**Negative charge and membrane-tethered viral 3B cooperate to recruit viral RNA dependent RNA polymerase 3D$^{pol}$**

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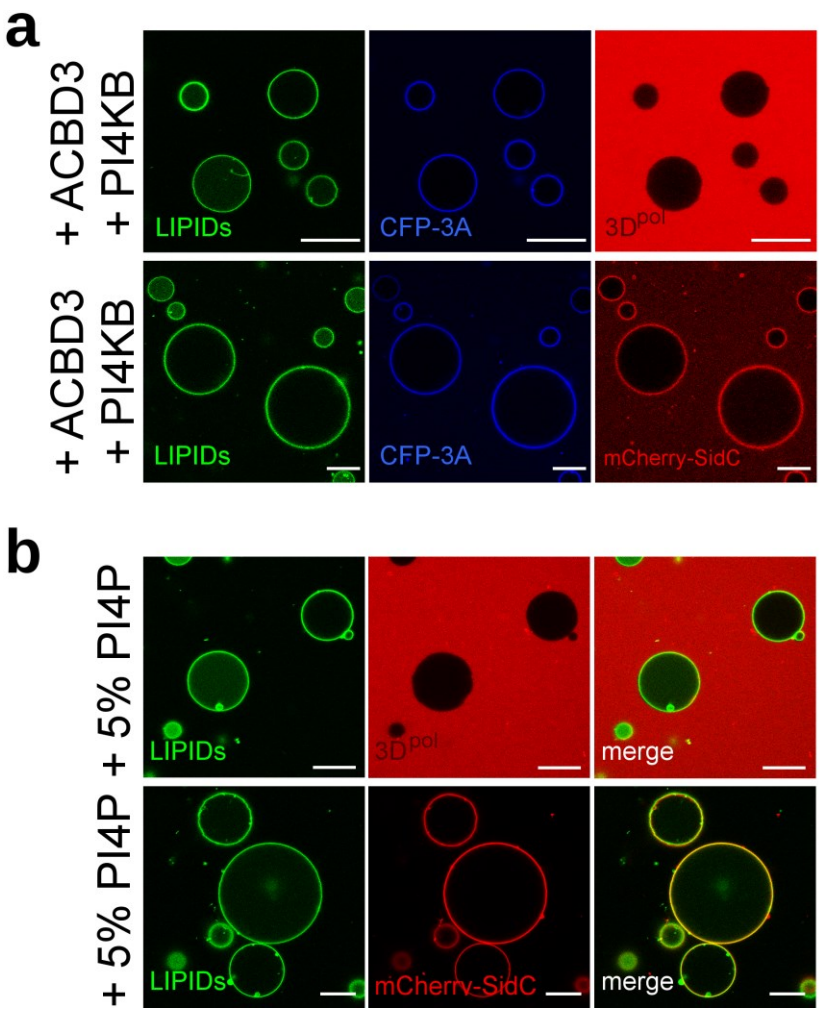
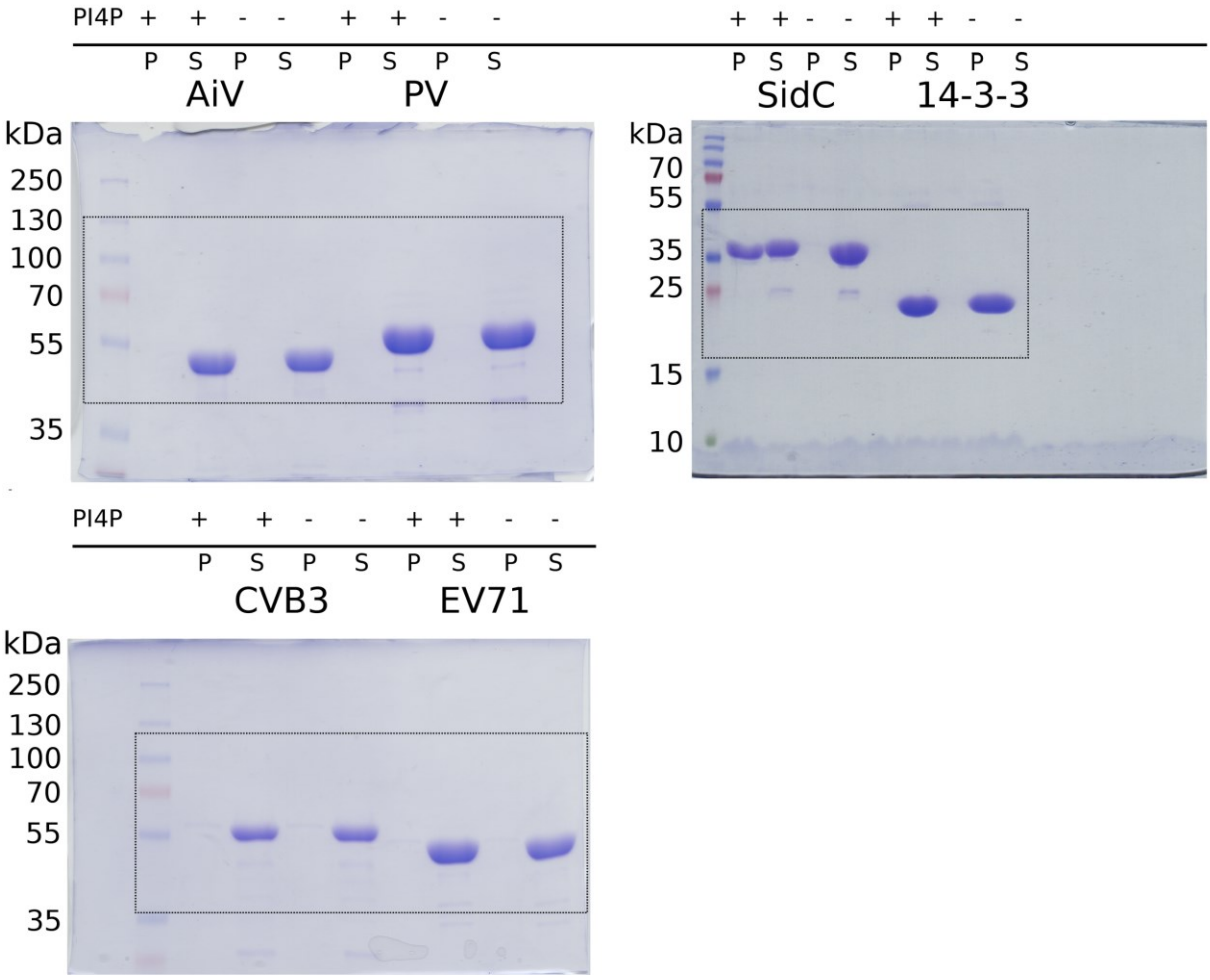
**Supplementary Figures**



