

1 The 3A protein from multiple picornaviruses utilizes the Golgi Adaptor Protein ACBD3  
2 to Recruit PI4KIII $\beta$

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10

## 11 **Abstract**

12

13 The activity of phosphatidylinositol 4-kinase class III beta (PI4KIII $\beta$ ) has been shown to  
14 be required for the replication of multiple picornaviruses, however it is unclear whether a  
15 physical association between PI4KIII $\beta$  and the viral replication machinery exists and if it  
16 does, whether association is necessary. We examined the ability of the 3A protein from  
17 18 different picornaviruses to form a complex with PI4KIII $\beta$  by affinity purification of  
18 Strep-tagged transiently transfected constructs followed by mass spectrometry and  
19 western blotting for putative interacting targets. We found that the 3A proteins of Aichi  
20 virus, bovine kobuvirus, poliovirus, Coxsackievirus B3, and human rhinovirus 14 all  
21 copurify with PI4KIII $\beta$ . Furthermore we found that multiple picornavirus 3A proteins  
22 copurify with with the Golgi adaptor protein acyl-CoA binding domain protein 3  
23 (ACBD3/GPC60), including those from Aichi virus, bovine kobuvirus, human rhinovirus

24 14, poliovirus, coxsackievirus B2, B3, and B5. Affinity purification of ACBD3  
25 confirmed interaction with multiple picornaviral 3As and revealed the ability to bind  
26 PI4KIII $\beta$  in the absence of 3A. Mass spectrometric analysis of transiently expressed  
27 Aichi virus, bovine kobuvirus, and human klassevirus 3A proteins demonstrated that the  
28 N-terminal glycines of these 3A proteins are myristoylated. Alanine scanning  
29 mutagenesis along the entire length of Aichi 3A followed by transient expression and  
30 affinity purification revealed that copurification of PI4KIII $\beta$  could be eliminated by  
31 mutation of specific residues, with little or no effect on recruitment of ACBD3. One  
32 mutation at the N-terminus, I5A, significantly reduced copurification of both ACBD3 and  
33 PI4KIII $\beta$ . The dependence of Aichi virus replication on the activity of PI4KIII $\beta$  was  
34 confirmed by both chemical and genetic inhibition. Knockdown of ACBD3 by siRNA  
35 also prevented replication of both Aichi virus and poliovirus. Point mutations in 3A that  
36 abrogate PI4KIII $\beta$  association sensitized Aichi virus to PIK93, suggesting that disruption  
37 of the 3A/ACBD3/PI4KIII $\beta$  complex may represent a novel therapeutic intervention  
38 target that would be complementary to the inhibition of the kinase activity itself.

39

40

41 Keywords:

42 Picornavirus

43 3A

44 Poliovirus

45 Aichi virus

46 Kobuvirus

- 47 Coxsackievirus
- 48 Human rhinovirus
- 49 PI4KIII $\beta$
- 50 ACBD3
- 51 GCP60
- 52 PI4KB
- 53

54 **Introduction**

55 Reorganization of cellular membranes has been recognized as a critical aspect of  
56 positive-stranded RNA viral replication<sup>(30)</sup>. Positive-stranded RNA viruses use  
57 membranes from distinct cellular organelles to concentrate and protect RNA replication  
58 machinery from cellular defenses. Among the picornaviruses, poliovirus and other  
59 enteroviruses devote their 3A and 2BC genes to reorganizing cellular membranes  
60 associated with the Golgi apparatus<sup>(38)</sup>. Consistent with reorganization of the Golgi, the  
61 3A proteins from multiple enteroviruses are also responsible for the shutdown in cellular  
62 secretion associated with enteroviral infection<sup>(8)</sup>. Recent work has suggested that the  
63 binding of the protein Golgi-specific brefeldin A-resistance guanine nucleotide exchange  
64 factor 1 (GBF1) by enteroviral 3A is required for the secretion phenotype and viral  
65 replication<sup>(2,22)</sup>.

66 Recent work has also demonstrated the importance of the phosphatidylinositol 4-  
67 phosphate (PI4P) composition of membranes associated with positive-stranded RNA  
68 replication<sup>(18)</sup>. This model suggests that GBF1 binding of poliovirus 3As is important  
69 vis-à-vis recruitment of phosphatidylinositol 4-kinase class III, catalytic subunit  $\beta$   
70 (PI4KIII $\beta$ ) to replication complex membranes. In this model, the change in  
71 phosphoinositol membrane lipid composition resulting from PI4 kinase activity is  
72 expected to directly recruit the viral RNA dependent RNA polymerase via its PI4P-  
73 binding domain. The requirement for PI4 kinase activity has also been demonstrated in  
74 enterovirus 71<sup>(1)</sup>. Furthermore, two known anti-enteroviral drugs have been shown to  
75 have anti-PI4K activity, supporting the notion that PI4KIII $\beta$  inhibitors may have promise  
76 as broad-spectrum picornavirus therapeutics. However, the Picornaviridae family is

77 highly diverse, and the relationship between other members of this family and PI4KIII $\beta$   
78 is unclear.

79         Among the most divergent animal picornaviruses are those that belong to the  
80 kobuvirus genus, including Aichi virus<sup>(33, 39)</sup>. Originally isolated in Japan from patients  
81 with oyster-associated gastroenteritis in 1991, Aichi virus has a worldwide reach with a  
82 relatively high seroprevalence<sup>(33, 39)</sup>. Several other kobuviruses including bovine,  
83 porcine, sheep, canine, murine, and chiroptera (bat) strains have been discovered from  
84 stool in the past decade, as well as a kobu-like agent of a closely-related *Picornaviridae*  
85 genus, klassevirus/salivirus<sup>(13, 15, 19, 23, 24, 31, 32, 40)</sup>.

86         In this study, we characterized associations between the picornavirus  
87 nonstructural protein 3A and host factors using a mass spectrometry-based proteomic  
88 approach. We found that transiently expressed strep-tagged 3A protein from Aichi virus  
89 and bovine kobuvirus both copurified PI4KIII $\beta$  and a Golgi adaptor protein, acyl-CoA  
90 binding domain protein 3 (ACBD3 or GCP60), under stringent capture and wash  
91 conditions. In the process, we found that Aichi virus 3A is myristoylated at its N-  
92 terminal glycine, despite the fact that it lacks a recognizable N-myristoyl-transferase  
93 (NMT) motif. By Western blot detection, we found ACBD3 to be stably associated with  
94 several picornaviruses, including poliovirus, Aichi virus, bovine kobuvirus, porcine  
95 kobuvirus, human rhinovirus 14, and Coxsackie B viruses. We also found that the  
96 association with PI4KIII $\beta$  and 3A proteins was more stable with Aichi virus and bovine  
97 kobuvirus 3A proteins than with other picornaviral 3A proteins. Affinity purification of  
98 ACBD3 in the absence of 3A revealed association with PI4KIII $\beta$ , and consistent with  
99 this, all picornaviral 3A proteins that could recruit PI4KIII $\beta$  also copurified with ACBD3.

