+	+	+
Assay 1	90,950 (91.0%)	3574 (3.6%)	3567 (3.6%)	1909 (1.9%)	300 (0.3%)
Assay 2	90,518 (90.5%)	3648 (3.6%)	3455 (3.5%)	2379 (2.4%)	352 (0.4%)
Assay 3	90,131 (90.1%)	4430 (4.4%)	3516 (3.5%)	1923 (1.9%)	346 (0.3%)
Average[a,b]	90,533 (90.5%)	3884 (3.9%)	3512 (3.5%)	2070 (2.1%)	332 (0.3%)

[a]On average 9.5 ± 0.2 % of the cells were fluorescent, calculated a (G+R+D)/(N+G+R+D).

[b]On average, among fluorescent cells, 21.9 ± 1.7 % were doubly fluorescent, calculated as D/(G+R+D).

[c]Calculated as E(D)=(G+D)×(R+D)/(N+G+R+D). The average observed count was 6.2-fold higher than the expected count.

Table S3. Flow cytometry counts of cultures inoculated with P_2* fractions harvested from cells coinfected with CVB3-EGFP and CVB-mCherry at 12 hpi, related to Figure 2.

		Observed count (%)			**Expected count[c] (%)**
Category	N	G	R	D	E(D)
EGFP	–	+	–	+	+
mCherry	–	–	+	+	+
Assay 1	87,414 (86.5%)	7574 (7.5%)	2375 (2.4%)	3670 (3.6%)	673 (0.7%)
Assay 2	84,248 (83.2%)	9365 (9.2%)	2970 (2.9%)	4736 (4.7%)	1072 (1.1%)
Assay 3	86,828 (85.6%)	8017 (7.9%)	2672 (2.6%)	3920 (3.9%)	776 (0.8%)
Average[a,b]	86,163 (90.5%)	8319 (3.9%)	2672 (3.5%)	4109 (2.1%)	840 (0.8%)

[a]On average 14.9 ± 1.0 % of the cells were fluorescent, calculated a (G+R+D)/(N+G+R+D).

[b]On average, among fluorescent cells, 27.2 ± 0.3 % were doubly fluorescent, calculated as D/(G+R+D).

[c]Calculated as E(D)=(G+D)×(R+D)/(N+G+R+D). The average observed count was 5.0-fold higher than the expected count.