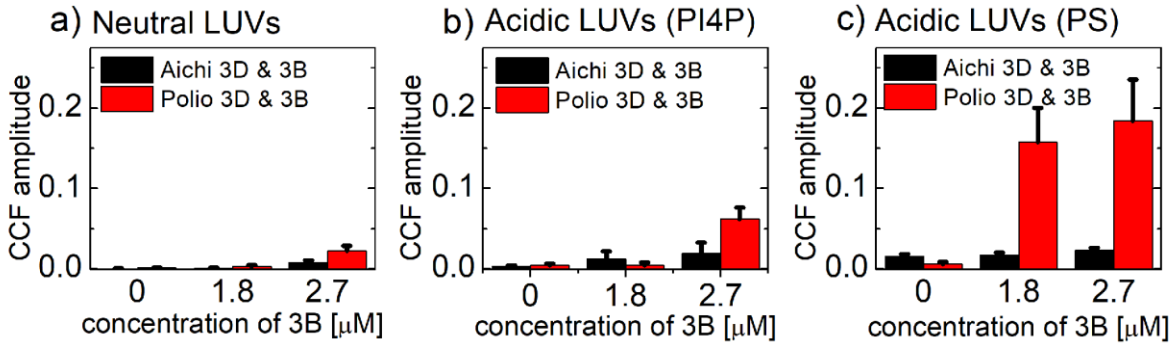

**SI Figure 1 – PI4P mediated recruitment of $3D^{pol}$ under conditions where the nanomolar PI4P binder is recruited**

A) **No recruitment observed upon membrane phosphorylation by the 3A:ACBD3:PI4KB protein complex.** Upper panel: GUVs were incubated with 250 nM 3A-CFP and PI4KB, 1 µM ACBD3 and 1 µM $3D^{pol}$ labeled by rhodamine. Lower panel: the same except that $3D^{pol}$ was replaced with 250 nM mCherry-SidC. GUVs contained 0.1% of PE-Fluorescein lipid (Avanti). Lipids are in green, CFP-3A is in blue, $3D^{pol}$ labeled by rhodamine or mCherry-SidC is in red. Representative image of two independent experiments. Scale bar = 20 µm.

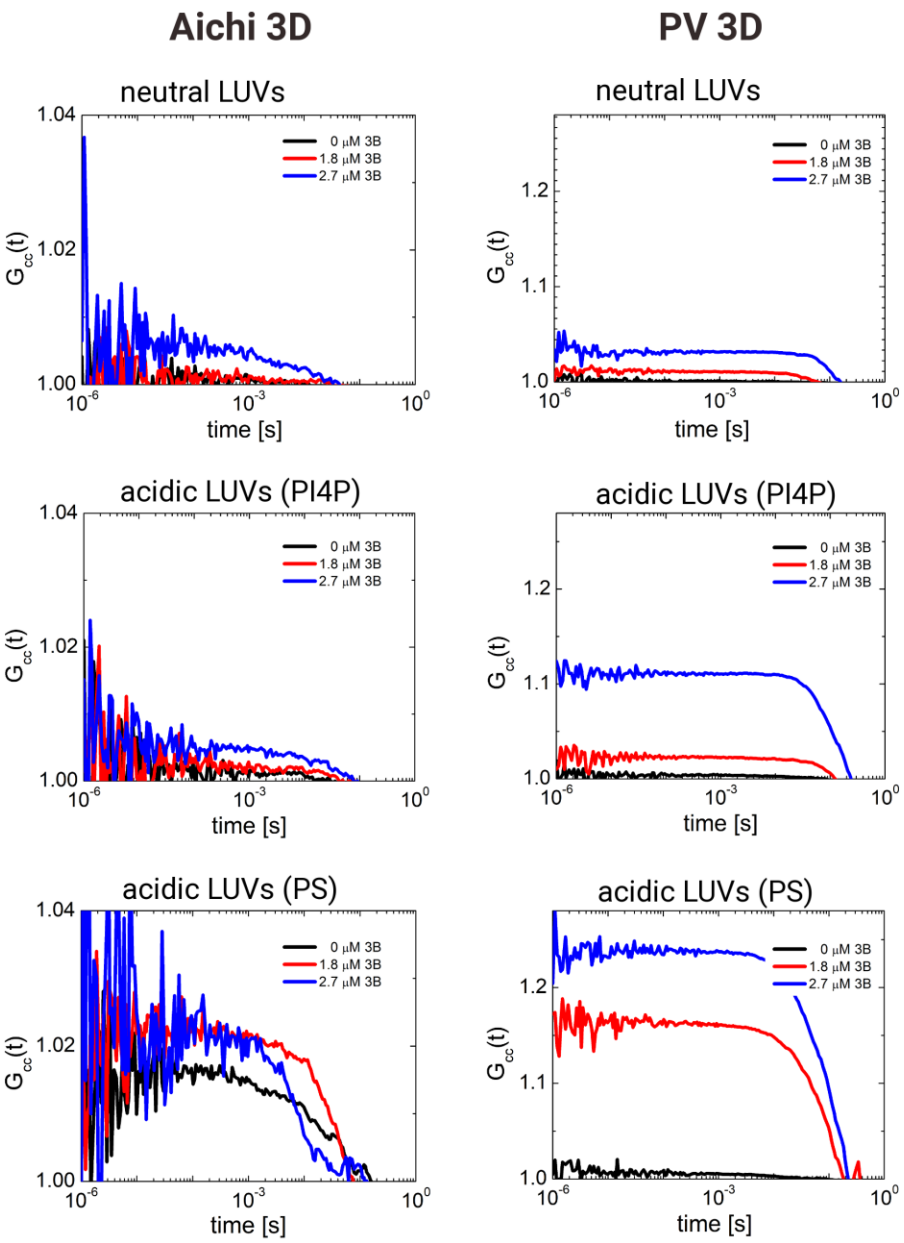
B) **No recruitment observed using GUVs containing 5 mol % of synthetic PI4P.** Upper panel: GUVs were incubated with 1 µM $3D^{pol}$ labeled by rhodamine. Lower panel: the same except that $3D^{pol}$ was replaced with 250 nM mCherry-SidC. GUVs contained 0.1% of PE-Fluorescein lipid (Avanti). Lipids are in green, CFP-3A is in blue, $3D^{pol}$ labeled by rhodamine or mCherry-SidC is in red. Representative image of two independent experiments. Scale bar = 20 µm.