100 By alanine-scanning mutagenesis of Aichi virus 3A, we subsequently identified residues  
101 within the protein necessary for interaction with PI4KIII $\beta$  or ACBD3. We further  
102 demonstrated the necessity of PI4KIII $\beta$  activity and association for Aichi virus  
103 replication through chemical and genetic means. Overall, our data suggest that multiple  
104 picornaviral 3As utilize ACBD3 to recruit PI4KIII $\beta$ .  
105

106 **Methods**

107 **Virus, cells, plasmid, cloning**

108           The complete Aichi virus genome (8280nt) was synthetically constructed (Bio  
109 Basic Inc., Canada) and inserted into a plasmid-based expression construct (pAV-UCSF,  
110 GenBank accession JQ281544), based on pAV-FL (Genbank AB040749)<sup>(35)</sup>, and is  
111 freely available upon request. 293T and HeLa cells were maintained in DMEM-H21  
112 media supplemented with 10% FBS and penicillin/streptomycin. All genes for transient  
113 transfections were cloned into a modified pcDNA4/TO vector with a C-terminal 2xStrep-  
114 tag<sup>(27)</sup> or Flag-tag as noted in the results.

115           Cloning of viral 3A genes was performed using the InFusion Advantage kit  
116 (Clontech) and sequence-confirmed using the BigDye system on an Applied Biosystems  
117 3130xl sequencer. Mutagenesis of single or multiple sites in Aichi 3A was performed as  
118 published previously, respectively<sup>(10, 14)</sup>. Primer sequences are provided in Supplemental  
119 Table 1.

120

121 **Affinity purifications**

122           Affinity purifications were executed as previously described<sup>(17)</sup>. Briefly, 10 µg  
123 of plasmid was transfected with FuGeneHD (Roche, Switzerland) into a 15 cm plate of  
124 log-phase 293T cells at 50% confluency. Cells were harvested 48-72 hours later in 10  
125 mM EDTA/PBS on ice, centrifuged for 5 min at 140 rcf in a Eppendorf 5410 centrifuge  
126 at 4 °C, and lysed with 1 hour of nutation at 4 °C in 2 ml of capture buffer: 50 mM Tris  
127 pH 7.4, 150 mM NaCl, 1 mM EDTA buffer containing 0.5% NP-40. DNA was pelleted  
128 for 15 min at max speed in an Eppendorf 5415D centrifuge at 4 °C. Lysate was

129 precleared by rotation for 1 hour at 4 °C with 50 µl of Protein G Sepharose 4 FastFlow  
130 (Amersham) resuspended 1:1 in the capture buffer above without added detergent.  
131 Preclarification beads were pelleted for 2 min at 2000 rpm and lysate was affinity  
132 purified by inversion mixing overnight at 4 °C with 45 µl of Strep-Tactin Sepharose  
133 resuspended 1:1 in detergent-free capture buffer. Beads were washed 3x in capture  
134 buffer containing 0.1% NP-40, with a final wash in detergent-free capture buffer for a  
135 total wash time of 20-30 minutes. Captured proteins were eluted with 1X D-desthiobiotin  
136 dissolved in 40 µl capture buffer by inversion mixing for 30 minutes at room temperature  
137 (IBA Technology, Germany). For magnetic bead affinity purifications, no preclearing  
138 was performed and 2 mL cell lysates were incubated with 40 µl of StrepTactin magnetic  
139 bead slurry (Qiagen) for 2 hours and then washed 3x in capture buffer containing 0.1%  
140 NP-40, with a final wash in detergent-free capture buffer for a total wash time of  
141 approximately 5-8 minutes. Proteins were eluted as above.

142 For Western blot analysis, 10 µl of eluate was run on a 4-12% Bis-Tris SDS-  
143 PAGE gel (Invitrogen; Carlsbad, CA), transferred to nitrocellulose membrane using the  
144 XCell Blot II system, and blotted with 1:300 PI4KIIIβ A-2 antibody (sc-166822, Santa  
145 Cruz), 1:250 ACBD3 518 antibody (sc-101277, Santa Cruz), 1:300 GBF1 25 antibody  
146 (sc-136240, Santa Cruz), 1:1000 anti-Strep-tag antibody (Qiagen), or 1:1000 anti-Flag  
147 tag antibody (Qiagen). Blots were stained in 1:10,000 Alexa680 anti-mouse secondary  
148 antibody (Invitrogen) and imaged on a Licor Odyssey and quantitated using Licor  
149 Odyssey 2.1 software.

150

151 **Protein Identification by Mass Spectrometry**

152 Protein identification from affinity-purified samples was performed using peptide  
153 sequencing by mass spectrometry. Affinity purified samples containing approximately  
154 10-20  $\mu\text{g}$  total protein were denatured with urea, reduced and alkylated with DTT and  
155 iodoacetamide, and subjected to in-solution digestion using sequencing grade porcine  
156 trypsin (Promega). For targeted post-translational modification analysis, alternate digests  
157 were additionally performed using GluC or AspN (Roche). The resulting peptide  
158 samples were desalted using C18 zip tips (Millipore). Secondary validation of ACBD3  
159 and PI4KIII $\beta$  proteins in affinity purified samples was obtained by in gel digestion of  
160 selected protein bands in silver stained SDS-PAGE gels using standard protocols (in gel  
161 digestion protocol, UCSF), and LC-MS/MS peptide sequencing.

162 Two systems were used for LC-MS/MS analysis; the first was an LTQ-FT mass  
163 spectrometer (Thermo) equipped with a 10,000 psi system nanoACUITY (Waters) UPLC  
164 for reversed phase chromatography with a C18 column (BEH130, 1.7  $\mu\text{m}$  bead size, 100  
165  $\mu\text{m}$  x 100 mm). The second system was a linear ion trap LTQ instrument (Thermo)  
166 equipped with an Ultimate HPLC and Famos autoinjector (LC Packings), and a self-  
167 packed C18 column (New Objective Inc., 5  $\mu\text{m}$  bead size, 100  $\mu\text{m}$  x 150 mm). The two  
168 LC systems were operated at either 600 or 300 nL/min flow rates respectively, and  
169 peptides were separated using a linear gradient over 42 min from 2% B to 30% B, with  
170 solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile. On  
171 the LTQ-FT instrument, survey scans were recorded over 310-1600 m/z range, and  
172 MS/MS was performed in data dependent acquisition mode with CID fragmentation on  
173 the six most intense precursor ions, measured in the ion trap. On the LTQ instrument,  
174 survey scans were taken over 320-1500 m/z, and the top three ions in the survey scan

175 were subjected to a high resolution MS scan of the precursor and then a CID  
176 fragmentation MS/MS scan.

177           Mass spectrometry peak lists were generated using in-house software called  
178 PAVA, and data were searched using Protein Prospector software v. 5.8.0<sup>(26,6)</sup>. Database  
179 searches were performed against the *Homo sapiens* plus *Picornaviridae* subset of the  
180 NCBI nr Refseq database (*January 14, 2011*), to which were added virus clone sequences  
181 missing from the public database, totaling 37,526 entries. This database was  
182 concatenated with a fully randomized set of 37,526 entries for estimation of false  
183 discovery rate<sup>(11)</sup>. Data were searched with a parent mass tolerance of 20 ppm on the  
184 LTQ-FT or 0.8 Da on the LTQ, and fragment mass tolerances of 0.8 Da for both  
185 instruments.

186           For database searching, peptide sequences were matched as tryptic peptides with  
187 no missed cleavages, and carbamidomethylated cysteines as a fixed modification.  
188 Variable modifications included oxidation of methionine, N-terminal pyroglutamate from  
189 glutamine, loss of methionine and N-terminal acetylation. For reporting of protein  
190 identifications from this database search, score thresholds were selected that resulted in a  
191 protein false discovery rate of 1.1%. The specific Protein Prospector parameters were:  
192 minimum protein score of 22, minimum peptide score of 15, and maximum expectation  
193 values of 0.02 for protein and 0.05 for peptide matches. Protein identification results from  
194 specific affinity purification experiments are reported with a spectral count as an  
195 approximation of protein abundance, along with percent sequence coverage and an  
196 expectation value for the probability of the protein identification<sup>(9,25)</sup>.

197 To address non-specificity of background interacting proteins in the affinity  
198 purifications, multiple capture experiments were performed for 91 unrelated picornavirus  
199 protein constructs selected from 21 subspecies and 11 different genes, totaling 293  
200 control datasets. The control proteins included both structural (VP0, VP1, VP2, VP3,  
201 VP4) and non-structural genes (L, 2A, 2C, 2D, 3C, 3D) from Aichi virus, poliovirus,  
202 theiloviruses, enterovirus, and klassevirus. These control experiments were used as a  
203 background model for defining interaction specificity of copurifying proteins for a given  
204 prey 3A protein. Using peptide counts as an approximation of protein abundance, Z-  
205 scores were calculated for all copurified proteins to represent their interaction specificity  
206 to the bait. For each interacting protein in a replicate, a population of peptide counts  
207 consisting of the observed counts in the replicate together with the observed counts in all  
208 each of the control experiments was used to derive a per-replicate protein Z-score by  
209 calculating the number of standard deviations that the protein's peptide counts in the  
210 replicate were above or below the population mean. Per-replicate protein Z-scores were  
211 then averaged to obtain a final Z-score for each prey protein. Z-scores for proteins  
212 interacting with Aichi 3A were calculated using four replicate analyses of the viral  
213 protein affinity purification results together with a background model of 293 control,  
214 non-3A datasets.

215 For mapping of potential post-translational modifications (PTMs) on the 3A bait  
216 proteins themselves, alternate digests using AspN or GluC instead of trypsin were  
217 analyzed using targeted analysis. In these searches, additional post-translational  
218 modifications were allowed, including N-myristoylation, phosphorylation of serine,  
219 threonine or tyrosine, and GlyGly as a signature of ubiquitylation on Lys. Non-specific

220 (one) and missed cleavages (increased to two) were also allowed, as would be consistent  
221 with GluC and AspN reaction conditions. The peptide expectation value threshold was  
222 increased to 0.001 and spectra matches were manually validated to eliminate false  
223 positive identifications. Complete mapping of Aichi 3A wild-type construct is reported  
224 in a peptide summary in Supplemental Table 2. The most significant biological PTMs  
225 were found to be myristoylation and acetylation of the N-terminal glycine. All identified  
226 myristoylated peptides in kobuvirus 3A proteins were manually verified, with example  
227 spectra provided in Figure 1.

228

#### 229 **Viral luciferase replicon assay**

230 Renilla luciferase replicons were created using the synthetic virus plasmid pAV-  
231 UCSF or XpA by replacing the capsid region of each virus with Renilla luciferase gene  
232 using InFusion Advantage cloning (Clontech, Takara Bio). Primer sequences are  
233 described in Supplemental Table 1. Two micrograms of plasmid were cleaved for 1-3  
234 hours at 37 °C using HindIII-HF for Aichi virus replicon and MluI for XpA replicon  
235 (New England BioLabs), and then purified over a Zymo DNA-5 column (Zymo  
236 Research). One microgram of cut plasmid was T7 amplified for 4 hours, Turbo DNase  
237 cleaved for 15 min, and purified over a Zymo RNA-25 column (Zymo Research).

238 For replicon assays, 20,000 293T or HeLa cells were plated the night before in  
239 100 µl complete media per well in an opaque, white 96-well plate (Grenier America,  
240 catalog 655075). Cells were transfected with 100 ng of T7-amplified RNA using TransIT  
241 mRNA Transfection kit (Mirus Bio) in 75 µL complete media mixed with 1X EnduRen  
242 live cell imaging substrate (Promega). Cells were maintained in an incubator and

243 analyzed for Renilla activity hourly on a Veritas Microplate Luminometer. After viral  
244 replication had peaked, total cell count was determined by CellTiterGlo assay (Promega),  
245 and luminometer readings were normalized to cell count.

246

#### 247 **shRNA knockdown**

248 Previously published shRNA oligos against PI4KIII $\beta$  and GFP were ligated and cloned  
249 into a modified pSicoR lentivirus packaging vector <sup>(4)</sup>. The sequence-confirmed shRNA-  
250 expressing pSicoR plasmids were cotransfected with pRSV and pVSG plasmids into  
251 293T cells, and lentivirus was harvested 72 hours after transfection. Lentivirus was used  
252 to infect 293T cells and shRNA-expressing clones were selected for with 1  $\mu$ g/ml  
253 puromycin and expression was confirmed by mCherry expression. Target gene  
254 knockdown was confirmed by Western blotting and qRT-PCR. For qRT-PCR, 2  $\mu$ g of  
255 total RNA from 293T cells was reverse transcribed using SuperScript III reverse  
256 transcriptase (Invitrogen) and oligo(dT)(20), and qRT-PCR was performed using the 480  
257 DNA SYBR Green I Master mix (Roche) on a LightCycler (Roche).

258

#### 259 **siRNA knockdown**

260 Control, PI4KIII $\beta$ , GBF1, and ACBD3 ON-TARGETplus siRNAs were purchased  
261 commercially (Dharmacon). In a 96-well plate, 3,000 log-phase HeLa cells were reverse  
262 wet-transfected in 50nM siRNA with 0.15  $\mu$ l of Dharmafect 1 transfection reagent and  
263 125 $\mu$ l total media per well. At the same time, 60,000 log-phase HeLa cells were reverse  
264 wet-transfected in 50nM siRNA with 3  $\mu$ l of Dharmafect 1 transfection reagent and  
265 2.5mL total media per well in a six-well plate to assay for gene knockdown. After 72

266 hours, cells in the 96-well plate were transfected with viral replicon RNA and measured  
267 for Renilla luciferase per above. After the viral replicon assay was finished (~12 hours),  
268 total cell count was normalized using CellTiterGlo to adjust for differences in growth  
269 among different siRNAs. All measurements are an average of six wells per viral RNA-  
270 siRNA pair and adjusted to subtract background Renilla signal from the first time point  
271 (t=50 min) due to higher Renilla luciferase background in HeLa cells.