**SI Figure 2 – Full length gels –** Full length gel accompanying Figure 4A – Dashed rectangles are shown in Fig. 4




**SI Figure 3 – Amplitudes of the functions that cross-correlate fluorescence signal of CFP-3D$^{pol}$ and of LUVs labeled with ATTO647-DOPE.** The amplitudes are shown for various LUV lipid compositions: a) neutral LUVs, b) acidic LUVs (PI4P), and c) acidic LUVs (PS), and for increasing amounts of membrane-tethered 3B. Black and red columns stand for Aichi and PV viral proteins, respectively. The error bar represents the standard error of the mean.




**SI Figure 4 – An independent set of experiments conducted in the same way as in Figure 4D (Cross-scorrelation curves of CFP – 3D$^{pol}$ and LUVs).** FCS curves representing temporal cross-correlation of the CFP-3D$^{pol}$ (Aichi 3D$^{pol}$ on the left panel and PV 3D$^{pol}$ on the right panel) and ATTO647N-DOPE labelled LUVs at various membrane lipid compositions (neutral, enriched by PI4P, and enriched by PS; see SI Table 1). The curves show the cooperative effect of the 3B peptide on 3D$^{pol}$ membrane recruitment. The concentration of the Aichi or PV 3B peptide (attached to the membrane surface by His-tag – 18:1 DGS-NTA(Ni) interaction) was 0 μM (black), 1.8 μM (red), and 2.7 μM (blue).

| Figure | Membrane type | POPC [%] | POPS [%] | PI [%] | PI4P [%] | cholesterol | DGS-NTA(Ni) [%] | Atto647N-DOPE [%] |
|---|---|---|---|---|---|---|---|---|
| 1 | GUVs | 54.9 | 10 | 10 | 0 | 20 | 5 | 0.1 |
| 2 | GUVs | 54.9 | 10 | 10 | 0 | 20 | 5 | 0.1 |
| 3 | GUVs | 54.9 | 10 | 10 | 0 | 20 | 5 | 0.1 |
| SI 1a | GUVs | 54.9 | 10 | 10 | 0 | 20 | 5 | 0.1 |
| SI 1b | GUVs | 54.9 | 5 | 10 | 5 | 20 | 5 | 0.1 |
| 4b neutral,4c | GUVs | 59.99 | 5 | 10 | 0 | 20 | 5 | 0.01 |
| 4b PI4P, 4c | GUVs | 54.99 | 5 | 10 | 5 | 20 | 5 | 0.01 |
| 4b PS, 4c | GUVs | 39.99 | 25 | 10 | 0 | 20 | 5 | 0.01 |
| 4a - PI4P | LUVs | 60 | 20 | 0 | 0 | 20 | 0 | 0 |
| 4a + PI4P | LUVs | 60 | 10 | 0 | 10 | 20 | 0 | 0 |
| 4d neutral | LUVs | 64.99 | 0 | 10 | 0 | 20 | 5 | 0.01 |
| 4d acidic (PI4P) | LUVs | 49.99 | 10 | 10 | 5 | 20 | 5 | 0.01 |
| 4d acidic (PS) | LUVs | 39.99 | 25 | 10 | 0 | 20 | 5 | 0.01 |

**SI Table 1: Membrane composition for each experiment.**