272

## 273 **Results**

### 274 **Aichi virus, bovine kobuvirus, and klassevirus 3A proteins are myristoylated.**

275 To identify proteins that interact with the main membrane reorganizing 3A  
276 protein of picornaviruses, we undertook an unbiased screen using single-step affinity  
277 purification of C-terminally strep-tagged 3A proteins in 293T cells followed by mass  
278 spectrometric peptide sequencing analysis of in solution trypsin digests. Careful  
279 inspection of the mass spectrometry data identified a myristoylation on the N-terminal  
280 glycine of Aichi 3A, bovine kobuvirus 3A, and klassevirus 3A (Figures 1A-E). The  
281 activity of N-myristoyltransferase (NMT) enzymes is strongly dependent on the first five  
282 residues, with an N-terminal glycine being absolutely required<sup>(28)</sup>. The N-terminal  
283 sequence of Aichi virus 3A is GNRVIDAE. Although algorithms such as NMT-The Myr  
284 Predictor (<http://mendel.imp.ac.at/myristate/SUPLpredictor.htm>) and ExPASy  
285 Myristoylator do not predict the N-terminus of any of the kobuviruses to be NMT  
286 substrates, *in vitro* experiments using human NMT1 have shown synthetic octapeptides  
287 such as GNRAAARR to be valid substrates with kinetics comparable to other known  
288 substrates<sup>(3, 34)</sup>.

289 To explore the functional effects of myristoylation, N-terminal mutations of the  
290 Aichi virus 3A protein were analyzed. Mutation of Aichi virus G1A abolishes  
291 myristoylation, as does N2A and R3A, while V4A, I5A, D6A, and E8A mutants retain N-  
292 terminal myristoylation as measured by mass spectrometry analysis of the affinity-  
293 purified protein. Mass spectra for the I5A and R3A mutations of Aichi virus 3A are  
294 shown in Figure 1D and E, and unique peptide identifications for this analysis are  
295 provided in Supplemental Table 3. Targeted analysis of MS spectra for affinity  
296 purifications of 3A proteins derived from Coxsackieviruses B2, B3, and B5, enterovirus  
297 71, poliovirus 1, and human rhinovirus 14 did not reveal evidence of N-terminal  
298 myristoylation despite recovering significant peptide counts for unmodified or acetylated  
299 N-termini. The cardioviruses, theilovirus strains TMDA, BeAn, and TMGDVII, and  
300 Saffold viruses UC6 or Saf2 have serine as their start residues and thus cannot be  
301 substrates for NMTs. For all 3A affinity purification experiments, additional searches  
302 were performed for other post-translational modifications, including phosphorylations,  
303 GlyGly signatures for ubiquitination, as well as broad mass range modifications of up to  
304 500 atomic mass units. With the exception of N-terminal acetylation, cysteine  
305 carbamidomethylation, pyroglutamylation of glutamine, and oxidation, no other  
306 modifications were detected in these experiments.

307 The 3A protein was also frequently observed to run as a doublet at 15 and 17 kDa  
308 by SDS-PAGE (for example, see Figure 2A) with detection by silver stain or by anti-  
309 Strep-tag antibody in Western blot format. In the case of Aichi 3A, mass spectrometric  
310 analysis confirmed that both bands contained full-length 3A protein, the lower band co-  
311 migrating with streptavidin. Despite extensive searches, no post-translational

312 modifications, including myristoylation, could be found that explain a mass shift in these  
313 bands, suggesting that these may be conformationally resolved forms of the protein. N-  
314 terminal and C-terminal peptides for wild-type and mutant Aichi 3A proteins are  
315 provided in Supplemental Table 4.

316

#### 317 **Method for determining specific interactions from mass spectrometry data**

318 To identify proteins that specifically interact with picornavirus 3A within the set  
319 of proteins identified by mass spectrometry, we first attempted to use a strep-tagged GFP  
320 as a negative control for non-specific interactions. The combined protein identifications  
321 across four replicate experiments for Aichi 3A and GFP, resulted in approximately 70  
322 and 40 putative interacting proteins respectively (Supplemental Tables 5 and 6). Based  
323 on these results, we determined that strep-GFP did not adequately sample the spectrum of  
324 non-specific interactions in this experimental system, consistent with observations by  
325 other groups <sup>(7, 17)</sup>.

326 Therefore, we chose instead to perform a comprehensive analysis of background  
327 proteins assessing their ability to interact with multiple unrelated viral proteins,  
328 consisting of 91 unique non-3A picornaviral bait proteins assayed in 293 individual  
329 experiments (Methods). The set of background interacting proteins was then used to  
330 derive the specificity for virus bait-host protein interactions, ranked using *Z*-scores. To  
331 minimize false positives, we report interactions that pass a highly conservative *Z*-score  
332 threshold of 10 and whose preys are represented by a minimum of 2 peptides in at least  
333 two biological replicates. To further strengthen confidence in the analysis, Aichi 3A  
334 affinity purifications were compared against those of 3A proteins from across 15 diverse

335 picornaviruses. The most specific protein interaction partners for each 3A protein were  
336 ranked using the *Z*-score metric, and resulted in a refined list of candidate interactions  
337 (Table 1). This methodology for scoring interactions was in part confirmed by the top  
338 ranking of GBF1 for poliovirus and Coxsackievirus 3A proteins. Prior to this study,  
339 GBF1 was the only confirmed protein to copurify with any picornaviral 3A. The  
340 complete table of *Z*-scores for all identified interacting proteins across all picornaviral 3A  
341 proteins tested is reported in Supplemental Table 7.

342

343 **AP-MS of C-terminally Strep-tagged Picornaviral 3A proteins copurify PI4KIII $\beta$**   
344 **and ACBD3.**

345 The top-ranked protein identified in affinity purifications with both Aichi virus  
346 3A and bovine kobuvirus 3A was PI4KIII $\beta$  (Table 1). PI4KIII $\beta$  interaction with Aichi  
347 virus 3A and bovine kobuvirus 3A was confirmed by Western blot (Figure 2A).  
348 Intriguingly, one peptide to PI4KIII $\beta$  was found in one replicate of Coxsackievirus B5  
349 affinity purification by MS, and a weak positive result by Western blot (Figure 2A) was  
350 also observed. To investigate whether other 3A proteins such as poliovirus 3A might still  
351 interact with PI4KIII $\beta$  more transiently, affinity purifications for selected enteroviral 3As  
352 were repeated under more rapid kinetic conditions, using short binding and washing steps  
353 and capture on magnetic StrepTactin beads (7). Using this more rapid procedure,  
354 PI4KIII $\beta$  was detected by affinity purification with 3As from poliovirus, human  
355 rhinovirus 14, and Coxsackievirus B3 (Figure 2B). We note that the 3A protein from  
356 rhinovirus 14 also captured GBF1, consistent with HRV sensitivity to Brefeldin-A<sup>(16)</sup>.

357           The second-ranking protein identified in the Aichi 3A affinity purifications was  
358 acyl-CoA binding domain protein 3 (ACBD3), also known as Golgi complex-associated  
359 protein GCP60 (Table 1). This protein was also affinity purified specifically by the 3A  
360 proteins of multiple picornaviruses, including poliovirus, Aichi virus, bovine kobuvirus,  
361 porcine kobuvirus, human rhinovirus 14, and Coxsackie B viruses. Although the 3A  
362 protein of EV71 did not copurify ACBD3 these conditions, we note that it did copurify  
363 with a different acyl-CoA binding protein, ACAD9. Although it was not detectable by  
364 Western blot (Figure 2A), a single peptide for ACBD3 was detected in an EV71 3A  
365 protein AP-MS experiment using rapid capture and wash steps on StrepTactin sepharose  
366 beads (data not shown), thus we cannot exclude interaction of EV71 with ACBD3.  
367 ACBD3 is a Golgi resident protein that has been implicated in multiple cell signaling  
368 systems, including Golgi complex maintenance, steroidogenesis, and apoptosis<sup>(12)</sup>.  
369 Interaction between ACBD3 and picornaviral 3As was confirmed by Western blot  
370 (Figure 2A). Furthermore, Aichi 3A and PI4KIII $\beta$  were both immunoprecipitated by  
371 anti-ACBD3 antibody in Aichi 3A transfected 293T cells as detected by mass  
372 spectrometry (Supplemental Table 8). However, in the absence of Aichi 3A, endogenous  
373 PI4KIII $\beta$  and ACBD3 did not co-precipitate with each other, suggesting that 3A  
374 specifically stabilizes the complex containing both of these proteins (Supplemental Table  
375 8).

376           To test whether ACBD3 and PI4KIII $\beta$  have direct interactions in the absence of  
377 Aichi virus 3A, C-terminally Strep-tagged ACBD3 was transiently transfected and  
378 affinity purified using the rapid binding and washing protocol with and without a series  
379 of 3A proteins from enterovirus and kobuviruses. In the absence of any transfected 3A

380 proteins, PI4KIII $\beta$  was found to co-purify with ACBD3 (empty vector lane, Figure 3)  
381 indicating that the interaction between ACBD3 and PI4KIII $\beta$  does not require 3A.  
382 Affinity purification of ACBD3 in the presence of 3A proteins captured 3A from  
383 poliovirus, CVB3, human rhinovirus 14, Aichi virus, and bovine kobuvirus. The only  
384 exception was EV71, consistent with the reciprocal affinity purification experiments  
385 discussed above. Surprisingly, a band consistent with a complex containing ACBD3-  
386 strep and Aichi-3A-Flag was observed (Figure 3A, marked by a black triangle, Aichi 3A  
387 lane) despite standard denaturing gel running conditions. Taken together, this reciprocal  
388 affinity capture experiment implies direct interaction between ACBD3, PI4KIII $\beta$ , and  
389 multiple picornaviruses.

390 Alignment of all Aichi 3A sequences in Genbank indicated >90% amino acid identity  
391 among the five sequences available, including 100% conservation in the N-terminal half  
392 of the protein.

393 **Site-directed mutagenesis of Aichi 3A identifies residues required for interaction**  
394 **with PI4KIII $\beta$  and ACBD3.**

395 To identify the critical residues for the interaction between Aichi 3A, PI4KIII $\beta$ , and  
396 ACBD3, we employed alanine scanning of the 95 amino acid Aichi 3A protein. In total,  
397 all 87 non-alanine residues were converted to alanine with a focus on single site mutants  
398 on the N-terminal half, where all Aichi 3A sequences in GenBank are 100% conserved,  
399 in addition to multi-site mutants on the C-terminus (Figure 3A). Of 87 positions mutated  
400 (grey squares, Figure 4B), approximately 20 residues (black squares, Figure 4B),  
401 clustered at the N-terminus, severely reduced or abolished copurification of PI4KIII $\beta$   
402 (<10% of wild type, normalized to expression of the 3A protein in each experiment). In

403 particular, mutation of R3A, I5A, NR2AA, NRV2AAA, and NRVI2AAAA abolished or  
404 reduced the amount of PI4KIII $\beta$  and ACBD3 interaction by more than 90%. Although  
405 the R3A mutation, along with G1A, both eliminate the N-terminal myristoylation of  
406 Aichi virus 3A, the lack of myristoylation does not account for the loss of PI4KIII $\beta$  and  
407 ACBD3 interaction, since N2A, which also eliminates the N-terminal myristoylation, has  
408 no effect on copurification of either of these proteins (Figure 3A). While 21 mutations  
409 could abrogate PI4KIII $\beta$  association without affecting association of ACBD3 with Aichi  
410 virus 3A, all mutations that had a negative impact on ACBD3 association also severely  
411 reduced or eliminated PI4KIII $\beta$  association. These results support the hypothesis that  
412 PI4KIII $\beta$  association with Aichi virus 3A requires ACBD3 and also implies that 3A  
413 association with ACBD3 either enhances or stabilizes the interaction with PI4KIII $\beta$ .

414

#### 415 **Chemical and genetic inhibition of PI4KIII $\beta$ blocks Aichi virus replication**

416 To assess whether the interaction between Aichi virus 3A and PI4KIII $\beta$  was of  
417 functional importance for viral replication, we measured a Renilla luciferase replicon  
418 Aichi virus construct in the presence of chemical and genetic inhibition of PI4KIII $\beta$ .  
419 PIK93 is a small molecule inhibitor of PI4KA and PI4KIII $\beta$  <sup>(20)</sup>. It has previously been  
420 shown to block the replication of poliovirus and hepatitis C virus replication with an EC<sub>50</sub>  
421 of 0.14 and 1.9  $\mu$ M, respectively <sup>(1)</sup>. The addition of 0.5  $\mu$ M and 1.0  $\mu$ M PIK93  
422 demonstrated a dose-dependent inhibition of Aichi virus replication similar to the dose-  
423 dependent decrease observed with poliovirus (Figure 5A&B).

424 Stable shRNAs were used to reduce or nearly eliminate PI4KIII $\beta$  expression in  
425 293T cells, using previously published shRNAs (Figure 5C&D) <sup>(5)</sup>. PI4KIII $\beta$  mRNA

426 transcript abundance was reduced by up to 98% as normalized to the expression of the  
427 ribosome gene RPL19 (Figure 5E). The shRNA-dependent knockdown PI4KIII $\beta$  protein  
428 expression was also confirmed by Western (Figure 5F). Only the most potent shRNA  
429 construct inhibited Aichi virus and poliovirus replication, while incomplete knockdown  
430 of PI4KIII $\beta$  did not significantly impact Aichi virus or poliovirus replication (Figure  
431 5C&D).

432 While siRNA knockdown of PI4KIII $\beta$  completely abolished Aichi virus and  
433 poliovirus replication, knockdown of ACBD3 demonstrated significantly reduced  
434 replication in both viruses (Figure 6A,B). Although Aichi virus replication has been  
435 reported to be insensitive to Brefeldin-A (36), an inhibitor of GBF1/Arf1, we were  
436 surprised to find that siRNA knockdown of GBF1 also abolishes Aichi virus replication  
437 (Figure 6B). Interestingly, the siRNA knockdown of GBF1 resulted in a loss of PI4KIII $\beta$   
438 similar to what was achieved with a directed siRNA knockdown of PI4KIII $\beta$  (Figure 6C).  
439 While these results demonstrate a requirement for GBF1, it is possible that the replication  
440 defect is actually due to a loss of PI4KIII $\beta$  indirectly caused by a loss of GBF1. These  
441 results support the hypothesis that the presence and activity of PI4KIII $\beta$  is essential for  
442 Aichi virus replication, similar to what has been shown previously for poliovirus<sup>(18)</sup>.  
443 These data also support the hypothesis that ACBD3 is functionally important for  
444 picornavirus replication presumably by facilitating the interaction with PI4KIII $\beta$ .

445

446 **Reduced recruitment of PI4KIII $\beta$  correlates with delayed or altered replication**

447 **kinetics of Aichi virus replicons**

448 To further assess the requirement of the association between Aichi virus 3A and  
449 PI4KIII $\beta$  for viral replication, we tested viral replication after replacing the wild-type 3A  
450 sequence with a series of point mutants based on our affinity purification results. No  
451 replication above background was measurable in the context of the Aichi virus replicon  
452 with the E11A mutation, which significantly disrupted copurification with PI4KIII $\beta$ , and  
453 reduced, but did not eliminate ACBD3 association (Figure 7A). Aichi virus with the  
454 G1A mutation also demonstrated no replication, though this is likely due to disruption of  
455 the P2-P3 3C proteolytic cleavage site. Myristoylation had a minimal impact on  
456 replication as the N2A mutant had near wild-type levels of replication, while the R3A  
457 mutant was slightly delayed (Figure 7B). Delayed replication was observed in the  
458 NR2AA, NRV2AAA, NRVI2AAAA, I5A, I12A, L20A, L21A, M24A, and HH26AA  
459 mutations, which significantly reduced copurification of PI4KIII $\beta$  by >90%, though  
460 several of the mutations retained the same maximal level of replication as the wild-type  
461 Aichi virus replicon (Figure 7C-F). The E22A mutation that retained wild-type PI4KIII $\beta$   
462 binding demonstrated replication kinetics slightly delayed but comparable with wild-type  
463 virus (Figure 7G). The P59A mutation that appeared to stimulate PI4KIII $\beta$  copurification  
464 (Figure 4A) demonstrated a significant delay in replication yet continued to produce  
465 luciferase signal over 10 hours post-transfection (Figure 7G).

466 To ascertain whether the delayed replication in viruses with mutations in 3A that  
467 inhibited PI4KIII $\beta$  association was due to the reduced PI4KIII $\beta$  association, we examined  
468 the EC<sub>50</sub> of PIK93 in these mutant viruses compared to wild-type virus. PIK93 had an  
469 EC<sub>50</sub> of 0.60  $\mu$ M at 330 minutes post-transfection in the wild-type Aichi viral replicon.  
470 Interestingly, lower concentrations of PIK93 did not reduce the total amount of viral

471 replication but only delayed viral replication in a dose-dependent fashion (Figure 8A).  
472 However, the  $EC_{50}$  of PIK93 was reduced more than 2-fold to 0.24  $\mu$ M in the I5A mutant  
473 and by almost 20-fold to 0.03  $\mu$ M in the NRVI2AAAA mutant (Figure 8B). These  
474 results support the notion that physical association of PI4KIII $\beta$  with Aichi virus 3A via  
475 ACBD3, is required for replication.  
476

477 **Discussion**

478

479 In this study, we demonstrated that the 3A protein from multiple picornaviruses,  
480 including Aichi virus, bovine kobuvirus, poliovirus, Coxsackievirus B, and human  
481 rhinovirus 14, associates PI4KIII $\beta$  and the Golgi adaptor protein ACBD3. We note that  
482 some picornaviruses that did not appear to associate with ACBD3. For example, we  
483 identified a Rap1A as a possible interaction in the case of cardioviruses, and ACAD9 in  
484 the case of EV71. Previous studies have shown PI4KIII $\beta$  is required for picornaviral  
485 replication and have created models of indirect recruitment of PI4KIII $\beta$  by 3A<sup>(18)</sup>.  
486 Through genetic and chemical inhibition, we have demonstrated a requirement for  
487 PI4KIII $\beta$  for Aichi virus replication, and we further defined the molecular determinants  
488 in Aichi virus 3A that are required for its physical association with ACBD3 and  
489 PI4KIII $\beta$ .

490 ACBD3 is localized in the Golgi apparatus and contains an acyl-CoA binding  
491 domain, a putative nuclear localization signal, and a GOLD lipid trafficking domain<sup>(12)</sup>.  
492 Mutation and over expression of ACBD3 can cause disruption of the Golgi, implicating it  
493 in the maintenance of Golgi structure and function<sup>(37)</sup>. It has also been implicated in a  
494 wide variety of cell signaling processing from lipid transport to apoptosis. Though a  
495 genetic interaction with acyl-CoA binding protein and bromovirus replication has been  
496 demonstrated in yeast, our study is the first to demonstrate a physical interaction between  
497 a picornavirus and a host protein involved in acyl-CoA binding<sup>(21)</sup>. The 3A protein of  
498 EV71, the one enteroviral 3A that did not strongly copurify ACBD3, instead copurified  
499 with another acyl-CoA binding protein, acyl-CoA dehydrogenase family member 9

500 (ACAD9). These interactions suggest a potential mechanism for the localization of viral  
501 replication complexes to the ER-Golgi. It further suggests a role for lipid signaling and  
502 trafficking for the replication of picornaviruses. It is notable that despite alanine  
503 scanning almost the entirety of the Aichi virus 3A protein, only two mutations, R3A and  
504 I5A, could significantly disrupt association with ACBD3. In addition, affinity  
505 purification of ACBD3 in the presence of Aichi 3A revealed the presence of an SDS- and  
506 DTT-resistant Aichi 3A-ACBD3 complex suggesting a highly stable interaction. Given  
507 the known roles of ACBD3 in Golgi structure and function, we hypothesize that this  
508 protein, in association with 3A, serves as a scaffold for the generation of viral replication  
509 complexes and membrane remodeling.

510         The discovery of a myristoylation on Aichi virus, bovine kobuvirus, and  
511 klassevirus 3A is the first demonstration of myristoylation on a non-structural  
512 picornavirus protein. The 3A protein is myristoylated despite the presence of a non-  
513 canonical N-terminal sequence. Many viral proteins have previously been shown to be  
514 myristoylated, including retrovirus gag protein, HIV nef protein, hepadnavirus L protein,  
515 arterivirus E protein, as well as the VP4 capsid protein of poliovirus and foot-and-mouth  
516 disease virus<sup>(29)</sup>. The myristoylation on at least two different picornaviral proteins is  
517 significant as it suggests a potential contributing mechanism for membrane association  
518 and reorganization, as well as a potential mechanism for concentrating picornaviral  
519 proteins and associating RNA replication with encapsidation. Nonetheless, we found that  
520 while myristoylation of 3A contributed somewhat to the binding of PI4KIII $\beta$ , this post-  
521 translational modification was not a determinant for binding of ACBD3. Interestingly,  
522 the binding of the cellular protein NCS-1 to PI4KIII $\beta$  has been shown to be dependent on

523 myristoylation of NCS-1 (18). It is plausible that myristoylation may serve as an  
524 enhancer of PI4KIII $\beta$  activity or recruitment, but its precise role remains to be  
525 determined.

526         Our results also suggest that while inhibition of PI4KIII $\beta$  may delay viral  
527 replication, even a very low affinity interaction between 3A, ACBD3, and PI4KIII $\beta$  or  
528 partial activity of the enzyme may be sufficient to support viral replication. Indeed,  
529 mutants such as L20A or M34A, which do not affinity purify with PI4KIII $\beta$  but retain  
530 their association with ACBD3, show only modest reductions in replication. Even more  
531 striking is the NRVI2AAAA mutant, which fails to recruit both PI4KIII $\beta$  and ACBD3  
532 and lacks the N-terminal myristoylation, and yet this mutant is still supports replication.  
533 Nevertheless, we have shown that the NRVI2AAAA mutant results in a 20-fold greater  
534 sensitivity to chemical inhibition of PI4KIII $\beta$ . This is similar to the effect observed in  
535 poliovirus, where the 3A-2 mutant, which has reduced ability to bind GBF1, replicates at  
536 near wild-type levels, and displays enhanced sensitivity to the GBF1 inhibitor brefeldin A  
537 (2). Furthermore, the ability to isolate 3A mutants that do not copurify with PI4KIII $\beta$   
538 suggests that 3A may influence ACBD3's ability to recruit PI4KIII $\beta$  and is not merely  
539 limited to recruitment of a native ACBD3-PI4KIII $\beta$  complex. From a therapeutic  
540 standpoint, our results suggest that chemical inhibitors that block association of PI4KIII $\beta$   
541 and viral 3A, or ACBD3 and viral 3A, would represent a complementary approach to  
542 simple inhibition of the kinase activity of PI4KIII $\beta$  itself.

543         While this paper was under review, Sasaki et al. published a paper on the  
544 interaction of Aichi 3A with ACBD3 and PI4KIII $\beta$  (36). The results of Sasaki et al., and

545 our own are highly complementary and the overall conclusions are consistent, with  
546 respect to Aichi virus, despite using different experimental approaches.

547         The investigation of multiple picornaviruses in our work demonstrates the broad  
548 importance of a common strategy for enterovirus and kobuvirus replication. Despite  
549 differences in their associations, such as enterovirus 3A binding of GBF1, these two  
550 genera ultimately operate through a common platform, the golgi adaptor ACBD3, to  
551 recruit PI4KIII $\beta$ .

552

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562

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- 697

698 **Tables**

699

700 Table 1 - Interacting proteins were identified using in-solution digestion of eluted  
701 proteins followed by mass spectrometry. Hits were weighted by the Z-score of the  
702 peptide counts of captured proteins from four biological replicate experiments against a  
703 control database of picornavirus protein affinity purifications that did not include 3A  
704 tagged proteins (Methods). Based on the Z-score of the peptide count, PI4KIII $\beta$  and  
705 ACBD3 were the top two interacting proteins with Aichi 3A.

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707

708 **Figures**

709

710 Figure 1. **The N-terminus of Aichi 3A, klassevirus and bovine kobuvirus 3A is**  
711 **myristoylated.** Strep-tagged kobuvirus3A proteins were transiently transfected into 293T  
712 cells and affinity purified using the Strep-tag system then subjected to mass spectrometric  
713 peptide sequencing.. Mass spectra from LC-MS/MS analysis show myristoylation of  
714 Aichi 3A (A), Klassevirus 3A (B), bovine kobuvirus 3A (C), and I5A mutant Aichi 3A  
715 (D); however only the acetylated form was observed for R3A mutant Aichi 3A (E).

716

717 Figure 2. **Picornaviral 3A proteins interact differentially with PI4KIII $\beta$  and ACBD3.**

718 A) Strep-tagged 3A proteins across the picornavirus family were affinity captured under  
719 fully equilibrated binding conditions, then blotted with anti-ACBD3, anti-PI4KIII $\beta$ , and  
720 anti-StrepTag antibodies, revealing stable interactions between kobuvirus and enterovirus

721 3A proteins and ACBD3. B) When the affinity capture conditions were changed to rapid  
722 capture and wash conditions (described in Methods), transient interactions were also  
723 observed for the enterovirus 3A proteins with PI4KIII $\beta$ .

724

725 **Figure 3. Affinity purification of strep-tagged ACBD3.** Strep-tagged ACBD3 and  
726 FLAG-tagged enterovirus and kobuvirus 3A proteins were transiently co-transfected into  
727 293T cells, and ACBD3 complexes were affinity captured under rapid kinetic conditions.  
728 Samples were then serially Western blotted for PI4KIII $\beta$ , strep-tag, and Flag tag. ACBD3  
729 co-purifies with PI4KIII $\beta$  in the absence or presence of picornavirus 3A. In the presence  
730 of 3A, ACBD3 co-purifies with 3A proteins from multiple entero and kobuviruses, with  
731 the exception of EV71. In the presence of Aichi virus 3A, an SDS resistant complex  
732 consistent with ACBD3 bound to 3A is visible (black triangle). A separate Western blot  
733 of the input samples was serially blotted for GAPDH and Flag-tag is shown in the lower  
734 panel to control for expression and loading levels.

735

736 **Figure 4. Site-directed mutagenesis of Aichi 3A identifies residues required for**  
737 **interaction with PI4KIII $\beta$  and ACBD3.** Alanine scanning of individual and multiple  
738 residues in Aichi 3A was performed. Mutated Aichi 3A was transiently transfected into  
739 293T cells and affinity purified using the Strep-tag system. Eluate was blotted with anti-  
740 PI4KIII $\beta$  and anti-ACBD3 and anti-Strep-tag to normalize for bait expression. B) Map of  
741 contributing residues to Aichi 3A and ACBD3 and PI4KIII $\beta$  interaction. Residues  
742 labeled in red abolished myristoylation when converted to alanine. The predicted  
743 transmembrane region is highlighted in purple and predicted alpha-helices are highlighted

744 in teal. Black boxes indicate residues that reduce binding to ACBD3 or PI4KIII $\beta$  by  
745 >90% when converted to alanine. Grey boxes indicate residues that do not change  
746 binding by >90% and white boxes indicate residues for which no information is available.  
747

748 **Figure 5. Chemical and genetic inhibition of PI4KIII $\beta$  decrease Aichi virus**  
749 **replication.** PIK93, an inhibitor of PI4KIII $\beta$ , demonstrates a dose-dependent block in  
750 poliovirus (A) and Aichi virus replication (B). Genetic inhibition of PI4KIII $\beta$  by stable  
751 shRNA blocks poliovirus (C) and Aichi virus replication (D) only when PI4KIII $\beta$  levels  
752 are strongly reduced. qRT-PCR (E) and Western (F) of shRNA constructs demonstrates  
753 10%, 90%, and 99% knockdown of PI4KIII $\beta$  transcript levels and 0%, 60%, and 98%  
754 knockdown of PI4KIII $\beta$  protein levels.

755  
756 **Figure 6 – Genetic inhibition of physically interacting genes reduces viral**  
757 **replication.** siRNA knockdown of GBF1, PI4KIII $\beta$ , and ACBD3 reduce poliovirus (A)  
758 and Aichi virus (B) replicon growth in HeLa cells relative to control siRNA. C) Western  
759 blot of GBF1, PI4KIII $\beta$ , ACBD3, and GAPDH in siRNA knockdown and control  
760 knockdown HeLa cells.

761  
762 **Figure 7 – Reduced recruitment of PIKIII $\beta$  correlates with delayed replication**  
763 **kinetics of Aichi virus replicons**  
764 3A mutants were cloned into Aichi virus replicon and examined for replication  
765 efficiency. A) Aichi 3A E11A mutant is unable to replicate. B) Aichi 3A N2A and R3A  
766 replicate, suggesting myristoylation of Aichi 3A is not required for replication. C-F)

767 Aichi 3A mutations that reduce PI4KIII $\beta$  binding by >90% are still are capable of  
768 replicating albeit with slower kinetics. G) Aichi 3A E22A mutation that maintains  
769 PI4KIII $\beta$  and ACBD3 binding demonstrates similar replication kinetics as wild-type  
770 while P59A mutation demonstrates significantly delayed and reduced replication kinetics.

771

772 **Figure 8 – Aichi virus replicons with 3A mutants with reduced ability to recruit**  
773 **PI4KIII $\beta$  are sensitized to PIK93 inhibition.** A) The EC<sub>50</sub> for PIK93 against wild-type  
774 Aichi virus replicon was 0.60  $\mu$ M at 330 minutes post-transfection. PIK93 inhibition of  
775 Aichi virus replication can be overcome at lower concentrations given a longer  
776 replication window. While 1  $\mu$ M and 0.5  $\mu$ M PIK93 significantly reduce and delay Aichi  
777 virus replication, lower concentrations of PIK93 merely delay replication of wild-type  
778 Aichi virus, though the virus eventually reaches the same level of replication. B)  
779 Mutations in 3A that significantly reduce binding of PI4KIII $\beta$  are sensitized to PIK93  
780 inhibition, reducing the EC<sub>50</sub> to 0.24  $\mu$ M in the I5A mutant and to 0.03  $\mu$ M in the  
781 NRVI2AAAA mutant.

782

783

**Table 1. Highest Specificity Picornavirus 3A-Human protein-protein interactions, ranked by Z-score.**

| <b>Bait 3A</b> | <b>Prey Acc#</b> | <b>Prey Protein</b>  | <b>mean z-score</b> | <b>replicate counts</b> |
|----------------|------------------|--|---------------------|-------------------------|
| Aichi          | 311771621        | phosphatidylinositol 4-kinase beta isoform 2   | 17.12               | 5 14 9 16               |
| Aichi          | 15826852         | Golgi resident protein GCP60   | 17.06               | 33 23 10 25             |
| Aichi          | 4504341          | histone acetyltransferase type B catalytic subunit                                   | 12.84               | 7 0 3 1                 |
| Hrv14          | 15826852         | Golgi resident protein GCP60   | 16.29               | 2 15 15 30              |
| Hrv14          | 296317339        | voltage-dependent anion-selective channel protein 2 isoform                          | 11.91               | 0 6 7 13                |
| Polio          | 313747582        | Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 isoform 2 | 17.12               | 30 19 15 14             |
| Polio          | 21361794         | cullin-associated NEDD8-dissociated protein 1  | 17.12               | 2 1 4 2                 |
| Polio          | 15826852         | Golgi resident protein GCP60   | 17.03               | 34 30 13 9              |
| klasse         | 82659109         | E3 ubiquitin-protein ligase UBR4   | 16.55               | 6 15 3 12               |
| klasse         | 5453998          | importin-7   | 14.09               | 2 1 1 6                 |
| klasse         | 16445419         | secretory carrier-associated membrane protein 3 isoform 1                            | 13.16               | 6 2 1 9                 |
| klasse         | 6912734          | transportin-3 isoform 1  | 12.84               | 3 2 0 7                 |
| klasse         | 229577398        | basic leucine zipper and W2 domain-containing protein 2                              | 12.84               | 2 1 0 6                 |
| klasse         | 21361794         | cullin-associated NEDD8-dissociated protein 1  | 12.84               | 1 2 0 5                 |
| Porc kobu      | 94721252         | vesicle-associated membrane protein-associated protein A                             | 17.12               | 12 13 19 22             |

|           |           |  |       |               |
|-----------|-----------|--|-------|---------------|
|           |           | isoform 2  |       |               |
| Porc kobu | 4504341   | histone acetyltransferase type B catalytic subunit | 17.12 | 3 1 7 6       |
| Porc kobu | 19923919  | receptor expression-enhancing protein 6            | 17.12 | 2 1 2 3       |
| Porc kobu | 82659109  | E3 ubiquitin-protein ligase UBR4                   | 17.11 | 67 44 97 94   |
| Porc kobu | 15826852  | Golgi resident protein GCP60                       | 17.11 | 42 46 39 46   |
| Porc kobu | 6005794   | PRA1 family protein 2                              | 14.72 | 2 2 3 3       |
| Bov kobu  | 311771621 | phosphatidylinositol 4-kinase beta isoform 2       | 17.12 | 17 17 14 19   |
| Bov kobu  | 19923919  | receptor expression-enhancing protein 6            | 17.12 | 5 6 5 7       |
| Bov kobu  | 164519076 | transmembrane 9 superfamily member 4 precursor     | 17.12 | 4 5 7 12      |
| Bov kobu  | 115430112 | receptor expression-enhancing protein 5            | 17.12 | 4 1 6 8       |
| Bov kobu  | 82659109  | E3 ubiquitin-protein ligase UBR4                   | 17.12 | 88 72 110 171 |
| Bov kobu  | 15826852  | Golgi resident protein GCP60                       | 17.11 | 49 52 51 57   |















